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

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Highlights

1) Perinatal pesticides links with long-term behavioral modifications in male mice.

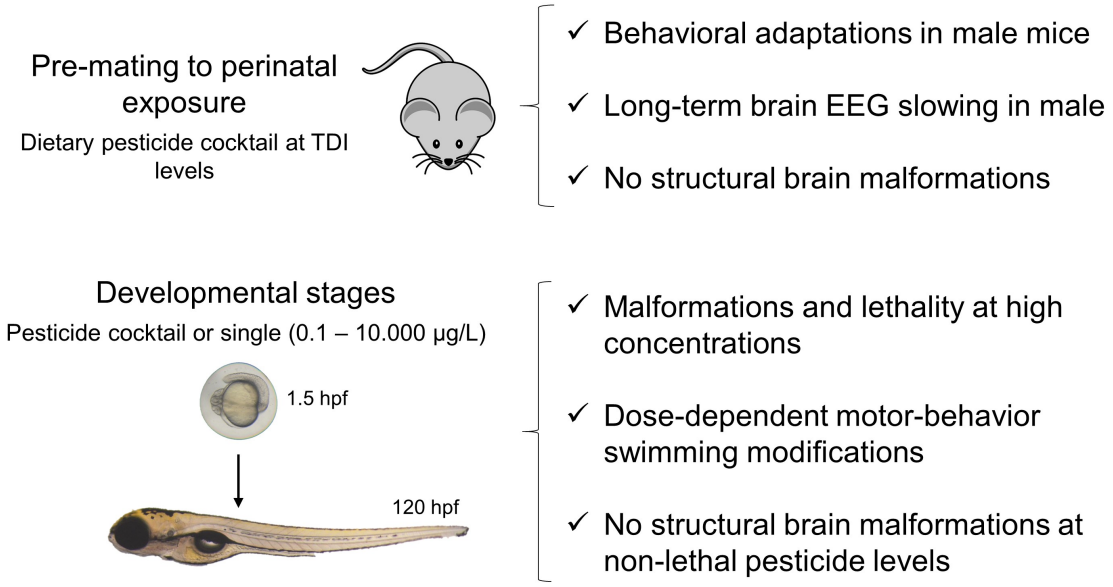
2) Behavioral changes are accompanied by electrographic brain slowing *in vivo*.

3) In zebrafish larvae, pesticide cocktail elicited dose-dependent motor-behavioral changes.

4) In zebrafish larvae, chlorpyriphos is a behavior-modifying pesticide contained in the cocktail.

5) Non-lethal prenatal pesticide cocktail does not provoke brain malformations.

Graphical abstract



Abstract

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

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

Introduction

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

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

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

Material and Methods

Animal Models and Husbandry

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

Mouse perinatal exposure protocol and pesticides chow preparation

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

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

prepared following the exact same steps, and without adding pesticides. Traces of methanol and acetone can be detected across pellet batches (e.g., < 0.05 g/Kg of methanol/pellet and 0.001 to 0.003 g/kg of acetone/pellet). These amounts are well below the levels associated with rodent maternal toxicity for solvents as reported by the U.S. Food and Drug Administration (http://academy.gmp-compliance.org/guidemgr/files/UCM073401.PDF), the European Chemical Agency (https://echa.europa.eu/registration-dossier/-/registered-dossier/15569/7/9/1; https://echa.europa.eu/registration-dossier/-/registered-dossier/15460/7/6/1) and by the French Agency for Food, Environmental and Occupational Health & Safety (https://www.anses.fr/fr/system/files/ALAN2007sa0013.pdf; https://www.dod.fr/PartageWeb/PartageWeb/Fiche_Tech/309266_fds_acetone.pdf). See discussion for further details. The resulting powder was incorporated into the pellet (SAAJ-RAF National Research Institute for Agriculture, Food and the Environment, INRAE). Pesticide levels in the pellet are re-analyzed by gas chromatography–tandem mass spectrometry and liquid chromatography–tandem mass spectrometry (Eurofins; Supplemental Table 1 and (Smith et al., 2020).

Behavioural testing

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

20cm height) for 10min. The center zone was defined as a virtual perimeter within 5cm from the sides of the arena. Experiments were videotaped and an observer scored the time spent in the center and the number of transitions in the center zone. Arena was cleaned with 70% ethanol after each mouse. *Spatial working memory, spontaneous alternation in the Y-maze*. Each mouse was placed at the end of one arm in a grey polyvinylchloride Y-maze and allowed to freely explore during a single 8min session. The series of arm entries, including possible returns into the same arm, was recorded visually. An alternation was defined as entries into all three arms on consecutive trials. The percentage of alternation was calculated as actual alternations/maximum alternations x 100. Measured parameters included the percentage of alternation (memory index) and total number of arm entries (exploration index). All arms were cleaned with 70% ethanol between two sessions. *Light/Dark Transition test*. The apparatus consists of a cage (21x42x25) divided into two chambers, dark and bright, of equal size by a partition with a door. Mice were allowed to move freely between the two chambers during 10min. The number of entries into the bright chamber and the duration of time spent there were measured by videotracking (Viewpoint, Lissieu, France). After each trial, all chambers were cleaned with 70% ethanol. *Elevated-Plus-Maze (EPM)*. The EPM is a 4-arm maze elevated 40cm above the floor. The 4 arms intersect and form a plus sign (dimension of each arm: length 30cm, width 5cm, and center region 5x5 cm^2). Two of the arms are closed on 3 sides by 15 cm high walls, and the other two are open. Animals were placed in the centre of the apparatus and allowed to freely explore the maze for 10min. Score for entries and time spent in open arms were measured. All arms were cleaned with 70% ethanol after each mouse entry. *Social Interaction*. The apparatus was a rectangular 3-chambered polycarbonate box. The test mouse was placed into

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

Freely moving telemetry Video-electroencephalography

A total of 16 male and 16 female C57Bl/6 mice were used at 2 and at 14 months (n= 4/group, pesticides and control), and monitored by using Video-EEG telemetry. Because of technical issues (defective implants) two male mice (1 control and 1 pesticide exposed; Figure 2) and two female mice (Supplemental Figure 2) were excluded from the analysis. As we previously reported (Ichkova et al., 2020; Klement et al., 2020; Runtz et al., 2018; Zub et al., 2019), animals were anesthetized using a intraperitoneal injection with chloral hydrate and xylazine (400 mg/kg; 4 mg/kg). Animals were placed on a heating pad during the procedure ensuring a physiological temperature. A telemetry implant (Physiotel transmitter ETA-F10, weight 1.6 g, 1.1 cm^3, Data Science international DSI, St Paul, MN, USA) was placed into a ventral pocket. The subcutaneous tissue covering the skull was separated and the periosteum cleaned. Two screws (one bipolar electrode) are inserted in the skull

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

Zebrafish pesticides exposure protocol

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

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

Zebrafish locomotor activity

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

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

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

Mouse brain histology.

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

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

Statistics

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

Results

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

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

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

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

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

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

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

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

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

Next, assessment of locomotor behavior at 120 hpf showed an increase in the distance travelled, average velocities (mean and maximum), body mobility, and number of rotations at 10 µg/L pesticide cocktail (Figure 4). Interestingly, a decrease

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Conclusions.

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

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

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

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

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

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

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

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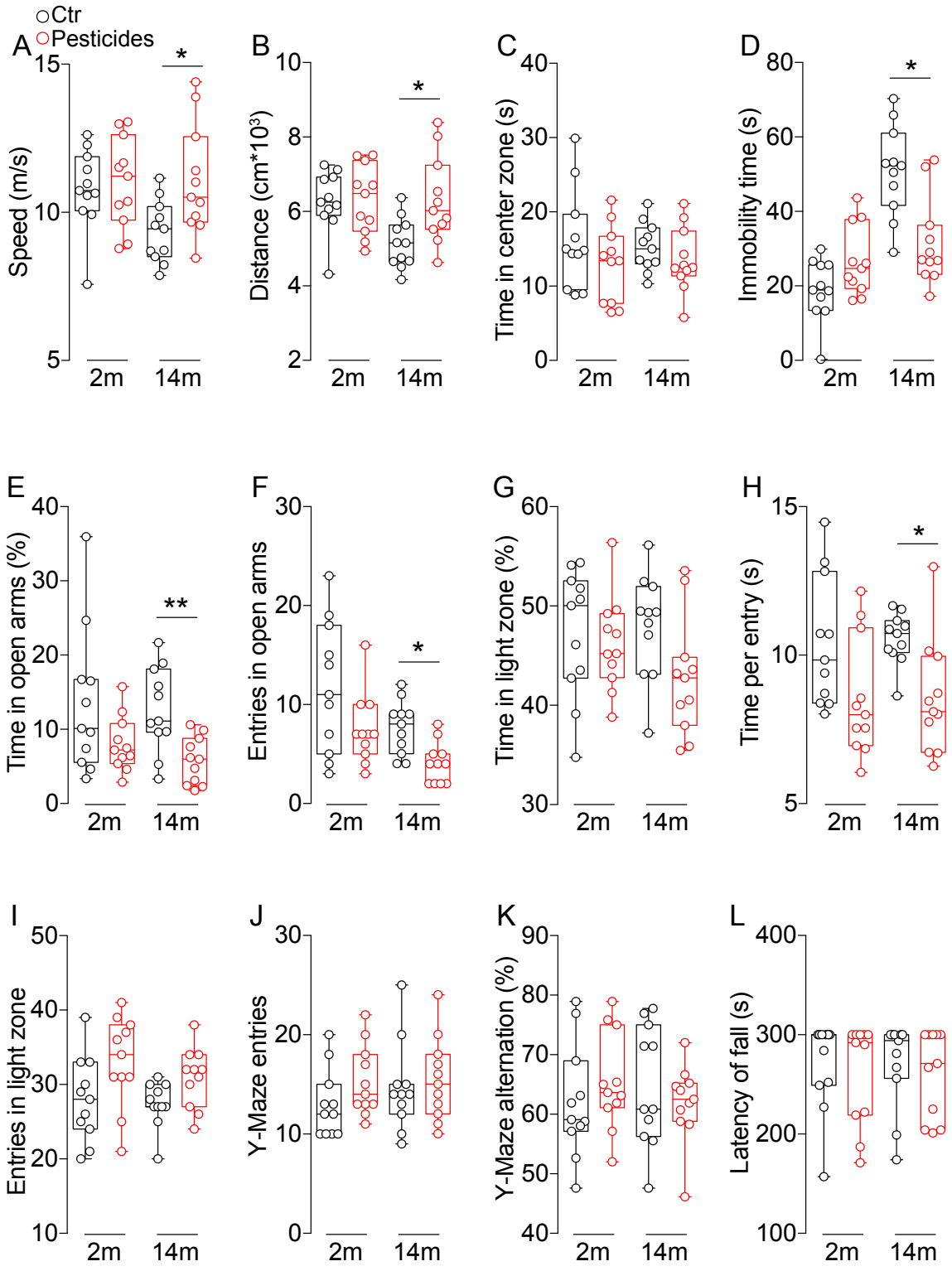


Figure 1: Perinatal exposure to dietary pesticides elicits an anxiety-like phenotype during aging in male mice. The panels indicate the behavioral parameters examined in males. Supplemental Figure 1 provides results obtained using female mice. *Open field test and locomotor activity*: (**A**) speed, (**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 per entry and (**I**) number of entries in the light zone. *Y-Maze test (working memory)*: (**J**) number of entries and (**K**) percentage of spontaneous alternation in the Y-Maze. Rotarod (procedural and motor learning): (**L**) Latency of fall (s) following a training period of 5 days. 11 male mice were used for each experimental group. Data are analyzed using the non-parametric Kruskal-Wallis ANOVA: (**A**) H_3=9.753, 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, (**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**) H_3=11.45, p=0.00995, (**I**) H_3=9.087, p=0.0282, (**J**) H_3=3.481, p=0.3232, (**K**) H_3=1.363, p=0.7142, (**L**) H_3=0.3088, p=0.9584. The Dunn's test was used for multiple comparisons between groups (Ctr 2m *vs* Pesticides 2m; Ctr 14m *vs* Pesticides 14m): *$p < 0.05$ and **$p < 0.01$.

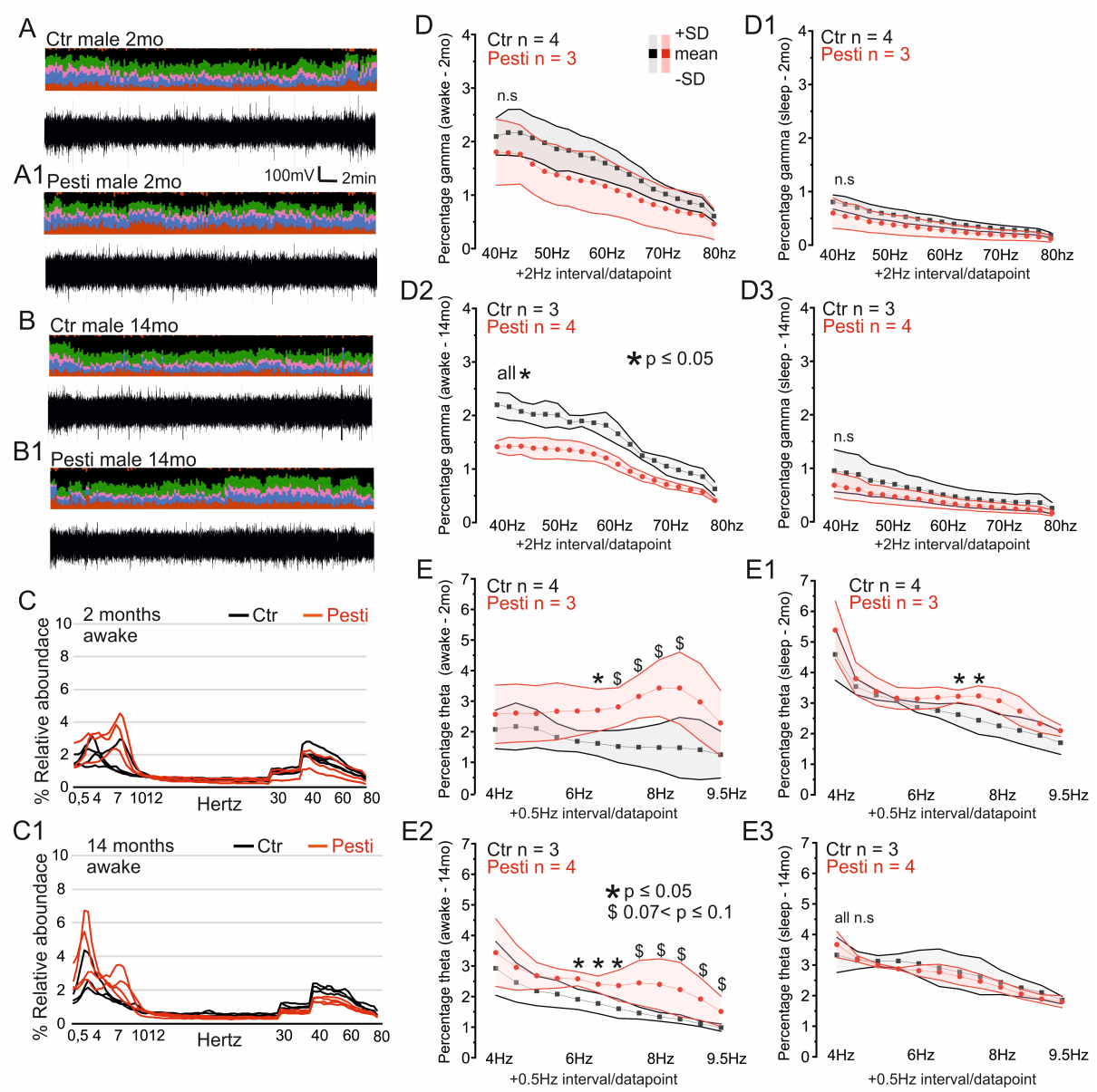


Figure 2. Time-dependent electroencephalographic modifications in freely moving male mice prenatally exposed to the pesticide cocktail. **A-B1)** Examples of EEG traces obtained at 2 and 14 months and relative color-coded wave distributions. Spike or seizure activity was not observed. **C-C1)** Examples 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

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

A

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 **

B

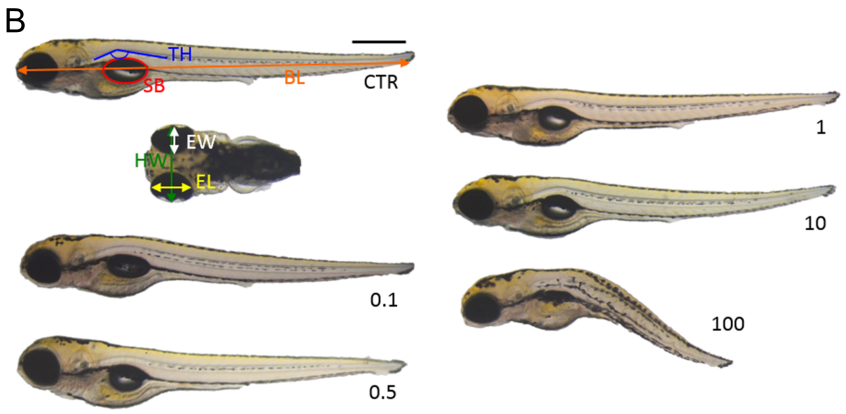


Figure 3. Morphological parameters, hatching rate, and mortality of zebrafish larvae exposed to a dose range pesticide cocktail. A) Morphological parameters: standard body length (BL, µm, F=1.292, p=0.2522), swim bladder (SB, μm^2, H_4=3.641, p=0.4567), head width (HW, µm, F=0.5741, p=0.6828), eye width (EW, µm, H_4=3.369, p=0.4981), eye length (EL, µm, H_4=4.116, p=0.3905) and trunk-head angle (TH, degrees, F=2.548, p=0.0536) of zebrafish larvae at 120 hpf, experiments conducted in duplicate (n=10/group; 1-way ANOVA followed by Dunnett's multiple comparisons test or Kruskal-Wallis with Dunn´s multiple comparisons test, $p < 0.05$). Mortality at 24 hpf, experiments conducted in duplicate (n=200/group; 1-way ANOVA, Dunnett's multiple comparisons test, F=3.094 p=0.0337). Cumulative hatching rate consecutively assessed at 72 and 96 hpf and expressed as (hatched eggs / total) * 100, experiments conducted in duplicate (n = 200/group; 2-way ANOVA, Sidak´s multiple comparisons test, $p< 0.05$). Data reported as means ± SD. **B)** Examples for morphological assessments. Scale bar: 500 µm.

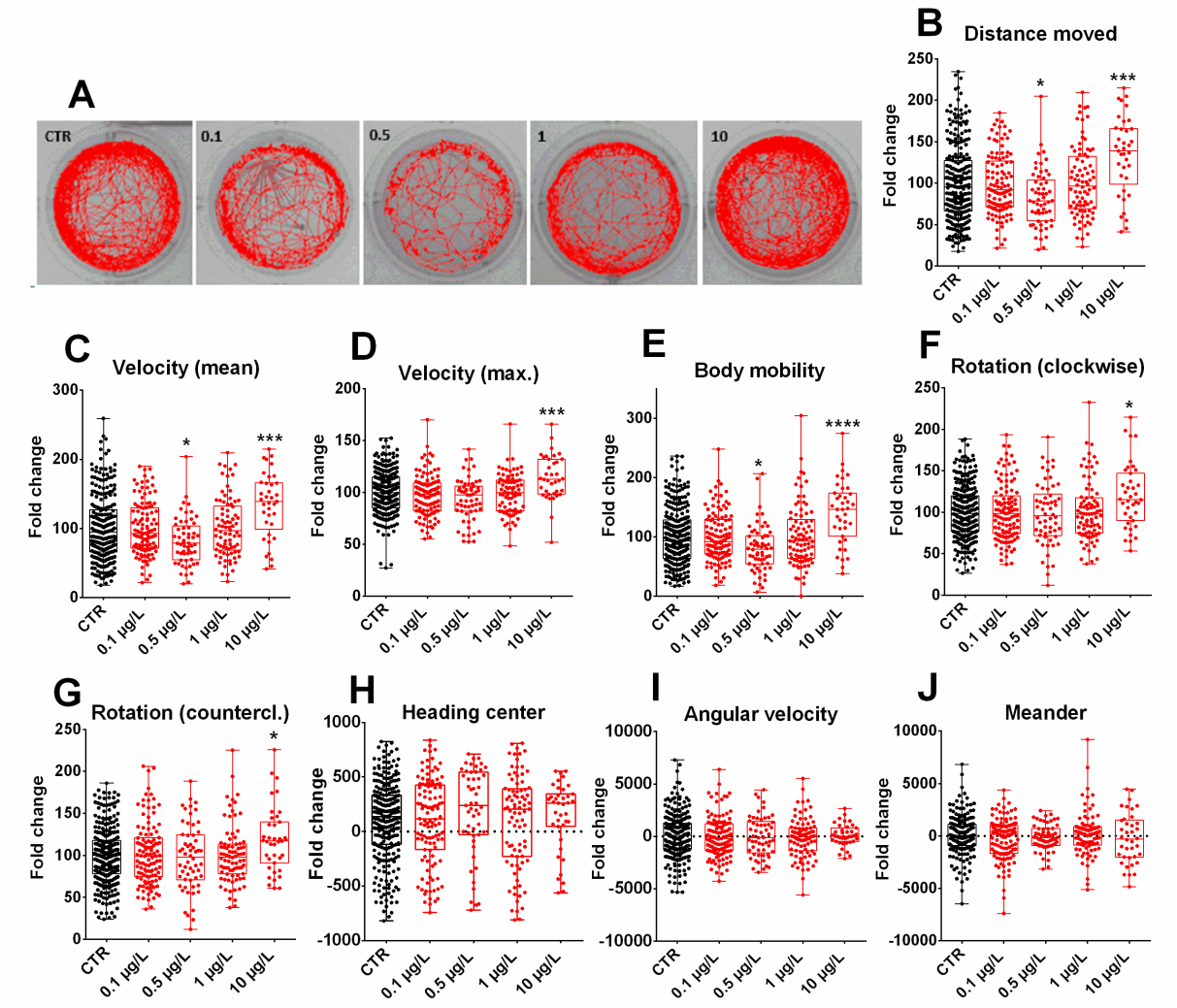


Figure 4. Motor-behavioral modifications in zebrafish larvae exposed to a dose range pesticide cocktail. A) Examples of 30-minute swimming paths for each experimental group; **B)** Distance (H_4=28.13, p<0.0001); **C)** Average velocity (H_4=28.25, p<0.0001); **D)** Maximum velocity (F=6.502, p<0.0001); **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 µg/L n=112; 0.5 µg/L n=57; 1 µg/L n=82; 10µg/L n=37).

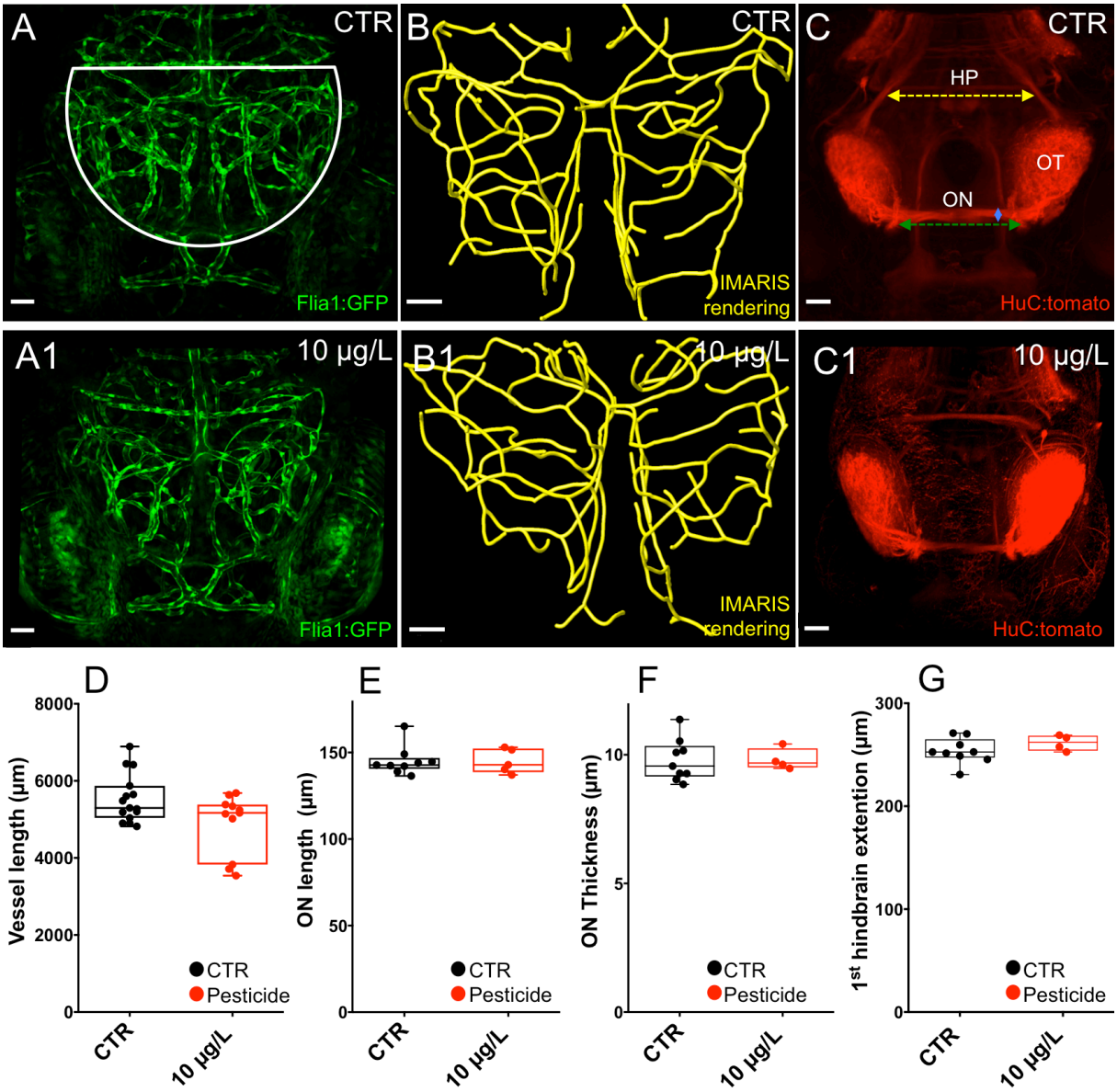


Figure 5. Neurovascular structures of zebrafish are generally preserved after exposure to the pesticide cocktail. A) Z-stack reconstructions of fli1a:GFP zebrafish showing brain vasculature in green for CTR and **(A1)** 10 µg/L (a behavior-modifying concentration, see Figure 4) cocktail pesticide (A1). 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.

D) Quantification of the total vasculature length (Mann-Whitney test, p=0.1449), experiments conducted in duplicate (CTR n=15, 10 µg/L n=10); **E)** Quantification of the ON length (Mann-Whitney test, p=0.8891), experiments conducted in duplicate (CTR n=10, 10 µg/L n=5); **F)** quantification of the ON thickness (Mann-Whitney test, p=0.7105), experiments conducted in duplicate (CTR n=10, 10 µg/L n=5); **G)** 1st hindbrain extension (Mann-Whitney test, p=0.2601), experiments conducted in duplicate (CTR n=10, 10 µg/L n=5).

Table 1. Food (g/day/mouse) and water (ml/day/mouse) intake during mating 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 (±SD)	5,34 (±0.43)	5,34 (±0.67)	4,07 (±1.27)	4,19 (±0.37)	4,61 (±0.46)	4,18 (±0.67)	4,15 (±1,64)	3,90 (±2.36)
	P-values	0.95812		0.2268		0.2606		0.3978	
WATER (mL)	Mean (±SD)	6,47 (±0.96)	5,90 (±0.51)	6,56 (±0.33)	5,78 (±0.50)	10,76 (±4.66)	6,18 (±1.24)	9,33 (±4.45)	7,94 (±2.14)
	P-values	0.18926		0.00741		0.42067		0.69854	

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

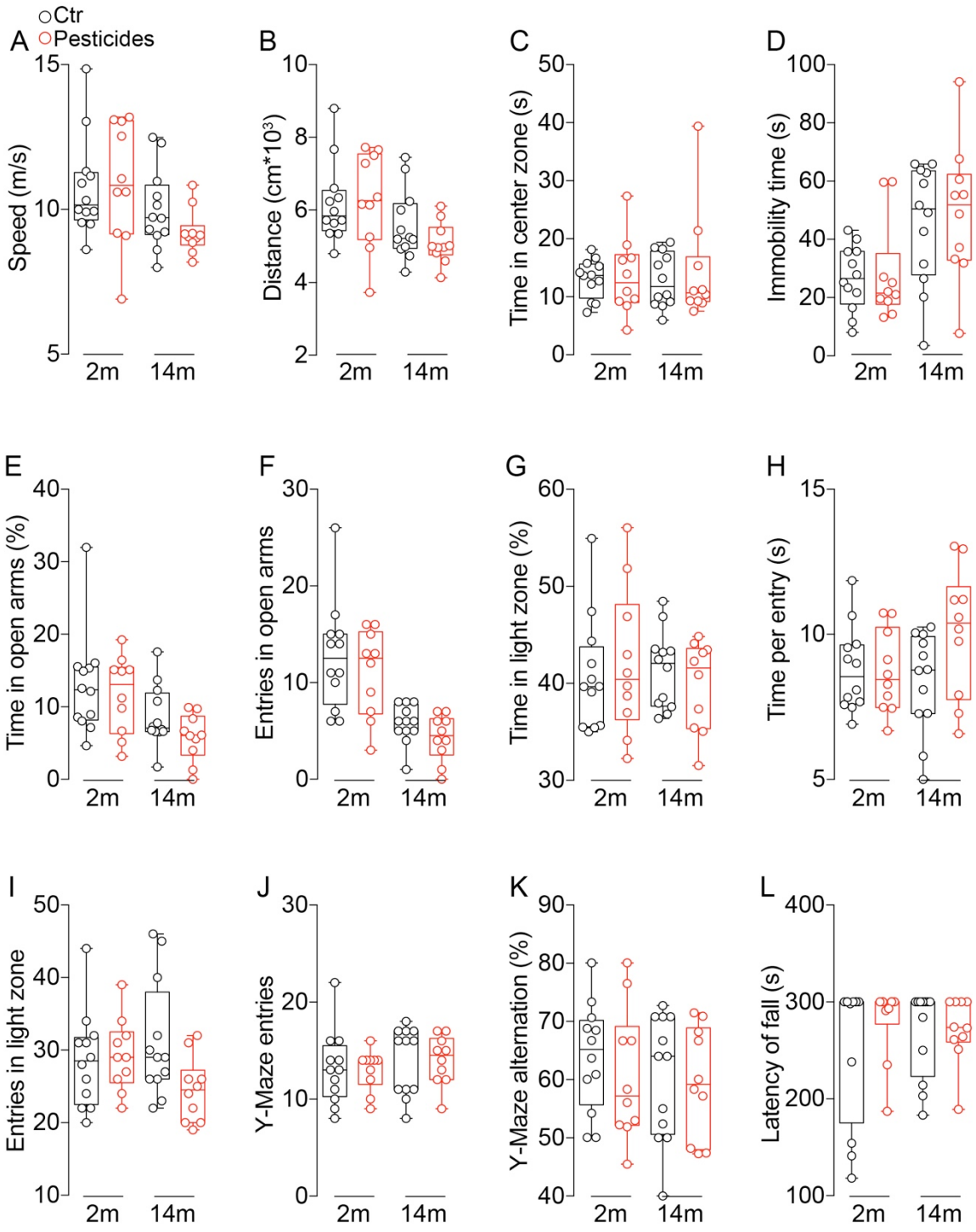
		SD	186.723	206.880	388.758	240.635
		P-values	0.39043		0.85161	
	CTX	Mean	1183.856	1008.267	1450.880	1430.572
		SD	321.621	257.484	440.657	187.208
		P-values	0.13136		0.46933	

Table 3. Motor-behavior outcomes in zebrafish exposed to a dose-range (0.1 to 10.000 µg/L) of individual pesticides. **A)** Boscalid (CTR n=154, 0.1 µg/L n=110, 0.5 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 (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 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 µg/L n=59, 100 µg/L n=21; 1000 µg/L n=49), **D)** Thiophanate (CTR n=188, 0.1 µg/L 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, 10.000 µg/L n=39), **E)** Thiacloprid (CTR n=286, 0.1 µg/L n=116, 0.5 µg/L n=57, 1 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)** 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 reported as mean ± SD (1-way ANOVA followed by Dunnett's multiple comparison test, or Kruskal-Wallis with Dunn´s multiple comparisons test, p<0.05).

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	-	-

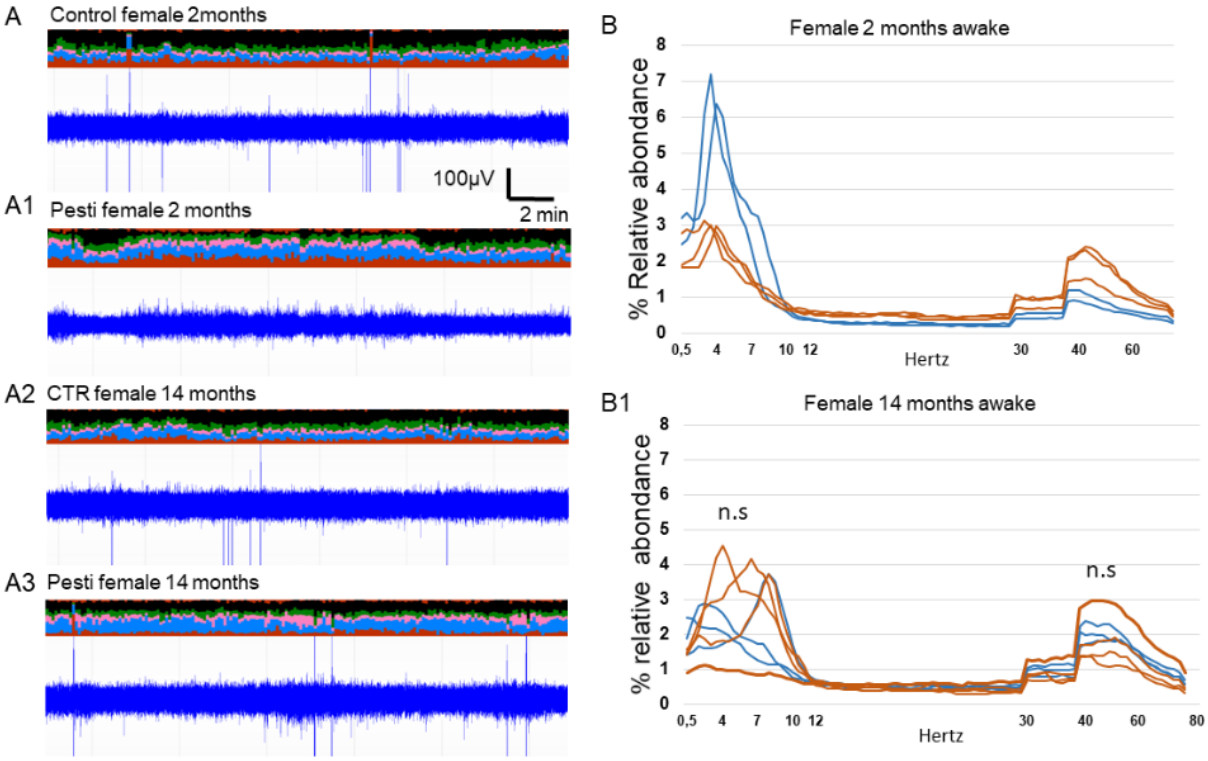
mobility								
Rotation (clockwise)	100 ± 31	110 ± 32 *	111 ± 34	119 ± 34 ****	93 ± 23	100 ± 56	-	-
Rotation (counter)	100 ± 30	110 ± 33 *	113 ± 32 *	121 ± 34 ****	98 ± 27	100 ± 56	-	-
Heading center	100 ± 844.7	21 ± 1181	68 ± 236	271 ± 1237	-474.4 ± 1791	18 ± 1708	-	-
Angular velocity	100 ± 3442	-132 ± 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- THIACLOPRID								
	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	-	-	-

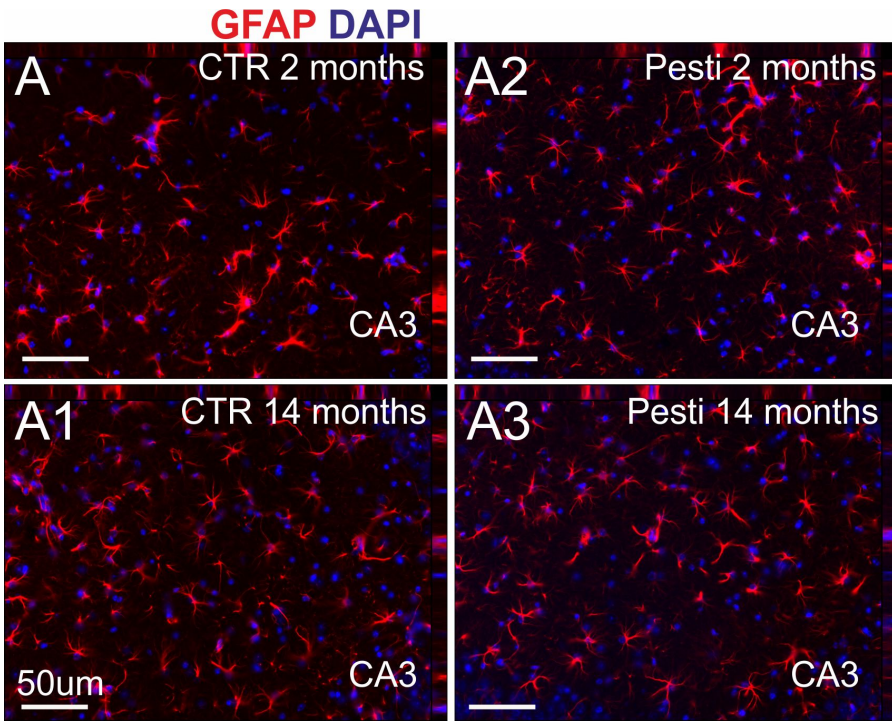


Supplemental Figure 1. Perinatal exposure to dietary pesticides does not alter behavior in adult female mice. The panels indicate the behavioral parameters measured. *Open field test and locomotor activity*: (**A**) speed, (**B**) distance travelled, (**C**) time spent in the center zone and (**D**) 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

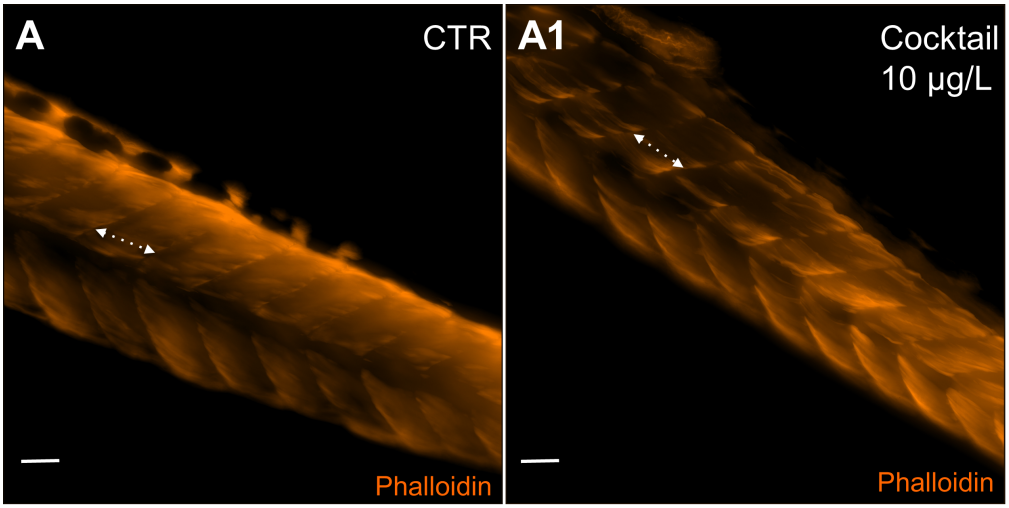
per entry and (**I**) number of entries in the light zone. *Y-Maze test (working memory)*: (**J**) number of entries and (**K**) percentage of spontaneous alternation in the Y-Maze. Rotarod (procedural and motor learning): (**L**) Latency of fall (s) following a training period of 5 days. 12 female control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



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



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.



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

Supplemental Table 1. Chemical families, functions, and tolerable daily intake (TDI) of each pesticide. Expected and measured pesticide concentrations (µg/kg food) in the food pellets. Adapted from Smith et. al 2020.

Pesticide name	Chemical family	Function	Tolerable Daily Intake (mg/kg BW/day)	Expected quantity [a] (µg/kg food)	Determined level (µg/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

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

[a] Expected quantity refers to the incorporated quantities of pesticides in mice pellets.

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