Evaluation of the effects of *Loxosceles intermedia's* venom in zebrafish

Ollavo Nogueira Tozzi [a],[1], Isabella Gizzi Jiacomini [a],[1], Thaís Sibioni Berti Bastos [a],
Laura Helena Cherem Netto Nicolazzi [a], Rebeca Bosso dos Santos Luz [a], Laís Cavalieri Paredes [b],
Luis Eduardo Gonçalves [b], Murilo Henrique Saturnino Lima [c], Waldiceu A. Verri Jr [d],
Niels Olsen Saraiva Camara [b], Helena Cristina Silva de Assis [e], Marisa Fernandes de Castilho [f],
Larissa Magalhaes Alvarenga [a], Tárcio Teodoro Braga [a],[c],[*]

[a] *Department of Basic Pathology, Federal University of Paraná, Curitiba, Brazil*
[b] *Department of Immunology, University of São Paulo, São Paulo, Brazil*
[c] *Graduate Program in Biosciences and Biotechnology, Instituto Carlos Chagas, Fiocruz, Curitiba, Brazil*
[d] *Department of Pathology, Biological Sciences Center, Londrina State University, Brazil*
[e] *Department of Pharmacology, Federal University of Paraná, Curitiba, Brazil*
[f] *Department of Physiology, Federal University of Paraná, Curitiba, Brazil*

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ABSTRACT

The zebrafish is an animal model of increasing use in many biomedical fields of study, including toxicology, inflammation, and tissue regeneration. In this paper, we have investigated the inflammatory effects of *Loxosceles intermedia*'s venom (LIV) on zebrafish, as well as the effects of Maresin 2 (Mar2) and Resolvin D5 (RvD5), two specialized pro-resolving mediators (SPMs), in the context of tissue regeneration after fin fold amputation. Furthermore, increasing concentrations of LIV (250–2000 ng) were assayed for their haemolytic effects *in vitro,* and, afterwards, the same concentrations were evaluated *in vivo,* when injected intraperitoneally. LIV caused haemolysis in human red blood cells (RBCs), but not in zebrafish RBCs. The survival curve was also not altered by LIV injection, regardless of venom dosage. Histological analysis of renal and hepatic tissues, as well as the whole animal, revealed no pathological differences between LIV-injected and PBS-injected groups. Fin fold regeneration was not altered between LIV-injected and control groups, nor in the presence of MaR2 and RvD5. Results of swimming behavioral analysis also did not differ between groups. Moreover, *in silico* data indicated differences between human and zebrafish cell membrane lipid constitutions, such as in phospholipases D preferred substrates, that could lead to the protection of zebrafish against LIV. Although our data implies that zebrafish cannot be used as a toxicological model for LIV studies, the absence of observed toxicological effects paves the way for the comprehension of the venom's mechanism of action in mammals and the fundamental evolutionary processes involved.

1. Introduction

Loxoscelism represents a serious public health issue in some regions of the world and specially in the Americas. The *Loxosceles intemedia* (*L. intermedia*) spider, also known as brown spider, is responsible for the greatest number of cases amongst the *Loxosceles* genus in Brazil, and its venom is widely studied. Venoms can be defined as complex mixtures of bioactive molecules with potential deleterious effects. Different components have been described in the Loxosceles sp. venoms, such as phospholipases, hyaluronidases, metalloproteinases and serineproteases, altogether responsible for the deleterious effects observed in loxoscelism [1]. The severity of the envenomation is strongly influenced by the inflammatory environment, directly and indirectly triggered by the toxins' activity [2]. Given the complexity and richness of the venom's composition, many *in vivo* and *in vitro* models have been employed to evaluate its toxins' biological properties, as well as the neutralizing ability of molecules that may pose as potential toxin inhibitors [3].

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* Corresponding author at: Department of Basic Pathology, Federal University of Paraná, Curitiba, Brazil.
  E-mail address: tarcio.braga@ufpr.br (T.T. Braga).
[1] These authors contributed equally.

Zebrafish (*Danio rerio)* have been largely used as a model to understand the activation of the immune system in different contexts [4–6]. Several factors favour zebrafish as a research model [7]. Such species is easily accessible and requires low maintenance costs when compared to other animal models, mainly due to animals' small size. The larvae are translucent, which in turn facilitates the visualization of internal structures under a simple optical microscope [8,9]. Notably, the zebrafish and human genome present approximately 70% of similarity, thus improving the model's reliability and indicating potential implications of the research results [10].

The effects of different venoms were also explored in zebrafish. Despite living in different environments, venom from jellyfish *Chrysaora* sp. induced several damages in *Danio rerio*, determined by histopathological analysis and through the measurement of the venom's haemolytic and phospholipase A2 activities. Sublethal concentrations of crude venom caused eye haemorrhage, gill hyperplasia and hypertrophy. Additionally, *Chrysaora* sp. venom also induced haemolysis, nuclear abnormalities and echinocytes [11].

Moreover, zebrafish have been used as a model for studying *Nemopilema nomurai* toxins, another species of jellyfish. Severe haemorrhage, and inflammation in cardiopulmonary regions of zebrafish were observed. In addition, the venom has altered the swimming behaviour of animals [12]. Lastly, *Bothrops alternatus* snake venom was also studied in zebrafish and the induction of necrosis, inflammation, and weight gain in animals were observed. Histological analysis show gills, liver, kidneys, and intestine alterations in animal groups treated with snake venom, indicating that zebrafish may serve as a useful model for understanding *Bothrops alternatus'* venom pathophysiology [13].

Zebrafish also possess an impressive regenerative ability in various organs and tissues, such as the fins, heart, spinal cord and brain [14]. Their caudal fin has crucial features that classify it as a good model for studying regeneration. It is easily accessed and presents a simple anatomy, including a bi-lobed morphology which allows evaluation of the growth rate along the medial-lateral axis [15]. Tissue regeneration is a process dependent on immune system activation [16] as an initial inflammatory response is required for lesion repair, followed by a resolving process [17]. Prolonged and unresolved inflammation would prevent regeneration and even induce fibrotic processes in the injured region [16]. Some molecules act as mediators of inflammation, inducing or inhibiting this process. In regeneration, specialized pro-resolving mediators (SPMs) appear to play a beneficial role in resolving and effectively stimulating this process [18]. SPMs, classified in families called lipoxins, resolvins, protectins, and maresins, are mediators biosynthesized from omega-6 or omega-3 fatty acids, and capable of limiting polymorphonuclear neutrophil infiltration and enhancing macrophage clearance of apoptotic cells, debris, and microbes [19,20]. These molecules apparently induce a macrophage phenotype switch from a pro-inflammatory to a non-inflammatory state, altering their metabolic status [18], hence contributing to the resolution of the inflammatory process and regeneration [21]. Finally, considering the inflammatory events triggered by *Loxosceles intermedia* spider venom and the zebrafish model's promising potential in inflammation and regeneration studies, we decided to investigate LIV pathophysiology using zebrafish, and aiming to circumvent venom effects, the SPMs Maresin 2 (Mar2) and Resolvin D5 (RvD5) were employed.

2. Materials and methods

2.1. Venom

Loxosceles intermedia venom (LIV) used in this study was kindly provided by the *Centro de produção e pesquisa de imunobiológicos* (CPPI), Piraquara, PR, Brazil. LIV was dissolved in different concentrations in phosphate buffered saline (PBS, pH 7.2, 0.576 mg.mL0–1) and administrated to zebrafish, as further specified t.

2.2. Animal maintenance

Adult zebrafish were maintained in the Pharmacology Department of the UFPR (Federal University of Paraná), Animals were kept in glass tanks, with mechanical air-bubbling supply, with a 10 h dark, 14 h light cycle, temperature of 28ºC, pH 7 7,2, plus ammonia and nitrite control. They were fed twice a day with *Nutravit* dry food (North Finchley, London, England) and with brine shrimp nauplii once a day. Tanks were cleaned weekly. All procedures were approved by the Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Parana (CEUA/BIO – UFPR), under the certification number RO 01/2020 (process number 23075.089273/2019-35).

2.3. Venom and PBS-injections

Animals were divided in 5 experimental groups and injected either with autoclaved phosphate saline buffer (PBS) or LIV, in increasing concentrations (250, 500, 1000 and 2000 ng). The number of animals in each experiment and group is described in the respective figure legends.

2.4. Maresin 2 and Resolvin D5

The SPMs Maresin 2 (13R,14S-dihydroxy-4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid) and Resolvin D5 (7S,17S-dihydroxy-4Z,8E,10Z,13Z,15E,19Z-docosahexaenoic acid) were purchased from Cayman Chemical.

2.5. Survival assay

Mortality was observed through the course of seven days by counting daily the number of fish left in each group tank from LIV and PBS-injected groups. A survival curve was then plotted using the GraphPad Prism Software v7.

2.6. Venom injections and caudal fin amputation

Adult zebrafish were anesthetized individually with 0.168 mg.mL^{-1} tricaine diluted in tank water, for approximately 5 min. The various venom dilutions (250–2000 ng) were prepared in autoclaved PBS. Each fish was injected intraperitoneally (i.p.) with a volume of 20 µL of *Loxosceles intermedia* venom (LIV) diluted in PBS or 20 µL of PBS alone. Subsequently, the animals were submitted to superior caudal fin amputation with a sterile scalpel blade and observed under a stereoscopic microscope (Zeiss Stemi 508) for accurate visualization and image capture. Lastly, fish were immediately placed into oxygenated water for recovery after each injection.

2.7. Microplate haemolysis assay

Blood from human healthy donors and zebrafish was collected in presence of citrate buffer to prevent clotting. The RBC containing fraction was isolated through centrifugation at 290 x *g*, for 15 min at RT. Human RBCs were washed three times with Ringer solution and spun at 688 x *g* for 5 min, at RT, between washes. Zebrafish cells were washed with filtered PBS pH 7.2. Subsequently, cells were counted in a Neubauer chamber and 10^5 erythrocytes/replicate of both human healthy donors and zebrafish were individually incubated with different amounts of LIV diluted in Ringer's Solution (250–2000 ng), in 96-rounded-well microplates, for 24 h at 37 °C and under gentle agitation. Cells were treated with distilled water for positive lysis control and, for the negative lysis control, human and zebrafish cells were incubated with Ringer solution or PBS alone, respectively. Next, cells were spun at 688 x g for 5 min at RT, and supernatant optical density was read at 570 nm in a spectrophotometer. The A_{570} of the positive control was considered as 100% lysis and the values of A_{570} of the other samples were used in relation to the positive control for their % haemolysis

calculation. The test's experimental groups were each evaluated in triplicate.

2.8. Regeneration analysis

For fin regeneration analysis, fish were again anesthetized individually with 0.168 mg.mL^{-1} tricaine diluted in tank water and the whole caudal fin was photographed at days 0, 3, 7 and 14 after injection + amputation, using a stereoscopic microscope (Zeiss Stemi 508). The area and relative percentage of caudal fin regeneration was determined utilizing the "measure" function on the Fiji software (ImageJ - http://rsb.info.nih.gov/ij/) and the following formula: % of regeneration = (area of regeneration in amputated fin/area of the proximal non-amputated fin) x 100. Measurements were performed three times for each animal for data normalization.

2.9. Behavioral analysis

A behavioral analysis was conducted in adaptation to the methods described previously by Barreto & Volpato [22]. First, adult zebrafish (n = 7/group) were isolated in separate aquaria and left to acclimate for a week. A grid containing 33 quadrants (11 × 3, of 7 cm^2 each) was drawn in each aquarium. Next, fish were anesthetized individually by submersion in 0.168 mg.mL^{-1} tricaine diluted in tank water for approximately 5 min, and then injected intraperitoneally with 20 μL of LIV (2000 ng – G3) or filtered PBS (PBS). Fish were filmed at days −1, 0 (3 hrs post-injection), 1, 3 and 7, for 10 min a day. Each animal's head position was logged at every10 seconds as an x/y coordinate in the drawn grid. Three variables were analysed: (1) Dispersion in aquaria – the average of the x/y values at each measurement constitutes a barycentric coordinate, from which dispersion (cm) was calculated [22]; (2) Relative aquaria longitudinal position – the average y value for each 10 min video analysed; (3) Time spent in locomotion – the calculated percentage of time in which the fish was actively swimming from the total 10 min. After filming at day 7, animals were killed following the protocol described by Blessing et al. [23] for subsequent histological analysis.

2.10. Histological analysis

Animals were immediately placed in FAA solution (68% Ethanol, 4% Paraformaldehyde and 5% Acetic Acid) for 24 h and then decalcified in 5% Trichloroacetic Acid (TCA; Sigma Aldrich.) for 6 days. Samples were successively dehydrated in a graded alcohol series of 70%, 80%, 90%, 95% and 99.99%. Next, they were diaphanized by impregnation with a 50% xylene and 50% ethanol solution and embedded whole in Paraplast® (Sigma-Merck, Massachusetts, USA). Samples were then sectioned at 7 μm using a microtome and histological analysis was performed after the tissue section were stained with haemoxylin and eosin and with Masson's trichrome stain as described by Liu et al. [24].

2.11. Different expressed genes (DEGs)

In order to evaluate the genomic response during an inflammatory process, we utilized GEO Datasets, a genomic repository with public access. The GSE 73223 dataset was identified and selected/. It contains 24 samples of microarray gene expression on zebrafish after either LPS induction or on a PBS control condition. We plotted the GSE 73223 datasets at GEO2R for DEG comparison. DEGs with $p > 0.05$ were selected to evaluate which genes were up or down regulated. Upregulated genes had a LogFC = 1 as a cut-off, and a cut-off of LogFC = −1 was stablished for downregulated genes. We also used the website Enrich R to run the enrichment of the selected genes. At Enrich R, we selected the Fish Enrich R platform. Lastly, the enriched pathways were analysed on KEGG.

2.12. Gene enrichment analysis (GSEA)

The GSEA assesses significantly statistic differences in a set of genes in two different biological states. Here, GSEA was performed to evaluate the expression of genes in the liver of zebrafish treated with LPS or PBS. As the IDs from GSE73223 dataset were generated by the Agilent for Zebrafish, we reach the gene symbol of each ID by running a script at R Studio. Subsequently, we converted each gene symbol to its human orthologs as GSEA only reads human, mouse, and mice gene symbols. Finally, each gene was aligned with their respective expression value in two different groups: control and LPS-treated. The false discovery rate (FDR) < 25% was set as a criterion for the choice of the genes enriched in the different pathways.

2.13. Statistical analyses

Data were expressed as the mean ± standard error of the mean (SEM). One-way ANOVA (Analysis of variance) was applied, followed by the Bonferroni test for statistical analysis. The GraphPad Prism Software v7.0 for MacOS (GraphPad Software, California, USA) was utilized and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. L. intermedia haemolysis assays

Loxoscelism is caused primarily by phospholipase D (PLD) enzyme toxins in the spider venom [25]. We aimed to establish zebrafish as a model system for studying the haemolytic capacity and histopathology of organs damaged by exposure to LIV. Aiming to investigate whether LIV could also lyse zebrafish erythrocytes, we adapted a direct haemolytic assay [4,26] to a 96-well microplate format, attempting to use a lower number of cells per replicate given the small blood volume of zebrafish. Human and zebrafish RBCs (10^5/replicate) were concomitantly assayed in the presence and absence of the LIV, considering PBS as a negative lysis control, and distilled water as a positive lysis control. Human RBCs are lysed in a venom dose-dependent fashion after 24 h of incubation, reaching values as high as 100% lysis (Fig. 1A). Zebrafish RBCs, however, show no detectable lysis regardless of LIV dose, as the points treated with venom have an O.D. at 570 nm. similar to the negative control (Fig. 1A).

3.2. Survival curve and fin fold regeneration upon LIV or SPM injection

Brown spider venom may lead to vascular disorders including leukocyte and platelet activation, disseminated intravascular coagulation, increase in vessel permeability and haemorrhage into the dermis [27]. Despite its harmful effects, LIV did not alter the survival rate of injected animals (Fig. 1B). Additionally, there were no differences between control and venom-injected groups at different concentrations (250, 500, 1000 and 2000 ng), as all groups demonstrated approximately a survival rate of 100% (Fig. 1A). When zebrafish fin fold is amputated, the wound epithelium is readily established and, a few hours after, the regrowth phase of regeneration begins [15,28]. In attempt to investigate the modulation of regeneration in zebrafish, we administrated LIV at different concentrations (250–2000 ng) followed by fin fold amputation. No significant differences were observed between venom-injected and PBS-injected groups at 3-, 7-, 10- and 14-days post-amputation (dpa) (Fig. 1C and D), as all groups exhibited approximately the same percentage of caudal fin regeneration.

Specialized pro-resolving mediators (SPMs) are a lipid superfamily with the capacity to modulate macrophages to a pro-resolutive phenotype, and to increase tissue regeneration at initial days post injury [29]. Despite no observed alterations upon LIV injection, we aimed to analyse if SPMs could modulate regeneration. RvD5 was selected given it has been shown to inhibit PLD activity, shifting macrophage phenotype to
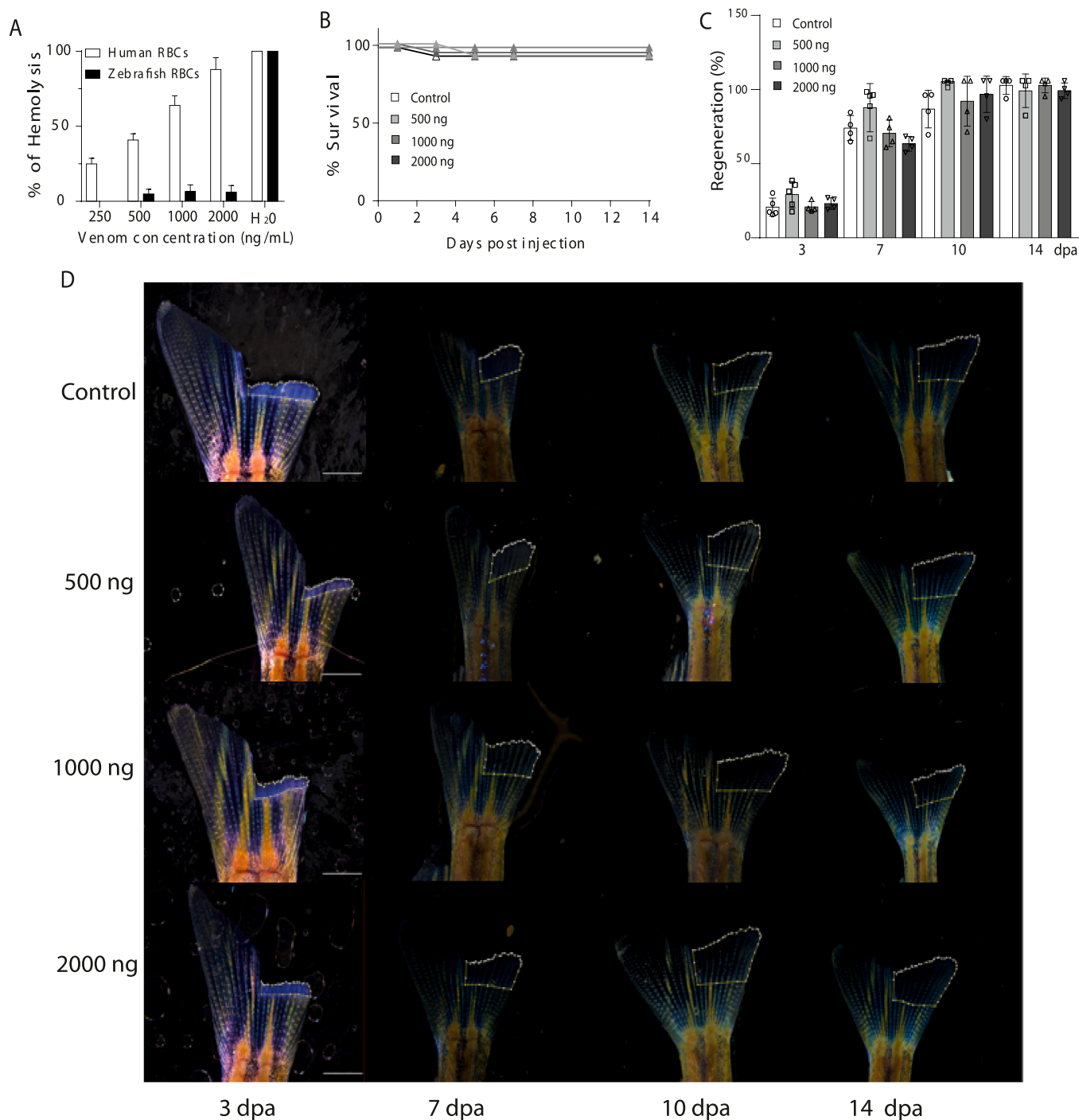
Fig. 1. Haemolysis assay, survival curve and caudal fin regeneration analysis. A) For the haemolysis assay, 10^5 human RBCs, as well as 10^5 zebrafish RBCs were diluted in either Ringer solution or filtered PBS, respectively, and incubated with various amounts of LIV (250, 500, 1000 and 2000 ng) in a 96-well plate (rounded wells). After 24 hrs, cells were spun at 688 x g for 5 min and the supernatant was read at 570 nm. For positive lysis control, cells were incubated with dH_2O and as a negative control, Ringer solution or PBS alone were used for human and zebrafish blood, respectively. Calculations of % haemolysis were made considering haemolysis caused by the dH_2O alone as 100% lysis. For *in vivo* experiments, fish were anesthetized individually, injected with increasing amounts of LIV (250, 500, 1000 and 2000 ng) or saline and then one side of the caudal fin was amputated. Depicted images were taken at 3, 7 and 14 after injection + amputation, using a stereoscopic microscope. B) Survival of injected/control animals between the different groups. C) Percentage of regeneration of the amputated fin in the different experimental groups. D) Evolution of fin regeneration across time in amputated animals in the indicated experimental groups. For the haemolysis assay, experiments were performed in three independent experiments and in three technical replicates. For survival experiments, n = 10 per group. For regeneration experiments, n = 4–6 per group and the experiments were performed three times.

M2 [30]. Although MaR1 is known to induce tissue regeneration [31], this activity had not been investigated for MaR2 yet. Thus, modulating tissue regeneration would be a novel finding for this SPM. This considered, animals were then injected with Maresin 2 (Mar2) or Resolvin D5

(RvD5). After injection, the caudal fin was partially amputated, animals were imaged, and the regenerated area was quantified as previously described (Sup. Fig. 1A and 1B). Again, comparisons between PBS-, MaR2- and RvD5-injected groups did not show any statistical difference

(Sup. Fig. 1B). Altogether, these results indicate no differences between regeneration processes observed after LIV or SPM injections during the described timeframe.

### 3.3. Histological and behavioural analysis after LIV injection

To histologically confirm the absence of any alterations following LIV injection, we collected zebrafish from control, 1000 and 2000 ng of LIV-injected groups at 7 days-post injection. Staining by haematoxylin-eosin and Masson's trichrome was performed and revealed no histopathological alterations in any organs, including kidney and liver, as demonstrated in Fig. 2.

Changes in zebrafish behaviour can indicate that the animal's body may be in contact with a foreign substance [13]. In this study, behavioral analysis was performed as adapted from Barreto & Volpato's method [22]: the mean Y position, time spent in locomotion, and

dispersion results are represented in Fig. 3B, C, and D, respectively. Animals with higher stress levels were expected to be closer to the water line, with low motility and concentrated in specific areas of the aquarium. In accordance with previous data, the mean Y positions demonstrated no alterations between LIV-and PBS-injected fish (Fig. 3B). Moreover, the dispersion presented relevant results only on day 1, with a lower dispersion on the group injected with 1000 ng of LIV, suggesting a higher stress level at a very early time point when compared to the control group (Fig. 3C). The time spent swimming, on the other hand, remained unaltered between both groups (Fig. 3D).

3.4. Predictive differences between human and zebrafish response to LIV as suggested by in silico studies

Attempting to explain the lack of LIV toxic effects in zebrafish, we compared human and zebrafish organisms regarding their cell
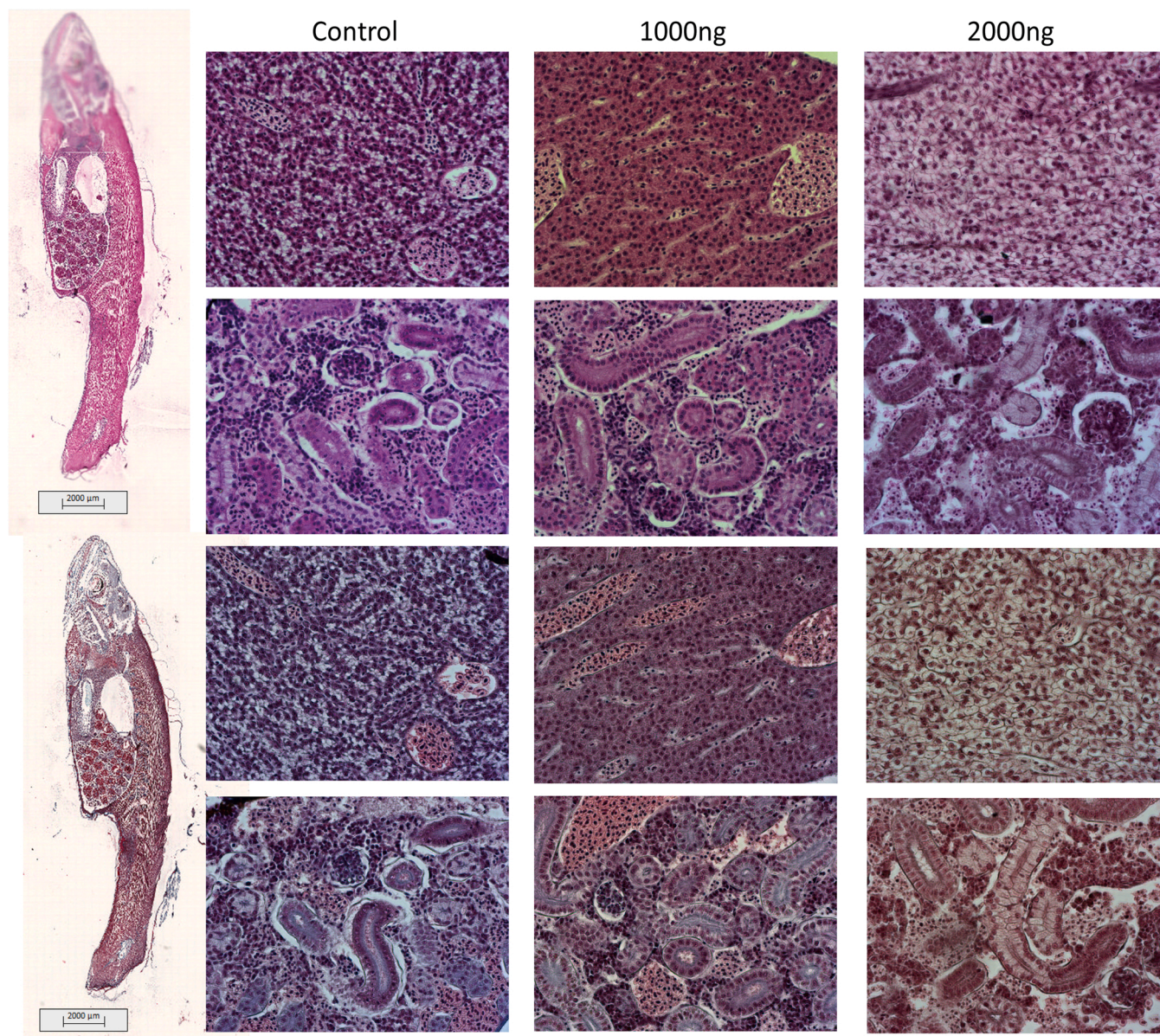
Fig. 2. Histological analysis. Animals injected with 1000 ng, 2000 ng of LIV or PBS (negative control) were killed and fixed in formalin-aceto-alcohol (FAA) solution, decalcified in trichloroacetic acid (TCA) and processed whole. Staining was carried out by haematoxylin-eosin and Masson's trichrome (MT) protocols. The two leftmost images are control fish (upper - HE; lower - MT; scale bar = 2000 μm). Following three rows (scale bar = 20 μm) are control, 1000 ng and 2000 ng, organs and staining, from top to bottom, are displayed as follows: kidney (HE), liver (HE), kidney (MT), liver (MT). Representative images are shown. n = 10 per group and 5–10 photomicrographs are analysed per animal.
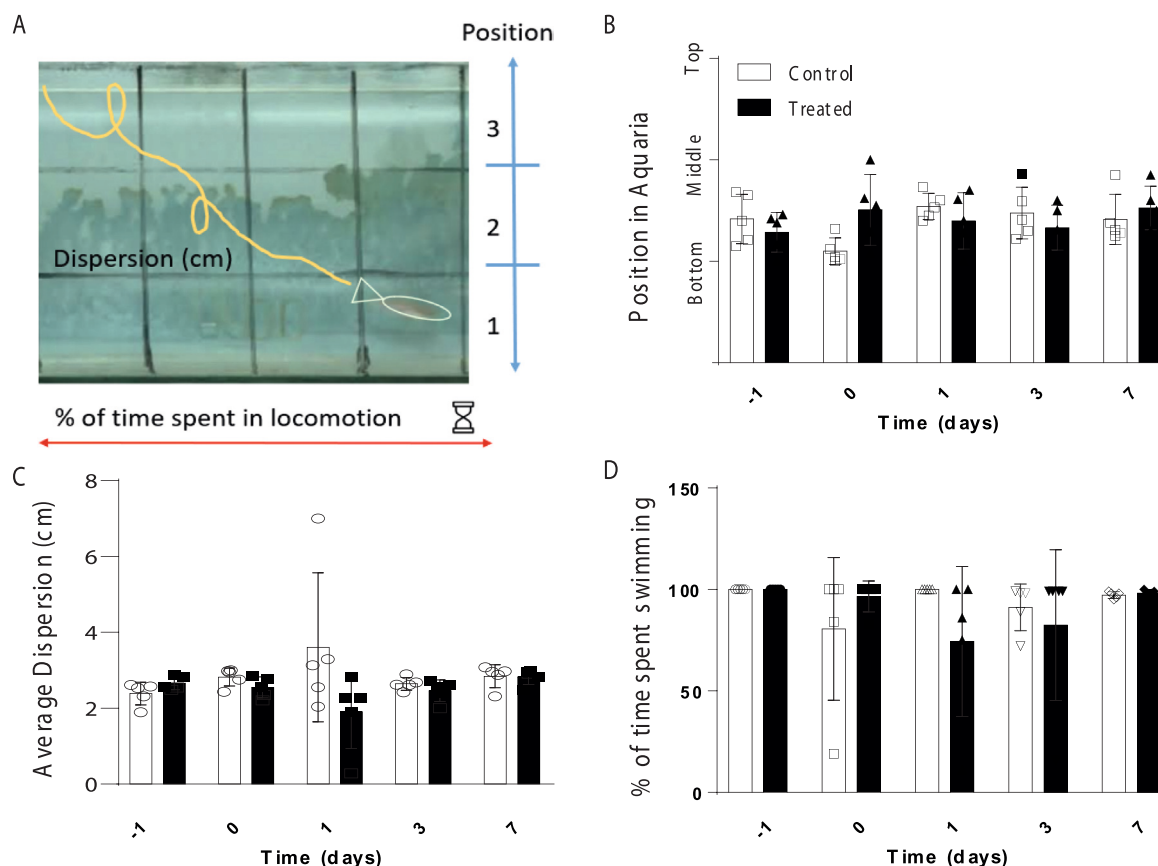
Fig. 3. Behavioural studies. Individualized zebrafish were left to acclimate in 11×3 cm aquaria for 15 days and were later filmed for 10 min in a timespan of 8 days: 1 day prior the LIV injection (1000 ng) and 0, 1, 3 and 7 days after LIV injections. A) Representative scheme of parameters analysed for each fish individualized in aquaria. Amongst the analysed parameters, dispersion, position in aquaria and percentage of time spent in locomotion were evaluated. B) Percentage of total time spent in locomotion: The time spent in locomotion was recorded as the total swimming time for each individual fish and is showed in relation to the total registration. C) Relative Fish Position in Aquaria: Fish longitude in relation to aquaria was calculated from the average measurements recorded for Y in each video. D) Average animal dispersion: The average of the x/y values at each measurement constitute a barycentric coordinate, from which dispersion (cm) was calculated. For behavioural analyses experiments were performed twice and n = 5 per group.

membrane constitution in an inflammatory environment, especially in terms of phospholipase D/sphingomyelinase substrates, as they represent the main venom constituents [25]. Considering no data was found on the literature on the use of zebrafish as a model for *L. intermedia* venom studies, we performed an *in silico* analysis in a LPS stimulus context. The GSE 73223 dataset consists in 24 samples of three different tissues (liver, kidney, and muscle), being liver samples the most affected by LPS injection. After GEO2R analyses, we identified ten differentially expressed genes, five upregulated and five downregulated (Fig. 4A). These results indicate the differential gene expression between LPS-treated and control groups of the GSE 73223 dataset, demonstrating the upregulation of biosynthesis of unsaturated fatty acids (Fig. 4A) which may counterbalance the inflammatory process. GSEA showed 9 pathways containing down or upregulated genes in the liver of zebrafish treated with LPS compared to the control group (Fig. 4B). As expected, among the pathways, most pathways are linked to an inflammatory response, *e.g.* interferon-γ and TNF-α pathways, TGF-β signaling pathway, Epithelial-to-Mesenchymal Transition pathway, and reactive oxygen species and hypoxia-related pathways (Fig. 4B).

4. Discussion

Zebrafish may be a highly useful biosensor for assessing the cytotoxic effects of venoms [13]. Animal venoms are well-known for altering different cell lines given their rich and diverse toxin composition. Said diversity is associated to the spider's defense strategies against different

predators, as well as prey capture and digestion [32]. In the context of loxoscelism, the crucial role of toxins such as phospholipases, hyaluronidases and metalloproteases has been studied in mammal envenomation [33,34]. Low mass toxins, such as knottins, also present in *Loxosceles* venom, have shown to affect different species of arthropods, thus showing an insecticidal properties [35]. Various *in vivo* and *in vitro* models have been employed for these characterizations. Inflammatory effects provoked by the venom's toxins have been studied in cell lines that showed the production and effect of cytokines in cell recruitment, complement system activation, cell injury and eventually death [4,36, 37]. In this scenario, zebrafish represent a suitable animal model especially considering macrophage dichotomy, inflammation, and tissue regeneration [28,38].

However, in the different tested concentrations, LIV does not seem to produce any detectable deleterious effects in this model. Nevertheless, *Loxosceles sp.* venoms have shown to have variable effects among different animal species. For example, the dermonecrosis provoked by the venom has been established in rabbits [39], but these effects were not observed in mice injected with the same toxins [40]. Nonetheless, the systemic effects, such as kidney and heart toxicity observed in human loxoscelism, can be studied in mice, as these animals seem to reproduce these injuries when challenged with *Loxosceles* venoms [33, 41]. Moreover, the red blood cells of different animal species have been studied in terms of their susceptibility to haemolysis induced by these toxins. Such studies have shown that the presence and abundance of certain phospholipids in the cell membrane composition could be
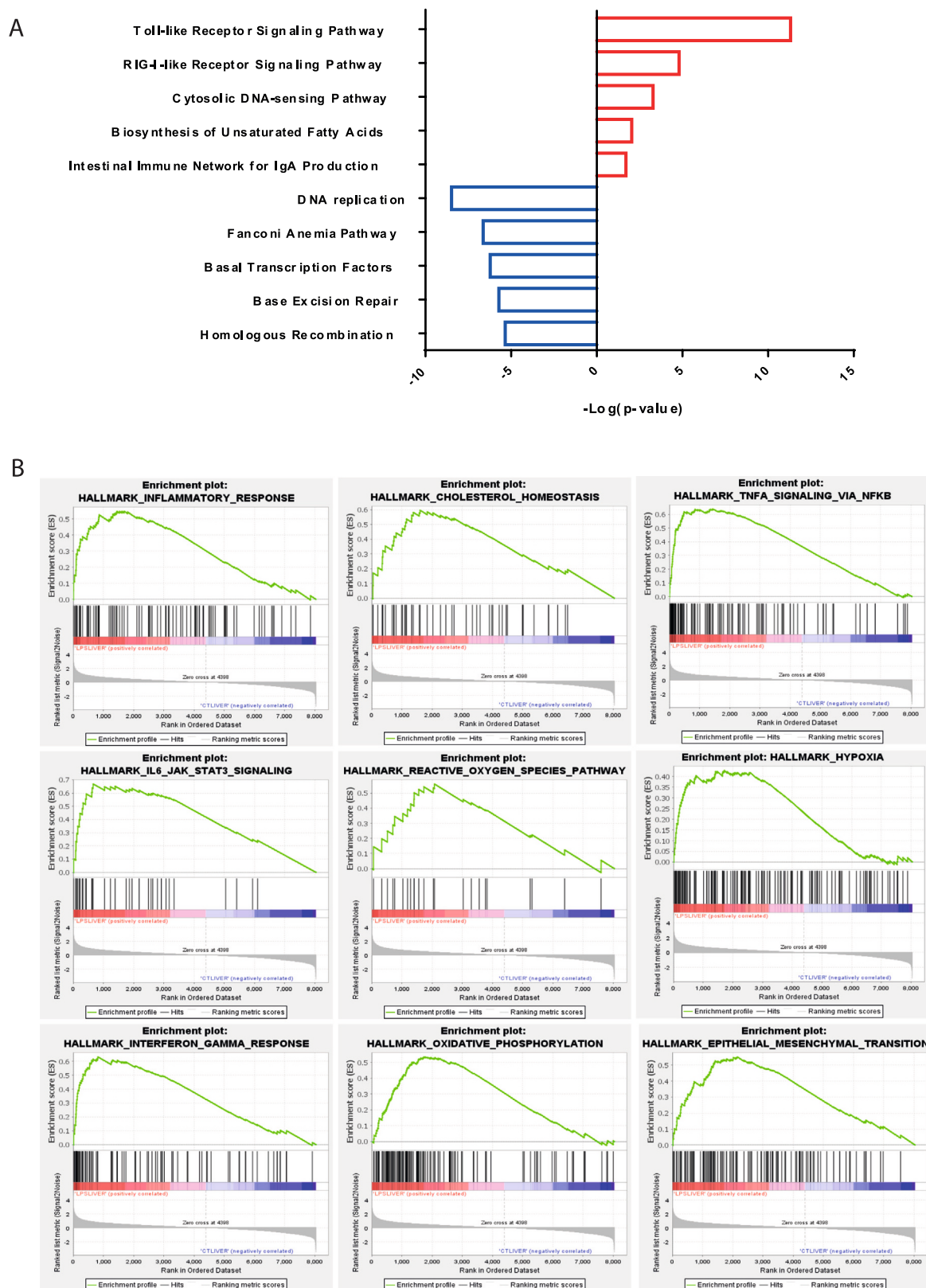
Fig. 4. *In silico* comparison between zebrafish and human response to LPS. A) GSE73223 showing 9 pathways contained genes down or upregulated in the liver of zebrafish treated with LPS, as compared to control group. Among the pathways, most of them are linked to an inflammatory response, for example, Interferon-γ pathway, TNF-α pathway *via* NF-κB, IL-6 pathway, Reactive Oxygen Species- and Hypoxia-related pathways, and Epithelial Mesenchymal Transition pathway. B) Gene pathways upregulated (red bars): Toll-like receptor signaling, RIG-I-Like receptor, cytosolic DNA-sensing, Biosynthesis of unsaturated fatty acids, and Intestinal Immune Network for IgA production; or down regulated (blue bars): DNA replication pathway, Fanconi anemia pathway, basal transcription factors, base excision repairs and homologous recombination.

responsible for increased sensitivity to lysis [22,42]. This might be associated to the differential preferences that Phospholipases D (PLDs) exhibit for phospholipid targets [25,43]. Upon substrate cleavage, phospholipases produce membrane injury and consequently, provoke the remodelling of the membrane structure [44], lipid raft formation [45], and produce inflammatory mediators responsible for cell recruitment, cell death and fibrosis [3]. In conclusion, cell membrane phospholipid composition, targeted directly by the PLD toxins, as well as the metabolites produced as a result of their enzymatic activity are paramount variables for the study and comprehension of the *Loxosceles sp.* toxins mechanism of action [46].

Zebrafish also represent a great model to understand regeneration processes since they present mainly epimorphic regeneration, a fast response able to cover the wound by epidermis and to form the blastema, a set of undifferentiated cells that differentiate over time [28, 47–49]. When tissue damage occurs, damage associated molecular patterns (DAMPs) and immune cells, such as neutrophils and macrophages, take part in the process [16,50,51]. Different subpopulations of macrophages, for instance, are necessary both at the beginning of the process, during the formation of the blastema, and later, at the conclusion of tissue regeneration and remodelling [52,53]. The original morphology is ultimately achieved due to positional memory, still the understanding of how the regenerative process occurs as a whole has not yet been elucidated [47]. We tested Maresin 2 (MaR2) and Resolvin D5 (RvD5), two immune resolvents included in the class of Specialized Pro-Resolving Mediators (SPMs).

Despite SPMs presenting anti-inflammatory and pro-resolving actions [54], RvD5 and MaR2 did not alter the effects of LIV, likely because LIV did not lead to an inflammatory profile. MaR2 is produced by macrophages, reduces neutrophil infiltration and enhances macrophage efferocytosis [55]. RvD5 belongs to the D-series of the Resolvin family acting *via* DRV1 receptor - which also binds to RvD1 and RvD3 [56] - and GPR32 receptor in order to stimulate neutrophil and macrophage phagocytosis and counter-regulate pro-inflammatory genes, such as NF-κB and TNF-α [57]. Recent analyses on the membrane components of zebrafish cells have suggested that in addition to fish being sources of omega-3 fatty acids and its derivatives, such as SPM, zebrafish have an abundance of corticosteroids on their membranes [58], which could help to explain the absence of LIV effects. Once SPMs have been poorly studied in zebrafish, our results suggest that future authors focus on a much earlier window of the regenerative process.

Due to the lack of studies regarding the use of LIV using zebrafish as a model, we performed *in silico* analyses comparing fish under the LPS stimulus. In the present study we found enriched pathways of DAMPs as ROS and HIF-1α. Some cytokines have major roles to maintain the inflammation at a non-pathological state, like IL-10 and TGF-β [59]. Our results demonstrated an enrichment in the TGF-β pathway raising the hypothesis that this cytokine may have a protective role in zebrafish under the event of *Loxosceles sp.* envenomation. Interestingly, cholesterol homeostasis is enriched as showed in the GSEA data. The hydrolysis of sphingomyelin, an important constituent of cell membrane lipid rafts, is one of the major elements in the pathophysiology of *Loxosceles sp.* envenomation by disrupting the membrane environment and leading to the activation of metalloproteinases of the ADAM family [45]. Thus, the gene expression changes in the cholesterol pathway in zebrafish could additionally be related to the lack of response to LIV.

Events such as inflammation and tissue regeneration have been widely studied and stablished for various pathological conditions in zebrafish and are essential in the context of envenomation. Although not useful for LIV studies, the employment of zebrafish as an animal model was possible for *Bothrops alternatus* snake venom [13], and jellyfishes *Nemopilema nomurai* [63] and from the *Chrysaora* genre [11]. In conclusion, the present study provides guidance in choosing the animal model most adapted to reproduce the cellular and systemic effects observed in loxoscelism.

CRediT authorship contribution statement

ONT, IGJ, TSBB, LHCNN, RBSL, LCP, LEG and MHSL contributed to Methodology, Formal analysis and Investigation. WAVJ, NOSC, HCSA and MFC contributed to Visualization and Project administration. LMA and TTB contributed to Visualization, Supervision, Writing – review & editing. TTB also contributed to Data Curation and Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.06.010.

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