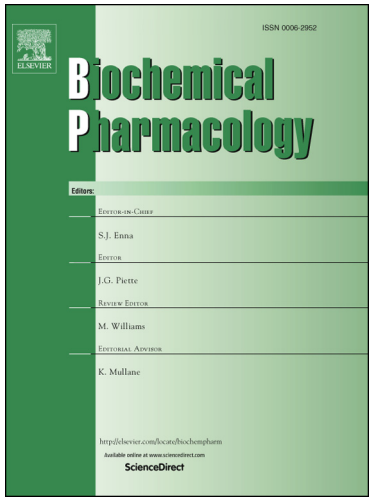
Journal Pre-proofs



Acetaminophen affects the survivor, pigmentation and development of craniofacial structures in zebrafish (*Danio rerio*) embryos

Vanessa P. Cedron, Andrea MJ Weiner, Manuel Vera, Laura Sanchez

PII: S0006-2952(20)30026-5
DOI: https://doi.org/10.1016/j.bcp.2020.113816
Reference: BCP 113816

To appear in: *Biochemical Pharmacology*

Received Date: 6 November 2019
Accepted Date: 15 January 2020

Please cite this article as: V.P. Cedron, A. MJ Weiner, M. Vera, L. Sanchez, Acetaminophen affects the survivor, pigmentation and development of craniofacial structures in zebrafish (*Danio rerio*) embryos, *Biochemical Pharmacology* (2020), doi: https://doi.org/10.1016/j.bcp.2020.113816

Manuscript, January 2020

Do not distribute without authors' permission

Acetaminophen affects the survivor, pigmentation and development of craniofacial structures in zebrafish (*Danio rerio*) embryos

Vanessa P. Cedron[1a], Andrea MJ Weiner[2a], Manuel Vera[1*], Laura Sanchez[1*]

[1] Departamento de Zoología Genética y Antropología Física. Facultad de Veterinaria. Universidade de Santiago de Compostela. Campus de Lugo. 27002 Lugo, Spain

[2] Instituto de Biología Molecular y Celular de Rosario (IBR), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) - Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), Ocampo y Esmeralda, (S2000EZP) Rosario, Argentina

Category: Toxicology

*Corresponding authors: Manuel Vera and Laura Sanchez.

Departamento de Zoología Genética y Antropología Física. Facultad de Veterinaria. Universidade de Santiago de Compostela. Campus de Lugo. 27002 Lugo, Spain. E-mails: manuel.vera@usc.es and lauraelena.sanchez@usc.es; Tel: +34 982 82 24 29; Fax: +34 982 82 24 28

[a] V.P. Cedron and A.M.J. Weiner have contributed equally to this work and are first co-authors.

Running title: Acetaminophen effects in zebrafish development

Declarations of interest: none

Abstract

In spite of its toxic effects, N-acetyl-p-aminophenol (APAP), also commonly known as acetaminophen or paracetamol, is one of the most widely used analgesic and antipyretic agents. It can be obtained without a medical prescription. To test the effect over the zebrafish embryonic development, a Fish Embryo acute Toxicity (FET) test was carried out with acetaminophen to establish the range of concentrations that cause a harmful effect on the zebrafish development. Diminished pigmentation (in embryos treated from 0 h post-fertilization) and blockage of melanin synthesis (in larvae treated from 72 h post-fertilization) were detected, suggesting the involvement of this compound in the development of black pigment cells as described recently for human epidermal melanocytes. Morphological abnormalities such as aberrant craniofacial structures, pericardial edemas, and blood accumulation were also found. All these effects could be due to higher levels of apoptotic cells detected in treated embryos. Therefore, teratogenic effects of acetaminophen cannot be ruled out, and its wide use should be taken with caution.

Keywords: acetaminophen; toxicity test; depigmentation; morphological abnormalities; embryonic development.

1. Introduction

The zebrafish (*Danio rerio*) has emerged as a popular vertebrate model in different areas of research such as genetics, pharmacology and toxicology [1–6]. The zebrafish has several features that make it attractive as an animal model. As an oviparous species, zebrafish has external fertilization and development, which allows easy detection of morphological alterations. The species show rapid growth, developing transparent embryos during the first 24-48 hours post-fertilization (hpf), with all main organs formed within 48-96 hpf, and they have high egg yield with large embryos, short generation time and low maintenance cost [7–9]. All these characteristics make the zebrafish embryos/larvae as a suitable model for pharmacological tests and screening for potential adverse effect of drugs, particularly during the developmental process [10–12].

N- acetyl-p-aminophenol (APAP, also commonly known as acetaminophen or paracetamol) is one of the most widely used analgesic and antipyretic agents [13]. Its action is focused on hypothalamic center, where acetaminophen regulates the temperature. In addition, acetaminophen inhibits the synthesis of prostaglandins in the Central Nervous System (CNS), blocking the generation of painful impulse at peripheral level [13–15]. The liver excretes the acetaminophen alongside sulphate, glucuronic acid, and the cytochrome P450 [16,17]. Thus, acetaminophen intoxication and/or overdose produce hepatic necrosis, which can cause renal insufficiency, myocardial damage, and hematological alterations [18–20].

The Fish Embryo Acute Toxicity (FET) test was developed and registered by the Organization for Economic Co-operation and Development (OECD) [21]. This test allows to analyze the effect of different drugs on zebrafish embryos in a short period of time. The test has a reduced cost and enables the analysis of a vast number of samples, which gives robust statistic results [22]. Also, the 3Rs rule (Reduction, Refinement, and Replacement) is one of the main objectives in animal experimentation, so using zebrafish embryos for toxicity test is

desirable and useful [23,24]. In a previous study, the embryotoxic effects of acetaminophen on the development of zebrafish were evaluated [10]. Acetaminophen induced anomalies at different levels of development in a dose-dependent manner, causing impairment in the early development, hatching, organogenesis, larval growth, tail and tail-fin formation, pigmentation, and larval behavior and survival. Nevertheless, authors did not report a profound cellular and molecular study, making superficial and descriptive observations on the causes of all the disruptions. Besides, a metabolic activation *D. rerio* teratogenic assay (m*Dar*T) was developed for acetaminophen [25]. By this assay, authors observed a concentration dependency for teratogenic effects (malformation of heart and tail) in embryos exposed to the drug.

The neural crest (NC) is a transient, pluripotent stem cell population whose formation occurs early in vertebrate development at the border of the developing neural tube. After closure of the neural tube, NC cells (NCC) experience an epithelial-to-mesenchymal transition in order to delaminate and migrate away [26]. NCC differentiate into a variety of derivatives, including neurons and glia of the enteric, sensory, and autonomic nervous system, pigment cells of skin, chromaffin cells, bone and cartilage of the face, endocrine cells, cardiac structures, smooth muscle cells, and tendons [27]. Mutations in NC genes lead to disease in humans, highlighting the importance of this cell population for human health. Animal models faithfully recapitulate these defects demonstrating functional conservation. The *soxE* genes encode Sry-related transcription factors and are expressed early in NC development as part of the network of NC specifiers [28]. Zebrafish mutants have revealed specific roles for *soxE* genes in development; *sox9b* is involved in craniofacial development [29] and *sox10* in the specification of all pigment cells, dorsal root ganglia, sympathetic and enteric neurons, and glia [30–33]. Regarding melanocyte development, several studies have shown that *sox10* can directly activate the expression of *mitf* (*microphtalmia-associated transcription factor*, the

key master gene for melanocyte specification), when then promote the activation of melanogenic enzymes [34–36]. However, in fish a decrease in *sox10* mRNA levels from 28 hpf onwards, involving a microRNA regulatory loop, is necessary to allow the expression of melanogenic enzyme genes [37,38].

The aim of the present study was to understand: (i) the toxicity and (ii) the effects of acetaminophen on the embryonic development of zebrafish. Toxicity was estimated via an acute toxicity test on zebrafish embryos; this test was used to establish the acetaminophen toxic range for carrying out the subsequent analyses of its effect in morphological traits. Several malformations as well as higher levels of apoptotic cells were detected in acetaminophen-treated larvae. After morphological tests, a lack of black pigmentation and aberrant craniofacial structures in treated embryos and larvae were observed. These results suggest that the compound affects normal embryonic development as well as some NCC derivatives and highlights the need for control in the use of acetaminophen.

2. Material and methods

2.1. Embryo production for the tests

The breeding stock of wild-type adult zebrafish was maintained under a photoperiod/light cycle condition of 14 light hours (from 8h to 22h) and 10 darkness hours (from 22h to 8h) on a recirculating water system. Water conditions were maintained at a constant 7-7.5 pH, 400-600µS/cm conductivity and 26-28ºC of temperature. Adult fish were fed three times each day, first with *Artemia sp.* (at 9 h), then with Tetramind compound (Tetramind®, at 12 h) and finally with Gemma Micro feed (Skretting, at 15 h). Individual fish used as breeders were also fed again with *Artemia sp.* at 17 h in the afternoon. Fish were macroscopically free of

apparent symptoms of disease and were not subjected to any pharmaceutical treatment for the previous two months to the spawning.

Breeder fishes were separated by sexes in 3 L tanks. The mean number of individuals per tank was seven. The day before obtaining the embryos, breeders were merged in a 10 L tank with a spawning cage. Fertilized eggs were deposited in the cage, which was lined with marbles to avoid their depredation by adult fish. Finally, these eggs were washed with autoclaved osmosis water to maintain their quality.

2.2. Toxicity experiments

Pure commercial acetaminophen (Sigma-Aldrich, A5000, CAS number 103-90-2, San Luis, MO, USA) was used for all the toxicity experiments. Dilutions were carried out using autoclaved osmosis water.

All these experiments were performed according to OECD 236-2013 guidelines (Fish Embryo Acute Toxicity Test). In each test, six increasing concentrations of acetaminophen (2.5, 3.5, 4.9, 6.9, 9.6, and 13.4 mM) and a control with autoclaved osmosis water were used. Before the exposure, all the embryos were carefully selected using a dissecting microscope. The type of exposure regimen started from one-cell stage (0 hpf) up to the stage evaluated. All tests were performed in triplicates using 24-well plates composed of 20 samples under treatment and 4 samples used as internal control (total number of embryos treated at 0 hpf = 7 concentrations (including negative control) x 3 replicates x 24 individuals = 504). The plates were checked at 24, 48, 72, and 96 hpf to identify the surviving embryos in each stage of the test and following OECD guidelines,.

All the experiments and protocols were approved by the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (CEEA-LU-003 and Directive 2012-63-UE).

The lethal concentrations (LC) of acetaminophen necessary to kill the 10% (LC10), 25% (LC25) and 50% (LC50) of zebrafish larvae at the end of the test were calculated following the OECD 236-2013 test guidelines and implemented in the ToxRat Professional ver. 3.2.1 software (ToxRat® Solutions GmbH). The 95% confidence intervals (CI) for each LC value were also calculated using the ToxRat software. Two-way ANOVA followed by pairwise comparisons and Dunnett's multiple comparison tests [39] were used to study the interaction between concentration and time exposure. The significance level for all the statistic tests was 95% ($\alpha = 0.05$). All these analyses were performed using the SPSS Statistics v.24.0 software (IBM).

2.3. Morphological studies

Morphological abnormalities (e.g. malformation of spine or yolk sac, pigmentation, blood accumulation) were tested for embryos treated at 0 hpf at concentrations under 2.5 and 4.9 mM (see results section). Negative controls exclusively reared with osmosis water were included. For all the different tests, embryo and larvae images were taken over a 1.5% (w/v) agar plate using an AZ-100 Nikon fluorescence stereomicroscope (Nikon), with 40X augments and compared with those taken for controls at the same stage.

2.4. Acridine Orange stainings

Forty-eight hpf live drug-treated and control embryos were manually dechorionated and stained with the vital dye Acridine Orange (Sigma-Aldrich, A6014-10G, CAS number 10127-02-3, San Luis, MO, USA) as previously described [40]. The *in vivo* staining was repeated two times in a total of 50 embryos. To perform a comparative analysis, fluorescent pictures were taken with an AZ-100 Nikon fluorescence stereomicroscope (Nikon) with the same exposure time (14.7 ms). Imaging analysis for quantification was performed with QuantiFish software (https://zenodo.org/record/1182791; 2017).

2.5. Melanin quantification and black pigment cell counting

Several concentrations of acetaminophen (2.5, 4.9, and 6.9 mM) were prepared. There were two types of exposure regimen: one group received exposure starting from 0 hpf until collection (24, 48, 72, and 96 hpf) and the other group from 72 hpf until collection (96, 120, 144, and 168 hpf). Twenty embryos from each stage and group were manually dechorionated and used to measure melanin levels as previously described [38,41]. Results were obtained and processed from five independent experiments for each condition. For melanocyte counts, ten larvae of the group that received exposure starting from 0 hpf were analyzed at 48 and 72 hpf under the microscope. Black pigment cells from the lateral stripe were counted in the tail alongside the yolk extension for each condition and controls.

2.6. Alcian blue staining

Five days post-fertilization larvae exposed to different concentrations from 0 hpf or 72 hpf were fixed in 4% (w⁄v) paraformaldehyde (PFA) in phosphate-buffered saline 1X (PBS) with 0.1% (v⁄v) Tween-20 (PBT 1X), and washed four times in PBT 1X. The protocol was performed as detailed elsewhere [42]. Pictures of cartilage-staining larvae were taken with an AZ-100 Nikon fluorescence stereomicroscope (Nikon). Cranial cartilages measurements were taken as reported [43,44], using the ImageJ software (National Institutes of Health, Bethesda, MD, USA) [45].

2.7. RT-qPCR assays

Total RNA from 10 embryos (controls and embryos exposed from 0 hpf to 4.9 mM of acetaminophen) was obtained using TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified RNA was incubated with RQ1 DNAse (Promega, Madison, WI, USA) and retro-transcribed with Revert Aid RT enzyme (Thermo Scientific, Waltham, MA, USA) and oligo(dT) primer. Quantitative PCR (qPCR) reactions

were performed using four different RNA purifications and three independent experiments using a Stratagene Mx3005P (Agilent Technologies, Santa Monica, CA, USA) and standard temperature protocol. Primer sequences were as follows: *dct* forward, TCTTCCCACCTGTGACCAAT; *dct* reverse, ACATAGCCGGTCTGTTGTCC; *mitfa* forward, CTGGACCATGTGGCAAGTTT; *mitfa* reverse, TGAGGTTGTGGTTGTCCTTCT; *sox9b* forward, GGACATCGGCGAGCTGAGCA; *sox9b* reverse, CTGGAGAACCCTGCACCGGC; *sox10* forward, AAAACACTGGGGAAGCTGTG; *sox10* reverse, ACGTGGCTGGTACTTGTACT. Predicted PCR products span at least one intron to ensure amplification solely from the cDNA and not from genomic DNA. *Ef1α* and *βactin* were used as endogenous control for normalization analysis [46]. Data were analyzed using qBase software version 2.2 and *t*-student tests were employed as indicated.

3. Results

3.1. Toxicity tests

Embryos from 0 hpf were exposed to several concentrations of acetaminophen (2.5, 3.5, 4.9, 6.9, 9.6, and 13.4 mM) until analysis (24, 48, 72, and 96 hpf). All ANOVA tests found significant differences for acetaminophen concentration ($F = 347.346$, $P < 0.001$), time ($F = 374.651$, $P < 0.001$), and their interaction ($F = 106.354$, $P < 0.001$) (Table 1). Dunnet tests showed significant differences ($P_{\text{Dunnet's test (I-J)}} < 0.001$) between the control and the three highest concentrations (i.e. 6.9, 9.6, and 13.4 mM) while no differences (P Dunnet's test (I-J) > 0.010) were found for the three lowest concentrations (2.5, 3.5, and 4.9 mM). Significant pair-wise differences were also found between the exposure times ($P < 0.001$), except for the pair 0 h and 24 h (Dunnet's test (I-J) = |1.9|, $P = 0.252$). Therefore, the survival percentage was constant until 24 h of exposure, where survival reduction started for all the treatments in

a similar way until 96 hpf. LC10, LC25, and LC50 values were 4.393 (95% Interval Confidence = 1.879 – 5.647), 5.353 (3.120 – 6.728), and 6.668 (95% Interval Confidence = 4.958 – 9.039), respectively. Thus, two different acetaminophen concentrations included in the LC10 interval which had no significant mortality differences with negative controls (i.e. 2.5 mM and 4.9 mM) were used for morphological studies.

3.2. Morphological abnormalities

Gross morphological abnormalities such as pericardial blood accumulation, pericardial and peritoneal edema, and spinal abnormalities (e.g.cifosis) were observed in embryos treated with 2.5 and 4.9 mM of acetaminophen from 0 hpf until 24, 48, 72, and 96 hpf (Fig. 1). Thus, these abnormalities were more difficult to identify in 13.4 mM treatments because they presented high mortality at early stages where anomalies had not manifested yet. The most important abnormality was the edema (both pericardial and peritoneal), which was observed even in the treatment with the lowest acetaminophen concentration (i.e. 2.5 mM) at the end of the test (96 hpf) (Table 2).

In zebrafish, acetaminophen treatment results in significant affections of tail fin development by apoptosis [10,25]. To explain the described malformations, we used Acridine Orange staining to see and quantify apoptotic cells in whole-mount 4.9 mM acetaminophen-treated embryos (Fig. 2A-C). At 48 hpf, treated embryos displayed significantly higher levels of apoptosis than controls all along the body region (Fig. 2D; N=50, *P < 0.0001). Besides, a greater number of apoptotic nuclei were observed in the tail fin of drug-exposed specimens (compare Fig. 2B’ with 2C’), as was previously reported [10]. These observations could explain the morphological phenotypes observed with drug treatment.

3.3. Effects of acetaminophen in neural crest derivatives

Zebrafish pigmentation is characterized by the presence of melanocytes, iridophores, and xanthophores, three different kinds of pigment cells derived from NCC [47]. Embryos exposed from 0 hpf to different concentrations (2.5, 3.5, 4.9, 6.9, and 9.6 mM) of acetaminophen showed no black pigmentation along the spinal column, yolk sac, head, and eyes (data not shown). To address the mechanisms underlying the observed phenotype in black pigment cell, melanin levels in controls as well as embryos treated with 2.5, 4.9, and 6.9 mM of pure acetaminophen from 0 hpf were measured at different developmental stages (Fig. 3A), following an established protocol [38,41]. All three concentrations showed significantly decreased levels of melanin when compared to controls at 48, 72, and 96 hpf, whereas differences in melanin levels based on drug concentration were undetectable (Fig. 3C).

Black pigment cells in zebrafish larvae at two developmental stages were counted to determine the impact of acetaminophen treatment on the number of melanocytes (Fig. 3A). A significant decrease in the number of black pigment cells in the lateral stripe alongside the yolk sac extension of embryos treated from 0 hpf with 2.5 and 4.9 mM of acetaminophen was observed (Fig. 3E-F). The reduction in the number of black pigment cells reinforced previous observations of melanin measurements as well as recently published cell culture results [48], suggesting a pigmentation problem due to defects in melanin synthesis and cell survival. When comparing all these results, the effect of acetaminophen on melanin production were not concentration-dependent, but the effect on cell number was. This observation could be attributable to the way the experiments were developed. Since melanin quantification were performed within a group of 20 embryos, this could have masked the concentration effects evidenced by counting black pigment cells individually. Additionally, it should be noted that melanin levels were measured in whole-mount specimens, whereas the counting of black pigment cells was performed in a portion of larvae tails.

Melanocytes showing black pigment melanin are evident by 25 hpf in zebrafish embryos [47]. At 72 hpf, larvae would have developed an important amount of black pigment cells, so we decided to test melanin levels in larvae from this stage. Larvae at 72 hpf were incubated with 2.5 and 4.9 mM of acetaminophen and melanin was measured at 96, 120, 144, and 168 hpf (Fig. 3B). From 120 hpf onwards, melanin levels were significantly reduced compared to the controls (Fig. 3D), suggesting an effect of acetaminophen in novel NCC differentiation and melanin synthesis. All these results suggest that acetaminophen treatment was affecting black pigment cell survival and melanin synthesis, regardless of the time-point at which the embryos or larvae came in contact with the compound.

Acetaminophen-treated larvae showed defects in head formation; therefore, we studied the craniofacial development by Alcian Blue staining (Fig. 4A). For all concentration treatments, defects in craniofacial development were detected, with defects becoming more aberrant at higher concentrations that do not allow a quantification analysis (Fig. 4B-G). Malformations were principally detected in lower mandible cartilages affected by 3.5 mM treatment (Fig. 4D), while the ethmoid plate and trabeculae appeared stained until 6.9 mM treatment (Fig. 4F). Meckel's cartilage and lower jaw were barely detectable at 4.9 mM treatment (Fig. 4E) and ceratobranchial arches 1–5 were difficult to distinguish in all concentration treatments (Fig. 4B-G). The progressive worsening of the phenotype from anterior to posterior arches may reflect the time course of cartilage differentiation, which progresses in an anterior-to-posterior wave [49]. The 2.5 mM treatment was the only one that could be quantified against the controls. This concentration treatment caused significant shortening in the lengths of Meckel (M), ceratohyal (CH) and hyosymplectic-palatoquadrate (PQ) cartilages (Fig. 4H, N=25). The Meckel area (defined as the area of triangle shaped by Meckel cartilages), the distance between ceratohyal cartilages joint and the lateral fins, and the cranial distance (measured as the distance from the anterior-most M to the lateral fins) also showed significant

reductions (Fig. 4H, N=25). Moreover, the angle formed by the ceratohyal cartilages was significantly more acute than in controls (Fig. 4I, N=25). These defects could be attributed to the observed increase in apoptotic cells during embryonic development. Besides, it is worth noting that all these structures are among the NCC derivatives, suggesting that compound treatment could affect NCC normal development and craniofacial structures in a dose-dependent manner.

Almost all-cranial cartilages had been developed in zebrafish larvae during the hatching period in zebrafish (48-72 hpf) [49]. By 72 hpf, the cranial cartilage differentiation process is completed and is followed by cell proliferation, new condensations and joints of the former ones [50]. The study of affectations over these pre-formed craniofacial structures when incubated with pure acetaminophen from 72 hpf was also carried out. Larvae at 72 hpf were incubated with 2.5 and 4.9 mM of acetaminophen, fixed at 120 hpf, and stained with Alcian Blue (Fig. 5A). Again, defects in craniofacial development were detected in both cases, with defects becoming more aberrant at 4.9 mM concentration (compared Fig. 5B with 5C-D). Malformations were principally detected in lower mandible cartilages; the ceratobranchial arches 3–5 were difficult to distinguish while the ethmoid plate and trabeculae appeared normal (Fig. 5C-D). Both treatments were quantifiable against controls, causing significant shortening of ceratohyal (CH) and hyosymplectic-palatoquadrate (PQ) cartilages lengths (Fig. 5E, N=25). The distance between the joints of ceratohyal cartilages and the lateral fins, and the cranial distance also showed significant reductions (Fig. 5E, N=25). However, the Meckel length and area (Fig. 5E), as well as the angles formed by the Meckel and ceratohyal cartilages, were not significantly disturbed (not shown). These results suggest that although craniofacial cartilage had already been differentiated by 72 hpf, the 48h treatment with acetaminophen (from 72 to 120 hpf) affected enlargement, condensation, and joints formation

during zebrafish larvae development. Besides, these effects seem to be predominant in the posterior cartilages, the ones that formed at the later stages of craniofacial development [49].

To further explain the abnormalities observed in NCC derivatives, we considered if the expression of NC marker genes could be affected by acetaminophen treatment. Thus, the expression of *sox9b* and *sox10* in embryos treated from 0 hpf with 4.9 mM of acetaminophen were examined by RT-qPCR at different developmental stages. *Sox9b* expression decreased significantly in acetaminophen-treated embryos at 24 hpf (Fig. 6A), thus reinforcing the presence of craniofacial phenotypic abnormalities. Conversely, *sox10* expression was significantly enhanced at the two stages analyzed (from 15 to 24 hpf; Fig 6B). To further explain the increase in *sox10* mRNA levels, the expression of other genes involved in melanocyte differentiation, such as *mitfa* and *dct* (*dopachrome tautomerase*, enzyme involved in melanin biosynthesis) were also analyzed. The expression of *mitfa* was significantly diminished in acetaminophen-treated embryos at 48 hpf (Fig. 6C) and a significant reduction of *dct* mRNA was detected in treated embryos at both 24 and 48 hpf (Fig. 6D). Taking this into account, results suggest that the pale phenotype observed in acetaminophen-treated embryos could be due to *sox10* up-regulation and, consequently, *dct* down-regulation. Our data indicate that acetaminophen treatment affects the relative abundance of NC marker genes, such as *sox9b* and *sox10* mRNAs, and thus its downstream associated effectors thus could be responsible for the craniofacial and pigmentation anomalies during zebrafish development.

4. Discussion

Since the 1980's, the use of zebrafish as vertebrate animal model has boomed due to its previously reported properties [8,51] and its suitability for many toxicity tests [1,10,52–55]. During FET toxicity tests, all embryos incubated with 13.4 mM of acetaminophen from 0 hpf

328 died at 96 hpf. When embryos were exposed to 9.6 mM, only 5% survived at the end of the
329 experiment. A recent study analyzing the protective role of the heptametoxyflavone isolated
330 from the weed *Sphaeranthus amaranthoides* showed the important effect of acetaminophen
331 toxicity on zebrafish embryos exposed from 0 hpf, although 100% mortality rate was reached
332 at 72 hpf at a concentration of 10 mM of compound [56]. Embryos exposed from 72 hpf were
333 more resistant to higher concentrations of acetaminophen treatments than embryos exposed
334 from 0 hpf. The explanation for this may be that hepatocytes are formed at 32 hpf and liver is
335 functional at 72 hpf [57]. Therefore, 72 hpf larvae are capable of metabolizing drugs [58],
336 suggesting that acetaminophen is metabolized and eliminated before causing fish death.
337 Similar to toxicity tests, zebrafish has also been used to evaluate teratogenic embryo
338 alterations [25,59]. In this study, all embryos exposed to acetaminophen showed
339 morphological alterations, including depigmentation, pericardial blood accumulation,
340 pericardial and peritoneal edemas, and craniofacial and spinal abnormalities (Fig. 1 and Table
341 2). It is known that this kind of alterations are produced by teratogenic effects [60]. Similar
342 spinal abnormalities have been found when zebrafish embryos are exposed to the teratogenic
343 compounds, chrome trioxide (CrO_3) and mercury chloride ($HgCl_2$) [25,61,62]. Teratogenic
344 antiepileptic drugs caused similar edemas in zebrafish embryos [63]. Peritoneal and
345 pericardial edemas can be consequences of toxin accumulation, which disturbs
346 osmoregulation system, leading to abdominal and pericardial hyperhydration [64,65]. Heart
347 and tail malformations were also identified in zebrafish embryos subjected to m*Dar*T assays
348 with acetaminophen in combination with a mammalian metabolic activation system [25]. All
349 these malformations could be explained by the increase in apoptotic cells observed in treated
350 embryos of zebrafish (Fig. 2).

351 Congenital abnormalities (i.e. those present at birth) are the cause of many childhood deaths
352 and pediatric hospitalizations, with environmental teratogenic factors (e.g. drugs, viruses,

physics, and chemical pollutants, etc.) being an important cause for these abnormalities [66]. Acetaminophen is the most used analgesic and antipyretic agent for patients who cannot receive opioids or non-steroidal anti-inflammatory drugs (NSAIDs). Moreover, acetaminophen can be obtained without a medical prescription [15]. This compound was initially considered as totally safe in therapeutic doses [67–69], even during pregnancy [70]. However, recent studies have described different teratogenic effects caused by acetaminophen, such as the development of asthma in children at early ages [71,72], changes in the endocrine system causing cryptorchidism in children, or alterations in the nervous system leading to attention deficit hyperactivity [13,73,74]. Therapeutic doses in humans are 10-15 mg/Kg of weight (representing a concentration of 0.93-1.39 mM for an individual of 70 Kg), while doses of 150 mg/Kg (13.9 mM) are considered potentially toxic [68]. These doses would be within the concentration range used in the present study, considering that acetaminophen concentration within embryos could be lower than those of treatments.

Pigmentation in the zebrafish embryos starts around 25 hpf in the retinal epithelium and is extended to the skin afterwards [47]. Reduced pigmentation in zebrafish embryos had been previously described in the presence of acetaminophen (3.3×10^{-4} mM), with a dose-dependent decrease of pigment distribution in the retinal epithelium and skin cells [10]. However, these authors did not quantify the observations nor describe the metabolic route(s) involved in this process. Another study carried out using human epidermal melanocytes had shown that acetaminophen concentrations ≥ 2.0 mM inhibits melanization in these cells by reducing tyrosinase activity and decreasing their melanin content [48]. In our study, a reduction in melanin concentration as well as absence of black pigment cells was observed in treated zebrafish larvae at concentrations between 2.5 and 6.9 mM, values included in the range analyzed by Wrzesniok et al. (2016) [48] (from 2.0 to 20.0 mM). However, we observed a higher decrease in the percentage of melanin detected between controls and drug-

treated larvae at 48, 72, and 96 hpf. Treatment of cells with acetaminophen at concentrations of 2.0 and 20.0 mM decreased in melanin content to 93% and 89%, respectively. Though, 4.9 mM treated larvae showed a decrease in melanin content of 83.9, 65.1, and 60.3% through development (at 48, 72, and 96 hpf, respectively). All these observations might suggest that in the whole embryo the treatment sffects not only the pathway of melanin synthesis but also the development of black pigment cells. Besides, the results demonstrated that larvae treated from 72 hpf are unable to increase their melanin levels, suggesting a blockage in black pigment cell differentiation and/or melanin synthesis. Although acetaminophen could also act in a similar way in zebrafish melanocytes, i.e. avoiding the metabolization of tyrosine necessary for the production of melanin [75], the alterations detected in the expression of genes involved in melanocyte specification and differentiation reinforce the notion that NC development is notably affected. Interestingly, effects on craniofacial development had not been reported to date. Here, we noted that craniofacial anomalies observed in acetaminophen-treated larvae are dose-dependent and *sox9b* expression is reduced in treated embryos. Moreover, craniofacial structures are also affected when larvae of 72 hpf are treated with acetaminophen, indicating that the compound affects the proliferation, composition, and joint formation of differentiated cranial NCC. In particular, we gathered data suggesting that acetaminophen treatment affects NCC specification and differentiation processes, principally by modifying the expression of *soxE* genes and impacting their derivatives.

Zebrafish is a good model for analyzing health risk in humans. In the present study we corroborated that acetaminophen affects embryonic development with some morphological and teratogenic effects. Further, our results support an influence of this compound on the development of zebrafish pigmentation and craniofacial structures. Studies based on NC gene expression analysis lead us to suggest an acetaminophen effect in NCC specification and

differentiation. Future studies focused on acetaminophen (even their metabolites) will be necessary to improve the knowledge about the toxicity of this globally used compound.

Acknowledgments

Authors wish to thank M. Otero and S. Gómez for their technical support. The authors are also indebted to Dr. J.G. Piette and two anonymous reviewers for their constructive comments on the earlier version of this manuscript. This study has been supported by funding from GI-1251-ACUIGEN research group of the University of Santiago de Compostela (Spain) and Xunta de Galicia project (ED431C 2018/28).

References

[1] S. Dasgupta, S.M. Vliet, A. Kupsco, J.K. Leet, D. Altomare, D.C. Volz, Tris(1,3-dichloro-2-propyl) phosphate disrupts dorsoventral patterning in zebrafish embryos, PeerJ. 5 (2017) e4156. doi:10.7717/peerj.4156.

[2] C.F. Howe, K., Clark, M.D., Torroja, J. Torrance, C. Berthelot, S. Muffato, M., Collins, J.E. Humphray, K. McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J.C. Barrett, R. Koch, G.-J. Rauch, S. White, D.L. Chow, W, The zebrafish reference genome sequence and its relationship to the human genome, Nature. 496 (2013) 498–503. doi:10.1038/nature12111.

[3] G.J. Lieschke, P.D. Currie, Animal models of human disease: zebrafish swim into view, Nat. Rev. Genet. 8 (2007) 353. https://doi.org/10.1038/nrg2091.

[4] S. Scholz, S. Fischer, U. Gündel, E. Küster, T. Luckenbach, D. Voelker, The zebrafish embryo model in environmental risk assessment - Applications beyond acute toxicity testing, Environ. Sci. Pollut. Res. 15 (2008) 394–404. doi:10.1007/s11356-008-0018-z.

[5] J. Guerra-Varela, P. Cabezas-Sainz, E. Yebra-Pimentel, C. Gutiérrez-Lovera, V.P. Cedrón, M.A. Otero Obarrio, A.A. Sciara, N. Rodríguez, J. Araujo, A. Millán, L. Sánchez, "A Zebra in the Water": Inspiring Science in Spain, Zebrafish. 13 (2016) 241–247. doi:10.1089/zeb.2015.1178.

[6] L. Ribas, F. Piferrer, The zebrafish (Danio rerio) as a model organism, with emphasis on applications for finfish aquaculture research, Rev. Aquac. 6 (2014) 209–240. doi:10.1111/raq.12041.

[7] D.K. Badyal, C. Desai, Animal use in pharmacology education and research: the changing scenario, Indian J. Pharmacol. 46 (2014) 257–265. doi:10.4103/0253-7613.132153.

[8] S. Sarasamma, M.M. Varikkodan, S.-T. Liang, Y.-C. Lin, W.-P. Wang, C.-D. Hsiao, Zebrafish: A Premier Vertebrate Model for Biomedical Research in Indian Scenario, Zebrafish. 14 (2017) 589–605. doi:10.1089/zeb.2017.1447.

[9] Z. Xu, Developmental Toxicity of Dextromethorphan and Acetaminophen in Zebrafish

Embryos/Larvae: Relevance of SULT-mediated Dextromethorphan/Acetaminophen Sulfation, Theses. Univ. Toledo. (2010). https://etd.ohiolink.edu/!etd.send_file?accession=toledo1271433014&disposition=inline.

[10] A. David, K. Pancharatna, Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, Danio rerio, J. Appl. Toxicol. 29 (2009) 597–602. doi:10.1002/jat.1446.

[11] C.A. MacRae, R.T. Peterson, Zebrafish as tools for drug discovery, Nat. Rev. Drug Discov. 14 (2015) 721. https://doi.org/10.1038/nrd4627.

[12] C. Maximino, A.W.B. da Silva, A. Gouveia, A.M. Herculano, Pharmacological analysis of zebrafish (Danio rerio) scototaxis, Prog. Neuro-Psychopharmacology Biol. Psychiatry. 35 (2011) 624–631. doi:10.1016/j.pnpbp.2011.01.006.

[13] A. Aminoshariae, A. Khan, Acetaminophen: Old Drug, New Issues, J. Endod. 41 (2015) 588–593. doi:10.1016/j.joen.2015.01.024.

[14] D.J. Douzjian, A. Kulik, Old Drug, New Route: A Systematic Review of Intravenous Acetaminophen After Adult Cardiac Surgery, J. Cardiothorac. Vasc. Anesth. 31 (2017) 694–701. doi:10.1053/j.jvca.2016.03.134.

[15] E. Roberts, V.D. Nunes, S. Buckner, S. Latchem, M. Constanti, P. Miller, M. Doherty, W. Zhang, F. Birrell, M. Porcheret, K. Dziedzic, I. Bernstein, E. Wise, P.G. Conaghan, Paracetamol: Not as safe as we thought? A systematic literature review of observational studies, Ann. Rheum. Dis. 75 (2016) 552–559. doi:10.1136/annrheumdis-2014-206914.

[16] J.E. Laine, S. Auriola, M. Pasanen, R.O. Juvonen, Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes, Xenobiotica. 39 (2009) 11–21. doi:10.1080/00498250802512830.

[17] U.B. Ghaffar, N.A. Tadvi, Paracetamol Toxicity : A Review Epidemiology of Toxicity Etiology and Pathophysiology of Paracetamol Toxicity, J Cont Med A Dent. 2 (2014) 12–15. doi:10.18049/jcmad/232.

[18] M. Blieden, L.C. Paramore, D. Shah, R. Ben-Joseph, A perspective on the epidemiology of acetaminophen exposure and toxicity in the United States, Expert Rev. Clin. Pharmacol. 7 (2014) 341–348. doi:10.1586/17512433.2014.904744.

[19] J. Nagai, Y. Uesawa, R. Shimamura, H. Kagaya, Characterization of the Adverse Effects Induced by Acetaminophen and Nonsteroidal Anti-Inflammatory Drugs Based on the Analysis of the Japanese Adverse Drug Event Report Database, Clin. J. Pain. 33 (2017) 667–675. doi:10.1097/AJP.0000000000000457.

[20] I.A. Sisamón, Acerca de la hepatotoxicidad del paracetamol, Rev. Del Hosp. Priv. Comunidad. 6 (2003) 4.

[21] OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test., OECD Guidel. Test. Chem. Sect. 2, OECD Publ. (2013) 1–22. doi:10.1787/9789264203709-en.

[22] T. Braunbeck, B. Kais, E. Lammer, J. Otte, K. Schneider, D. Stengel, R. Strecker, The fish embryo test (FET): origin, applications, and future, Environ. Sci. Pollut. Res. 22 (2014) 16247–16261. doi:10.1007/s11356-014-3814-7.

[23] Z.C. Dang, L.T.M. van der Ven, A.S. Kienhuis, Fish embryo toxicity test, threshold approach, and moribund as approaches to implement 3R principles to the acute fish toxicity test, Chemosphere. 186 (2017) 677–685. doi:10.1016/j.chemosphere.2017.08.047.

[24] M.R. Embry, S.E. Belanger, T.A. Braunbeck, M. Galay-Burgos, M. Halder, D.E. Hinton, M.A. Léonard, A. Lillicrap, T. Norberg-King, G. Whale, The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research, Aquat. Toxicol. 97 (2010) 79–87.

doi:10.1016/j.aquatox.2009.12.008.
[25] S. Weigt, N. Huebler, T. Braunbeck, F. von Landenberg, T.H. Broschard, Zebrafish teratogenicity test with metabolic activation (mDarT): Effects of phase I activation of acetaminophen on zebrafish Danio rerio embryos, Toxicology. 275 (2010) 36–49. doi:10.1016/j.tox.2010.05.012.
[26] E. Theveneau, R. Mayor, Neural crest delamination and migration: From epithelium-to-mesenchyme transition to collective cell migration, Dev. Biol. 366 (2012) 34–54. doi:10.1016/j.ydbio.2011.12.041.
[27] E. Dupin, N.M. Le Douarin, The neural crest, A multifaceted structure of the vertebrates, Birth Defects Res. Part C - Embryo Today Rev. 102 (2014) 187–209. doi:10.1002/bdrc.21080.
[28] P. Betancur, M. Bronner-Fraser, T. Sauka-Spengler, Genomic code for Sox10 activation reveals a key regulatory enhancer for cranial neural crest, PNAS. 107 (2010) 3570–3575. doi:10.1073/pnas.0906596107.
[29] Y.-L. Yan, J. Willoughby, D. Liu, J. Gage Crump, C. Wilson, C.T. Miller, A. Singer, C.B. Kimmel, M. Westerfield, J.H. Postlethwait, A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development, Development. 132 (2005) 1069–1083. doi:10.1242/dev.01674.
[30] R.N. Kelsh, J.S. Eisen, The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives., Development. 127 (2000) 515–25. http://www.ncbi.nlm.nih.gov/pubmed/10631172.
[31] K.A. Dutton, A. Pauliny, S.S. Lopes, S. Elworthy, T.J. Carney, J. Rauch, R. Geisler, P. Haffter, R.N. Kelsh, Zebra sh colourless encodes sox10 and speci es non-ectomesenchymal neural crest fates, Development. 128 (2001) 4113–4125. doi:10.1093/hmg/4.12.2407.
[32] S. Elworthy, J.P. Pinto, A. Pettifer, M.L. Cancela, R.N. Kelsh, Phox2b function in the enteric nervous system is conserved in zebrafish and is sox10-dependent, Mech. Dev. 122 (2005) 659–669. doi:10.1016/j.mod.2004.12.008.
[33] T.J. Carney, K.A. Dutton, E. Greenhill, M. Delfino-Machin, P. Dufourcq, P. Blader, R.N. Kelsh, A direct role for Sox10 in specification of neural crest-derived sensory neurons, Development. 133 (2006) 4619–4630. doi:10.1242/dev.02668.
[34] S. Elworthy, J. a Lister, T.J. Carney, D.W. Raible, R.N. Kelsh, Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development., Development. 130 (2003) 2809–2818. doi:10.1242/dev.00461.
[35] M. Lee, J. Goodall, C. Verastegui, R. Ballotti, C.R. Goding, Direct regulation of the microphthalmia promoter by Sox10 links Waardenburg-Shah syndrome (WS4)-associated hypopigmentation and deafness to WS2, J. Biol. Chem. 275 (2000) 37978–37983. doi:10.1074/jbc.M003816200.
[36] C. Verastegui, K. Bille, J.P. Ortonne, R. Ballotti, Regulation of the Microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, SOX10, J. Biol. Chem. 275 (2000) 30757–30760. doi:10.1074/jbc.C000445200.
[37] E.R. Greenhill, A. Rocco, L. Vibert, M. Nikaido, R.N. Kelsh, An iterative genetic and dynamical modelling approach identifies novel features of the gene regulatory network underlying melanocyte development, PLoS Genet. 7 (2011) e1002265. doi:10.1371/journal.pgen.1002265.
[38] A.M.J. Weiner, N.L. Scampoli, T.J. Steeman, C.M. Dooley, E.M. Busch-Nentwich, R.N. Kelsh, N.B. Calcaterra, Dicer1 is required for pigment cell and craniofacial development in zebrafish, Biochim. Biophys. Acta - Gene Regul. Mech. 1862 (2019) 472–485. doi:10.1016/j.bbagrm.2019.02.005.
[39] C. Dunnett, New Tables for Multiple Comparisons with a Control, Biometrics. 20

(1964) 482–491.
[40] A.M.J. Weiner, M.L. Allende, T.S. Becker, N.B. Calcaterra, CNBP mediates neural crest cell expansion by controlling cell proliferation and cell survival during rostral head development, J. Cell. Biochem. 102 (2007). doi:10.1002/jcb.21380.
[41] B. Fernandes, T. Matamá, D. Guimaraes, A. Gomes, A. Cavaco-Paulo, Fluorescent quantification of melanin, Pigment Cell Melanoma Res. (2016) 1–6. doi:10.1111/pcmr.12535.
[42] K.S. Solomon, Zebrafish foxi1 mediates otic placode formation and jaw development, Development. 130 (2003) 929–940. doi:10.1242/dev.00308.
[43] M.S. Porcel De Peralta, V.S. Mouguelar, M.A. Sdrigotti, F.A. Ishiy, R.D. Fanganiello, M.R. Passos-Bueno, G. Coux, N.B. Calcaterra, Cnbp ameliorates Treacher Collins Syndrome craniofacial anomalies through a pathway that involves redox-responsive genes, Cell Death Dis. 7 (2016) e2397. doi:10.1038/cddis.2016.299.
[44] D.R. Van Gijn, A.S. Tucker, M.T. Cobourne, Craniofacial development: Current concepts in the molecular basis of Treacher Collins syndrome, Br. J. Oral Maxillofac. Surg. 51 (2013) 384–388. doi:10.1016/j.bjoms.2012.09.008.
[45] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ : 25 years of image analysis, Nat. Methods. 9 (2012) 671–675. doi:10.1038/nmeth.2089.
[46] R. Tang, A. Dodd, D. Lai, W.C. McNabb, D.R. Love, Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization, Acta Biochim. Biophys. Sin. (Shanghai). 39 (2007) 384–390. doi:10.1111/j.1745-7270.2007.00283.x.
[47] R.N. Kelsh, M. Brand, Y. Jiang, C. Heisenberg, S. Lin, P. Haffter, J. Odenthal, M.C. Mullins, F.J.M. Van Eeden, M. Furutani-seiki, M. Granato, M. Hammerschmidt, D.A. Kane, R.M. Warga, D. Beuchle, L. Vogelsang, C. Nüsslein-volhard, Zebrafish pigmentation mutations and the processes of neural crest development, Development. 123 (1996) 369–389.
[48] D. Wrześniok, A. Beberok, M. Otreba, E. Buszman, M. Oprzondek, A. Hechmann, Effect of paracetamol on melanization process in human epidermal melanocytes, Acta Pol. Pharm. - Drug Res. 73 (2016) 653–658.
[49] T.F. Schilling, C.B. Kimmel, Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryoN, Development. 2960 (1997) 2945–2960.
[50] C.B. Kimmel, H.C. Snyder, K.D. Larison, B. Ullmann, G. Kruze, C.T. Miller, R.A. BreMiller, The Shaping of Pharyngeal Cartilages during Early Development of the Zebrafish, Dev. Biol. 203 (1998) 245–263. doi:10.1006/dbio.1998.9016.
[51] H.J. Johnston, R. Verdon, S. Gillies, D.M. Brown, T.F. Fernandes, T.B. Henry, A.G. Rossi, L. Tran, C. Tucker, C.R. Tyler, V. Stone, Adoption of in vitro systems and zebrafish embryos as alternative models for reducing rodent use in assessments of immunological and oxidative stress responses to nanomaterials, Crit. Rev. Toxicol. (2017) 1–20. doi:10.1080/10408444.2017.1404965.
[52] A. Babayigit, A. Ethirajan, M. Muller, B. Conings, Toxicity of organometal halide perovskite solar cells, Nat. Mater. 15 (2016) 247–251. doi:10.1038/nmat4572.
[53] C. Chakraborty, A.R. Sharma, G. Sharma, S.S. Lee, Zebrafish: A complete animal model to enumerate the nanoparticle toxicity, J. Nanobiotechnology. 14 (2016) 1–13. doi:10.1186/s12951-016-0217-6.
[54] K.Y. Lee, G.H. Jang, C.H. Byun, M. Jeun, P.C. Searson, K.H. Lee, Zebrafish models for functional and toxicological screening of nanoscale drug delivery systems: promoting preclinical applications, Biosci. Rep. 37 (2017) BSR20170199. doi:10.1042/bsr20170199.
[55] R.A. Hauser-Davis, J.A.N. Silva, R.C.C. Rocha, T. Saint'Pierre, R.L. Ziolli, M.A.Z. Arruda, Acute selenium selenite exposure effects on oxidative stress biomarkers and

essential metals and trace-elements in the model organism zebrafish (Danio rerio), J. Trace Elem. Med. Biol. 33 (2016) 68–72. doi:10.1016/j.jtemb.2015.09.001.

[56] Y. Swarnalatha, Protective role of heptametoxyflavone against acetaminophen induced hepatotoxicity in zebrafish, Indian J. Exp. Biol. 55 (2017) 805–810.

[57] J. Chu, K. Sadler, A New School in Liver Development: Lessons from Zebrafish, Hepatology. 50 (2009) 1656–1663. doi:10.1038/jid.2014.371.

[58] M. Driessen, A.S. Kienhuis, J.L.A. Pennings, T.E. Pronk, E.J. Van De Brandhof, M. Roodbergen, H.P. Spaink, B. Van De Water, L.T.M. Van Der Ven, Exploring the zebrafish embryo as an alternative model for the evaluation of liver toxicity by histopathology and expression profiling, Arch. Toxicol. 87 (2013) 807–823. doi:10.1007/s00204-013-1039-z.

[59] P. McGrath, C.Q. Li, Zebrafish: a predictive model for assessing drug-induced toxicity, Drug Discov. Today. 13 (2008) 394–401. doi:10.1016/j.drudis.2008.03.002.

[60] J. Bin Chen, H.W. Gao, Y.L. Zhang, Y. Zhang, X.F. Zhou, C.Q. Li, H.P. Gao, Developmental toxicity of diclofenac and elucidation of gene regulation in zebrafish (Danio rerio), Sci. Rep. 4 (2014) 1–7. doi:10.1038/srep04841.

[61] F. Prieto, O.A. Báez, Concentraciones de arsénico en agua potable y sus implicaciones de bioacumulaciíon y teratogénesis en el pez cebra (Danio rerio), Av. En Ciencias e Ing. 2 (2011) 37–50.

[62] I.V. Rivera Castillo, Determinación de la frecuencia de malformaciones en columna vertebral, opérculo y aleta en Danio rerio Hamilton, 1822, como posibles biomarcadores en la valoración de daño teratogénico., Tesis Lic En Biol. (2006) 1–86.

[63] S.H. Lee, J.W. Kang, T. Lin, J.E. Lee, D. Il Jin, Teratogenic potential of antiepileptic drugs in the zebrafish model, Biomed Res. Int. 2013 (2013). doi:10.1155/2013/726478.

[64] L. Cook, C. Paradise, B. Lom, The pesticide malathion reduces survival and growth in developing Zebrafish., Environ. Toxicol. Chem. 24 (2005) 1745–1750. doi:10.1897/04-331R.1.

[65] H. Hollert, S. Keiter, N. König, M. Rudolf, M. Ulrich, T. Braunbeck, A new sediment contact assay to assess particle-bound pollutants using zebrafish (danio rerio) embryos, J. Soils Sediments. 3 (2003) 197–207. doi:10.1065/jss2003.09.085.

[66] T. Mazzu-Nascimento, D.G. Melo, G.G. Morbioli, E. Carrilho, F.S.L. Vianna, A.A. Da Silva, L. Schuler-Faccini, Teratogens: A public health issue – A Brazilian overview, Genet. Mol. Biol. 40 (2017) 387–397. doi:10.1590/1678-4685-GMB-2016-0179.

[67] L.F. Prescott, P. Roscoe, N. Wright, S.S. Brown, Plasma-Paracetamol Half-Life and Hepatic Necrosis in Patients With Paracetamol Overdosage, Lancet. 297 (1971) 519–522. doi:10.1016/S0140-6736(71)91125-1.

[68] T. Roldán, Á. López, Intoxicación por acetaminofén en pediatría: aproximación y manejo, Univ. Médica. 53 (2012) 56–67.

[69] J. Sthephen Forte, Paracetamol: Safety versus Toxicity, Chronic Ill. 6 (2002) 12–16. http://www.mcppnet.org/publications/issue06-6.pdf.

[70] J. Janes, P. Routledge, Recent developments in the management of phaeochromocytoma, Drug Saf. 7 (1992) 170–177. doi:10.1016/b978-0-407-02317-8.50017-3.

[71] M. Jozwiak-Bebenista, J.Z. Nowak, Paracetamol: Mechanism of action, applications and safety concern, Acta Pol. Pharm. - Drug Res. 71 (2014) 11–23.

[72] K. Thiele, T. Kessler, P. Arck, A. Erhardt, G. Tiegs, Acetaminophen and pregnancy: Short- and long-term consequences for mother and child, J. Reprod. Immunol. 97 (2013) 128–139. doi:10.1016/j.jri.2012.10.014.

[73] Z. Liew, B. Ritz, C. Rebordosa, P.-C. Lee, J. Olsen, Acetaminophen Use During Pregnancy, Behavioral Problems, and Hyperkinetic Disorders, JAMA Pediatr. 168

(2014) 313–320. doi:10.1001/jamapediatrics.2013.4914.
[74] I. Reuter, S. Knaup, M. Romanos, K.P. Lesch, C. Drepper, C. Lillesaar, Developmental exposure to acetaminophen does not induce hyperactivity in zebrafish larvae, J. Neural Transm. 123 (2016) 841–848. doi:10.1007/s00702-016-1556-z.
[75] N.M. Vad, G. Yount, D. Moore, J. Weidanz, M.Y. Moridani, Biochemical Mechanism of Acetaminophen (APAP) Induced Toxicity in Melanoma Cell Lines, J. Pharm. Sci. 98 (2009) 1409–1425. doi:https://doi.org/10.1002/jps.21505.

Figure legends

Figure 1. Representative pictures of abnormalities caused in zebrafish embryos exposed from 0 hpf to different acetaminophen concentrations until 24, 48, 72, and 96 hpf. Arrows indicate the malformations produced: **(A)** pericardial edema, **(B)** spinal abnormalities and **(C)** pericardial blood accumulation.

Figure 2. (A) Scheme of experimental procedure**.** Apoptosis analysis on 4.9 mM acetaminophen treated-embryos **(C)** compared with control ones **(B)** at 48 hpf. (**B'** and **C'**) Amplifications from original pictures of tail fin regions. **(C)** Graph showing the integrity intensity values of acridine orange images, statistically significant difference was found (unpaired t-test, *$p < 0.0001$).

Figure 3. (A-B) Scheme of experimental procedure**. (A)** Embryos at 0 hpf were selected and incubated with different acetaminophen concentrations until collected for melanin detection (at 24, 48, 72, and 96 hpf; 20 embryos each timepoint) or black pigment cell counting (at 48 and 72 hpf; 10 embryos each timepoint). **(B)** Embryos at 72 hpf were selected and incubated with different acetaminophen concentrations until collected for melanin detection (at 96, 120, 144, and 168 hpf; 20 embryos each timepoint). **(C)** Levels of melanin in controls and embryos

treated from 0 hpf with different acetaminophen concentrations (2.5, 4.9, and 6.9 mM) measured at 24, 48, 72, and 96 hpf (+S.E.M., n=5). Statistically significant differences were found at 48, 72, and 96 hpf (ANOVA, post-hoc Tukey test, *p<0.0001). **(D)** Levels of melanin in controls and larvae treated from 72 hpf with different acetaminophen concentrations (2.5 and 4.9 mM) measured at 96, 120, 144, and 168 hpf (+S.E.M., n=5). Statistically significant differences were found at 120, 144, and 168 hpf (ANOVA, post-hoc Tukey test, *p<0.0001). **(E-F)** Number of melanocytes in the lateral stripe of the posterior trunk in controls and embryos treated from 0 hpf with different acetaminophen concentrations (2.5 and 4.9 mM) counted at 48 and 72 hpf. Statistically significant differences were found as indicated (±S.E.M., n=3; ANOVA, post-hoc Tukey test, *p<0.0001). Pictures alongside each graph are representative for each condition.

Figure 4. (A) Scheme of experimental procedure**.** Embryos at 0 hpf were selected and incubated with different acetaminophen concentrations until collected for Alcian Blue staining at 120 hpf. **(B-G)** Alcian Blue staining of head cartilages from control **(B)**, 2.5 mM **(C)**, 3.5 mM **(D)**, 4.9 mM **(E)**, 6.9 mM **(F)**, and 9.6 mM **(G)** of representative 5-dpf larvae (treated from 0 hpf). **(H)** Bar graph showing the quantification, in arbitrary units (AU) and +S.E.M., of craniofacial parameters measured in 5-dpf control and larvae treated from 0 hpf with 2.5 mM of acetaminophen (two-tailed statistical t-test; *p<0.001). Representative pictures over the graph illustrate the measurements taken. Meckel length: distance between Meckel cartilage and ceratohyal cartilages joint; Meckel area: area of the inner triangle defined by the Meckel cartilage; CH length: length of ceratohyal cartilage; PQ length: length of palatoquadrate+hyosymplectic cartilages; CH distance: distance between ceratohyal cartilages joint and lateral fins (white dashed line); Cranial distance: distance between the most anterior Meckel and lateral fins (white dashed line). **(H)** Bar graph showing the quantification of Meckel and ceratohyal cartilage angles and +S.E.M. in 5-dpf control and

larvae treated from 0 hpf with 2.5 mM of acetaminophen (two-tailed statistical t-test; *p<0.001). **(I)** Bar graph showing the quantification, in arbitrary units (AU) and +S.E.M., of craniofacial parameters measured in 5-dpf control and larvae treated from 72 hpf with 2.5 or 4.9 mM of acetaminophen (two-tailed statistical t-test; *p<0.001). **(J-L)** Alcian Blue staining of head cartilages from control **(J)**, 2.5 mM **(K)**, and 4.9 mM **(L)** of representative 5-dpf larvae (treated from 72 hpf). All larvae in ventral views, cephalic to the left.

Figure 5. (A) Scheme of experimental procedure**.** Embryos at 72 hpf were selected and incubated with different acetaminophen concentrations until collected for Alcian Blue staining at 120 hpf. **(B-D)** Alcian Blue staining of head cartilages from control **(B)**, 2.5 mM **(C)**, and 4.9 mM **(D)** of representative 5-dpf larvae (treated from 72 hpf). All larvae in ventral views, cephalic to the left. **(E)** Bar graph showing the quantification, in arbitrary units (AU) and +S.E.M., of craniofacial parameters measured in 5-dpf control and larvae treated from 72 hpf with 2.5 or 4.9 mM of acetaminophen (two-tailed statistical t-test; *p<0.001).

Figure 6. Relative levels of mRNA amount of *sox9b* **(A)**, *sox10* **(B)**, *mitfa* **(C)**, and *dct* **(D)** genes in embryos treated from 0 hpf with 4.9 mM acetaminophen; bars represent values measured at each developmental stage normalized to the amount of the corresponding mRNA measured in controls at 15, 24, and 48 hpf (+S.E.M., n=4; two-tailed statistical t-test; *p<0.05).

713 **Table 1**. Two-way ANOVAs (concentration and time of exposure) results for FET test.

Factor	Sum of squares type III	Freedom degrees	Square mean	F	Sig.
Concentration	26299.048	6	4383.175	347.346	0.000
Time of exposure	18910.952	4	4727.738	374.651	0.000
Concentration * Time of exposure	32205.714	24	1341.905	106.340	0.000

714

715 **Table 2.** Morphological abnormalities present in embryos treated from 0 hpf with concentrations of 2.5 and 4.9 mM of acetaminophen.

Morphological abnormality	Time of exposure	Treatment		
		Control (N = 6)	2.5 mM (N = 6)	4.9 mM (N = 6)
Edema (pericardial/peritoneal)	72 h	0 (0 %)	0 (0 %)	6 (100 %)
	96 h	0 (0 %)	5 (83 %)	6 (100 %)
Blood accumulation	72 h	0 (0 %)	0 (0 %)	3 (50 %)
	96 h	0 (0 %)	0 (0 %)	0 (0 %)
Spinal abnormalities	72 h	0 (0 %)	0 (0 %)	4 (67 %)
	96 h	0 (0 %)	0 (0 %)	4 (67 %)

Highlights

- Acetaminophen is a widely used analgesic and antipyretic agent
- Acetaminophen effect was tested during the development of zebrafish embryos
- Depigmentation and blockage of melanin synthesis was detected in treated embryos
- Cranial malformations and higher levels of cell death were found in treated embryos
- Acetaminophen affects specification and differentiation of neural crest cells

Author contributions (following the CRediT format: https://www.elsevier.com/authors/journal-authors/policies-and-ethics#authorship)

Vanessa P. Cedron: Conceptualization, Methodology, Formal Analysis, Investigation, Software, Data Curation, Writing- Review & Editing. **Andrea M.J. Weiner:** Conceptualization, Formal Analysis, Investigation, Software, Data Curation, Writing- Original Draft. **Manuel Vera:** Conceptualization, Methodology, Investigation, Software, Resources, Data Curation, Writing- Original Draft, Visualization, Supervision, Project administration. **Laura Sanchez:** Conceptualization, Methodology, Investigation, Resources, Writing- Review & Editing, Visualization, Supervision, Project administration, Funding Acquisition.