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1 Varying modalities of perinatal exposure to a pesticide cocktail
2 elicit neurological adaptations in mouse and zebrafish.

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27

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37 **Highlights**

- 38 1) Perinatal pesticides links with long-term behavioral modifications in male mice.
39 2) Behavioral changes are accompanied by electrographic brain slowing *in vivo*.
40 3) In zebrafish larvae, pesticide cocktail elicited dose-dependent motor-behavioral
41 changes.
42 4) In zebrafish larvae, chlorpyrifos is a behavior-modifying pesticide contained in
43 the cocktail.
44 5) Non-lethal prenatal pesticide cocktail does not provoke brain malformations.

45

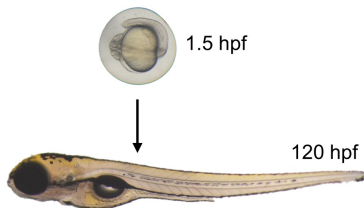
46 **Graphical abstract**

Pre-mating to perinatal exposure
Dietary pesticide cocktail at TDI levels



- ✓ Behavioral adaptations in male mice
- ✓ Long-term brain EEG slowing in male
- ✓ No structural brain malformations

Developmental stages
Pesticide cocktail or single (0.1 – 10.000 µg/L)



- ✓ Malformations and lethality at high concentrations
- ✓ Dose-dependent motor-behavior swimming modifications
- ✓ No structural brain malformations at non-lethal pesticide levels

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54 **Abstract**

55

56 Epidemiological indications connect maternal and developmental presence or
57 exposure to pesticides with an increased risk for a spectrum of neurological
58 trajectories. To provide pre-clinical data in support of this hypothesis, we used two
59 distinct experimental models. First, female and male mice were fed immediately prior
60 to mating, during gestation and lactation periods using chow pellets containing a
61 cocktail of six pesticides at tolerable daily intake levels. Male and female offspring
62 were tracked for behavioral and *in vivo* electrophysiological adaptations. Second, a
63 zebrafish model allowed us to screen toxicity and motor-behavior outcomes
64 specifically associated with the developmental exposure to a low-to-high
65 concentration range of the cocktail and of each individual pesticide. Here, we report
66 anxiety-like behavior in male mice maternally exposed to the cocktail, as compared to
67 age and gender matched sham animals. In parallel, *in vivo* electrocorticography
68 revealed a decrease in gamma (40-80 Hz) and an increase of theta (6-9 Hz) waves,
69 delineating a long-term neuronal slowing. Neurological changes were not
70 accompanied by brain structural malformations. Next, by using zebrafish larvae, we
71 showed an increase of all motor-behavioral parameters resulting from the
72 developmental exposure to 10 µg/L of pesticide cocktail, an outcome that was not
73 associated with midbrain structural or neurovascular modifications as assessed by *in*
74 *vivo* 2-photon microscopy. When screening each pesticide, chlorpyrifos elicited
75 modifications of swimming parameters at 0.1 µg/L, while other components provoked
76 changes from 0.5 µg/L. Ziram was the single most toxic component inducing
77 developmental malformations and mortality at 10 µg/L. Although we have employed
78 non-equivalent modalities and timing of exposure in two dissimilar experimental

79 models, these outcomes indicate that presence of a pesticide cocktail during varying
80 perinatal periods represents an element promoting behavioral and neurophysiological
81 modifications. The study limitations and the pertinence of our findings to
82 ecotoxicology and public health are critically discussed.

83

84 **Introduction**

85

86 Epidemiological evidence traces a link between presence of environmental
87 contaminants and a risk for the development of central nervous system (CNS)
88 negative trajectories (Baghdadli et al., 2019; Bouchard et al., 2011; Coker et al.,
89 2017; De Felice et al., 2016; Engel et al., 2016; Eskenazi et al., 2004; Matsuki et al.,
90 2020; Philippat et al., 2018, 2017; Rauh et al., 2011; Ricceri et al., 2006; Roberts et
91 al., 2019; Sagiv et al., 2019; Von Ehrenstein et al., 2019). Considering the existence
92 of pesticides in agricultural crops and in environmental matrices such as water,
93 investigating the impact of contaminants on brain functions is relevant to public health
94 and ecotoxicology. However, contemporary epidemiological alarms are not
95 adequately paralleled by experimental studies examining the neurological outcome
96 resulting from the maternal or perinatal exposure to pesticides, particularly when
97 these are combined as a cocktail and at dose ranges relevant to dietary or
98 environmental settings. Most of the available results originated from investigations of
99 supra-threshold dosages of single pesticide(s), mimicking intoxications. In rodents,
100 doses exceeding the tolerable daily intake (TDI) have been used to study how pre-
101 and neonatal exposure to the herbicide glyphosate causes behavioral impairments in
102 adulthood (de Souza et al., 2019; Gallegos et al., 2016). Other reports showed long-
103 term anxiety-like behaviors following perinatal exposure to high concentration of

104 chlorpyrifos (Braquenier et al., 2010; Silva et al., 2017). Furthermore,
105 neurodevelopmental toxicity was reported when testing high levels of boscalid in
106 zebrafish (Qian et al., 2018).

107

108 Here, by using two distinct mouse and zebrafish models we provide evidence
109 for an impact of maternal or developmental exposure to dietary and environmental
110 pesticides on neuronal functions. In one model, C57BL6/j mice were fed immediately
111 prior to and during mating, during pregnancy and lactation with chow pellets
112 containing a cocktail of six pesticides, with relevance to agriculture (EFSA, 2018;
113 Klement et al., 2020; Lukowicz et al., 2018; Smith et al., 2020) and exposing mice to
114 the TDI for each pesticide. Using male and female offspring, we tracked behavior and
115 *in vivo* brain electrophysiological outcomes. In a second model, we systematically
116 exposed zebrafish larvae during development to either the complete pesticide
117 cocktail or individual components, at a wide range of concentrations (0.1 to 10.000
118 µg/L) which includes the varying water thresholds as defined by current international
119 guidelines (see Methods). The zebrafish larvae model allowed us to screen and
120 identify the components of the cocktail possibly inducing neurological modifications.
121 These analyses were integrated with *ex vivo* and *in vivo* 2-photon microscopy brain
122 morphological and cellular assessments. By using this two experimental approaches,
123 we have found that varying perinatal exposures to pesticides promote discrete
124 neurophysiological and behavioral modifications in the absence of apparent structural
125 brain malformations.

126

127

128 **Material and Methods**

129

130 *Animal Models and Husbandry*

131

132 Investigations were performed according to the institutional guidelines for
133 laboratory animal usage [European Union Council September 22, 2010 (2010/63)].
134 Research using mice is covered by an accepted protocol (Apafis#13145-
135 2018012216217153 v3). C57BL/6j mice were housed in a 12h light/dark cycle,
136 minimizing discomfort. Adult zebrafish (*Danio rerio*, AB wild-type strain) were
137 maintained under standardized conditions ($28 \pm 0.5^{\circ}\text{C}$, pH 7.0, 14h light/10h dark
138 photoperiod) in a recirculating system (Techniplast) and fed *ad libitum* twice per day
139 with dry pellet. Embryos were generated by natural spawning once a week. After
140 fertilization, eggs were collected, cleaned in E3 medium and dead eggs were
141 removed. Next, 50 embryos were transferred in each petri dish containing 50 mL of
142 E3 medium and kept inside an incubator ($28 \pm 0.5^{\circ}\text{C}$). E3 medium was renewed
143 every day. At the end of the experiments, embryos were euthanized by immersion
144 using an overdose of tricaine methane sulfonate (300 mg/L; MS222, Sigma-Aldrich).

145

146 *Mouse perinatal exposure protocol and pesticides chow preparation*

147

148 We used a total of 32 female and 16 male mice (F0) to obtain synchronous
149 pregnancy, birth and similar age range in each group (within 6 days). This protocol
150 delivered an adequate number of male and female mice to be used for behavioural,
151 telemetry electrocorticography, and histological analyses. Male and female C57BL/6j
152 mice were fed with pesticide or control pellet immediately prior to and during mating
153 (total of 1 week) to ensure presence of pesticide from fecundation. Animals were
154 randomly divided into 2 groups, and one male and two females were housed in each

155 cage. Pregnant females were separated and housed in single dedicated cages.
156 During this phase, food (mg/day) and water (ml/day) intake were closely monitored,
157 and no significant differences were observed among the two groups (Table 1). This
158 protocol was previously used by our research consortium (Smith et al., 2020). At
159 weaning, all offspring were fed using standard control pellet and animals were kept in
160 cages of 4 individuals. The generation of the chow pellet containing pesticides is
161 identical to the one we have previously described and used in (Klement et al., 2020;
162 Lukowicz et al., 2018; Smith et al., 2020). The selected cocktail contains fungicides
163 and insecticides (ziram, thiophanate, captan, chlorpyrifos, boscalid, and thiacloprid)
164 that are relevant to agriculture, as suggested by the European Food Safety Authority
165 reports (EFSA, 2018; Klement et al., 2020; Lukowicz et al., 2018; Smith et al., 2020).
166 Pregnant mice were exposed to the tolerable daily intake (TDI; mg/kg body
167 weight/day, See Supplemental Table 1). TDI is defined for humans by the EFSA and
168 by the Joint Food and Agriculture Organization (United Nations, World Health
169 Organization). TDI levels defined for human exposure were adjusted to mice body
170 weight (BW). In our study, we considered a mouse BW of 30 grams and a daily chow
171 intake of 5 g. Importantly, pesticide and sham pellets underwent the exact same
172 protocol of preparation, except for the incorporation of pesticides. This method is
173 routinely executed at a dedicated facility (Animal Feed Preparation Unit) and it was
174 used by this research consortium. As we previously reported (Klement et al., 2020;
175 Lukowicz et al., 2018; Smith et al., 2020), the pesticides were dissolved in a 9:1
176 volume/volume methanol:acetone. The solution was dispersed on a Vitaminic powder
177 mixture-200 (Scientific Animal Food Engineering) and homogenized using a
178 Rotavapor (Laborota 4000™; BUCHI Switzerland; 45 °C) and then kept at room
179 temperature to evaporate the volatile solvents. Control diet was concomitantly

180 prepared following the exact same steps, and without adding pesticides. Traces of
181 methanol and acetone can be detected across pellet batches (e.g., < 0.05 g/Kg of
182 methanol/pellet and 0.001 to 0.003 g/kg of acetone/pellet). These amounts are well
183 below the levels associated with rodent maternal toxicity for solvents as reported by
184 the U.S. Food and Drug Administration ([http://academy.gmp-](http://academy.gmp-compliance.org/guidemgr/files/UCM073401.PDF)
185 [compliance.org/guidemgr/files/UCM073401.PDF](http://academy.gmp-compliance.org/guidemgr/files/UCM073401.PDF)), the European Chemical Agency
186 (<https://echa.europa.eu/registration-dossier/-/registered-dossier/15569/7/9/1>;
187 <https://echa.europa.eu/registration-dossier/-/registered-dossier/15460/7/6/1>) and by
188 the French Agency for Food, Environmental and Occupational Health & Safety
189 (<https://www.anses.fr/fr/system/files/ALAN2007sa0013.pdf>;
190 https://www.dod.fr/PartageWeb/PartageWeb/Fiche_Tech/309266_fds_acetone.pdf).

191 See discussion for further details. The resulting powder was incorporated into the
192 pellet (SAAJ-RAF National Research Institute for Agriculture, Food and the
193 Environment, INRAE). Pesticide levels in the pellet are re-analyzed by gas
194 chromatography–tandem mass spectrometry and liquid chromatography–tandem
195 mass spectrometry (Eurofins; Supplemental Table 1 and (Smith et al., 2020).

196

197 *Behavioural testing*

198

199 Mice were tested starting at 2 and 14 months of age. We analysed n=11
200 pesticide-diet male, n=11 standard-diet male (Figure 1). Two groups of female mice
201 were tested: n=10 pesticide diet, n=12 standard diet and results are provided in
202 Supplemental Figure 1. As we previously reported and described in (Klement et al.,
203 2020) we performed: *Open field (OF)*. Spontaneous exploratory behaviour was
204 monitored in an OF (opaque black plastic arena with 35cm width x 50cm length x

205 20cm height) for 10min. The center zone was defined as a virtual perimeter within
206 5cm from the sides of the arena. Experiments were videotaped and an observer
207 scored the time spent in the center and the number of transitions in the center zone.
208 Arena was cleaned with 70% ethanol after each mouse. *Spatial working memory,*
209 *spontaneous alternation in the Y-maze.* Each mouse was placed at the end of one
210 arm in a grey polyvinylchloride Y-maze and allowed to freely explore during a single
211 8min session. The series of arm entries, including possible returns into the same
212 arm, was recorded visually. An alternation was defined as entries into all three arms
213 on consecutive trials. The percentage of alternation was calculated as actual
214 alternations/maximum alternations x 100. Measured parameters included the
215 percentage of alternation (memory index) and total number of arm entries
216 (exploration index). All arms were cleaned with 70% ethanol between two sessions.
217 *Light/Dark Transition test.* The apparatus consists of a cage (21x42x25) divided into
218 two chambers, dark and bright, of equal size by a partition with a door. Mice were
219 allowed to move freely between the two chambers during 10min. The number of
220 entries into the bright chamber and the duration of time spent there were measured
221 by videotracking (Viewpoint, Lissieu, France). After each trial, all chambers were
222 cleaned with 70% ethanol. *Elevated-Plus-Maze (EPM).* The EPM is a 4-arm maze
223 elevated 40cm above the floor. The 4 arms intersect and form a plus sign (dimension
224 of each arm: length 30cm, width 5cm, and center region 5x5 cm²). Two of the arms
225 are closed on 3 sides by 15 cm high walls, and the other two are open. Animals were
226 placed in the centre of the apparatus and allowed to freely explore the maze for
227 10min. Score for entries and time spent in open arms were measured. All arms were
228 cleaned with 70% ethanol after each mouse entry. *Social Interaction.* The apparatus
229 was a rectangular 3-chambered polycarbonate box. The test mouse was placed into

230 the middle chamber and allowed to explore freely for 10min. The test was video
231 tracked (Viewpoint). Time spent in each chamber was analysed to measure the side
232 preference bias. Each of the 2 sides contained an inverted empty wire cup. After the
233 habituation period, the test mouse was retired, an unfamiliar mouse was introduced
234 into one of the empty wire cups. The test mouse was reintroduced and time spent
235 sniffing each wire cup was analysed for 10min. *Rotarod*. Balance and motor
236 coordination as well as motor learning were assessed using an acceleration rotarod.
237 Mice were placed on the rotating drum accelerating from 4 to 40 rpm over 5min for 3
238 trials a day and for 5 consecutive days. The trial interval was 45min for all mice.
239 Rotarod sessions were scored for latency to fall.

240

241 *Freely moving telemetry Video-electroencephalography*

242

243 A total of 16 male and 16 female C57Bl/6 mice were used at 2 and at
244 14 months (n=4/group, pesticides and control), and monitored by using Video-EEG
245 telemetry. Because of technical issues (defective implants) two male mice (1 control
246 and 1 pesticide exposed; Figure 2) and two female mice (Supplemental Figure 2)
247 were excluded from the analysis. As we previously reported (Ichkova et al., 2020;
248 Klement et al., 2020; Runtz et al., 2018; Zub et al., 2019), animals were anesthetized
249 using a intraperitoneal injection with chloral hydrate and xylazine (400 mg/kg;
250 4 mg/kg). Animals were placed on a heating pad during the procedure ensuring a
251 physiological temperature. A telemetry implant (Physiotel transmitter ETA-F10,
252 weight 1.6 g, 1.1 cm³, Data Science international DSI, St Paul, MN, USA) was placed
253 into a ventral pocket. The subcutaneous tissue covering the skull was separated and
254 the periosteum cleaned. Two screws (one bipolar electrode) are inserted in the skull

255 corresponding to the fronto-parietal neocortex. Screws were attached to the
256 transmitter wires. Dental cement was used to fix the two screws to the skull. The
257 incision was closed using a non-reabsorbable 6/0 suture material (prolene
258 polypropylene, Ethicon) and stitches. Mice were allowed to recover for 1 week. Mice
259 were recorded (Dataquest ART software DSI) for at least 24 h, equally distributed
260 between day and night. Signals were acquired at 500 Hz and analyzed using
261 Neuroscore. Video analysis was executed by an operator to rule out motion artifacts
262 (e.g., eating, drinking or chewing). Trace portions (1–5 min/extract, for a total of
263 30 min/mouse) were chosen considering light vs. dark phases, awake/exploratory vs.
264 sleep/immobility, and avoiding all motion artifacts. Periodogram Power Bands
265 (Neuroscore) was calculated for all EEG and the relative abundance of each 0.5 Hz
266 increment (0.5–80 Hz) was quantified (Boussadia et al., 2018, 2016; Ichkova et al.,
267 2020). As we routinely perform and we previously described (Ichkova et al 2020, Zub
268 et al 2019), spike or seizure analysis was executed as follow: 1) setting a threshold
269 for spike detection of 2.5 x times the standard deviation of the baseline; 2) minimum
270 and maximum spike duration (1 and 100 ms); 3) minimum train-spike duration 5s with
271 a minimum number of spikes of n= 5 (at least 1Hz). Minimum separation between
272 two seizures: 1 second. Peak detection was visually re-checked *a posteriori*.

273

274 *Zebrafish pesticides exposure protocol*

275

276 Zebrafish embryos were exposed to the same cocktail used for mice, or to
277 each of the single components. The exposition was from 1.5 hours post-fertilization
278 (hpf) to 120 hpf. The concentrations of pesticides tested across experiments were:
279 0.1, 0.5, 1, 10, 100, 1.000 and 10.000 µg/L. These concentrations were chosen

280 based on: i) the European drinking water directive (98/83/EC) and the ground water
281 directive (2006/118/EC) which set a maximum concentration of 0.1 µg/L for an
282 individual pesticide and 0.5 µg/L for total pesticides; ii) US aquatic life benchmarks
283 for registered pesticides ([https://www.epa.gov/pesticide-science-and-assessing-
284 pesticide-risks/aquatic-life-benchmarks-and-ecological-risk](https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/aquatic-life-benchmarks-and-ecological-risk)); iii) reported
285 environmental concentrations (Morrissey et al., 2015; Qian et al., 2021, 2018;
286 Thompson et al., 2020; Velisek and Stara, 2018; Vu et al., 2016) and previous
287 publications (Cao et al., 2019; Jia et al., 2020; Lulla et al., 2016; Osterauer and
288 Köhler, 2008; Qian et al., 2021; Wang et al., 2020; Zhou et al., 2019). All water
289 solutions were renovated every day. The treatments were performed in duplicate
290 using 50 larvae in each petri dish. Dimethyl sulfoxide (DMSO) was used as solvent
291 carrier for the pesticides, with a maximum concentration of 100 µL/L [0.01 % (v/v)].
292 DMSO did not induce embryotoxicity at the concentration used (*data not shown*), as
293 also reported by others (Christou et al., 2020; Hallare et al., 2006; Hong and Zha,
294 2019; Kais et al., 2013; Maes et al., 2012). All pesticides were dissolved with DMSO
295 to obtain stock solutions of 100 mg pesticide/L, and aliquots were kept at -20°C.
296 Stock solutions were diluted with E3 medium to obtain the working concentrations.
297 For the cocktail, 100 µL from stock solution of each pesticide were mixed and then
298 diluted to the targeted final concentration. The presence of single pesticide in the
299 cocktail was confirmed by the Phytocontrol Agrifood Laboratory (Nimes, France)
300 using liquid chromatography-mass spectrometry (LC-MS/MS) for boscalid,
301 thiacloprid, and thiophanate (not shown). Gas chromatography-mass spectrometry
302 (GC-MS/HS) was used for captan, chlorpyrifos, and ziram (not shown).

303 *Zebrafish locomotor activity*

304

305 Locomotor activity was analyzed as we previously described (Forner-Piquer et
306 al., 2020) using 120 hpf zebrafish embryos, exposed to cocktail or single pesticide.
307 We examined: i) distance traveled; ii) mean velocity; iii) maximum velocity; iv) body
308 mobility; v) rotation clockwise; vi) rotation counterclockwise; vii) heading center-point;
309 viii) angular velocity; ix) meander. Data are expressed as fold change: [(replicate /
310 mean CTR group) * 100].

311

312 *Morphological assessments, zebrafish phalloidin staining of actin fibers, in vivo 2-*
313 *photon microscopy brain analysis.*

314

315 Morphological parameters were measured at 120 hpf for zebrafish exposed to
316 0.1, 0.5, 1 and 10 µg/L of cocktail pesticide (duplicate, n=10/group) as we previously
317 described (Forner-Piquer et al., 2020). Mortality rate at 24 hpf and cumulative
318 hatching at 96 and 120 hpf were also evaluated (duplicate, n = 200/group). In
319 zebrafish, 120 hpf larvae from control and 10 µg/L cocktail groups were stained using
320 Phalloidin-iFluor 594 (muscle fibers; Abcam ab176757) in duplicate, n=10/group; see
321 (Forner-Piquer et al., 2020). For *in vivo* 2-photon brain analyses, 96-hpf
322 *Tg(fli1a:GFP)y1Tg* (duplicate, CTR n=15, 10 µg/L n=10) and *Tg(HuC:Tomato)*
323 (duplicate, CTR n=10, 10 µg/L n=5) transgenic zebrafish larvae were studied as we
324 previously described (Forner-Piquer et al., 2020).

325

326

327

328 *Mouse brain histology.*

329

330 We tested the presence of structural-to-cell modifications in the mouse brain.
331 To this end we used available tissues from i) n=8 pesticide diet; n=6 standard diet
332 male mice at 2 months; ii) n=8 pesticide diet, n=7 standard diet male mice at 14
333 months. After intracardial perfusion with PBS, brains were dissected and fixed in PFA
334 4% solution. Fixed brains were immersed in sucrose 15% for 24h followed by
335 sucrose 30%. Brains were then snap frozen and stored at -80°C. Slices (20 µm) were
336 obtained using a cryostat. Immunohistochemistry was performed after PBS washes.
337 Slices were added with blocking solution (PBS, triton 0.5%, horse serum 20%) at
338 room temperature for 1h. Primary antibodies (anti-GFAP chicken Abcam Ab4674
339 1/300 or anti-CD13 rat Abcam ab33489 1/100) were diluted in blocking solution and
340 slices incubated overnight at 4°C. After PBS washes, Jackson ImmunoResearch
341 secondary antibody [Donkey anti-chicken Alexa Fluor Cy3 (703-165-155) or Donkey
342 anti-rat Cy3 (712-165-153)] was added in PBS for 2h at room temperature (dilution
343 1/500). After PBS washes, slices were mounted using Vectashield containing DAPI.
344 For all quantifications, 20X Z-stack images (Z=12 to 15 planes, each of 1µm) were
345 analysed using Fiji. Two slices were examined for each mouse to quantify signals in
346 constant regions of interest (ROI) CA1, CA3, DG, white matter (WT) and CTX, as
347 identified by DAPI maps (Table 2). Prior to analysis, all Z-stacks images were
348 combined (Z-project, sum). GFAP quantification: images were converted to RGB
349 stack format. Signal threshold was adjusted to 200 units for each image. Area of
350 GFAP signal was calculated setting threshold sensitivity equal for each image. GFAP
351 data are expressed as a percentage of ROI total pixels. CD13 vessel tracing
352 quantification: a skeleton plug-in was used. The analysis is automated setting
353 threshold sensitivity identical for each image. Length is calculated as pixel. Finally,
354 DAPI cartographies of the parietal cortex and underlying dorsal hippocampi were

355 obtained by a montage of 10X images. Cortical thickness (1st cortical layer to white
356 matter; μm) and hippocampal size (distance between CA1 pyramidal neurons and
357 dentate gyrus granular cells) were quantified using Fiji (line tool) performing multiple
358 (3 to 4) measurements for each mouse.

359

360 *Statistics*

361

362 All data were analyzed using Prism 8.3.1. When data fulfilled the criteria for
363 applying a parametric test, one-way ANOVA was used followed by Dunnett's multiple
364 comparisons test, otherwise, Kruskal-Wallis (non-parametric) followed by Dunn's
365 multiple comparisons test was used (e.g., body morphology, mortality, locomotor
366 test). For hatching analysis, two-way ANOVA followed by Sidak's multiple
367 comparison test was applied. When two experimental groups were examined (e.g.,
368 mouse behavior and EEG read-outs) an unpaired t-test (parametric with Welsh
369 correction) or a Mann-Whitney test (nonparametric) were used. Histological mouse
370 data were analysed using nonparametric test (Table 2). Significance threshold was
371 set at $p < 0.05$. Superscript asterisks (*) evidenced statistical differences among
372 groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Results are reported as
373 box and whiskers plot with single points. All mouse experiments were blinded as
374 three researchers were implicated (mouse housing, neurological explorations,
375 histology). Zebrafish investigations were performed by one researcher.

376

377

378 **Results**

379

380 *In mice, maternal exposure to a pesticide cocktail associates with long-term*
381 *behavioral adaptations and brain electrographic slowing.*

382

383 We specifically examined whether mice maternally exposed to a pesticide
384 cocktail, at TDI levels (Supplemental Table 1), showed behavioral changes during
385 adulthood or aging. While no significant modifications were observed in young adult
386 mice (2-3 months), exposure to pesticides triggered long-term (14 months)
387 modifications in male mice. We report an increase in spontaneous locomotor activity
388 (Figure 1A, B, D) as compared to control. At this time point, pesticides-exposed mice
389 also showed increased anxiety-like behavior when tested in the elevated plus maze
390 (EPM), with a reduction in time spent and number of entries in the open arms (Figure
391 1E, F). This phenotype was further confirmed when mice were tested in a light/dark
392 box, a behavioral assay that relies on the innate aversion of rodents to explore
393 unfamiliar areas. As shown in Figure 1G-I, male mice exposed to the pesticide
394 cocktail spent slightly less time in the light zone (Figure 1G) and showed reduced
395 time per entry (Figure 1H), despite a similar number of entries (Figure 1I). These
396 results indicate that maternal exposure to pesticide cocktail elicits a long-term
397 anxiety-like behavior. No significant modifications were observed when testing female
398 mice (Supplemental Figure 1).

399

400 Next, we asked whether such exposure to pesticides modifies memory-related
401 functions. To this end, we performed the Y-Maze (working memory) and the rotarod
402 (procedural and motor learning and memory) tests. We observed no significant
403 impairments in working memory and procedural learning, as all groups showed a
404 similar alternation index in the Y-Maze (Figure 1J, K) and similar motor learning and

405 memory (Day1 vs Day5, Figure 1L). These results indicate that the anxiety-like
406 behavior observed in male mice exposed perinatally to pesticides does not
407 generalize into memory deficits. No significant modifications were found when
408 analyzing female mice (Supplemental Figure 1).

409

410 To extend this study, we performed *in vivo* electrocorticography explorations
411 (Figure 2 and Supplemental Figure 2). Spike activity or epileptiform modifications
412 were not identified under these experimental conditions (Figure 2A1-B1). We next
413 quantified theta and gamma EEG waves during awake/exploratory and
414 sleep/immobility phases, as electrographic biomarkers of neurological dysfunction
415 (Ishii et al., 2018; Milikovsky et al., 2019). Figure 2C-C1 shows examples of entire
416 EEG frequency spectrograms (0.5-80 Hz) calculated for individual mice. A significant
417 decrease in the percentage of gamma waves (40-80 Hz) was measured at 14 months
418 during awake periods in male mice perinatally exposed to the pesticides cocktail
419 (Figure 2D2). A typical decrease of gamma waves was recorded during
420 sleep/immobility periods (Figure 2D1-D3) with no significant difference between any
421 of the experimental groups. Within the theta wave range, the percentage of specific
422 frequencies (6-9 Hz) was increased during awake periods at 2 and 14 months in
423 pesticide exposed male mice (Figure 2E-E3). Explorations performed in female mice
424 did not identify significant EEG patterns or robust signatures when comparing control
425 and pesticide exposed groups (Supplemental Figure 2).

426

427 Finally, we asked whether structural brain malformations could have
428 influenced the aforementioned outcomes. No significant changes (DAPI maps, see
429 Methods) were observed between pesticide exposed animals and their sex, age-

430 matched controls (control female hippo 561.8 ± 184.5 ; control male hippo $559.9 \pm$
431 266.5 ; pesticide female hippo 510.2 ± 140 ; pesticide male hippo 570 ± 197.3 ; control
432 female cortex 1173 ± 169.9 ; control male cortex 1439 ± 231.8 ; pesticide female
433 cortex 1192 ± 177.5 ; pesticide male cortex $1090 \pm 193.9 \mu\text{m}$; mean \pm SD, $P > 0.05$,
434 Kruskal-Wallis). We further examined the regional distribution of GFAP glial cells and
435 CD13 pericyte-capillary length in dorsal hippocampal sub-regions (CA1, CA3 and
436 DG), somatosensory cortex, and white matter. We report no significant differences
437 (Table 2 and Supplemental Figure 3). Taken together, these results indicate that a
438 dietary perinatal exposure to a low-dose pesticide cocktail is associated with
439 behavioral and neurophysiological modifications in aging male mice and in the
440 absence of major structural brain malformations.

441

442 *In zebrafish larvae, pesticide cocktail triggers dose-dependent locomotor behavioral*
443 *modifications.*

444

445 We used a zebrafish model to examine the effects elicited by ranging pesticide
446 cocktail concentrations (0.1 to 10.000 $\mu\text{g/L}$, see Methods) during development. We
447 initially screened mortality, hatching rate, and morphological outcomes (Figure 3). No
448 significant modifications were observed at cocktail concentrations from 0.1 to 10 $\mu\text{g/L}$
449 (Figure 3A). Concentrations $>10 \mu\text{g/L}$ induced gross morphological malformations in
450 the notochord and delayed development (Figure 3B). Furthermore, mortality was
451 significantly increased from 10 $\mu\text{g/L}$ and the hatching rate was diminished.

452 Next, assessment of locomotor behavior at 120 hpf showed an increase in the
453 distance travelled, average velocities (mean and maximum), body mobility, and
454 number of rotations at 10 $\mu\text{g/L}$ pesticide cocktail (Figure 4). Interestingly, a decrease

455 of specific motor parameters occurred at 0.5 $\mu\text{g/L}$ (Figure 4B, C and E). To examine
456 whether the behavioral modifications, observed at 10 $\mu\text{g/L}$, could have been
457 associated with structural brain malformations we reconstructed the neuronal and
458 cerebrovascular architectures. *Tg(fli1a:GFP)yTg* transgenic zebrafish larvae,
459 expressing green fluorescent protein (GFP) in cerebral vessels, were analyzed by
460 using *in vivo* 2-photon microscopy. Figure 5A and 5A1 shows examples of z-stack
461 images. 3D skeleton analysis (Figure 5B and 5B1) of the cerebrovascular tree
462 indicates that 10 $\mu\text{g/L}$ of cocktail does not modify midbrain total vascular length
463 (Figure 5D). By using a zebrafish reporter line *Tg(HuC:Tomato)*, we examined
464 whether 10 $\mu\text{g/L}$ cocktail affected brain neuronal structures (Figure 5C and 5C1). No
465 significant changes were detected in optic nerve length (Figure 5E), optic nerve
466 thickness (Figure 5F), and length of the 1st hindbrain axon projection (Figure 5G). As
467 a corollary, we ruled out the possible contribution of muscular modifications to the
468 observed locomotor changes by staining F-actin fibers with phalloidin (Supplemental
469 Figure 4). We report no difference in muscle fiber length (CTR: 80 ± 2.7 ; 10 $\mu\text{g/L}$: 81
470 ± 6.1 ; t-test, $p = 0.6429$). These data indicate that, in zebrafish larvae, developmental
471 exposure to the pesticide cocktail modifies locomotor activities in a dose-dependent
472 manner.

473

474

475

476 *Screening the effects of single pesticide(s) on zebrafish larval locomotor behavior.*

477

478 The use of zebrafish model allowed us to systematically screen the effect
479 elicited by each single pesticide contained in the cocktail, testing a broad range of
480 concentrations (Table 3). Ziram provoked mortality impeding behavioral testing at
481 concentrations >10 µg/L. Distance, mean velocity and body mobility were all
482 significantly impacted starting from 0.5 µg/L (Table 3F). Thiophanate and thiacloprid
483 were not lethal and locomotor modifications occurred following exposure to 10, 100
484 and 10.000 µg/L (Table 3D and 3E). Boscalid and chlorpyrifos were lethal at the
485 highest concentration tested (Table 3A, 3C; 10.000 ug/L). In our experimental
486 conditions, chlorpyrifos significantly modified all motor-behavior parameters at a low
487 concentration of 0.1 µg/L. Boscalid decreased selected parameters such as distance,
488 velocity and body mobility at 0.5 µg/L. Captan (Table 3B) induced mortality at 1000
489 and 10000 µg/L, while a reduction in distance, velocities and body mobility occurred
490 at 10 µg/L. Collectively, these results outline the varying and dose-dependent impact
491 of each pesticide, unveiling an effect of chlorpyrifos at a low concentration.

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496

497 **Discussion**

498

499 While there is no direct equivalence between the mouse and the zebrafish
500 larvae models and protocols, this particular integrative experimental design may help
501 understanding the varying brain effects associated with the exposure to specific
502 pesticides, as a cocktail or single, within dietary and environmentally relevant
503 settings. Introduction of a pesticide cocktail, at TDI levels from pre-mating to
504 weaning, associates with sex-dependent behavioral and *in vivo* electrographic
505 modifications unfolding long-term in mice. Furthermore, the zebrafish larval model
506 allowed us to systematically screen low-to-high pesticide concentrations, as a
507 cocktail or alone, and subsequently determine that levels generally higher than
508 current environmental EU limits (European Directives 98/83/EC and 2006/118/EC)
509 and during development can provoke significant behavioral modifications.

510

511 *Perinatal exposure to dietary pesticide cocktail: a risk factor for neurological*
512 *sequelae?*

513

514 Recent experimental studies have traced a link between maternal, perinatal or
515 developmental exposure to food contaminants and cognitive deficits during adulthood
516 (de Souza et al., 2019; Gallegos et al., 2016; Venerosi et al., 2015). Furthermore,
517 epidemiological studies have indicated that maternal or postnatal exposure to
518 pesticides (i.e chlorpyrifos, carbamate) can be associated with neurodevelopmental
519 alterations, attention deficit hyperactivity disorder and motor or social defects
520 (Fortenberry et al., 2014; Guo et al., 2019; Zhang et al., 2020). By using a mouse
521 model, we report sex- and age-dependent neurological modifications, thus supporting

522 a long-term trajectory. Male mice were more susceptible to this selected pesticide
523 cocktail as compared to female, in accordance to previously reported behavioral
524 modifications resulting from the prolonged dietary exposure to this cocktail (Klement
525 et al., 2020). Here, we report significant *in vivo* electrocorticographic changes,
526 specifically the decrease in fast gamma waves and the increase in slow theta waves
527 during aging, at 14 months, a finding that is coherent with the emerging notion of an
528 electrophysiological signature characterized by the slowing of the cortical neuronal
529 network in experimental cognitive dysfunctions (Milikovsky et al., 2019). Our results
530 are consistent with clinical monitoring of gamma waves as a potential biomarker for
531 neurological pathophysiology (Iaccarino et al., 2016; Kitchigina, 2018; Mably and
532 Colgin, 2018), while enhanced slow theta waves have been proposed to associate
533 with an abnormal, or accelerated, aging (Ishii et al., 2018). In our study, female mice
534 did not present with robust behavioral and electrographic changes, supporting the
535 hypothesis of a dimorphic impact of pesticides on neuronal functions. We
536 acknowledge that, although all physiological explorations were performed at
537 consistent time schedules, we did not account for the estrous cycle in female mice, a
538 potential pitfall as estradiol levels can impact behavioral outcomes (Azcoitia et al.,
539 2010).

540

541 Here, the pesticide cocktail was used at tolerable daily intake (TDI) levels in an
542 attempt to model a consumer relevant exposure. This is a significant departure from
543 previous work that has mainly focused on the effects elicited by supra-threshold and
544 high concentrations of single pesticides. For instance, mice perinatally exposed to
545 elevated concentrations of malathion developed anxiety-like traits and presented with
546 impairment of social interactions (Ouardi et al., 2019). Perinatal exposure to the

547 insecticide permethrin was associated with an incomplete adaptation to a novel
548 environment, as well as an impairment in learning and memory in adult males (Saito
549 et al., 2019). Exposure to high concentrations of glyphosate during gestation and
550 lactation, delayed innate reflexes and caused a deficit in motor development (Ait-Bali
551 et al., 2020), as well as autism spectrum disorder-like behaviors (Pu et al., 2020).
552 Male pups exposed to high concentration of chlorpyrifos displayed an enhanced
553 investigative response to unfamiliar social stimuli, whereas females showed a lack of
554 reaction to social novelty (Venerosi et al., 2015). Another study indicated that
555 gestational exposure to chlorpyrifos induces social deficits in adult mice in a sex-
556 dependent manner (Lan et al., 2019).

557

558 It is worth to mention that our experimental design does not allow to isolate a
559 single and specific mechanism responsible for the observed sex-specific and long-
560 term neurological changes. Importantly, this research consortium reported sex-
561 dependent perturbations of the urinary and fecal metabolic fingerprints in response to
562 this protocol of perinatal pesticides exposure (Smith et al., 2020). Together with the
563 data here presented, this evidence supports the hypothesis of a gut-brain extra-
564 physiological mechanism in this experimental settings (Cryan et al., 2019).
565 Furthermore, the reported neuronal defects support the notion of unfavorable
566 neurophysiological trajectories in conditions where an early insult was delivered. A
567 concept of initial priming, here applied to pesticide exposure, was previously
568 postulated for other pathological conditions and insults, such as brain or peripheral
569 infections, or early trauma, where a progressive brain pathology unfolds over time
570 (Estes and McAllister, 2016; Ichkova et al., 2020; Patterson, 2011). Although our
571 results indicate preserved brain structures and cell distributions, the possibility of

572 specific molecular activations or sub-cellular inflammatory processes remain to be
573 examined (Giannoni et al., 2020; Marchi et al., 2011; Oliviero et al., 2020). In
574 summary, our data suggest that a perinatal exposure to a pesticide cocktail at low,
575 TDI, levels is sufficient to elicit long-term neurophysiological changes or adaptations,
576 at least in male rodents. Clinical and epidemiological studies are required to
577 understand the significance and applicability of these findings to humans.

578

579 *Screening the effects of environmental pesticide(s): clues from a zebrafish larval*
580 *model.*

581

582 Zebrafish larvae display a rich behavior and a high sensitivity to environmental
583 pollutants, making this aquatic vertebrate a pertinent model for neurotoxicology (Cao
584 et al., 2019; Hong and Zha, 2019; Stewart et al., 2014). Here, we outline significant
585 motor-behavioral defects elicited by the pesticide cocktail at a concentration (10 µg/L)
586 greater than the one allowed in water (European Directive 98/83/EC). However,
587 discrete motor modifications also occurred at a specific concentration of 0.5 µg/L.
588 When testing single pesticides, we found that chlorpyrifos significantly modified all
589 motor-behavior parameters at the environmental concentration of 0.1 µg/L.
590 Intriguingly, chlorpyrifos induced effects were significant at low and high dosages, an
591 outcome that requires further examinations, also in light of non-monotonic responses
592 previously reported for other environmental contaminants (Vandenberg, 2015;
593 Vandenberg et al., 2012). Further evidence for an adverse effect of chlorpyrifos
594 arises from rodent studies, showing sex-dependent neuroendocrine responses and a
595 sex-dimorphic social behavior in response to *in utero* and neonatal exposure.
596 Importantly, selected pesticides may act as neuro-endocrine disrupting chemicals,

597 interfering with hormones homeostasis, hypothalamic neuropeptides, and brain
598 maturation (Venerosi et al, 2012). Furthermore, pesticides are frequently applied as
599 combinations (Covert et al., 2020) and the impact of a mixture is rather unpredictable
600 because combined chemicals can generate additive, independent, synergistic, or
601 antagonist effects (Rizzati et al., 2016). Our results are in agreement with the latter
602 concept, as the pesticide cocktail induced dissimilar patterns of locomotor response
603 as compared to the single components (Pérez et al., 2013; Wang et al., 2018, 2017).

604

605 Emerging evidence has linked pesticide cocktail exposure to
606 neurodevelopmental disorders (Liu and Schelar, 2012; Roberts et al., 2019).
607 Zebrafish exposed to 50 nM ziram (15 µg/L) and high concentrations of boscalid (0.3,
608 0.6 and 1.2 mg/L) displayed a decrease in locomotor activity (Lulla et al., 2016; Qian
609 et al., 2018). In accordance with our results, previous studies indicated that
610 chlorpyrifos, or other organophosphate compounds (i.e. diisopropylflorophosphate),
611 reduced swimming activity (Cao et al., 2018; Richendrfer and Creton, 2015; Tilton et
612 al., 2011; Brenet et al, 2020). To our knowledge, the data presented in Table 3
613 provide previously unavailable indications of locomotor defects triggered by varying
614 concentrations of captan, thiophanate and thiacloprid. We also report that this
615 specific pesticide cocktail (Figure 3), or ziram alone (data not shown), provoked gross
616 body malformations at a concentration > 10 µg/L. Previous studies indicated that
617 ziram is highly toxic for zebrafish larvae, inducing mortality in a dose-dependent
618 manner, diminishing hatching rate and causing deformities at 100 and 1000 nM (0.30
619 mg/L) (Cao et al., 2019). Here, captan elicited mortality at ≥ 100 µg/L, in accordance
620 to recent studies showing a reduced hatching rate and increased developmental
621 deformities at 0.58 mg and 0.66 mg captan/L, respectively (Zhou et al., 2019).

622 *Conclusions.*

623

624 While our study outlines variations in neurophysiological outcomes in
625 response to early-stages pesticides exposure in two experimental models, the
626 cellular and molecular underpinnings of the reported changes remain to be
627 deciphered. As previously mentioned, hypotheses include the role of the gut-brain
628 axis (Smith et al., 2020) along with an inflammatory track in response to pesticides,
629 within dietary or environmentally relevant experimental conditions. Existing evidence
630 points to inflammation unfolding during, or in response to, the exposure to
631 contaminants (Banks and Lein, 2012; Cao et al., 2019; Gómez-Giménez et al., 2017;
632 Hossain et al., 2017; Zhang et al., 2015). The latter notion is significant considering
633 the pathological impact that inflammation exerts on brain neuronal transmission from
634 developmental into adulthood and aging (Dallérac and Rouach, 2016; Pekny et al.,
635 2016; Suzuki et al., 2011).

636

637 We here underscore that our study presents a number of limitations and, by
638 design, experimental dissonances. First, mice and zebrafish have highly dissimilar
639 timing and modalities of development. If embryonic mice develop in their mothers'
640 womb and are thus exposed to the pesticide via their mothers' dietary intake,
641 zebrafish fertilization and embryonic development occur externally. Therefore, it is
642 necessary to directly treat the developing zebrafish embryos to determine whether
643 the pesticide cocktail affects this process. In zebrafish, the exposure to pesticides is
644 direct from the water and the chorion is the only protection afforded to the larvae. The
645 extent of maternal biotransformation of pesticides and the protection granted by the

646 placental barrier in rodents during pregnancy remains to be examined. As a result,
647 dosages and routes of exposure between mouse and zebrafish are not comparable.

648 The possibility exists that, in our experimental design, gametogenesis could
649 have been already impacted even if the presence of low, TDI, levels of pesticides.
650 The latter represent an unexplored field of investigations. Importantly, available
651 literature indicates spermatogenesis/oogenesis damage resulting from the exposure
652 to pesticides, although at concentrations higher than the ones used here. For
653 instance, oral exposure to ziram (1 – 8 mg / kg/ day) disrupts fetal Leydig cell
654 development and inhibits several steroidogenic enzymes, negatively affecting male
655 reproduction (Guo et al., 2017; Liu et al., 2018). The neonicotinoid thiacloprid (22.5
656 mg / kg / day) impairs sperm quality, induces abnormal spermatozoa morphology and
657 irregular seminiferous tubules (Kammoun et al., 2017). When chlorpyrifos is
658 administered by gavage (2.7 – 37 mg / kg/ day), spermatogenesis is arrested, the
659 number of Leydig cells decreases as well as the sperm quality. Furthermore, the
660 levels of sex steroids are disrupted and morphological alterations in seminiferous
661 tubules are reported (Babazadeh and Najafi, 2017; Farag et al., 2010; Joshi et al.,
662 2007; Sai et al., 2014). When female albino rats are exposed to chlorpyrifos (0.1 and
663 2.5 mg/kg/day), several alterations were found such as an unbalanced estrous cycle,
664 morphological modifications in the ovarian and uterine surface epithelium, and
665 follicular atresia (Nishi and Hundal, 2013).

666

667 Whether the defective locomotor behavior observed in zebrafish exposed to
668 the pesticide cocktail persists into adulthood should be examined. Whether there is a
669 combinatory effect between 2 or 3 specific molecules within the pesticide cocktail
670 remains to be determined and this exceeds the capacity of our current research

671 effort. Finally, although present at low trace amounts in both diets (see Methods), the
672 possibility exists that remaining solvents may introduce a confounding factor if, yet
673 unidentified, synergistic effects between such low levels of methanol, acetone, and
674 TDI pesticides occur.

675

676 In conclusion, we provide the indication that varying protocols of perinatal
677 exposure to a pesticide cocktail induces model dependent, neurological adaptations
678 in the absence of structural brain modifications. The mechanistic and molecular
679 underpinnings of such neurophysiological manifestations remain to be elucidated.

680

681

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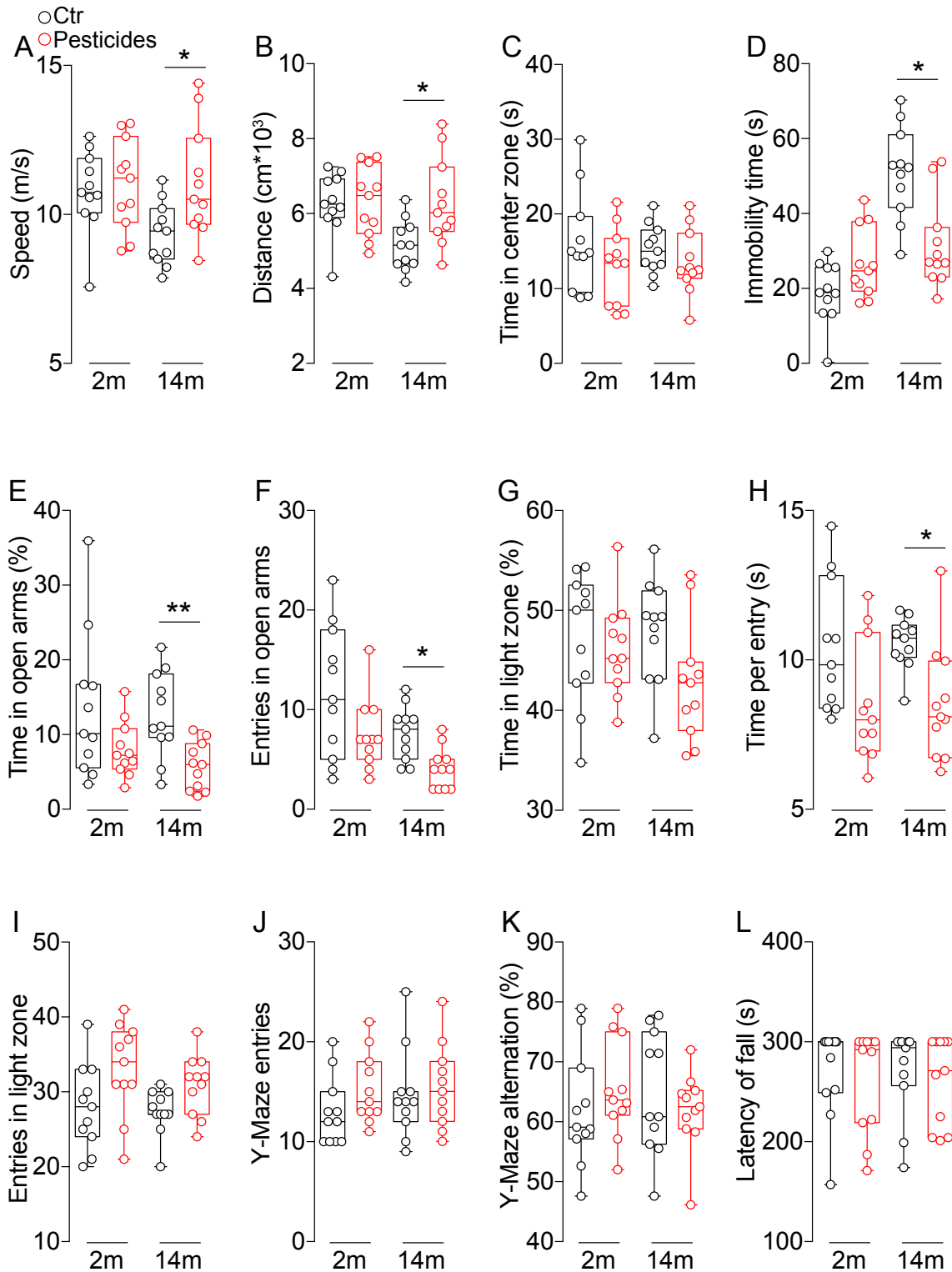
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997 **Figure 1: Perinatal exposure to dietary pesticides elicits an anxiety-like**

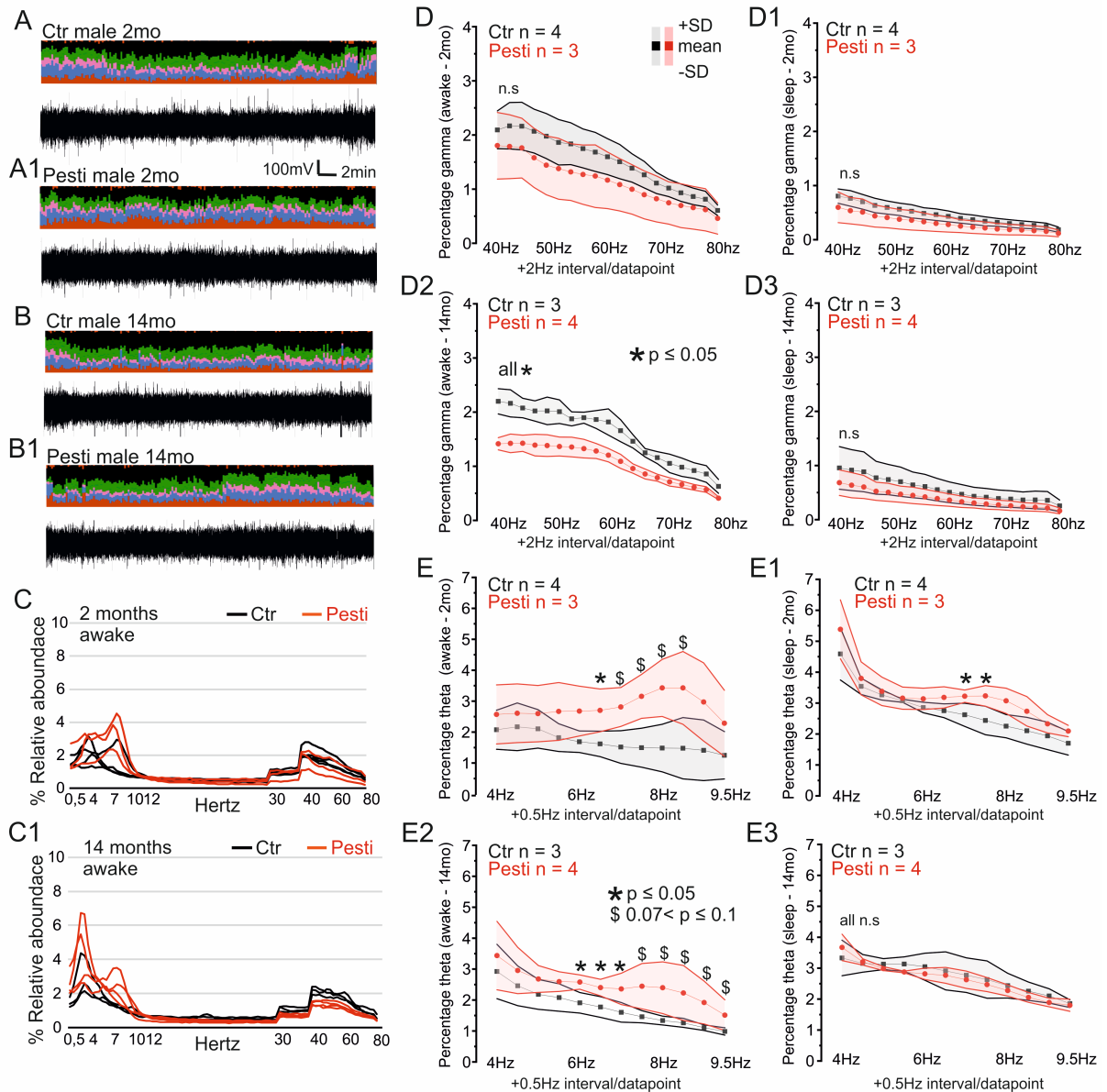
998 **phenotype during aging in male mice.** The panels indicate the behavioral

999 parameters examined in males. Supplemental Figure 1 provides results obtained

1000 using female mice. *Open field test and locomotor activity:* (A) speed, (B) distance

1001 travelled, (C) time spent in the center zone and (D) immobility time in the open field

1002 test. *Elevated-plus maze* (EPM): **(E)** Time spent and **(F)** number of entries in the
1003 open arms during the EPM test. *Light/Dark box*: **(G)** time spent in the light zone, **(H)**
1004 time per entry and **(I)** number of entries in the light zone. *Y-Maze test (working*
1005 *memory)*: **(J)** number of entries and **(K)** percentage of spontaneous alternation in the
1006 Y-Maze. Rotarod (procedural and motor learning): **(L)** Latency of fall (s) following a
1007 training period of 5 days. 11 male mice were used for each experimental group. Data
1008 are analyzed using the non-parametric Kruskal-Wallis ANOVA: **(A)** $H_3=9.753$,
1009 $p=0.0208$, **(B)** $H_3=11.34$, $p=0.010$, **(C)** $H_3=2.512$, $p=0.4730$, **(D)** $H_3=23.74$, $p<0.0001$,
1010 **(E)** $H_3=10.55$, $p=0.0144$, **(F)** $H_3=13.07$, $p=0.0045$, **(G)** $H_3=4.793$, $p=0.1876$, **(H)**
1011 $H_3=11.45$, $p=0.00995$, **(I)** $H_3=9.087$, $p=0.0282$, **(J)** $H_3=3.481$, $p=0.3232$, **(K)**
1012 $H_3=1.363$, $p=0.7142$, **(L)**
1013 $H_3=0.3088$, $p=0.9584$. The Dunn's test was used for multiple comparisons between
1014 groups (Ctr 2m vs Pesticides 2m; Ctr 14m vs Pesticides 14m): * $p < 0.05$ and ** $p <$
1015 0.01 .



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Figure 2. Time-dependent electroencephalographic modifications in freely moving male mice prenatally exposed to the pesticide cocktail. A-B1) Examples of EEG traces obtained at 2 and 14 months and relative color-coded wave distributions. Spike or seizure activity was not observed. C-C1) Examples of whole frequency spectrograms (0.5–80 Hz; black line: control; red line: prenatal pesticides). One line corresponds to one mouse. D-D1) Percentage gamma waves (40-80Hz) recorded during awake/exploratory and sleep/immobility phases in control and prenatal pesticides exposed mice at 2 and (D2-D3) at 14 months. E-E1) Percentage theta waves (4-9.5Hz) recorded during awake/exploratory and sleep/immobility phases in control and prenatal pesticides exposed mice at 2 and (E2-E3) at 14 months. Supplemental Figure 2 provides results obtained using female mice. Data

1028 were analyzed using non-parametric Mann-Whitney or parametric t-test with Welch
1029 correction, * $p < 0.05$; § $0.07 < p \leq 0.1$.

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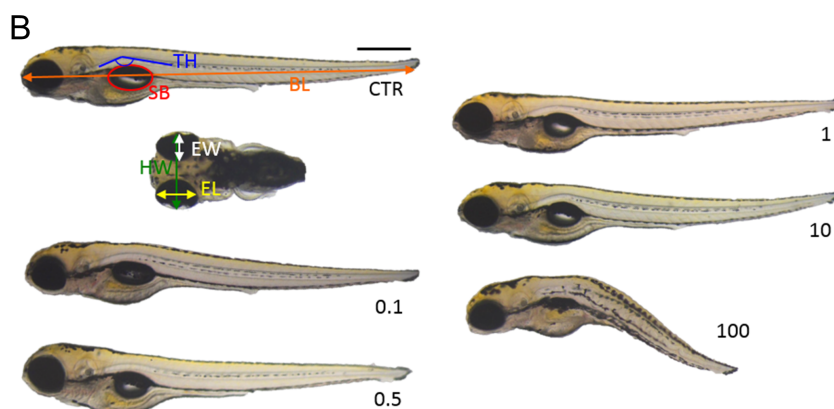
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COCKTAIL		CTR	0.1 µg/L	0.5 µg/L	1 µg/L	10 µg/L
Body length (µm)		3732 ± 148.7	3600 ± 186.6	3678 ± 75.60	3670 ± 144.6	3620 ± 109.9
Swim bladder area (µm ²)		63901 ± 6764	66396 ± 6308	58370 ± 18138	60818 ± 13739	61140 ± 3020
Head width (µm)		602.3 ± 16.89	615.1 ± 23.42	608.4 ± 33.30	612.8 ± 28.70	597.7 ± 42.36
Eye width (µm)		235.5 ± 6.81	235.7 ± 5.65	238.4 ± 22.72	239.0±8.58	231.3 ± 11.27
Eye length (µm)		334.2 ± 4.86	335.6 ± 6.15	325.1 ± 31.13	329.6 ± 12.05	325.0 ± 11.98
Trunk-head angle (deg)		155.2 ± 3.69	155.4 ± 2.66	156.5 ± 2.78	153.9 ± 5.93	151.4 ± 2.94
Mortality (%)		15.57 ± 2.52	18.60 ± 6.49	13.70 ± 11.80	20.08 ± 8.77	31.29 ± 5.00*
Hatching rate (%)	96 hpf	84.09 ± 17.15	70.99 ± 16.47	93.40 ± 9.43	90.68 ± 12.88	64.53 ± 15.51 *
	120 hpf	99.67 ± 0.87	92.85 ± 3.23	95.90 ± 4.96	95.48 ± 5.28	78.63 ± 6.70 **



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1048 **Figure 3. Morphological parameters, hatching rate, and mortality of zebrafish**1049 **larvae exposed to a dose range pesticide cocktail. A) Morphological parameters:**1050 standard body length (BL, µm, F=1.292, p=0.2522), swim bladder (SB, µm²,1051 H₄=3.641, p=0.4567), head width (HW, µm, F=0.5741, p=0.6828), eye width (EW,1052 µm, H₄=3.369, p=0.4981), eye length (EL, µm, H₄=4.116, p=0.3905) and trunk-head

1053 angle (TH, degrees, F=2.548, p=0.0536) of zebrafish larvae at 120 hpf, experiments

1054 conducted in duplicate (n=10/group; 1-way ANOVA followed by Dunnett's multiple

1055 comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test, p < 0.05).

1056 Mortality at 24 hpf, experiments conducted in duplicate (n=200/group; 1-way ANOVA,

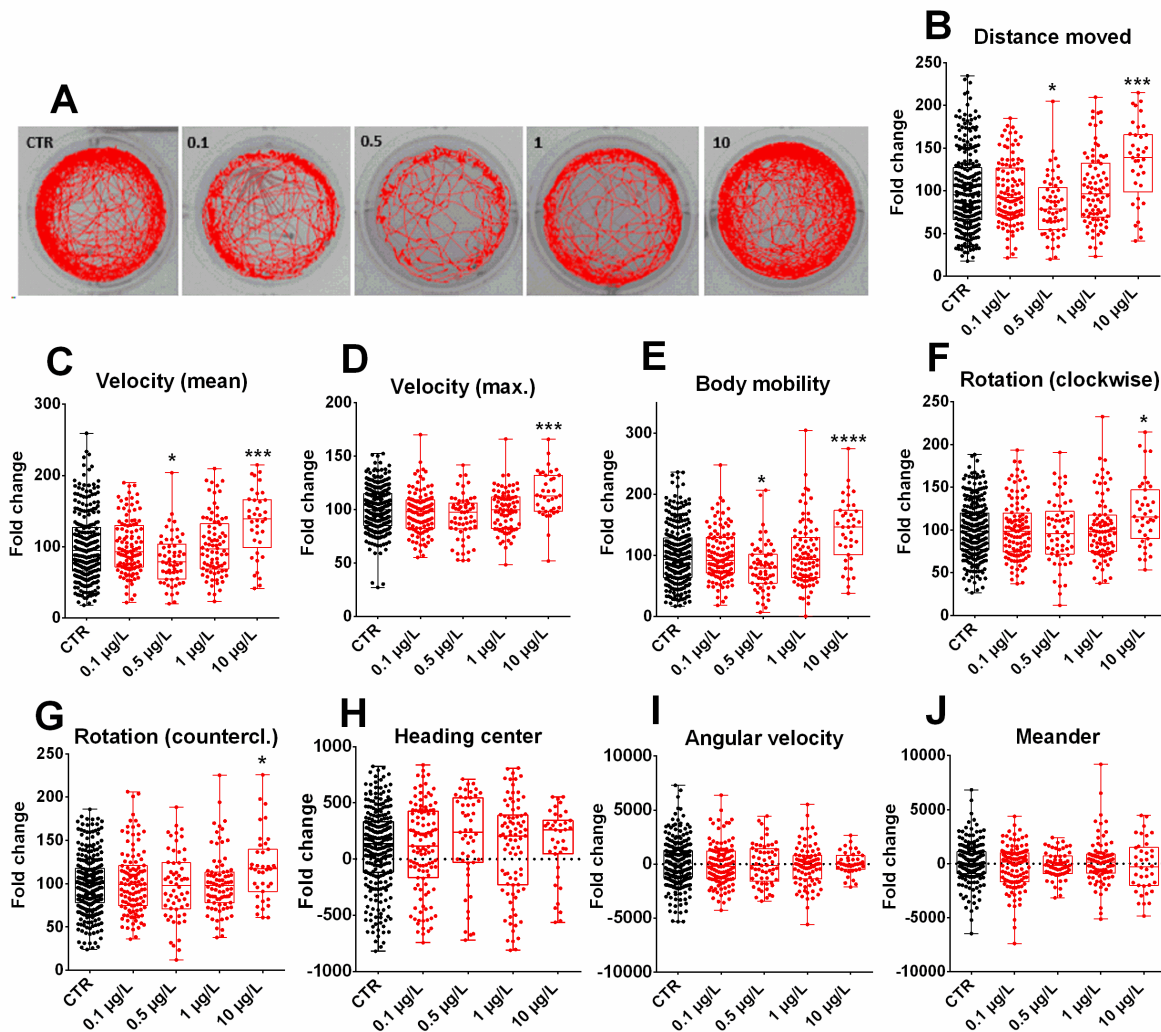
1057 Dunnett's multiple comparisons test, F=3.094 p=0.0337). Cumulative hatching rate

1058 consecutively assessed at 72 and 96 hpf and expressed as (hatched eggs / total) *

1059 100, experiments conducted in duplicate (n = 200/group; 2-way ANOVA, Sidak's

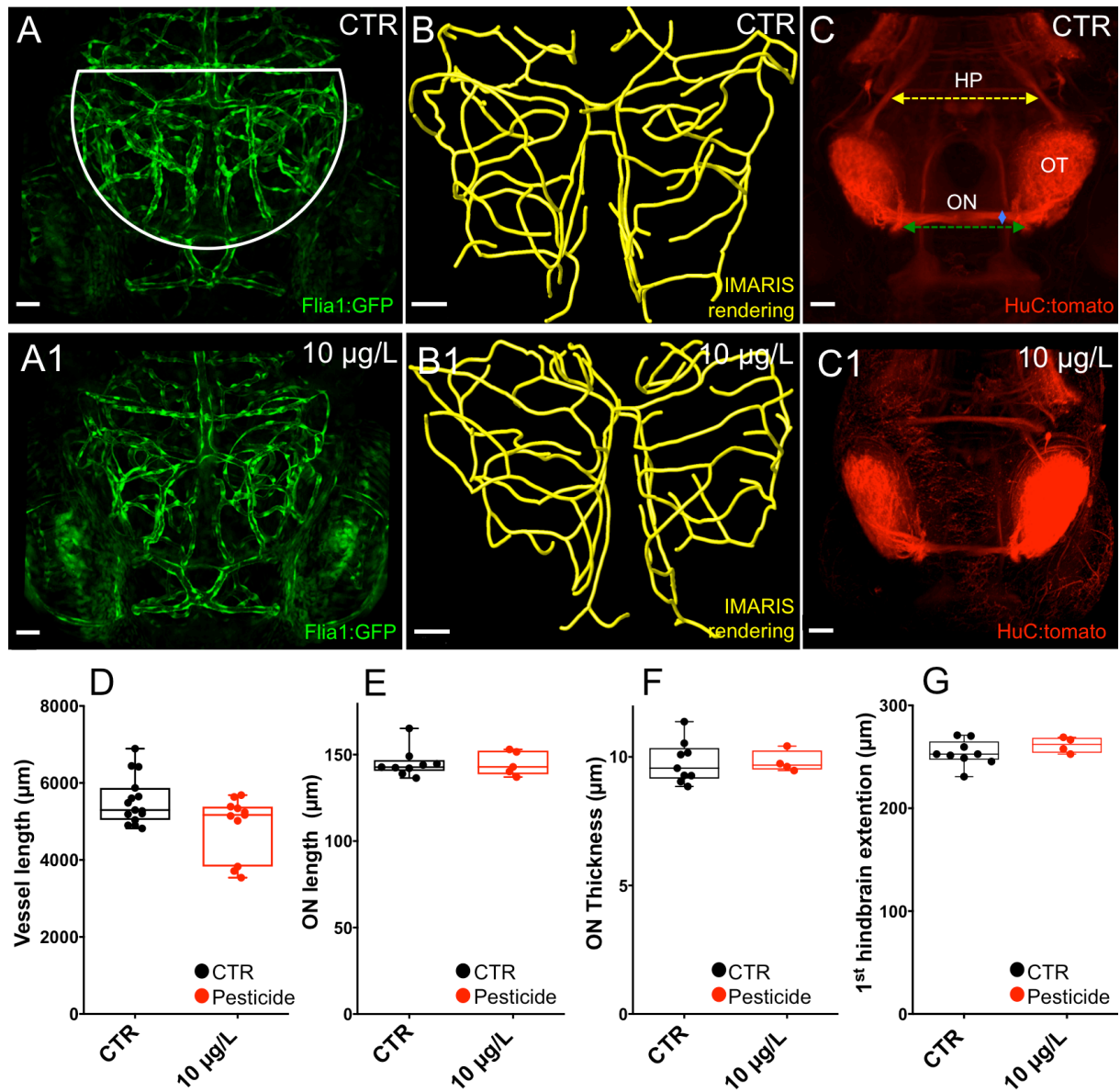
1060 multiple comparisons test, p< 0.05). Data reported as means ± SD. **B) Examples for**

1061 morphological assessments. Scale bar: 500 µm.



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Figure 4. Motor-behavioral modifications in zebrafish larvae exposed to a dose range pesticide cocktail. **A)** Examples of 30-minute swimming paths for each experimental group; **B)** Distance ($H_4=28.13$, $p<0.0001$); **C)** Average velocity ($H_4=28.25$, $p<0.0001$); **D)** Maximum velocity ($F=6.502$, $p<0.0001$); **E)** Body mobility ($H_4=32.56$, $p<0.0001$); **F)** Rotation clockwise ($H_4=9.384$, $p=0.0522$); **G)** Rotation counterclockwise ($H_4 = 8.298$, $p=0.0812$); **H)** Heading center-point ($H_4=4.011$, $p=0.4045$); **I)** Angular velocity ($H_4=1.695$, $p=0.7916$); **J)** Meander ($H_4=2.329$, $p=0.6756$). Results are reported as box and whiskers plots with single points and indicating median, interquartile ranges and min-max range. Experiments conducted in duplicate (CTR $n=270$; $0.1 \mu\text{g/L}$ $n=112$; $0.5 \mu\text{g/L}$ $n=57$; $1 \mu\text{g/L}$ $n=82$; $10\mu\text{g/L}$ $n=37$).



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Figure 5. Neurovascular structures of zebrafish are generally preserved after exposure to the pesticide cocktail. **A)** Z-stack reconstructions of *fli1a*:GFP zebrafish showing brain vasculature in green for CTR and **(A1)** 10 µg/L (a behavior-modifying concentration, see Figure 4) cocktail pesticide (A1). Scale bar: 30 µm. The studied area is delimited by a white dashed semi-circle. **B)** Examples of Imaris 3D skeleton reconstruction of the midbrain vasculature in CTR and **(B1)** 10 µg/L cocktail pesticide. Scale bar: 30 µm. **C)** Z-stack images of a pan-neuronal *HuC*:tomato zebrafish for CTR and **(C1)** 10 µg/L cocktail pesticide. OT: optic tectum, ON: optic nerve, HP: hindbrain axon projection. Dashed lines with arrows indicate the structures quantified: yellow (length of the 1st hindbrain axon projection), green (length of the optic nerve) and blue (thickness of the optic nerve). Scale bar: 40 µm.

1086 **D)** Quantification of the total vasculature length (Mann-Whitney test, $p=0.1449$),
1087 experiments conducted in duplicate (CTR $n=15$, $10\ \mu\text{g/L}$ $n=10$); **E)** Quantification of
1088 the ON length (Mann-Whitney test, $p=0.8891$), experiments conducted in duplicate
1089 (CTR $n=10$, $10\ \mu\text{g/L}$ $n=5$); **F)** quantification of the ON thickness (Mann-Whitney test,
1090 $p=0.7105$), experiments conducted in duplicate (CTR $n=10$, $10\ \mu\text{g/L}$ $n=5$); **G)** 1st
1091 hindbrain extension (Mann-Whitney test, $p=0.2601$), experiments conducted in
1092 duplicate (CTR $n=10$, $10\ \mu\text{g/L}$ $n=5$).

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1119 **Table 1. Food (g/day/mouse) and water (ml/day/mouse) intake during mating**
 1120 **and pregnancy.**

		Week 1 (mating)		Week 2 (gestation)		Week 3 (gestation)		Week 4 (gestation)	
		CTRL	PESTI	CTRL	PESTI	CTRL	PESTI	CTRL	PESTI
FOOD (g)	Mean (\pm SD)	5,34 (\pm 0.43)	5,34 (\pm 0.67)	4,07 (\pm 1.27)	4,19 (\pm 0.37)	4,61 (\pm 0.46)	4,18 (\pm 0.67)	4,15 (\pm 1,64)	3,90 (\pm 2.36)
	P-values	0.95812		0.2268		0.2606		0.3978	
WATER (mL)	Mean (\pm SD)	6,47 (\pm 0.96)	5,90 (\pm 0.51)	6,56 (\pm 0.33)	5,78 (\pm 0.50)	10,76 (\pm 4.66)	6,18 (\pm 1.24)	9,33 (\pm 4.45)	7,94 (\pm 2.14)
	P-values	0.18926		0.00741		0.42067		0.69854	

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1145 **Table 2. At the structural level, glial and pericyte-capillaries are generally**
 1146 **preserved.** No significant differences exist between pesticides exposed and aged-
 1147 matched control diet mice. Data refer to male animals, where neurological changes
 1148 occur long-term (see also Figures 1 and 2). Non-parametric Mann-Whitney test was
 1149 used. CA: cornu ammonis, DG: dentate gyrus, WM: white matter, CTX:
 1150 somatosensory cortex).

		2 months		14 months		
		WT	PEST	WT	PEST	
		n	6	8	7	8
Astrocytes (%Fluorescence GFAP)	CA3	Mean	8.577	7.763	13.123	12.208
		SD	3.214	2.229	2.646	1.945
		P-values	0.64297		0.32321	
	CA1	Mean	7.608	7.528	12.859	13.945
		SD	2.537	1.487	1.928	1.677
		P-values	0.86439		0.16617	
	DG	Mean	12.477	9.662	14.358	16.506
		SD	4.610	2.750	3.323	2.304
		P-values	0.09934		0.10858	
	WM	Mean	8.539	8.802	13.495	14.287
		SD	2.564	2.433	2.951	3.091
		P-values	0.67832		0.46276	
	CTX	Mean	0.585	0.830	1.358	1.258
		SD	0.730	1.027	0.827	0.788
		P-values	0.7781		0.57681	
Length of brain capillary-pericyte	CA3	Mean	1054.334	1005.660	1282.064	1158.651
		SD	293.744	258.223	292.065	247.557
		P-values	0.72771		0.25296	
	CA1	Mean	950.786	936.182	1587.720	1400.617
		SD	152.363	208.722	543.064	269.139
		P-values	0.59343		0.32861	
	DG	Mean	1115.695	1187.384	1613.480	1348.747
		SD	201.621	286.891	376.880	288.251
		P-values	0.41655		0.07573	
	WM	Mean	927.078	845.637	914.180	867.817

		SD	186.723	206.880	388.758	240.635
		P-values	0.39043		0.85161	
	CTX	Mean	1183.856	1008.267	1450.880	1430.572
		SD	321.621	257.484	440.657	187.208
		P-values	0.13136		0.46933	

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1178 **Table 3. Motor-behavior outcomes in zebrafish exposed to a dose-range (0.1 to**
1179 **10.000 µg/L) of individual pesticides. A)** Boscalid (CTR n=154, 0.1 µg/L n=110, 0.5
1180 µg/L n=59, 1 µg/L n=95, 10 µg/L n=60, 100 µg/L n=40; 1000 µg/L n=40) **B)** Captan
1181 (CTR n=213, 0.1 µg/L n=70, 0.5 µg/L n=50, 1 µg/L n=89, 10 µg/L n=18, 100 µg/L
1182 n=24), **C)** Chlorpyrifos (CTR n=265, 0.1 µg/L n=109, 0.5 µg/L n=60, 1 µg/L n=118, 10
1183 µg/L n=59, 100 µg/L n=21; 1000 µg/L n=49), **D)** Thiophanate (CTR n=188, 0.1 µg/L
1184 n=99, 0.5 µg/L n=40, 1 µg/L n=97, 10 µg/L n=40, 100 µg/L n=40; 1000 µg/L n=40,
1185 10.000 µg/L n=39), **E)** Thiacloprid (CTR n=286, 0.1 µg/L n=116, 0.5 µg/L n=57, 1
1186 µg/L n=116, 10 µg/L n=60, 100 µg/L n=60; 1000 µg/L n=60, 10.000 µg/L n=58), **F)**
1187 Ziram (CTR n=218, 0.1 µg/L n=87, 0.5 µg/L n=50, 1 µg/L n=87, 10 µg/L n=39). Data
1188 reported as mean ± SD (1-way ANOVA followed by Dunnett's multiple comparison
1189 test, or Kruskal-Wallis with Dunn's multiple comparisons test, p<0.05).
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A- BOSCALID								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 33	89 ± 36	78 ± 30 ***	85 ± 29 *	117 ± 45	113 ± 41	118 ± 43	-
Velocity (mean)	100 ± 33	90 ± 38	78 ± 30 ***	86 ± 31 *	117 ± 45	118 ± 46	120 ± 44	-
Velocity (max.)	100 ± 20	94 ± 17	98 ± 18	97 ± 19	91 ± 19 **	91 ± 20	90 ± 17*	-
Body mobility	100 ± 39	87 ± 39	73 ± 28 ****	83 ± 33*	122 ± 50*	125 ± 54	128 ± 56	-
Rotation (clockwise)	100 ± 29	104 ± 32	104 ± 35	97 ± 39	111 ± 31	109 ± 33	111 ± 31	-
Rotation (counter)	100 ± 29	104 ± 32	100 ± 34	98 ± 39	113 ± 32 *	112 ± 35	112 ± 31	-
Heading center	100 ± 1353	156 ± 1755	157 ± 361	187 ± 1670	-23 ± 2187	-1067 ± 1746 ***	-351 ± 2090	-
Angular velocity	100 ± 579	-7 ± 635	403 ± 1024	1 ± 626	41 ± 322	-25 ± 357	-25 ± 394	-
Meander	100 ± 1789	143 ± 1844	1208 ± 2724	152 ± 1353	37 ± 579	13 ± 447	-59 ± 621	-
B- CAPTAN								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 38	98 ± 39	95 ± 38	102 ± 42	51 ± 16 ****	82 ± 40	-	-
Velocity (mean)	100 ± 38	98 ± 39	95 ± 38	102 ± 42	57 ± 29 ****	81 ± 41	-	-
Velocity (max.)	100 ± 20	97 ± 18	101 ± 21	97 ± 18	88 ± 8 *	89 ± 14	-	-
Body	100 ± 44	101 ± 43	102 ± 41	105 ± 44	51 ± 26 ****	76 ± 36	-	-

mobility								
Rotation (clockwise)	100 ± 31	110 ± 32 *	111 ± 34	119 ± 34 ****	93 ± 23	100 ± 56	-	-
Rotation (counter)	100 ± 30	110 ± 33 *	113 ± 32 *	121 ± 34 ****	98 ± 27	100 ± 56	-	-
Heading center	100 ± 844.7	21 ± 1181	68 ± 236	271 ± 1237	-474.4 ± 1791	18 ± 1708	-	-
Angular velocity	100 ± 3442	-132 ± 3443	486 ± 4498	-179 ± 3120	-14 ± 1083	355 ± 614	-	-
Meander	100 ± 4063	-69 ± 3850	-865 ± 5538	611 ± 3633	-21 ± 367	150 ± 361	-	-
C- CHLORPYRIFOS								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 44	75 ± 46 ****	81 ± 39 **	81 ± 39 **	89 ± 35	77 ± 54	73 ± 33 ***	-
Velocity (mean)	100 ± 44	75 ± 46 ****	78 ± 40 **	81 ± 39 **	89 ± 35	77 ± 54	73 ± 33***	-
Velocity (max.)	100 ± 24	92 ± 24 *	82 ± 29 ****	90 ± 29 *	99 ± 25	94 ± 34	93 ± 28 *	-
Body mobility	100 ± 49	75 ± 57 ****	77 ± 40 **	78 ± 44 ****	72 ± 35 ***	85 ± 75 **	61 ± 31 ****	-
Rotation (clockwise)	100 ± 33	83 ± 40 ***	94 ± 31	95 ± 43	99 ± 29	70 ± 56 **	80 ± 28 **	-
Rotation (counter)	100 ± 32	84 ± 41 ***	94 ± 29	97 ± 44	99 ± 29	71 ± 57 **	82 ± 27 **	-
Heading center	100 ± 1439	-73 ± 1811	540 ± 2124	248 ± 1485	84 ± 670	31 ± 843	99 ± 719	-
Angular velocity	100 ± 1305	-142 ± 1658	-153 ± 1742	-51 ± 1340	103 ± 1132	-341 ± 1195	-158 ± 1207	-
Meander	100 ± 1147	-55 ± 1164	107 ± 1330	13 ± 1181	9 ± 455	14 ± 443	-25 ± 453	-
D- THIOPHANATE								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 47	102 ± 49	96 ± 30	87 ± 36	66 ± 33 ****	65 ± 24 ***	100 ± 26	57 ± 32 ****
Velocity (mean)	100 ± 47	100 ± 48	94 ± 30	85 ± 36	66 ± 33 ****	67 ± 31 **	100 ± 26	56 ± 32 ****
Velocity (max.)	100 ± 22	107 ± 24	114 ± 26 *	111 ± 23 **	108 ± 19	105 ± 20	112 ± 26	80 ± 22 ***
Body mobility	100 ± 48	101 ± 55	97 ± 30	87 ± 39	74 ± 50 **	88 ± 58	103 ± 38	71 ± 64 ****
Rotation (clockwise)	100 ± 38	97 ± 31	114 ± 32	93 ± 37	67 ± 33 ****	84 ± 36	103 ± 30	56 ± 39 ****
Rotation (counter)	100 ± 38	95 ± 30	111 ± 30	92 ± 35	67 ± 32 ****	85 ± 36	100 ± 32	56 ± 36 ****
Heading center	100 ± 664	-27 ± 566	98 ± 487	-9 ± 648	79 ± 844	81 ± 856	119 ± 837	-71 ± 818
Angular velocity	100 ± 1179	-132 ± 1097	237 ± 749	-57 ± 1281	-125 ± 1711	69 ± 1475	-255 ± 1734	-83 ± 1799

Meander	100 ± 2853	395 ± 3337	302 ± 2070	161 ± 3069	539 ± 2787	-338 ± 3269	259 ± 4645	-150 ± 1858
E- THIACTOPRID								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 45	82 ± 33 **	73 ± 40 ****	86 ± 43 **	72 ± 24 ***	73 ± 39 ***	90 ± 43	94 ± 34
Velocity (mean)	100 ± 45	82 ± 33 **	73 ± 40 ****	86 ± 43 **	72 ± 24 ***	73 ± 39 ***	90 ± 42	96 ± 39
Velocity (max.)	100 ± 22	100 ± 26	101 ± 23	97 ± 22	95 ± 21	98 ± 23	97 ± 23	103 ± 24
Body mobility	100 ± 46	87 ± 40	74 ± 45 ****	84 ± 44 **	79 ± 35 **	87 ± 50 *	90 ± 43	102 ± 48
Rotation (clockwise)	100 ± 33	95 ± 32	83 ± 33 **	101 ± 37	97 ± 31	82 ± 35 ***	98 ± 27	108 ± 30
Rotation (counter)	100 ± 33	97 ± 33	82 ± 33 **	99 ± 35	96 ± 31	82 ± 36 ***	97 ± 28	109 ± 31
Heading center	100 ± 1412	140 ± 1576	431 ± 1914	-101 ± 1617	110 ± 723	-99 ± 814	-46 ± 777	72 ± 769
Angular velocity	100 ± 1378	41 ± 1304	1 ± 1663	173 ± 1463	66 ± 1200	-76 ± 1110	156 ± 1436	111 ± 1153
Meander	100 ± 2316	-64 ± 1885	-167 ± 1495	281 ± 2344	390 ± 3022	144 ± 2633	93 ± 2946	-71 ± 2653
F- ZIRAM								
	CTRL	0.1	0.5	1	10	100	1000	10000
Distance	100 ± 34	100 ± 31	70 ± 34 ****	75 ± 32 ****	71 ± 52 **	-	-	-
Velocity (mean)	100 ± 34	100 ± 31	70 ± 34 ****	75 ± 32 ****	71 ± 52 **	-	-	-
Velocity (max.)	100 ± 19	99 ± 17	99 ± 18	97 ± 18	79 ± 20 ****	-	-	-
Body mobility	100 ± 38	108 ± 39	73 ± 37 ****	76 ± 35 ****	77 ± 57 *	-	-	-
Rotation (clockwise)	100 ± 30	128 ± 29 ****	82 ± 38 *	95 ± 34	82 ± 47	-	-	-
Rotation (counter)	100 ± 29	128 ± 30 ****	82 ± 39 *	96 ± 32	83 ± 48	-	-	-
Heading center	100 ± 529	82 ± 671	57 ± 255	-77 ± 800	3.8 ± 1023	-	-	-
Angular velocity	100 ± 3392	-82 ± 2910	332 ± 4493	464 ± 3394	-81 ± 813	-	-	-
Meander	100 ± 4028	268 ± 3816	-363 ± 4777	109 ± 3066	14 ± 376	-	-	-

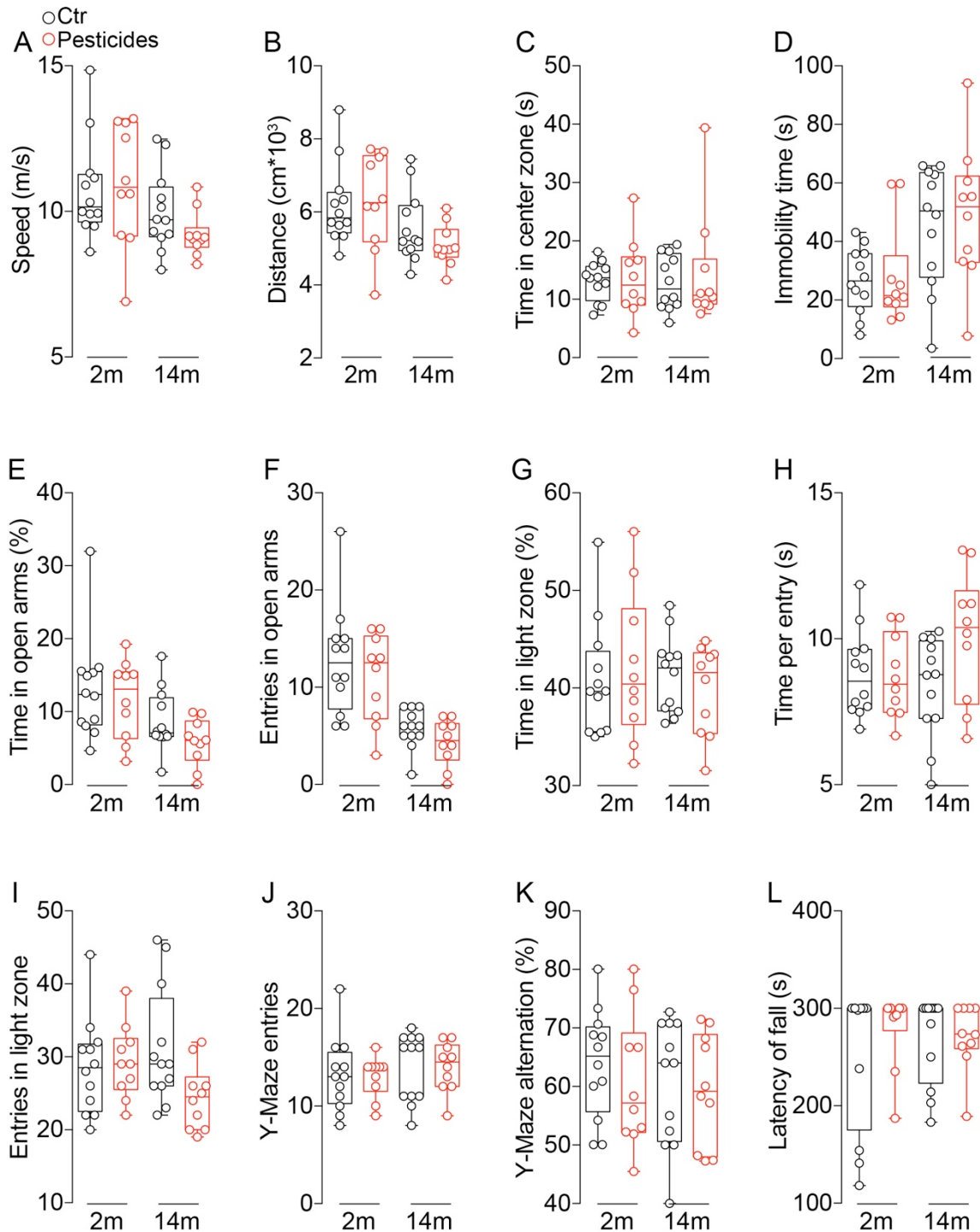
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Supplemental Figure 1. Perinatal exposure to dietary pesticides does not alter behavior in adult female mice. The panels indicate the behavioral parameters measured. *Open field test and locomotor activity:* (A) speed, (B) distance travelled, (C) time spent in the center zone and (D) immobility time in the open field test. *Elevated-plus maze (EPM):* (E) Time spent and (F) number of entries in the open arms during the EPM test. *Light/Dark box:* (G) time spent in the light zone, (H) time

1203 per entry and **(I)** number of entries in the light zone. *Y-Maze test (working memory)*:
1204 **(J)** number of entries and **(K)** percentage of spontaneous alternation in the Y-Maze.
1205 Rotarod (procedural and motor learning): **(L)** Latency of fall (s) following a training
1206 period of 5 days. 12 female control and 10 female pesticide exposed mice were used.
1207 Data are analyzed using the non-parametric Kruskal-Willis ANOVA: **(A)** $H_3=9.66$,
1208 $p=0.0217$, **(B)** $H_3=9.608$, $p=0.0222$, **(C)** $H_3=0.1231$, $p=0.9889$, **(D)** $H_3=10.3$,
1209 $p=0.0162$, **(E)** $H_3=11.17$, $p=0.0108$, **(F)** $H_3=20.94$, $p=0.0001$, **(G)** $H_3=0.6544$,
1210 $p=0.8839$, **(H)** $H_3=4.026$, $p=0.2587$, **(I)** $H_3=5.957$, $p=0.1137$, **(J)** $H_3=1.96$, $p=0.5807$,
1211 **(K)** $H_3=1.142$, $p=0.7670$, **(L)** $H_3=0.6178$, $p=0.8924$. The Dunn's test was used for
1212 multiple comparisons between groups (Ctr 2m vs Pesticides 2m; Ctr 14m vs
1213 Pesticides 14m): not significant for all panels, $p>0.05$.

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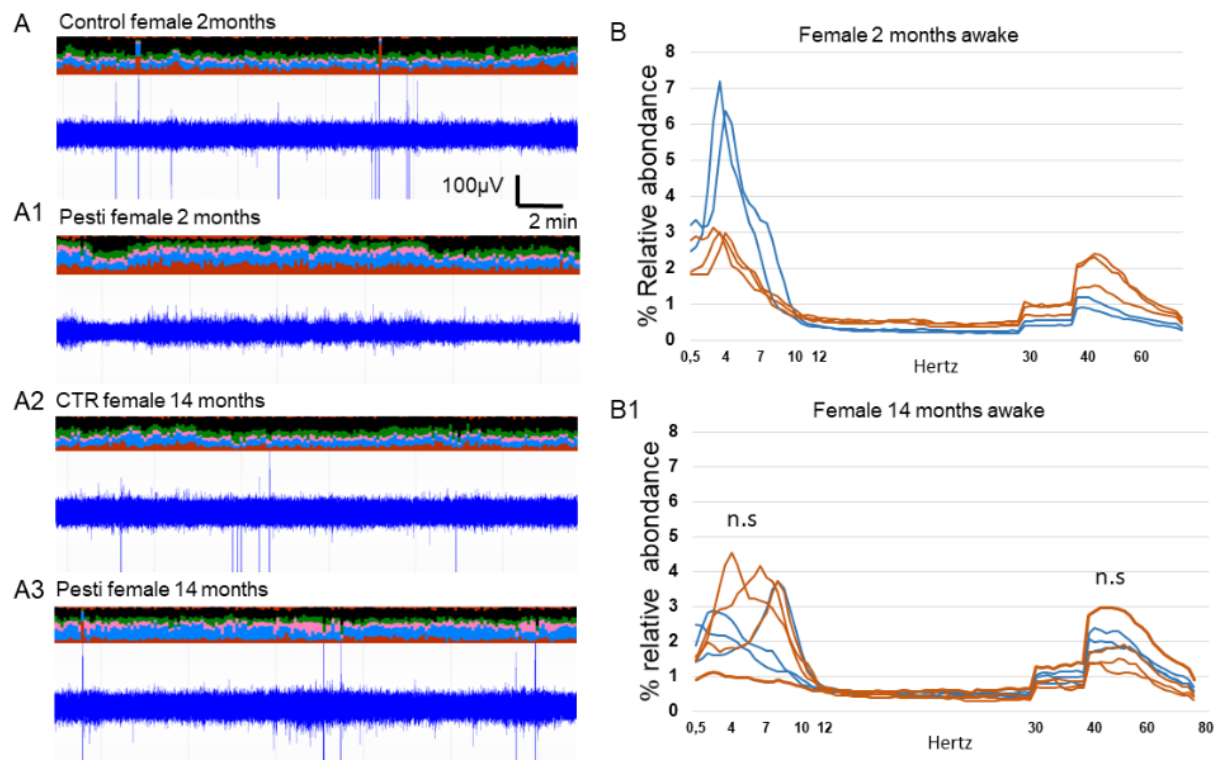
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1225 **Supplemental Figure 2. Longitudinal electroencephalographic monitoring in**
 1226 **freely moving female mice prenatally exposed to pesticide cocktail. A-A3)** No
 1227 significant electroencephalographic modifications (e.g., spike activity) are reported in
 1228 female mice prenatally exposed to pesticide cocktail. **B-B1)** Examples of EEG data
 1229 acquired during awake/exploration periods. No robust changes were recorded long-
 1230 term at 14 months.

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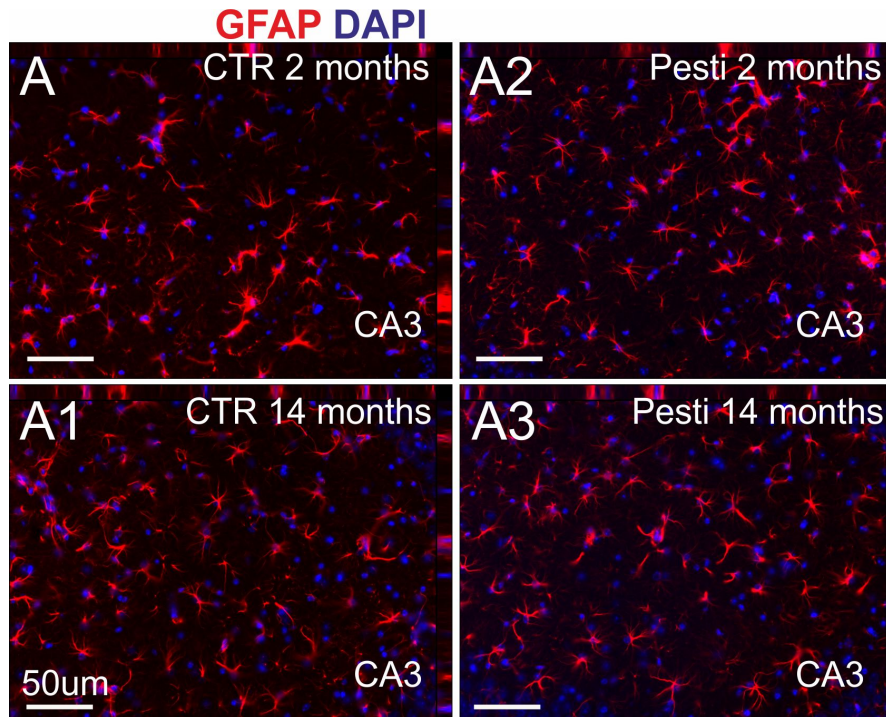
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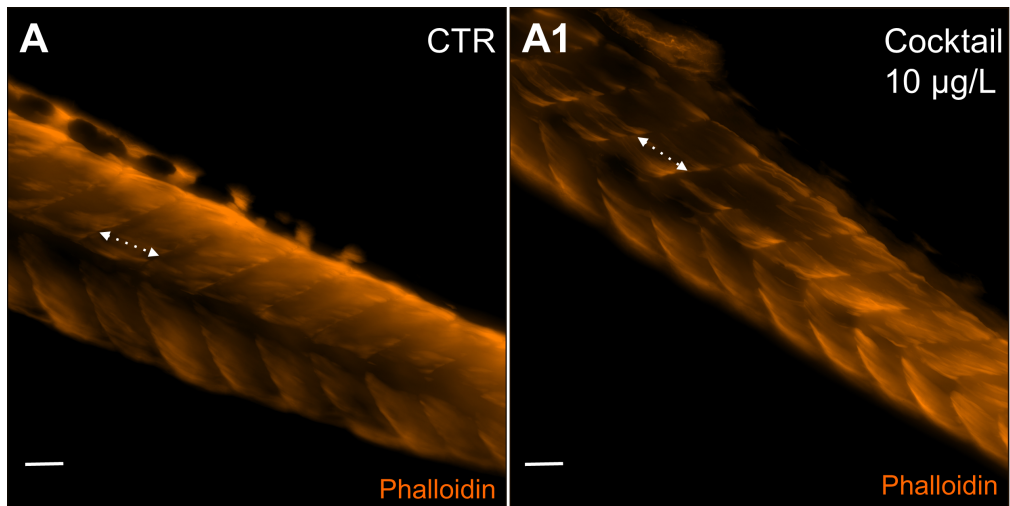
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Supplemental Figure 3. Lack of major structural brain malformations long-term.
A-A3) Histological examples of GFAP astrocytes and DAPI cell distributions in the dorsal hippocampus. See Table 2 for quantifications and statistics.



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1273 **Supplemental Figure 4. No structural muscle differences exist between control**
1274 **and pesticides exposed zebrafish.** Lateral views of trunk somites in zebrafish
1275 larvae stained with phalloidin. Examples of CTR (**A**), 10 µg/L cocktail pesticide (**A1**).
1276 Dashed lines with arrows indicated the length of a typical fiber measured. No
1277 significant changes were observed (quantifications are provided in the Results).
1278 Experiments conducted in duplicate (n=10/group). Scale bar: 50 µm.

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1289 **Supplemental Table 1.** Chemical families, functions, and tolerable daily intake (TDI)
 1290 of each pesticide. Expected and measured pesticide concentrations ($\mu\text{g}/\text{kg}$ food) in
 1291 the food pellets. Adapted from Smith et. al 2020.

Pesticide name	Chemical family	Function	Tolerable Daily Intake (mg/kg BW/day)	Expected quantity ^a ($\mu\text{g}/\text{kg}$ food)	Determined level ($\mu\text{g}/\text{kg}$ food)
Ziram	Dithiocarbamate	Fungicide	0.006	36	ND ^b
Chlorpyrifos	Organophosphorus	Insecticide	0.01	60	57
Thiacloprid	Neonicotinoid	Insecticide	0.01	60	63
Boscalid	Carboxamide	Fungicide	0.04	240	200
Thiofanate	Benzimidazole	Fungicide	0.08	480	260
Captan	Dicarboximide	Fungicide	0.1	600	230

1292 Note: BW, Body weight; ND, not determined; (<http://www.agritox.anses.fr/>).

1293 ^a Expected quantity refers to the incorporated quantities of pesticides in mice pellets.

1294 ^b Ziram was not present at a detectible level (< 0.01 mg/kg).

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