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Influence of waterborne gallic and pelargonic acid exposures on biochemical and reproductive parameters in the zebrafish (*Danio rerio*)

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Zebrafish, chronic toxicity, phytochemicals, gallic acid, pelargonic acid.

Abstract

Gallic and pelargonic acids are biologically derived substances receiving a growing interest as eco-friendly biocides with potential applications in freshwater system management. However, some data gaps remain to address their chronic ecotoxicity issue, particularly for fish. This work aimed at investigating the sublethal effects of a long-term waterborne exposure of zebrafish to these compounds. Mature fish were exposed to gallic or pelargonic acid at the concentrations of 0, 0.05, 0.5 and 5 mg/L during one month under semi-static conditions. Fecundity, hatching rate and median hatching time were regularly evaluated. Circulating sex hormone levels (11 ketotestosterone 211 KT, 17 bestradiol -E2-), plasma vitellogenin (Vtg), and gonad histology were monitored in males and females after exposure. Lactate dehydrogenase (LDH), total glutathione peroxidase (GPx) and glutathione-S transferase (GST) activities were assessed as enzymatic biomarkers of exposure in fish liver. Significant increases of GPx activity were reported in females exposed to both type of chemicals regardless the contamination level. Moreover, 5 mg/L gallic acid induced a decrease in 11-KT levels for males. For fish exposed to pelargonic acid, decreases in circulating hormone levels were reported respectively at 0.05 and 5 mg/L for 11-KT in males, and at 0.5 mg/L for E2 in females. However, no histological alteration in gonads neither significant variation in reproductive performances were detected following zebrafish exposure to gallic or pelargonic acid. Additional investigations concerning the mode of application and the environmental fate of these substances may warrant their further use in freshwater systems at concentrations compatible with biocidal/allelochemical effects.

1. Introduction

Biologically active plant-derived compounds such as gallic or pelargonic acid [Fig. 1(A,B)] have experienced an increased interest over the last three decades as a consequence of their presumed health benefits and properties for environmental safety (Balandrin et al., 1985; Ow and Stupans, 2003; Jones et al., 2004; Lederer et al., 2004). Gallic acid (3,4,5 trihydroxybenzoic acid) is actually one of the most publicized polyphenol ingredient in functional foods (Ow and Stupans, 2003; Sethiya et al., 2014). Traditionally recognized as a medicinally important component in ayurvedic herbs and formulations, this water-soluble polyphenol has been thoroughly studied for its free-radical scavenging activity that notably leads to anti-inflammatory and cytoprotective effects in mammal models (Ow and Stupans, 2003; Abdelwahed et al., 2007; Sethiya et al., 2014).

Pelargonic acid, also known as nonanoic acid due to its nine carbon-saturated linear aliphatic chain, has been approved by the United States Food and Drug Administration as well as the Council of Europe for food use, mainly as an artificial flavoring substance and food preservative (FCT, 1978; US EPA, 2000).

Relying on their molecular structures [Fig. 1(A,B)], both gallic and pelargonic acids can demonstrate antimicrobial, as well as, algicidal or herbicidal properties (Breeuwer et al., 1997; Nakai et al., 2000; Ow and Stupans, 2003; Lederer et al., 2004; Nakai et al., 2005; Abdelwahed et al., 2007; MMWD, 2008; Wang et al., 2008; Rai and Chikindas, 2011; Borges et al., 2013). Consequently, the growing need for eco-compatible technology development has enlarged their scope of applications over these last years (Shao et al., 2013; Sun et al., 2014). As an illustration, the increased demand for more environmentally friendly agrochemicals, safer for human and

animal health, has expanded the use of pelar-gonic acid as an adjuvant for “green” pesticide formulations (Coleman and Penner, 2008). Indeed, this representative of odd numbered medium-chain fatty acid has been shown to possess nonspecific herbicidal properties toward terrestrial weeds. Its mode of action would mainly rely on its capacity to intercalate into thylakoid and plasma membranes, enhancing ultimately reactive oxygen species (ROS) formation and lipid-membrane peroxidation prior to plant cell death (Lederer et al., 2004). More recently, laboratory tests for appropriate use of pelargonic acid in order to control invasive tendencies of floating aquatic plants like duckweeds (*Lemna* spp.) has yielded promising results, and commercial formulations are in progress (Webber, 2009; Webber et al., 2014). Besides, several researchers highlighted its critical role as a bioactive phytochemical exhibiting allelopathic activity on the harmful algal bloom (HAB) forming cyanobacteria *Microcystis aeruginosa*, with a 50% growth inhibitory concentration (IC₅₀) as low as 0.5 mg/L of substance (Nakai et al., 2005). Concerning gallic acid, this secondary plant metabolite with its three adjacent aromatic phenoxy groups has been also shown to act as a potent anticyanobac-terial allelochemical (toward *M. aeruginosa*), at an average IC₅₀ ranging from 1 to ~5 mg/L (Nakai et al., 2000; Wang et al., 2008). With this in mind, gallic and pelargonic acids show great potential for the development of novel eco-friendly and efficient water treatment process, with regards to the control of microalgae blooms or aquatic weed proliferations in freshwater systems. However, implementing integrated freshwater system management that would involve the use of biologically active phytochemicals would also bring up presumably enhanced environmental exposure issues for other nontarget aquatic organisms (Shao et al., 2013). Currently, the information available related to aquatic ecotoxicity risks of gallic or pelargonic acid exposure is very scarce, particularly for fish (European Food Safety Authority, 2013; Melo et al., 2009). As a matter of fact, only three studies have been listed in the ECOTOX Database (US EPA) relating the acute toxicity of pelargonic acid (in the range of 91 to >105 mg/L) toward three fish species, i.e., the bluegill (*Lepomis macrochirus*), the rainbow trout (*Oncorhynchus mykiss*), and the fathead minnow (*Pimephales promelas*) (Brooke et al., 1984; US EPA, 2013), whereas none has been referenced for gallic acid. However, several authors raised the ecotoxicity issue for freshwater organisms by reporting cellular morphological alterations and decrease in bivalve digestive

gland cell viability after 48 h in vivo exposure to gallic acid and other tannic acid derivatives (Labieniec et al., 2003, 2007). Still, fish are aquatic vertebrates also directly exposed to water enriched in such molecules and they possess an antioxidant system highly sensitive to a great variety of contaminants. More recently, Techer et al., (2015) determined the 96 h lethal concentration (LC₅₀) of pelargonic and gallic acids being respectively of 81.2 and >100 mg/L of substance using the zebrafish (*Danio rerio*). In this study, modulation of sublethal endpoints through antioxidant enzyme activity induction was also reported which raised the question of long-term toxicity issue for both compounds.

This study was designed to investigate the effects of chronic waterborne exposure to the current-use phytochemi-cals gallic and pelargonic acids on zebrafish (*Danio rerio*). This work was achieved in order to address data gaps related to their long-term aquatic ecotoxicity risks before any large-scale field application trial in a framework of water ecotechnology development. *Danio rerio* is a small freshwater fish species which is particularly suitable as an experimental model for long-term toxicity assessment (Hill et al., 2005). For this purpose, mature males and females were exposed to each type of contaminants under semi-static conditions for 1 month. Since breeding success is critical for fish species sustainability, population fecundity of the exposed adult zebrafish, with subsequent hatching time and rate were regularly evaluated. Then, a panel of biochemical, physiological, and histological biomarkers was assessed. Plasma sex steroid hormonal levels (11-ketotestosterone, 11-KT; 17 β estradiol, E2), circulating vitellogenin (Vtg) concentrations, testicular, and ovarian histology were monitored respectively in males and females at the end of experiments to investigate more thoroughly any potential impact on reproductive function. Moreover, selected liver enzyme activities were assessed as exposure biomarkers. The latter included lactate dehydrogenase (LDH) which is involved into energy production (Diamantino et al., 2001; Coelho et al., 2011), glutathione-S-transferase (GST) which plays a key role in phase II biotransformation of endogenous and xenobiotic substances, and glutathione peroxidase (GPx) that protects cells against ROS formation (van der Oost et al., 2003; Hermes-Lima, 2004).

2. Material and Methods

2.1. Chemicals

Stock solutions of 8 g/L gallic acid (Acros Organics, 98% purity) were prepared and renewed weekly in dechlorinated tap water. Pelargonic acid was obtained from Alfa Aesar, Johnson Matthey Company. In a liquid form (97% purity) at room temperature, it was directly used for appropriate dilution in glass container tanks. Tricaine methane sulphonate (MS-222), NaHCO₃, and chemicals required for LDH assays were of analytical grade and were procured from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Regarding histology preparation of gonads, OTTIX solution and Diawax paraffin substitute were provided by DIAPATH SpA, Italy/ MM France. Hematoxylin Gill III and phloxine were provided by VWR International, Briare, France, whereas Regaud's iron hematoxylin was brought from Merck & Co., Whitehouse

Station, NJ, USA, and Ponceau-Fushine of Masson and light green from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. For the determination of plasma 11-KT and E2 levels, enzyme-linked immunosorbent assay (ELISA) kits were bought from Cayman Chemical Company, Ann Arbor, USA, whereas Zebrafish Vitellogenin Elisa Kit (V01008402) were obtained from Biosense Laboratories AS (Bergen, Norway) for Vtg measurements. Other enzyme activity assay kits used for glutathione peroxidase and glutathione S-transferase measurements were purchased from Cayman Chemical Company (Ann Arbor, USA).

2.2. Animals

All fish treatment and procedures used in this study were in accordance with the general guidelines of the Council of

European Communities (European Union, No 2010/63/CEE) and the French Animal Care Guidelines. Wild-type Tuebin-gen strain zebrafish were purchased from GIS AMAGEN (UMS 3504 CNRS/UMS 1374 INRA, Gif-sur-Yvette, France) at 3 months of age. They were housed for three additional months in the laboratory in a fish containment unit (HP Aquarium, France) filled with dechlorinated and aerated tap water (at $26 \pm 1^\circ\text{C}$ on a 12 h:12 h light:dark cycle). They were daily fed with newly hatched *Artemia nauplii* and once a week with additional commercial pellets (BioMar, France). The same rearing conditions were followed during the exposure period.

2.3. Experimental Design

Two weeks before experiments, groups of randomly selected 5 males and 5 females, based on morphological differences (i.e., shape of abdomen from gravid females vs males), were introduced into experimental tanks (15 L whole-glass containers filled with 10 L of tap water, i.e., 1 fish/L). Thereafter, fish were exposed either to gallic or pelargonic acid for 1 month under semistatic conditions, i.e., 70% daily water exchange was made using peristaltic pumps connected to appropriate stock solution reservoirs of gallic/pelargonic acid (or of noncontaminated freshwater). Debris and feces were removed daily with a siphon and additional 80% water volume was exchanged two times a week to ensure steady concentrations of test compounds in each exposure tank. For each type of test compounds, the following nominal exposure concentrations were established: 0.05, 0.5, and 5 mg/L of substance (and control groups of 0 mg/L). Such a geometric progression aimed at covering a broad range of contamination levels that may be encountered when applying pelargonic or gallic acid to inhibit microalgae growth in freshwater systems in a framework of water ecotechnology development (Nakai et al., 2000, 2005; Wang et al., 2008; Shao et al., 2013). These concentrations also encompassed the theoretical toxicological reference value (TRV) of 0.46 mg/L pelargonic acid which has been established by the "Marin Municipal Water District" (MMWD) United States government agency for water quality monitoring (MMWD, 2008). Treatments, i.e., exposure concentrations, were randomly assigned to glass tanks, with $n = 3$ replicate tanks per treatment including control group (non-exposed fish in freshwater only). During all the exposure period, temperature ($25.6 \pm 0.1^\circ\text{C}$), pH (8.2 ± 0.0), conductivity ($223.1 \pm 0.5 \text{ mS/cm}$), and dissolved oxygen ($6.9 \pm 0.1 \text{ mg/L}$) remained within the ranges that have been reported for *D. rerio* rearing (Lawrence, 2007).

2.4. Spawning, Embryonic Development, and Hatching Time

Reproductive performance of zebrafish was evaluated twice a week during the exposure period. For this purpose, a spawning box equipped with a plastic mesh to prevent egg predation was introduced into each exposure tank, so that spawning boxes contained exposure solutions or clean water only for control tanks, depending on the exposure condition previously assigned to the exposure tank. Plastic plant imitations were fixed to the mesh so as to serve as spawning substrates (Lawrence, 2007). Males ($n = 5$) and females ($n = 5$) were placed separately (using a plastic barrier) into the spawning box in the evening. The next morning, fish were allowed to mix together just before light illumination, after which mating and spawning occurred progressively. Three hours after the light had been turned on, while fish are kept into exposure tanks,

spawned eggs were collected using a 10 mL sterile Falcon pipette, rinsed, and counted. Since several males and females were grouped together into the spawning box, spawning and nonspawning females could not be distinguished when collecting eggs, and thus the total number of females per exposure tank was used for mean fecundity calculations. Consequently, population fecundity was determined in this work as the average number of eggs per female per successful breeding experiment (Penglase et al., 2014). Moreover, in order to rule out any mistake on gender identification, gonad examination was conducted on all fish at the end of experiments using a dissecting microscope. Following fish spawning, coagulated eggs which were milky white were immediately discarded. The remaining eggs from a spawning box were pooled and transferred into Petri dishes (in several batches of 20 eggs each) containing clear water only. Developing embryos were regularly examined under a dissecting microscope for potential abnormalities such as spinal deformities (including spine bending), delayed yolk sac absorption, and pericardial edema (Westerfield, 1995). Mean malformation percentage of newly hatched fry was determined for each treatment over the reproductive test period. Newly hatched fry were recorded daily to evaluate the hatching rate and the median hatching time (expressed in days post fecundation or dpf). Hatching rate (%), median hatching time (dpf), and developmental abnormalities (%) were calculated for each exposure tank where a successful breeding experiment, i.e., egg production, was noticed. Mean values were then calculated over the entire exposure period according to the treatment modality (control group or exposure to gallic or nonanoic acid), and the total number of successful breeding experiments which have been reported for each treatment modality.

2.5. Anesthesia and Biometric Measurements

After 1 month of exposure, all fish were deeply anesthetized with NaHCO_3 , and total length (mm) and wet weight (mg) were recorded to calculate condition factor indices according to Fulton's formula (Nash et al., 2006):

$$K = \left[\left(\text{weight (mg)} / \text{length (mm)}^3 \right) \right] \times 100 \quad (1)$$

Fish were then killed by exsanguination for blood collection prior to circulating hormones and vitellogenin levels determination.

2.6. Blood Collection, Plasma Hormones, and Vitellogenin Level Determination

Blood was collected from each individual by cardiac punctures using 10 mL pipette tip coated with heparin (5000 U/ mL), and immediately centrifuged ($10,000 \times g$, 10 min). Plasma was removed and stored at -80°C until analysis. Measurements of circulating hormone levels (11-KT for males, E2 for females) and Vtg (in females but also in males to investigate putative feminization property of the molecules) were achieved following appropriate plasma dilutions in coating buffers according to the manufacturer's instructions. All measurements from each fish were assayed using commercial

ELISA kits and results were calculated from means of duplicates for each sample.

2.7. Gonad Histology

Midpart of two males and two females per exposure tank (ntotal = 12 specimens per treatment) were fixed in Bouin–Holland solution for 1 week, washed once with water, twice with 70% ethanol, and stored in absolute ethanol (Abdulfa-tah et al., 2011). Fragments of 5 mm thick slices were cut from each individual, dehydrated in OTTIX solution and embedded in the Diawax paraffin substitute (adapted from Langeron, 1942 and Ben Ammar et al., 2014). For females, sections of 6mm were cut from the Diawax block and stained with a trichrome: hematoxylin Gill III, 0.5% phloxine B, and 0.5% light green (Rinchar and Kestemont, 1996). Ovarian maturity was evaluated under light microscopy with respect to oocytes in the following four general categories (Selman et al., 1993): Primary growth (type I): protoplasmic oocytes with enlarged and vacuole-free basophilic cytoplasm [Fig. 2(A)]; Early cortical alveolus stage (type II): appearance of oil droplets or yolk vesicles, occupying 2 or 3 rings in the peripheral zone of cytoplasm [Fig. 2(B)]; Late cortical alveolus stage (type III): presence of the cortical alveoli occupying all the cytoplasm, with irregular shape, cuboidal follicle cells and differentiated cellular layers [Fig. 2(C)]; Vitellogenic follicles (type IV): include follicles in the primary, secondary or tertiary yolk stage with accumulation of crystalline yolk globules in the center of oocytes and the periphery of the cytoplasm, also includes mature follicles [Fig. 2(D)]. The number of oocytes at each developmental stage was counted using an optical microscope (Nikon Eclipse Ni-U) equipped with a DS-Fi2 digital camera associated to the software NIS BR (Nikon France, Champigny-sur-Marne, France), and expressed as relative percentage of total follicle number in the whole section examined. For male gonad examination, sections of 4 µm of fixed midpart of specimens in Diawax blocks were cut and also stained with a trichrome (Ben Ammar et al., 2014): Regaud's iron haematoxylin, 1% Ponceau-Fushine of Masson, and 0.1% light green. Testes cells were classified according to three general sperm germ cell categories (Fig. 3) in accordance with the Histology and Histopathology Atlas of the Zebrafish (<http://zfishtoxpat.comoj.com>) and the work of Huszno and Klag (2012): Spermatogonia: the largest of the spermatogenic cells with relatively voluminous and irregular nucleus with poorly condensed chromatin surrounded by acidophilic cytoplasm; Spermatocytes: include early and late spermatocytes, with relatively smaller cytoplasm than spermatogonia (with no affinity for dye) and easily identified by their densely stained chromatin; Spermatids and spermatozoa: dense, dark and round nuclei, spermatozoa also recognized sometimes by their light colored hair-like tails. For estimating the relative percentage of each sperm cell category, a stereological approach was followed (Huszno and Klag, 2012). Briefly, photographs of a whole gonad section were taken under the microscope objective 40X. Then, a mesh with 87 X 65 points was superimposed on each photograph using the ImageJVR software (v.1.48). The number of points lying on the cysts of a specified cell category was counted and reported to the total number of points embedded in one of the three cell types of the section examined. Results were finally expressed as relative percentage for each testis germ cell category.

2.8. Biochemical Analysis

2.8.1. Liver Isolation and Homogenate Preparation

Livers were collected from 6 fish per exposure tank (ntotal = 18 specimens for a treatment, including males and females), using fine forceps and a dissecting microscope. Livers were homogenized individually in 1.5 mL screw-cap microtubes using a Bullet Blender (Hoyt, 2009). In each microtube, prior to agitation, a mass of silica beads equals to twofold the mass of liver was introduced with 25 µL of ice-cold homogenization buffer (100 mM Tris, 150 mM KCl, 1 mM EDTA, 0.1 mM PMSF, and 10% v/v glycerol; pH 7.5). Tubes were agitated for 1 min at maximum speed and kept for 1 min on ice. Then, 90 µL of homogenization buffer were added followed by two more minutes of agitation. The resulting homogenate was centrifuged 13,000 X g, 20 min (4°C). The supernatant, corresponding to the S9 fraction, was aliquoted into 25 µL fractions and rapidly frozen (-80°C) until further use for protein content and enzymatic activity analyses.

2.8.2. Total Protein Determination

The volume of recovered supernatant from each liver homogenate was determined to estimate the total liver protein content. Protein content of S9 fraction was then measured on a 10-fold dilution of the recovered supernatant, according to the method developed by (Bradford, 1976) and using bovine serum albumin as a standard (Thermo Scientific Pierce Protein Assay kit 23236). Absorbance was read at 595 nm (Labsystem Multiskan EX, LabSystems Inc., Franklin, MA, USA) after an incubation period of samples and reagents for 15 min (258C) in microtiter plates.

2.8.3. Enzyme Activity Determination

Lactate dehydrogenase, glutathione transferase as well as total glutathione peroxidase activities were determined on S9 fraction spectrophotometrically using 96-well microtiter plates (Labsystem Multiskan EX, LabSystems Inc., Franklin, MA, USA).

LDH activity (EC 1.1.1.27) was evaluated according to the protocol described by Jesus et al., (2013) with slight modifications. The reaction mixture contained 147 mL of NADH (0.24 mM) and 23 mL of pyruvate (10 mM) in a Tris–NaCl buffer (0.1 M, pH 7.2). The decrease in absorbance at 340 nm due to the oxidation of NADH was continuously monitoring for 6 min.

For GST activity (EC 2.5.1.18) determination, the procedure of Habig and Jakoby (1981) was followed, using 1-chloro-2,4-dinitrobenzene (CDNB) as the model substrate (Sigma-Aldrich CS0410). The increase in absorbance due to the conjugation of the glutathione thiol group to CNDB was monitored at 340 nm for 6 min.

The measurement of total GPx activity (EC 1.11.1.9) was based on the standard procedure of Drotar et al., (1985) using cumene hydroperoxide as substrate. The oxidation of reduced glutathione (GSH) to its oxidized form (GSSG) by GPx is coupled to the recycling of GSSG to GSH. The decrease in NAPDH concentration, used as a co-factor, was monitored at 340 nm for 8 min (Cayman Chemical 703102).

All enzyme activities were determined in duplicates for each liver homogenate, using 20 mL of diluted homogenate samples per microplate well. The mean obtained from duplicate measurements for one fish liver sample was used for further calculations (total 518 per enzyme activity per treatment). All enzyme activities were finally expressed as U/mg protein. One unit corresponded to the amount of enzyme hydrolyzing one nanomole of substrate per minute.

2.9. Statistical Treatment

3. Results

3.1. Fish Condition Index

First of all, no dead fish neither any abnormality in animal behavior (swimming, feeding) could be reported during all the exposure period. Moreover, condition index was evaluated at the end of exposure as a general marker of overall fish health status (Table I). No differences were found among control and treatment groups regardless contaminant type and organisms gender (Table I).

3.2. Liver Enzyme Activities

In order to figure out eventual biological changes concomitant to the long-term exposure to each phytochemical, several biochemical biomarkers were further determined on total liver homogenates of both control and exposed fish. As reported in Table I, for both male and female specimens exposed to gallic or pelargonic acid, no significant variations were observed in total protein content, nor in LDH and GST activities. In contrast, higher levels of total GPx activity have been measured in males and females exposed to both type of substances compared to control regardless the exposure concentration (Table I). These increases in GPx activity were significant for female zebrafish exposed to 0.05, 0.5, and 5 mg/L of gallic acid on the one hand and to 0.05 and 5 mg/L of pelargonic acid on the other hand (Table I).

3.3. Plasma Steroid Hormones and Vitellogenin (Vtg) Levels

Additional biochemical parameters were assessed in order to investigate potential impacts of each phytochemical on zebrafish reproductive physiology. Results concerning plasma sex hormones and Vtg levels after exposure to gallic acid are presented in Figure 4. Males exposed to gallic acid showed a decrease in 11-KT levels but this decrease was significant only for fish exposed to the highest concentration of 5 mg/L [Fig. 4(A)]. Despite lower amounts of plasma estrogen levels measured in females exposed to gallic acid [Fig. 4(C)], no significant differences could be observed between treatments. Additional measurements of circulating Vtg indicated plasma levels which were about four degrees of magnitude higher in females [Fig. 4(D)] than in males [Fig. 4(B)] for all gallic acid treatments (including control). Besides, no significant

All data were expressed as mean \pm standard error. Median hatching time was determined using REGTOX Excel macro (v.7.0.6) based on nonlinear regression (Hill model) (Vindimian, 2012). Percentages were arcsin square root transformed prior to statistical analyses. Normality and homogeneity of variance assumptions were respectively checked using Kolmogorov–Smirnov and Levene's test. Differences between groups were then determined by one-way ANOVAs for parametric distributions or Kruskal–Wallis for nonparametric ones, followed by Student Newman–Keuls post-hoc tests ($p < 0.05$). In the case of inequality between the treatment group size, the Dunns' test was used. All analyses were carried out at a significance level of $p < 0.05$ using Statistica v.10.

variations could be observed between treatments for either females or males [Fig. 4(D,B)].

Results related to pelargonic acid exposed fish are illustrated in Figure 5. Overall, similar patterns could be reported respectively for hormone [Fig. 5(A,C)] and Vtg levels [Fig. 5(B,D)] of pelargonic acid exposed organisms. A significant decrease in 11-KT was noticed for males at the exposure concentrations of 0.05 and 5 mg/L of substance [Fig. 5(A)], whereas significantly lower levels of E2 were measured for females exposed to 0.5 mg/L of compound compared to control [Fig. 5(C)]. Regarding Vtg concentrations, there was no statistical difference for plasma levels in males [Fig. 5(B)]. On the contrary, mean Vtg concentration was significantly higher for females exposed to 5 mg/L of pelargonic acid compared to control [Fig. 5(D)].

3.4. Gonad Development

Subsequent histological examination of gonad development was conducted (Figs. 2 and 3) and results are summarized in Figure 6. Overall, testes and ovaries with typical organizational architecture were observed for all fish specimens from control and treatment groups. Moreover, the presence of all germ cell types could be reported in gonad sections from males and females exposed either to gallic acid [Fig. 6(A,B)] or to pelargonic acid [Fig. 6(C,D)]. Besides, a general trend has been noticed in the relative proportions of germ cells regardless contaminants' nature and concentration. Indeed, testes were mainly composed of cell types which were at their latest stage of development (spermatids and spermatozooids) and to a lesser extent in the spermatocyte stage [Fig. 6(A,C)]. The opposite pattern was observed for ovaries with a higher relative frequency of primary growth stage oocytes (type I) with typical enlarged basophilic cytoplasm [Figs. 2(A) and 6(B,D)], whereas the relative number of cortical alveolus stages (types II and III) and vitellogenic and mature follicles (grouped under "type IV" category) were lower, ranging from 10% to 15% in average. Furthermore, no significant variation was detected in relative germ cell types for both sex and contaminant exposure conditions [Fig. 6(A–D)].

3.5. Fecundity, Hatching Rate, and Embryo-Larval Development

As reported in Table II, there was no statistical difference in mean fecundity between control and treatments either for gallic or pelargonic acid exposed female zebrafish. The evaluation of overall hatching rates indicated that the lowest egg hatchability was observed for fish exposed to 0.5 mg/L of

gallic acid and to 5 mg/L of pelargonic acid, with, respectively, $32.0 \pm 8.2\%$ and $36.7 \pm 14.7\%$ of total egg survival (Table II). In addition, no significant differences were found between control and treatments regarding embryonic development malformation rate (Table II).

4. Discussion

In order to figure out any potential sublethal effects of gallic or pelargonic acid, several cellular enzyme activities were first evaluated as exposure biomarkers, as it is usually recommended to avoid misconception about cellular damages that xenobiotics could cause (van der Oost et al., 2003; Zorita et al., 2008). Taking into account that liver is the primary organ where detoxification occurs, it is also sensitive to oxidative stress damages subsequent to the intake of toxic substances (van der Oost et al., 2003). Thus, in this work, liver total protein content was primary determined as a form of intermediate metabolic substrate (Sancho et al., 2010), followed by enzyme activities such as LDH as an indicator of cytotoxicity (Diamantino et al., 2001; Coelho et al., 2011), GST and GPx as primary antioxidant defense mechanisms involved respectively in Phase II biotransformation and in radical species neutralization (van der Oost et al., 2003). From all these biochemical parameters, only GPx activity was responsive to chronic exposure to gallic or pelargonic acid (Table I). GPx is a part of the primary cell defense system to cope with an excess of free radical production and subsequent oxidative damages (van der Oost et al., 2003). In mammals, gallic acid has been shown to act as an antioxidant phenolic acid by leading to the induction of hepatic GPx, which in turn was believed to protect against adverse oxidative damages and to promote health protective effects (Yeh and Yen, 2006; Hsu and Yen, 2007). Whether the same protective mechanisms could be hypothesized for fish in our experimental conditions deserves further investigations, particularly in a context of ecofriendly surface water quality management. Regarding GPx induction under pelargonic acid exposure (Table I), the mode of action of this nonspecific herbicide has been well described when sprayed onto terrestrial weeds (Lederer et al., 2004). Because of its medium chain length, this aliphatic acid would be particularly efficient to intercalate into plasma cell membrane, resulting into chlorophyll pigment dislocation and subsequent outer thylakoid ROS production along with lipid peroxidation in chloroplast (Lederer et al., 2004). Concerning exposed zebrafish, similar phenomenon may also occur at the cellular or mitochondrial level that may lead to enhanced fish hepatic ROS formation and concomitant GPx induction as to prevent cells against oxidative stress.

Besides antioxidant enzyme variation, chronic exposure to both type of phytochemicals lead to modifications into reproductive hormones and circulating Vtg levels (Figs. 4 and 5). The most significant variation following gallic acid exposure was a decrease in plasma 11-KT level in males at the highest compound concentration of 5 mg/L [Fig. 4(A)]. Gallic acid autoxidation products, resulting from natural oxidation of the polyphenol in alkaline medium (Nogales et al., 2011), have been shown to possess antigonadotropic activity in vitro (Burczyk et al., 1996). Indeed, inhibition of basal or of LH-stimulated testosterone secretion have been reported by Burczyk et al., (1996) using cultured Leydig cells from a mouse model (Swiss strain) at exposure concentrations varying from 0.1 to 10 mg/L of oxidation products. In this study, autoxidation

of gallic acid also presumably occurred taking account of the alkaline pH of 8.2 in exposure tanks (see Materials and Methods part). Thus, one may also hypothesize that similar inhibition of 11-KT synthesis took place in the long term in fish due to the presence of gallic acid oxidation products; however, the investigation was not designed to discriminate between the biological effects of gallic acid by itself and its autoxidation products considering that both molecular forms would occur in a field scale context. Furthermore, in spite of 11-KT plasma concentration variations in gallic acid exposed males [Fig. 4(A)], further investigation indicated circulating Vtg levels in far higher concentrations in females than in males [Fig. 4(D,B)], as it is usually reported in the literature for fish only exposed to freshwater (Van der Ven et al., 2003). Moreover, since no significant variations were reported between treatments either for females or males [Fig. 4(D,B)], these results indicated thus an apparent absence of estrogen-like activity of gallic acid.

On the other hand, waterborne exposure to pelargonic acid was related to a decrease in 11-KT and E2 levels, respectively, in males and females [Fig. 5(A,C)], along with a dose-dependent increase in Vtg levels in exposed females [Fig. 5(D)]. Pelargonic acid is a fatty acid naturally occurring in several plants and animals (FCT, 1978; US EPA, 2000). It is usually not assumed to possess any estrogen-like activity or endocrine disrupting potency (Federal Register, 2003; MMWD, 2008). Indeed, this compound can be metabolized into a fatty acid-CoA complex and then enters the Krebs's cycle for energy production in mitochondria (FCT, 1978; Federal Register, 2003). Nevertheless, as recently shown by Stempin et al., (2013) using in vitro experiments, derivatives of short-chain fatty acids such as 2-(2-propynyl)-nonanoic acid (a derivative of valproic acid with pelargonic acid) could activate the estrogen receptor α (ER α) in stably transfected human cell line (HEK293). Moreover, there may be several ways in which xenobiotics, including nonsteroido-genic chemicals, could interact with the endocrine and reproductive systems of vertebrates, i.e., not only through direct interactions with nuclear receptors but also through modulation of metabolizing enzyme activities, or interferences with hormone binding serum proteins and other nongenomic pathways (Foradori et al., 2008; De Coster and van Lare-beke, 2012). Further investigations would be required to better assess the mode of action of pelargonic acid regarding the modulation of steroidal hormone and Vtg secretion as observed in the chronically exposed zebrafish [Fig. 5(A,C,D)].

Analyses of physiological endpoints were completed with gonad histological examination in both sexes in order to get more insights into potential reproductive consequences of the hormone and Vtg level modulation. Overall, all testes and ovaries developmental stages could be observed in control and exposed specimens, thereby confirming ongoing gametogenesis in fish which were all sexually mature. Besides, zebrafish are asynchronous spawners able to reproduce all the year round under laboratory conditions with very

short mean interspawning intervals of 1.5 days (Spence and Smith, 2006). In the context of our study, regular spawns (two times a week) were conducted during 1 month and all fish were sacrificed the day after the last spawning event. The naturally high fecundity of zebrafish in conjunction with our experimental design relying on the maintenance of reproductively active females may explain the observed oogenesis patterns characterized by a predominance of oogonia [Fig. 6(B,D)]. Finally both male and female gonad development patterns as reported in this study were in accordance with previous work assessing reproductive performance of this fish species (Daouk et al., 2011), therefore, indicating that exposure to gallic and pelargonic acids did not impact the progress of gametogenesis.

According to Penglase et al., (2014), the alternate method of fecundity determination in the present study may be used to estimate population fecundity relevant for the assessment of ecological impacts of contaminant exposure. Great variations in the spawning behavior considered on replicate tanks scale

could be noticed during breeding experiments regardless the exposure condition (personal observations), possibly explaining discrepancies observed between treatments concerning fecundity data (Table II). Indeed, mated fish did not spawn systematically and egg production could differ substantially from one spawning event to another for the same spawning box. Nevertheless, such observations were in accordance with the literature when groups of male and female zebrafish are used for mating (Sanden et al., 2012; Penglase et al., 2014). Our primary objective was to evaluate the influence of phytochemicals on the reproductive performances of *D. rerio* as realistically as possible in accordance with native fish ecology (Lawrence, 2007). Increasing the exposure period may help to mitigate the differences in spawning success and egg production from one replicate tank to another (Lawrence et al., 2012), and to better address the eventual effects of contaminants on fecundity as well. However, regardless of the reproductive performances, there was no difference in median-hatching time and developmental abnormality rates of larvae (Table II), indicating that eggs which developed until hatching were overall of similar "good" quality.

5. Conclusion

In this study, we showed that long-term waterborne exposure to gallic or pelargonic acid can lead to antioxidant enzyme activity induction at the cellular level, with significant increases in GPx activity which were generally reported for females exposed at concentrations as low as 0.05 to 5 mg/L for both compounds. At the physiological level, waterborne gallic acid exposure leads to a marked decrease of male steroid hormone (11-KT) at the highest tested concentration of 5 mg/L, whereas no variation could be observed in female estrogen (E2) plasma level. Similar results were obtained with fish exposed to pelargonic acid, with slightly more pronounced decreases in male and female hormone levels, respectively, at 0.05 and 5 mg/L on one hand, and 0.5 mg/L of the middle-chain fatty acid on the other hand. To our knowledge, this is the first report that gallic and pelargonic acids could influence some reproductive

parameters in zebrafish. However, these results had to be mitigated since histological examination indicated that all fish were reproductively mature, and without any alteration in relative germ cell types. Nevertheless, the possibility for gamete production and egg viability to get affected for longer exposure period than 1 month cannot be completely ruled out. To sum up, slight detrimental biological effects could be reported depending on gallic or pelargonic acid exposure at concentration ranges which were compatible with potential water treatment ecotechnology development based on the direct use of these compounds. In order to warrant more environmental safety, we suggest to take into account the selected mode of application and environmental fate of phytochemicals (e.g., application rate, sequestration or adsorption phenomena onto organic matter, etc.) prior to any large-field-scale trial.

Table 1. Fish condition index, liver total protein content, and enzyme activities of male (M) and female (F) zebrafish after 1 month exposure to gallic or pel-argonic acid, and the control group (0 mg/L of test substance)

		Control	Gallic acid			Pelargonic acid		
		0 mg/L	0.05 mg/L	0.5 mg/L	5 mg/L	0.05 mg/L	0.5 mg/L	5 mg/L
Fish condition index	M	0.80 ± 0.03	0.73 ± 0.03	0.79 ± 0.02	0.80 ± 0.03	0.76 ± 0.02	0.81 ± 0.03	0.78 ± 0.02
	F	0.82 ± 0.02	0.88 ± 0.02	0.83 ± 0.03	0.90 ± 0.06	0.85 ± 0.03	0.82 ± 0.02	0.91 ± 0.02
Liver total protein content (mg/g fresh weight)	M	77.5 ± 16.8	88.7 ± 13.6	96.3 ± 6.4	84.0 ± 14.3	93.6 ± 4.9	106.0 ± 11.0	82.0 ± 5.1
	F	86.2 ± 8.5	62.8 ± 13.6	77.2 ± 11.6	49.2 ± 7.8	88.5 ± 5.6	77.1 ± 4.4	80.1 ± 9.3
LDH activity (U/mg protein)	M	1839 ± 416	1322 ± 135	1922 ± 182	2116 ± 143	2448 ± 185	3269 ± 653	2517 ± 272
	F	512 ± 100	661 ± 57	446 ± 40	516 ± 75	893 ± 252	516 ± 62	578 ± 142
GST activity (U/mg protein)	M	359 ± 50	225 ± 16	286 ± 29	332 ± 31	368 ± 31	369 ± 34	446 ± 56
	F	168 ± 11	223 ± 27	169 ± 19	175 ± 16	211 ± 31	174 ± 12	139 ± 15
GPX activity (U/mg protein)	M	446 ± 111	507 ± 58	593 ± 97	668 ± 101	667 ± 94	608 ± 61	639 ± 98
	F	39 ± 12	214 ± 87*	171 ± 56*	214 ± 61*	277 ± 73*	110 ± 31	234 ± 78*

Table 2. Evaluation of reproductive parameters in zebrafish over 1 month exposure to gallic or pelargonic acid in the concentration range of 0, 0.05, 0.5, and 5 mg/L of test substance

	Control	Gallic acid			Pelargonic acid		
	0 mg/L	0.05 mg/L	0.5 mg/L	5 mg/L	0.05 mg/L	0.5 mg/L	5 mg/L
Fecundity (mean egg number/female/spawning event)	12.1 ± 3.3	8.0 ± 1.5	16.5 ± 4.5	24.4 ± 4.9	16.3 ± 6.4	35.9 ± 8.1	26.0 ± 8.3
Median hatching time (dpf)	3 ± 0.3	4.1 ± 0.3	4.5 ± 0.3	4.4 ± 0.3	3.7 ± 0.3	3.6 ± 0.3	4.1 ± 0.1
Hatching rate (%)	49.7 ± 18	65.9 ± 17	32.0 ± 8.2	59.4 ± 10	61.1 ± 10.4	47.2 ± 13.1	36.7 ± 14.7
Developmental abnormalities (%)	13.7 ± 8.0	3.6 ± 0.8	10.0 ± 3.5	5.3 ± 1.7	1.0 ± 0.5	12.1 ± 4.5	8.7 ± 3.5

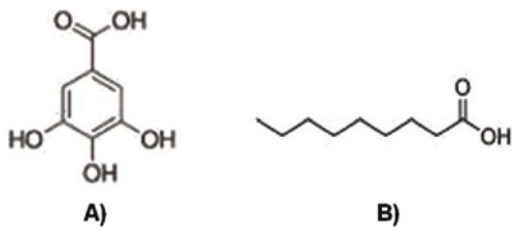


Figure 1. Molecular structures of (A) gallic acid (CAS 149-91-7) and (B) pelargonic acid (CAS 112-05-0).

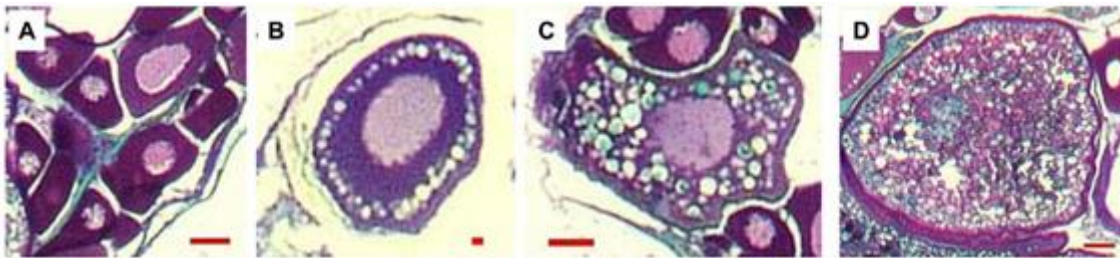


Figure 2. Typical germ cell types observed during the oogenesis in zebrafish. (A) Primary growth stage (type I). (B) Early cortical alveolus stage (type II). (C) Late cortical alveolus stage (type III). (D) Vitellogenic follicle (type IV). Bar = 10 μ m in A, B, C, and D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

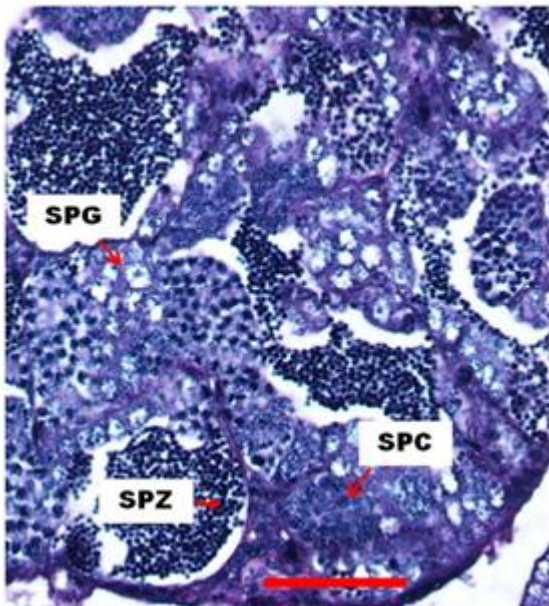


Figure 3. Typical germ cell types observed during the spermatogenesis in zebrafish. SPG, spermatogonia; SPC, spermatocytes; SPZ, spermatids and spermatozoa. Bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

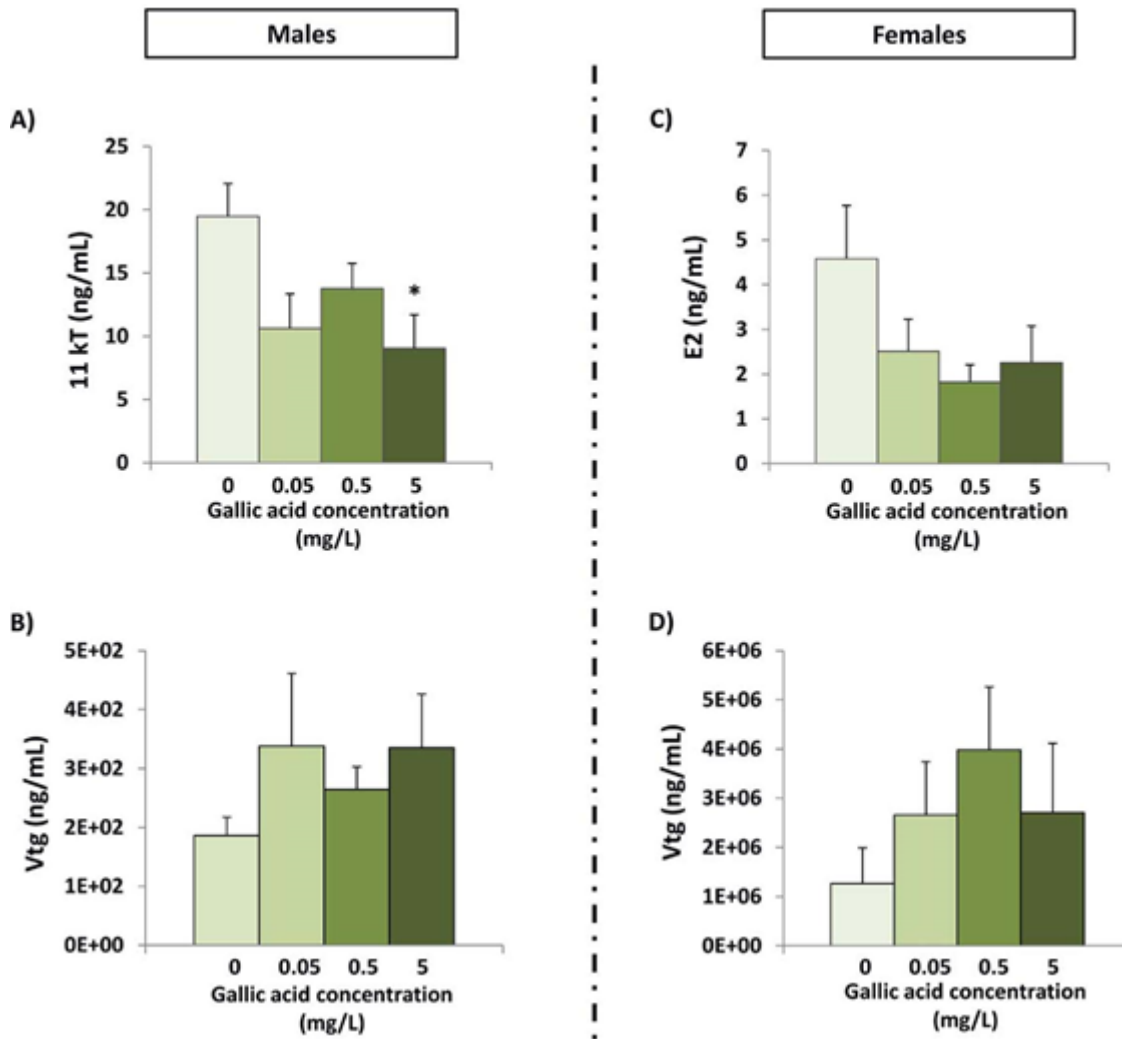


Figure 4.

Plasma levels of (A) 11 kT and (B) Vtg in male zebrafish; (C) E2 and (D) Vtg in female zebrafish after 1 month exposure to gallic acid in the concentration range of 0, 0.05, 0.5, and 5 mg/L. Data are expressed as mean \pm 6 SE. Asterisks indicate significant differences against control ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

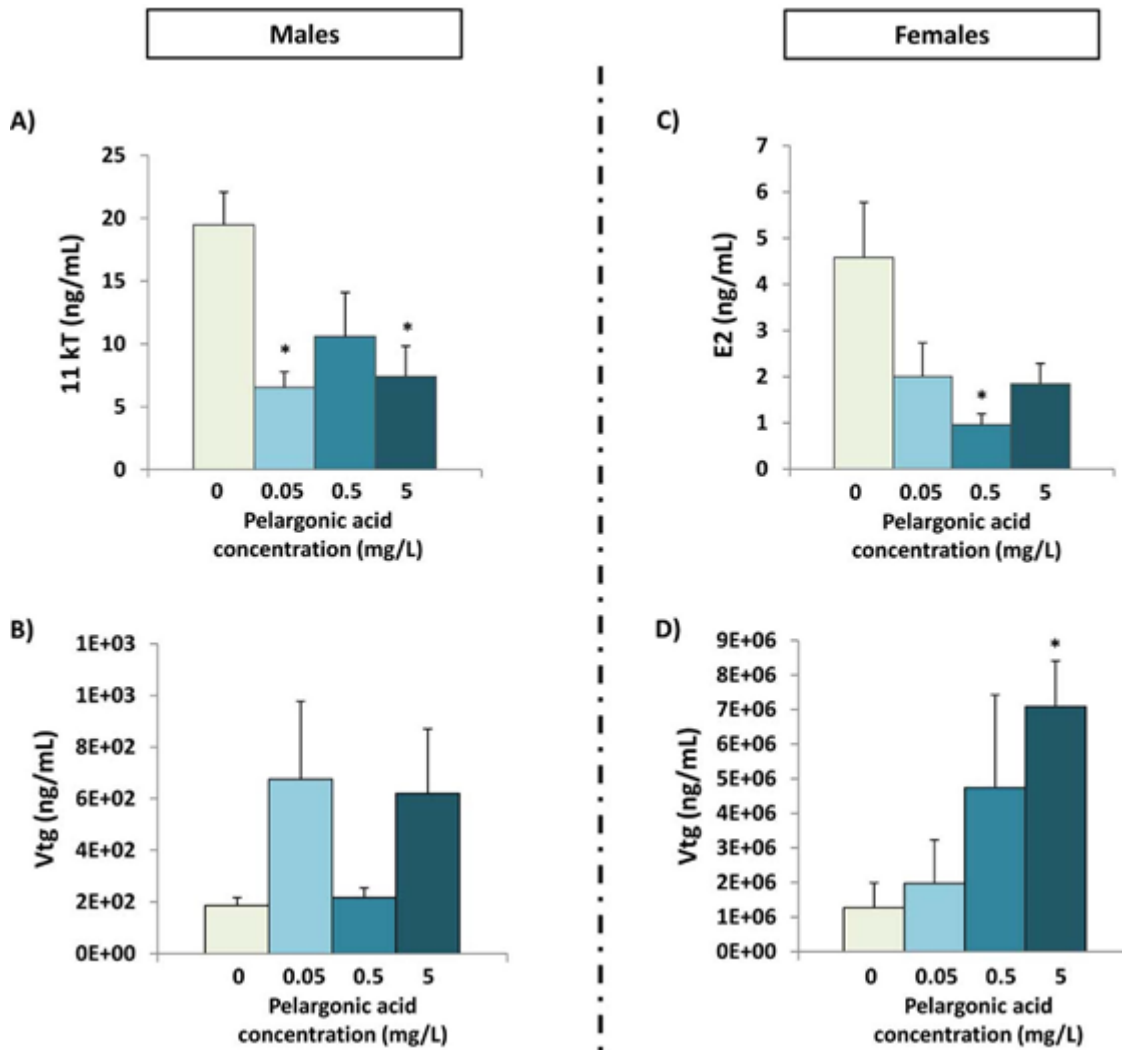


Figure 5.

Plasma levels of (A) 11 kT and (B) Vtg in male zebrafish; (C) E2 and (D) Vtg in female zebrafish after 1 month exposure to pelargonic acid in the concentration range of 0, 0.05, 0.5, and 5 mg/L. Data are expressed as mean \pm 6 SE. Asterisks indicate significant differences against control ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

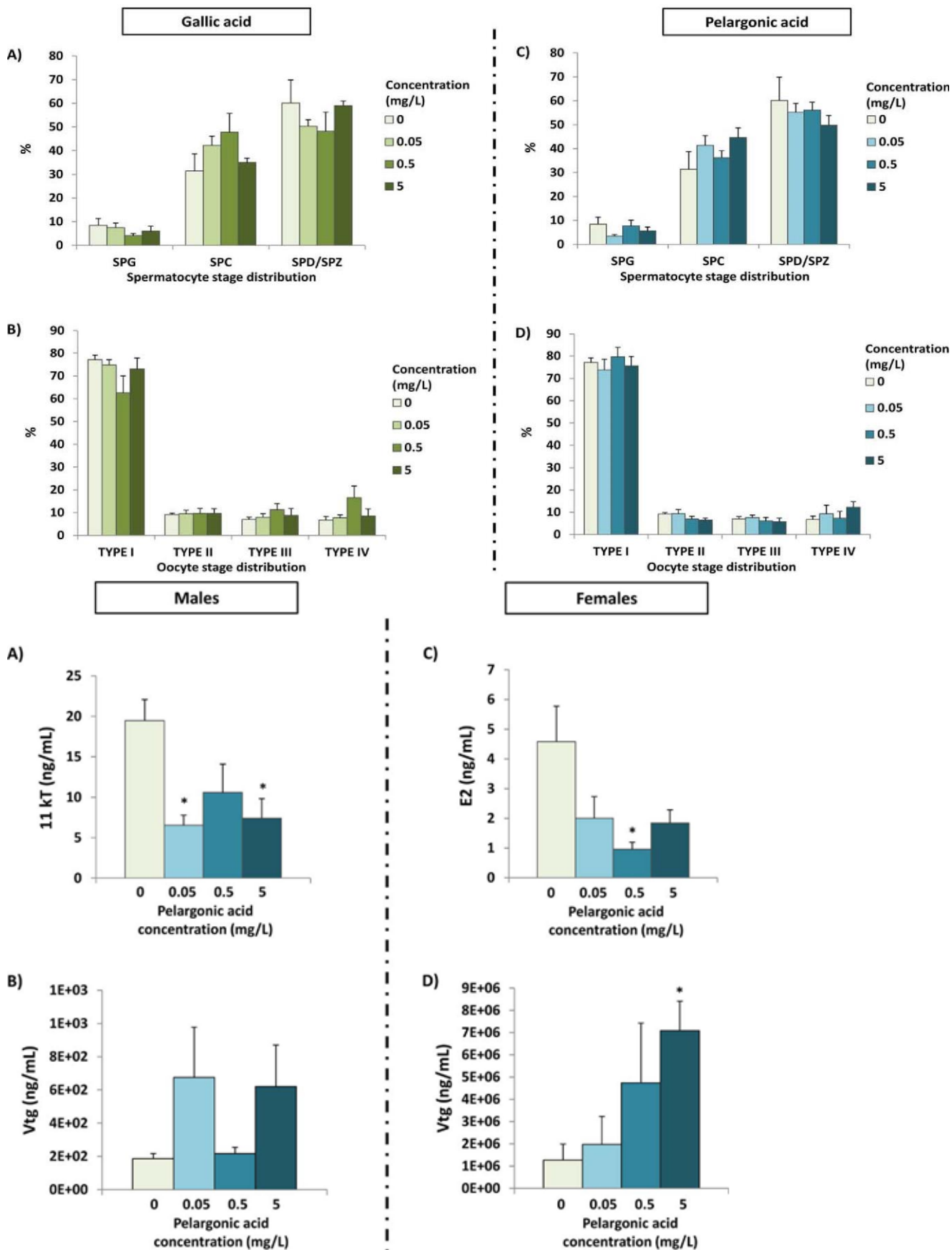


Figure 6. Relative distribution of germ cell types (%) in male and female zebrafish: (A and C) Percentage of spermatogonia (SPG), spermatocytes (SPC), spermatids, and spermatozooids (SPD/SPZ) in testis of male zebrafish respectively after gallic and pelargonic acid exposure. (B and D) Percentage of type I, II, III, and IV ovarian stages in female zebrafish respectively after gallic and pelargonic acid exposure. [Color figure can be viewed in the online issue, which is available at wileyonlineli-brary.com.]

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