

Article

Reproductive Toxicity of 3,4-dichloroaniline (3,4-DCA) on Javanese Medaka (*Oryzias javanicus*, Bleeker 1854)

Musa Adamu Ibrahim ^{1,2}, Syaizwan Zahmir Zulkifli ^{1,3,*}, Mohammad Noor Amal Azmai ^{1,4},
Ferdaus Mohamat-Yusuff ^{3,5} and Ahmad Ismail ¹

¹ Department of Biology, Faculty of Science, Universiti Putra Malaysia, UPM Serdang, Selangor 43400, Malaysia; moosaad8@gmail.com (M.A.I.); mnamal@upm.edu.my (M.N.A.A.); aismail@upm.edu.my (A.I.)

² Department of Biological Sciences, Faculty of Science, University of Maiduguri, Maiduguri 600230, Nigeria

³ International Institute of Aquaculture and Aquatic Sciences (i-AQUAS), Universiti Putra Malaysia, Batu 7, Jalan Kemang 6, Teluk Kemang, Port Dickson 71050, Malaysia; ferdius@upm.edu.my

⁴ Institute of Bioscience, Universiti Putra Malaysia, UPM Serdang, Selangor 43400, Malaysia

⁵ Department of Environmental Sciences, Faculty of Environmental Studies, Universiti Putra Malaysia, UPM Serdang, Selangor 43400, Malaysia

* Correspondence: syaizwan@upm.edu.my; Tel.: +60-13-3975787

Simple Summary: Compound 3,4-dichloroaniline (3,4-DCA) is a degradation product of some herbicide and intermediate industrial chemicals of many products. Unregulated use of 3,4-DCA affects the reproduction of non-target organisms. In this study, the potential reproductive toxicity of 3,4-DCA on Javanese medaka, a native sentinel organism distributed around aquatic ecosystems of Southern Asia, was evaluated. Low fecundity was observed in female exposed fish to 250 µg/L of 3,4-DCA. Gonad tissue alternation and interference of gametes development were observed both in male and female exposed fish. The response pattern indicated that a low concentration of 3,4-DCA exerted endocrine disruption in Javanese medaka. This finding would provide additional data on the toxicity effect of 3,4-DCA, and it will strengthen the potential of native species in monitoring local and regional aquatic environments.

Abstract: Compound 3,4-dichloroaniline (3,4-DCA) is a metabolite of several urea herbicides and intermediate chemical of several industrial products. Moreover, 3,4-DCA has been frequently detected in aquatic ecosystems around the world. This aniline is more toxic than the parent chemicals, and it affects non-target organisms. This study evaluated a 21-day reproductive response of an emerging aquatic vertebrate model, Javanese medaka (*Oryzias javanicus*), exposed to 3,4-DCA. Fecundity and gonads histopathology were observed. The spawning rate and fertilisation reduced significantly in the highest exposed-group (250 µg/L). Gonadosomatic index (GSI) was significantly low in females exposed to 250 µg/L. No substantial structural alteration of male gonads. However, oocyte development and ovarian cell structure were disrupted in 250 µg/L exposed females. The gonadal developmental was not affected in the males; however, a significant reduction in the developmental of female gonads was observed at 250 µg/L. These results show that 3,4-DCA interfere with the reproduction of Javanese medaka through fecundity and alteration of gonadal tissues.

Keywords: reproduction; fecundity; gonad; histopathology; fertilisation



Citation: Ibrahim, M.A.; Zulkifli, S.Z.; Azmai, M.N.A.; Mohamat-Yusuff, F.; Ismail, A. Reproductive Toxicity of 3,4-dichloroaniline (3,4-DCA) on Javanese Medaka (*Oryzias javanicus*, Bleeker 1854). *Animals* **2021**, *11*, 798. <https://doi.org/10.3390/ani11030798>

Academic Editor: Francesco Fazio

Received: 1 February 2021

Accepted: 10 March 2021

Published: 12 March 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The aniline, 3,4-dichloroaniline (3,4-DCA), is a metabolite of diuron and some herbicides [1] that reach the aquatic ecosystem in considerable concentrations through agriculture and industrial production of other parent chemicals [2,3]. In water, the concentration of 3,4-DCA is usually more than diuron in aquatic organisms; fish and crustaceans are susceptible to 3,4-DCA [4,5]. The Organisation for Economic Co-operation and Development (OECD) recognised 3,4-DCA as a reference chemical for toxicity testing [1,6].

Anilines prevent the metabolic pathway of unspecific membrane in the cell [1,6]. Embryotoxicity was observed in Javanese medaka [7] and zebrafish (*Danio rerio*) exposed to sublethal concentration of 3,4-DCA [8] embryos exposed to 3,4-DCA. Moreover, 3,4-DCA has prolonged toxicity in different organisms [9,10]. It is also toxic to humans [9]. Various studies have demonstrated that 3,4-DCA has nephrotoxicity and reproductive toxicity to mammals [2]. In addition, 3,4-DCA, as a metabolite, is frequently detected in the environmental compartments compared to its mother chemicals (diuron, propanil, etc.) [2]. Concentrations of 567 µg/L in water and 119 mg/kg in soil were detected in some environmental samples [11]. Aquatic pollutants (endocrine-disrupting chemicals; EDCs) can affect reproduction, development, immunity, and other physiological mechanisms [12,13], which eventually threaten fish's survival. Reproduction and development in fish are essential factors that predict its population dynamics [14].

Numerous anilines can disrupt reproductive and endocrine processes [3]. Importantly, 3,4-DCA acts as an antagonist; it competes with testosterone to bind with the androgen receptor (AR) [15]. At 100 and 200–400 µg/L, 3,4-DCA affected the secondary sex characters (male colour intensity and courtship behaviour) and androgen of *Gasterosteus aculeatus* 96-h post-exposure [1]. It was reported that 3,4-DCA is an endocrine-disrupting compound (EDCs) [3,7,8,16].

There is a worldwide increase in interest in EDCs. The quantity and types of EDCs released into the aquatic environment from different sources, such as agricultural runoff, waste treatment plants, and paper mill plants, can affect the reproduction of aquatic organisms [17]. EDCs affect the development and activities of both male and female reproductive structures [18]. However, the oestrogenic activities and health status of organisms exposed to some EDCs are transgenerational [12,16,19]. Estrogenic EDCs originate from both anthropogenic and natural sources [12].

Javanese medaka (*Oryzias javanicus*) inhabit estuarine ecosystems of Peninsular Malaysia, Singapore, Indonesia, and Thailand [7,20,21]. Javanese medaka possessed significant features that make it relevant to the widely used model fish [21–24]. To date, several studies on the toxicity of various chemicals on Javanese medaka have been conducted [25]. Generally, there is limited research on the reproduction of Javanese medaka. The size of Javanese medaka, concentration, and exposure period (usually ≤ 21 days) are adequate for toxicity response in the fish short-term reproduction assay (FSTRA) of endocrine-active substances. Imai et al. [26] observed that at least 16 ng/L of one of the well-known endocrine active chemicals, 7β-estradiol, disrupt the reproduction of Javanese medaka (*Oryzias javanicus*).

However, as an emerging model fish in Southern Asia, there are limited toxicological studies of organic chemicals on its reproduction. There is a limited report on the effects of 3,4-DCA on estuarine species [27] as compared to freshwater [19,28–30] and marine organisms. The differences in sensitivity of organisms to toxicant would provide possible response across diverse ecosystems. Therefore, more toxicological data is needed to strengthen the response pattern of euryhaline species to 3,4-DCA. Thus, this study aimed to evaluate the fecundity and alterations of gonads changes of Javanese medaka exposed to sublethal concentration of 3,4-DCA.

2. Materials and Methods

2.1. Source of Chemicals

Analar grade of 3,4-dichloroaniline (CAS: 95-76-1, purity: 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The solvent (DMSO, CAS: 67-68-5, purity: 99.9%) was obtained from Friedemann Schmidt Chemical (Kajang, Selangor, Malaysia).

2.2. Javanese Medaka Maintenance

F₂-generation (laboratory culture) of wild strains of Javanese medaka were collected from a water recirculation system in Javanese Medaka Mass Culture Laboratory, Department of Biology, Faculty of Science, Universiti Putra Malaysia. Water condition was maintained at 26 ± 1 °C, pH: 7.8–8.0, DO: 5.5–6.0 mg/L, salinity: $0.54 \pm 0.05\%$, light cycle:

14 h light:10 h dark. The fish were fed twice daily (9:30–10:00 a.m. and 5:00–5:30 p.m.) ad libitum with freshly hatched nauplii of *Artemia* sp. The water recirculation system was cleaned: ~80% of the water was changed at intervals of 3 weeks to avoid unnecessary removal of beneficial microflora that prevent accumulation of toxic ammonia and nitrite from excreta and uneaten food debris.

2.3. Selection of Reproductively Active Adults

Reproductively active adults (3 months post-hatched, total length: 31.5 ± 0.2 mm; body weight: 0.26 ± 0.02 g) were selected in a ratio of 1 male:3 female (12 fish) per glass tank in all the treatment groups (i.e., a total of 252 fish).

2.4. Preparation of Test Chemicals and Exposure

Five exposure groups (10, 25, 50, 125, and 250 $\mu\text{g/L}$) of 3,4-DCA were prepared from a stock concentration (100 mg/L). The experiment was conducted along with solvent control (0.01% v/v DMSO) and dilution water control (dechlorinated tap water) [31]. The highest concentration was selected based on the no observed effect concentration (NOEC) of the 96 h LC₅₀ acute toxicity. The treatments (5 exposure groups and 2 control groups) were done in triplicates with glass aquaria (37.5 cm \times 17.5 cm \times 18 cm) containing 6.0 L of a given treatment. The treatments were changed every 48 h. The exposure duration was 21 days.

2.5. Morphometric and Somatic Indices

The fish were euthanised by immersion in 75 mg/L of tricaine methanesulfonate (MS-222) for ~1–2 min and blotted with paper towels on the day of termination (21 days post-exposure). The average total body length (mm), body weight (mg), the weight of gonads, and liver weight of the fish in all the treatment groups were determined. Hepatosomatic index ($\text{HSI} = [\text{liver weight}/\text{body weight}] \times 100$), gonadosomatic index ($\text{GSI} = [\text{gonad weight}/\text{body weight}] \times 100$), and the condition factor ($(K) = [W100/L^3]$; where W = body weight (mg) and L = total length (mm)) (Bannister, 1976) were determined. The gonads were removed and weighed with an analytical balance (± 0.0001 g).

2.6. Fecundity

Eggs attached to the urogenital opening and the bottom of the glass tanks were collected with a plastic pipette ~30–45 min after the light turned on. The total number of spawned eggs, fertilisation (mean fertilised eggs), and fertility rate ($= [\text{fertilised eggs}/\text{total eggs spawned}] \times 100$) of each treatment tank was determined daily.

2.7. Gonad Histopathology

Whole body of individual of the euthanised fish (one male and one female/replicate from each experimental unit: glass tank) were immersed in Davidson's solution for 24 h, later transferred to 10% buffered formalin solution. The fish were dehydrated and embedded individually due to their sizes with Technovit 7100 (Heraeus Kuizer GmbH, Wehrheim, Germany). The transverse section (~5 μm) of the tissues was prepared with a microtome. Sectioned-tissues were stained with haematoxylin and eosin (H & E) and mounted with Eukitt (O. Kinder, Freiburg, Germany). The sections were examined under a light microscope.

2.8. Statistical Analysis

The normality was verified using Shapiro–Wilk normality test. One-way ANOVA followed by Dunnett's multiple comparisons test was performed between controls and the treatment groups, where the assumption failed, Kruskal–Wallis test was used. All the analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA) at 95% confidence interval ($p < 0.05$). Values are presented as mean \pm standard error of mean ($\bar{x} \pm \text{SEM}$).

3. Results

Figure 1A shows that the average total eggs spawned significantly reduced ($p = 0.007$) at 250 $\mu\text{g/L}$ compared with the control group. No significant difference in the fertility (%) ($p = 0.990$) (Figure 1B) and number of fertilised eggs ($p = 0.557$) between all the treatment groups (Figure 1C) was observed.

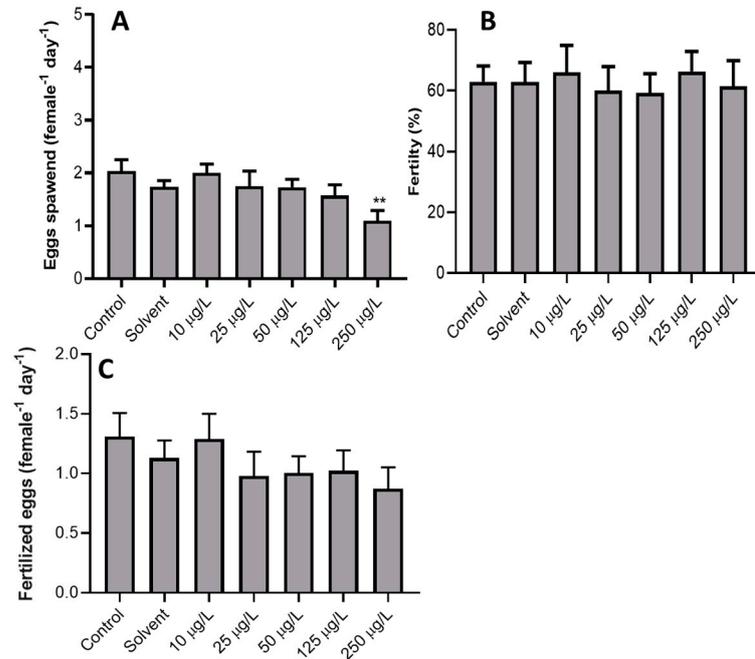


Figure 1. Fecundity of Javanese medaka ($n = 3$) exposed to sublethal concentration of 3,4-dichloroaniline (3,4-DCA) at 21 days exposure: (A) total eggs spawned, (B) fertility, and (C) fertilised eggs ** indicates a significant difference with control at $p < 0.01$. Error bar = \pm standard error of mean (SEM).

There was no significant difference in the average total body length ($p = 0.596$) and body weight ($p = 0.505$) between the treatment groups. However, the condition factor (K) did not substantially differ in male and female fish from different treatment groups (Table 1). Reduction in K implies depletion in reserved energy in fish due to stress factor, thereby disrupting normal physiological processes.

Table 1. Morphometric and condition factor of Javanese medaka ($n = 3$) at 21 days post-exposure to sublethal concentration of 3,4-dichloroaniline (3,4-DCA).

Concentration ($\mu\text{g/L}$)	Total Length (mm)	Body Weight (mg)	Condition Factor (K)
Control	31 ± 3.7	213.6 ± 11.9	0.72
Solvent	28 ± 0.4	214.6 ± 18.8	1.01
10	28 ± 1.2	209.8 ± 29.9	0.94
25	27 ± 0.6	209.1 ± 11.2	1.04
50	25 ± 0.3	233.2 ± 9.6	1.49
125	27 ± 0.3	210.3 ± 3.5	1.07
250	29 ± 1.5	260.3 ± 33.3	1.07

Table 2 shows that there was no significant difference in the HSI of males ($p = 0.995$) and females ($p = 0.054$) from all the treatment groups. The GSI of males in the exposed and control groups were not significantly different ($p = 0.068$). However, the GSI of females exposed to 250 $\mu\text{g/L}$ was significantly low ($p = 0.012$) between all the treatment groups.

Table 2. Hepatosomatic (HSI) and gonadosomatic (GSI) of Javanese medaka (n = 3) at 21 days post-exposure to sublethal concentration of 3,4-DCA.

Concentration ($\mu\text{g/L}$)	HSI (%)		GSI (%)	
	Male	Female	Male	Female
Control	3.65 ± 0.9	4.14 ± 0.8	2.44 ± 0.1	4.19 ± 0.2
Solvent	3.89 ± 1.3	6.34 ± 0.4	3.55 ± 0.8	4.92 ± 0.4
10	4.12 ± 0.8	4.15 ± 0.8	2.78 ± 0.5	3.64 ± 0.5
25	4.39 ± 1.3	6.03 ± 0.4	3.94 ± 0.3	3.79 ± 0.6
50	4.62 ± 1.4	5.11 ± 0.4	2.87 ± 0.2	4.36 ± 0.4
125	3.87 ± 1.6	5.16 ± 0.7	2.12 ± 0.6	2.84 ± 0.4
250	4.55 ± 0.3	5.90 ± 0.5	1.99 ± 0.4	$2.47 \pm 0.3^*$

* indicates a significant difference with both control at $p < 0.05$.

The gonads show all developmental stages of spermatogenic cells (spermatogonia; SG, spermatocytes; SY, spermatids; SD, spermatozoa; SZ) in all the treatment groups. However, there was a proliferation of Leydig cells in the 125 $\mu\text{g/L}$ exposed-group. There were substantial SG and SC domination at the highest exposure level (250 $\mu\text{g/L}$) (Figure 2).

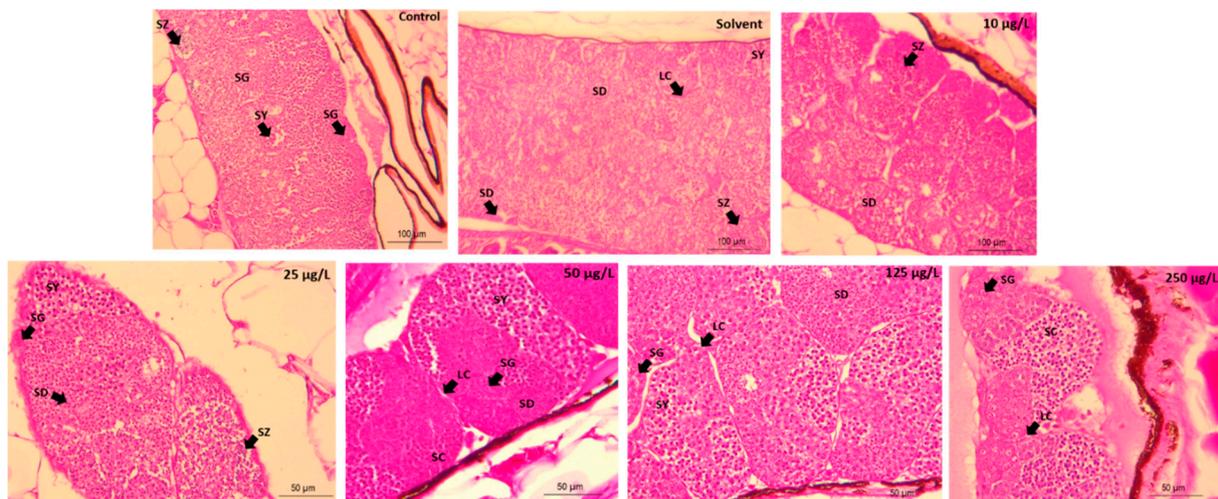


Figure 2. Microphotograph (H&E stain, mag. $\times 40$) of Javanese medaka testes at 21 days post-exposure to sublethal concentration of 3,4-DCA. SC, Sertoli cell; LC, Leydig cell; IPF, intravascular proteinaceous fluid, spermatogonia; SG, spermatocytes; SY, spermatids; SD, spermatozoa; SZ.

The development stages of the oocytes from different treatment groups show synchronous development in control groups (dilution water and solvent) and 25 and 125 $\mu\text{g/L}$, while 50 and 250 $\mu\text{g/L}$ oocytes indicate synchronous development. However, at 250 $\mu\text{g/L}$, the secondary vitellogenic stage (Vt2) dominates the ovary, no primary vitellogenic stage (Vt1) was observed, i.e., degeneration of ovarian cells and high proportion of perinucleolar oocytes (Figure 3).

Table 3 shows the gonadal development stage (median) of both sexes and ovarian tissue lesions from different treatment groups. The median stage was low (late spermatogenic; stage 3) at 50 and 250 $\mu\text{g/L}$. However, there was no significant difference ($p = 0.301$) between all the treatment groups. However, development in females exposed to 250 $\mu\text{g/L}$ were significantly low ($p = 0.069$) (mid-development; stage 2). The proportion (%) of atretic oocytes was significantly higher ($p = 0.034$) at 250 $\mu\text{g/L}$, however, there was no significant difference in oocyte retraction ($p = 0.141$) and karyoptic clumping ($p = 0.222$) in all the treatment groups.

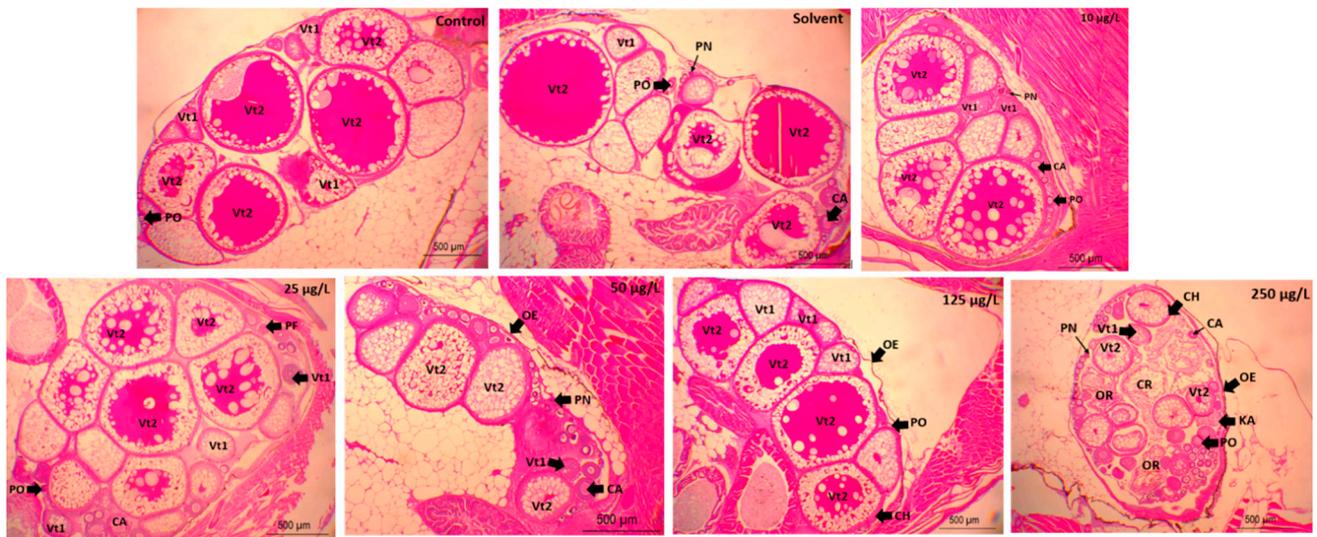


Figure 3. Microphotograph (H&E stained, ×40) of Javanese medaka ovaries at 21 days post-exposure to sublethal concentration of 3,4-DCA. AO, atretic oocyte OE, ovarian epithelium; BV, blood vessels; PF, primordial follicle; LF, large follicle; GR, granulosa cell; OC, ovarian cavity; OE, ovarian epithelium; OC, ovarian cavity; GF, growing follicle; LF, large follicle; CH, chorion; DM, Vt1, primary vitellogenic oocyte; Vt2, secondary vitellogenic oocyte, PN, perinucleolar oocytes; CA, cortical alveolar oocytes; PF, postovulatory follicle.

Table 3. Gonadal staging and ovarian lesions (n = 3) in Javanese medaka at 21 days post-exposure to 3,4-DCA.

Treatment (µg/L)	Staging (Median)		Lesions (%)		
	¹ Male	² Female	Atretic OOCYTE	Oocyte Retraction	Karyoptic Clumping
Control	4	4	0.0	0.0	0.0
Solvent	4	4	0.0	0.0	0.0
10	4	4	0.0	0.0	1.8
25	4	3	2.1	5.2	7.3
50	3	4	4.9	1.5	3.0
125	4	4	1.3	0.0	5.7
250	3	2 *	11.2 *	5.2	4.7

* = significant difference at $p < 0.05$, % = percentage alteration in oocytes per section. ¹ The guideline recommends the following gonadal staging scale for male: 0 = undeveloped, 1 = early spermatogenic, 2 = mid-spermatogenic, 3 = late spermatogenic, and 4 = spent. ² The guideline recommends the following gonadal staging scale for female: 0 = undeveloped, 1 = early development, 2 = mid-development, 3 = late development, 4 = late development/hydrated, and 5 = post-ovulatory.

Figure 4 shows the proportion (%) of the major developmental stages of the female fish's ovarian cells from different groups. The result shows that there was no significant difference in the number of primary oocytes ($p = 0.244$), previtellogenic oocytes ($p = 0.367$), and vitellogenic oocytes ($p = 0.174$) in all the treatment groups. The highest number of primary oocytes was $71.67 \pm 13.3\%$ in 250 µg/L exposed group. For the previtellogenic oocyte, 125 µg/L was the highest ($35.37 \pm 5.2\%$), and the lowest vitellogenic oocyte was $10 \pm 2.7\%$ at 50 µg/L.

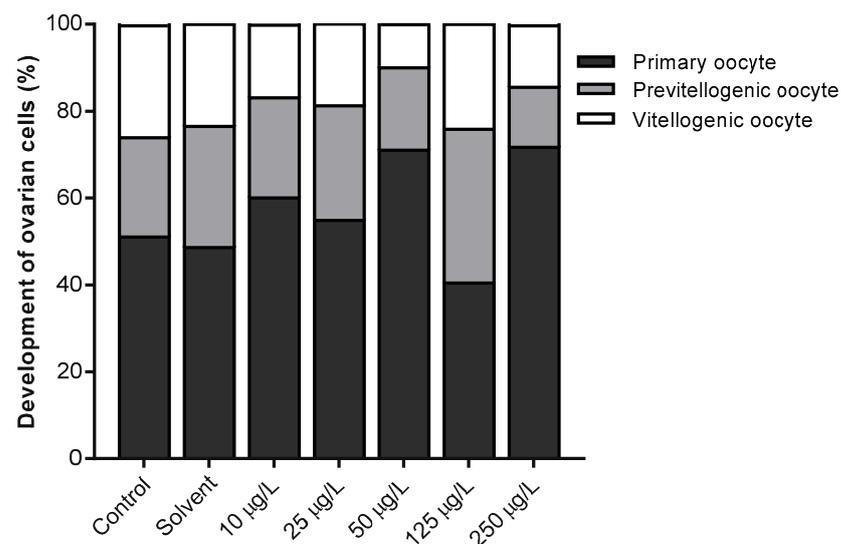


Figure 4. Development of oocyte in Javanese medaka (n = 3) at 21 days post-exposure to 3,4-DCA.

4. Discussion

In this study, the reproductive response of Javanese medaka exposed to sublethal concentration of 3,4-DCA was evaluated. Reproduction is a vital process that regulates population dynamics and functioning ecological communities. Biomarkers like GSI, fecundity rate, fertilisation rate, and eggs hatchability are used as diagnostics tools for the potential hostile effects of xenobiotics on fish reproduction [32–34]. At the same time, HSI indicates the health status of fish in a given environment. Estrogenic substances disrupt gonad morphology, fertility, fecundity, and behaviour [35]. The temporal (3 weeks) decrease in fecundity in Javanese medaka post-exposed to 3,4-DCA with both linear and non-linear patterns indicates toxicity stress that affects the normal physiological activities and a potential threat to its population structure. In this study, the fertility (%) of spawned eggs of Javanese medaka exposed to different treatments of 3,4-DCA was not significantly different. Fecundity is one of the factors that predict the functions of the gonads and mating behaviour in fish [36]. There was a decreased in reproduction of three euryhaline species, i.e., western mosquitofish (*Gambusia affinis*), sailfin molly (*Poecillia latipinna*), and least killifish (*Heterandria formosa*), in low salinity [37,38]. The result of this study is contrary to Glinka et al. [38], who reported that 5 α dihydrotestosterone (an EDC) did not affect egg production of an estuarine fish, Mummichog (*Fundulus heteroclitus*) at low salinity (1.0–2.5‰). Homeostasis to balance osmotic regulation and reproduction and other physiological activities might have been disrupted [37,38].

Similarly, in a 3-week exposure, 1.27–89.4 mg/L of 4-nonylphenol (NP) did not affect egg spawning and fertility of Japanese medaka [39]. In another study, 500 μ g/L of 3-(4-Methylbenzylidene) camphor (4-MBC) causes reproductive toxicity by reducing fecundity and fertility of Japanese medaka [14]. In water flea (*Daphnia magna*), 3,4-DCA exerted reproductive toxicity at a concentration of 0.02 mg/L and 20 pg/L [40]. Increased in time of exposure influences the effect of low concentrations of endocrine-disrupting chemicals [41]. Additionally, 3,4-DCA interferes with hormonal activities and ovulation in zebrafish (*Danio rerio*) at 3-week post-exposure [28]. Similarly, in a viviparous fish, *Chapalichthys pardalis* exposed to 3,4-DCA at 3.3 and 2.5 mg/L lost all their embryos [42]. The osmoregulatory mechanism of homeostasis in Javanese medaka influenced the toxicity response to 3,4-DCA.

The morphometric parameters (total body weight and total length) and K of Javanese medaka exposed to the sub-lethal concentrations of 3,4-DCA were not significantly affected in the tests in this study. This finding implied that the treatments tested had no significant effect on the morphometrics observed. In another study, octocrylene did not significantly affect body length and the total body weight of Japanese medaka at a concentration up to 500 μ g/L [14]. Likewise, Imai et al. [43] reported that estrone (E1) had no effect on Javanese

medaka on total body weight and length. The size of Javanese medaka, concentration, and exposure period (usually ≤ 21 days) are adequate for toxicity response in the fish short-term reproduction assay (FSTRA) of endocrine-active substances [44], as observed in this study and related studies reported in the literature. Chronic or full-lifecycle exposure to 3,4-DCA may exert responses from what is observed in this study.

Oryzias javanicus and *Oryzias dancena* revealed high adaptability to sudden changes of the ambient osmotic pressure in both directions, i.e., from freshwater to seawater or vice versa [45]. The “chloride cells” of the eel gills is typical of the euryhaline teleosts, and is the primary mechanisms involved in the active regulation of osmotic concentration [46]. The salinity of the exposure media affects the uptake and reproduction toxicity of EDCs on the exposed fish [38].

The exposure concentrations used in this study did not affect the HSI in both males and females as earlier reported in Javanese medaka exposed to concentrations up to 5000 ng/L estrone, an EDC [43]. The GSI of males and females exposed to the highest concentration (250 $\mu\text{g/L}$) was lower, which indicated a stress symptom. The decrease in GSI might be associated with endocrine-active substances [47,48]. Similarly, GSI was not affected in Javanese medaka exposed to estrone, an EDC [43]. The condition factor (K), HIS, and GSI provide information on anthropogenic stress and health [49,50]. However, the male GSI in this study is not in line with the finding of Pereira et al. [19] that observed a decrease in GSI of male Nile tilapia (*Oreochromis niloticus*) exposed to 200 ng/L 3,4-DCA for 25 days. The reduction in GSI, germ cell production, and increased gonadal tissue abrasion was detected in teleost fish after exposure to different metabolites of diuron [19].

Histological changes in the gonads of male and female exposed Javanese medaka reveal stress symptoms. Domination of some gametophytic cells in exposed males is attributed to 3,4-DCA toxicity stress during gametogenesis. Gametogenesis, spermiation, and spawning are essential indicators of reproduction status in living organisms [51]. Inhibition of the gametogenic cells is associated with endocrine-disrupting effects [48,52]. Histopathological damages in the liver and testis were also observed in animal models; mice [2], and rats 30 days after exposure to 3,4-DCA [2,15].

There was no considerable difference in the stages of gamete development in male Javanese medaka. Similarly, there was no difference in gametes' development in male Nile tilapia (*Oreochromis niloticus*) collected from lakes contaminated by oestrogenic compounds [53]. Moreover, 3,4-DCA may exert toxicity of male gonadal cells [15]. In this study, the significant reduction in oocyte development was due to toxicity stress by 3,4-DCA during follicular growth and differentiation. However, gonad tissue abrasions in Mysid (*Mesopodopsis slabberi*) 48 h post-exposure to 3,4-DCA were observed [27]. The substantial dose-dependent increase in primary oocytes in this study is in line with Rodgers-Gray et al. [54], who reported that the gonads of wild roach (*Rutilus rutilus*) that were exposed to treated sewage effluent for 150 days were affected similarly.

The three different oogenic stages observed (primary, previtellogenic, and vitellogenic oocytes) were not affected in a concentration-dependent trend. Non-linear (non-monotonic) patterns of the oocyte development are associated with endocrine disruption [55] by 3,4-DCA during oogenesis, which interferes with homeostasis and hormonal activities. It was previously reported that the disintegration of vitellogenic oocytes in female gonads exposed to EDCs [41,56]. High proliferation of atretic oocytes at higher concentration signifies cytotoxicity effect of 3,4-DCA in the gonads. Increase in atresia in the gonads of zebrafish exposed to 4 ng/L of 17 α -ethinylestradiol (EE2), 50 $\mu\text{g/L}$ of Fadrozole, and their mixture was observed at 90 days post-exposure [35]. Histological alteration indicates the bioaccumulation of 3,4-DCA on the gonadal tissues [27]. In fish exposed to xenobiotics, atretic oocyte indicates a pathological disorder [35].

5. Conclusions

The endpoints reported in this study have demonstrated that the subchronic exposure of Javanese medaka to 3,4-dichloroaniline (3,4-DCA) affects fecundity and fertilisation and

alters the structure and development of both male and female gonads in a non-monotonic (biphasic)-like pattern. The non-monotonic dose-response of some of the endpoints is associated with endocrine disruption observed in other aquatic organisms in similar studies. This effect might be reversible/irreversible or multigenerational, depending on the mode of action at the enzymatic pathways that need further studies.

Author Contributions: Conceptualization, S.Z.Z., M.N.A.A., and A.I.; methodology, M.A.I. and S.Z.Z.; software, M.A.I.; validation, S.Z.Z., M.N.A.A., A.I., and F.M.-Y.; formal analysis, S.Z.Z. and M.A.I.; investigation, S.Z.Z.; resources, S.Z.Z. and F.M.-Y.; data curation, M.A.I.; writing—original draft preparation, M.A.I.; writing—review and editing, M.A.I., S.Z.Z., and M.N.A.A.; visualization, M.A.I., S.Z.Z., and M.N.A.A.; supervision, S.Z.Z., M.N.A.A., and A.I.; project administration, S.Z.Z. and F.M.-Y.; funding acquisition, S.Z.Z. and F.M.-Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Universiti Putra Malaysia (UPM) under Geran Putra Isiatif Putra Siswazah (GP-IPS), Project No. GP-IPS/2017/9577500, Vot. 9577500.

Institutional Review Board Statement: The fish used in this experiment were sampled, handled, and treated following the guidelines and regulations approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (AUP No.: R006/2016).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Philippe, C.; Grégoir, A.F.; Thoré, E.S.J.; Brendonck, L.; De Boeck, G.; Pincheel, T. Acute sensitivity of the killifish *Nothobranchius furzeri* to a combination of temperature and reference toxicants (cadmium, chlorpyrifos and 3,4-dichloroaniline). *Environ. Sci. Pollut. Res.* **2018**, *25*, 10029–10038. [[CrossRef](#)]
2. Eissa, F.I.; El Makawy, A.I.; Badr, M.I.; Elhamalawy, O.H. Assessment of 3,4-Dichloroaniline Toxicity as Environmental Pollutant in Male Mice. *Eur. J. Biol. Sci.* **2012**, *4*, 73–82. [[CrossRef](#)]
3. Bhuiyan, M.N.H.; Kang, H.; Kim, J.H.; Kim, S.; Kho, Y.; Choi, K. Endocrine disruption by several aniline derivatives and related mechanisms in a human adrenal H295R cell line and adult male zebrafish. *Ecotoxicol. Environ. Saf.* **2019**, *180*, 326–332. [[CrossRef](#)]
4. Carena, L.; Proto, M.; Minella, M.; Ghigo, G.; Giovannoli, C.; Brigante, M.; Mailhot, G.; Maurino, V.; Minerio, C.; Vione, D. Evidence of an Important Role of Photochemistry in the Attenuation of the Secondary Contaminant 3,4-Dichloroaniline in Paddy Water. *Environ. Sci. Technol.* **2018**, *52*, 6334–6342. [[CrossRef](#)]
5. Tasca, A.L.; Fletcher, A. State of the art of the environmental behaviour and removal techniques of the endocrine disruptor 3,4-dichloroaniline. *J. Environ. Sci. Health Part A* **2018**, *53*, 260–270. [[CrossRef](#)]
6. Philippe, C.; Hautekiet, P.; Grégoir, A.F.; Thoré, E.S.; Brendonck, L.; De Boeck, G.; Pincheel, T. Interactive effects of 3,4-DCA and temperature on the annual killifish *Nothobranchius furzeri*. *Aquat. Toxicol.* **2019**, *212*, 146–153. [[CrossRef](#)]
7. Ibrahim, M.A.; Zulkifli, S.Z.; Azmai, M.N.A.; Mohamat-Yusuff, F.; Ismail, A. Embryonic toxicity of 3,4-dichloroaniline (3,4-DCA) on Javanese medaka (*Oryzias javanicus* Bleeker, 1854). *Toxicol. Rep.* **2020**, *7*, 1039–1045. [[CrossRef](#)] [[PubMed](#)]
8. Vieira, L.R.; Hissa, D.C.; De Souza, T.M.; Sá, C.A.; Evaristo, J.A.M.; Nogueira, F.C.S.; Carvalho, A.F.U.; Farias, D.F. Proteomics analysis of zebrafish larvae exposed to 3,4-dichloroaniline using the fish embryo acute toxicity test. *Environ. Toxicol.* **2020**, *35*, 849–860. [[CrossRef](#)] [[PubMed](#)]
9. Du, X.; Zhan, P.; Gan, N.; Cao, Y.; Li, T.; Sang, W.; Wang, L. Highly Selective Molecular Recognition and Ultrasensitive Detection of 3,4-dichloroaniline Based on Molecularly Imprinted Sol-Gel Film Combined with Multi-Walled Carbon Nanotubes. *J. Electrochem. Soc.* **2013**, *160*, H742–H748. [[CrossRef](#)]
10. Qu, X.; Yan, B. Zn(ii)/Cd(ii)-based metal-organic frameworks: Crystal structures, Ln(iii)-functionalized luminescence and chemical sensing of dichloroaniline as a pesticide biomarker. *J. Mater. Chem. C* **2020**, *8*, 9427–9439. [[CrossRef](#)]
11. Xiao, H.; Kuckelkorn, J.; Nüßler, L.K.; Floehr, T.; Hennig, M.P.; Roß-Nickoll, M.; Schäffer, A.; Hollert, H. The metabolite 3,4,3',4'-tetrachloroazobenzene (TCAB) exerts a higher ecotoxicity than the parent compounds 3,4-dichloroaniline (3,4-DCA) and propanil. *Sci. Total Environ.* **2016**, *551–552*, 304–316. [[CrossRef](#)]
12. Pinto, P.I.S.; Estêvão, M.D.; Power, D.M. Effects of Estrogens and Estrogenic Disrupting Compounds on Fish Mineralized Tissues. *Mar. Drugs* **2014**, *12*, 4474–4494. [[CrossRef](#)] [[PubMed](#)]
13. Kellock, K.A.; Moore, A.P.; Bringolf, R.B. Chronic nitrate exposure alters reproductive physiology in fathead minnows. *Environ. Pollut.* **2018**, *232*, 322–328. [[CrossRef](#)] [[PubMed](#)]

14. Yan, S.; Liang, M.; Chen, R.; Hong, X.; Zha, J. Reproductive toxicity and estrogen activity in Japanese medaka (*Oryzias latipes*) exposed to environmentally relevant concentrations of octocrylene. *Environ. Pollut.* **2020**, *261*, 114104. [[CrossRef](#)]
15. Bai, J.; Han, H.; Wang, F.; Su, L.; Ding, H.; Hu, X.; Hu, B.; Li, H.; Zheng, W.; Li, Y. Maternal linuron exposure alters testicular development in male offspring rats at the whole genome level. *Toxicology* **2017**, *389*, 13–20. [[CrossRef](#)] [[PubMed](#)]
16. Pereira, T.S.B.; Boscolo, C.N.P.; Felício, A.A.; Batlouni, S.R.; Schlenk, D.; De Almeida, E.A. Estrogenic activities of diuron metabolites in female Nile tilapia (*Oreochromis niloticus*). *Chemosphere* **2016**, *146*, 497–502. [[CrossRef](#)]
17. Frankel, T.E.; Meyer, M.T.; Kolpin, D.W.; Gillis, A.B.; Alvarez, D.A.; Orlando, E.F. Exposure to the Contraceptive Progestin, Gestodene, Alters Reproductive Behavior, Arrests Egg Deposition, and Masculinizes Development in the Fathead Minnow (*Pimephales promelas*). *Environ. Sci. Technol.* **2016**, *50*, 5991–5999. [[CrossRef](#)] [[PubMed](#)]
18. Brehm, E.; A Flaws, J. Transgenerational Effects of Endocrine-Disrupting Chemicals on Male and Female Reproduction. *Endocrinology* **2019**, *160*, 1421–1435. [[CrossRef](#)] [[PubMed](#)]
19. Pereira, T.S.B.; Boscolo, C.N.P.; Da Silva, D.G.H.; Batlouni, S.R.; Schlenk, D.; De Almeida, E.A. Anti-androgenic activities of diuron and its metabolites in male Nile tilapia (*Oreochromis niloticus*). *Aquat. Toxicol.* **2015**, *164*, 10–15. [[CrossRef](#)]
20. Yusof, S.; Ismail, A.; Alias, M.S. Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): A potential tropical test fish. *Mar. Pollut. Bull.* **2014**, *85*, 494–498. [[CrossRef](#)] [[PubMed](#)]
21. Aziz, Z.; Zulkifli, S.Z.; Mohamat-yusuff, F.; Azmai, M.N.A.; Ismail, A. An Histological Study on Mercury-Induced Gonadal Impairment in Javanese Medaka (*Oryzias javanicus*) An Histological Study on Mercury-Induced Gonadal Impairment. *Turk. J. Fish. Aquat. Sci.* **2017**, *17*, 621–627. [[CrossRef](#)]
22. Salleh, A.F.M.; Amal, M.N.A.; Nasruddin, N.S.; Zulkifli, S.Z.; Yusuff, F.M.; Ibrahim, W.N.W.; Ismail, A. Water pH effects on survival, reproductive performances, and ultrastructure of gonads, gills, and skins of the Javanese medaka (*Oryzias javanicus*). *Turk. J. Vet. Anim. Sci.* **2017**, *41*, 471–481. [[CrossRef](#)]
23. Amal, M.N.A.; Roseli, M.F.A.M.; Sutra, J.; Roslan, S.; Mazlan, S.N.K.; Basri, L.; Yie, L.J.; Mohamad, N.; Ismail, A.; Saad, M.Z.; et al. Javanese Medaka (*Oryzias javanicus* Bleeker, 1854) as Potential Model Organism for Aeromoniasis and Vibriosis Study in Fish. *Pertanika J. Trop. Agric. Sci.* **2019**, *42*, 1049–1065.
24. Rusni, S.; Sassa, M.; Takehana, Y.; Kinoshita, M.; Inoue, K. Correlation between cytochrome P450 1A (*cyp1a*) mRNA expression and ambient phenanthrene and pyrene concentration in Javanese Medaka *Oryzias javanicus*. *Fish. Sci.* **2020**, *86*, 605–613. [[CrossRef](#)]
25. Lee, B.-Y.; Park, J.C.; Kim, M.-S.; Choi, B.-S.; Kim, D.-H.; Lim, J.-S.; Yum, S.; Hwang, U.-K.; Nah, G.J.; Lee, J.-S. The genome of the Java medaka (*Oryzias javanicus*): Potential for its use in marine molecular ecotoxicology. *Mar. Pollut. Bull.* **2020**, *154*, 111118. [[CrossRef](#)]
26. Imai, S.; Koyama, J.; Fujii, K. Effects of 17 β -estradiol on the reproduction of Java-medaka (*Oryzias javanicus*), a new test fish species. *Mar. Pollut. Bull.* **2005**, *51*, 708–714. [[CrossRef](#)] [[PubMed](#)]
27. Sardo, M.; Azeiteiro, U.M.; Pereira, L.; Morgado, F.; Soares, M. Histological evaluation of the exposure to 3,4-dichloroaniline in the estuarine mysid *mesopodopsis slabberi*, under experimental conditions. *Fresenius Environ. Bull.* **2005**, *14*, 579–583.
28. Bhuiyan, N.H.; Kang, H.; Choi, J.; Lim, S.; Kho, Y.; Choi, K. Effects of 3,4-dichloroaniline (3,4-DCA) and 4,4'-methylenedianiline (4,4'-MDA) on sex hormone regulation and reproduction of adult zebrafish (*Danio rerio*). *Chemosphere* **2021**, *269*, 128768. [[CrossRef](#)]
29. Li, W.-M.; Yin, D.-Q.; Zhou, Y.; Hu, S.-Q.; Wang, L.-S. 3,4-Dichloroaniline-induced oxidative stress in liver of crucian carp (*Carassius auratus*). *Ecotoxicol. Environ. Saf.* **2003**, *56*, 251–255. [[CrossRef](#)]
30. Boscolo, C.N.P.; Pereira, T.S.B.; Batalhão, I.G.; Dourado, P.L.R.; Schlenk, D.; De Almeida, E.A. Diuron metabolites act as endocrine disruptors and alter aggressive behavior in Nile tilapia (*Oreochromis niloticus*). *Chemosphere* **2018**, *191*, 832–838. [[CrossRef](#)] [[PubMed](#)]
31. OECD Test No. 229: Fish short term reproduction assay. In *OECD Guidelines for the Testing of Chemicals, Section 2*; Organisation for Economic Development and Cooperation: Paris, France, 2012; Volume 40. [[CrossRef](#)]
32. Naderi, M.; Wong, M.Y.; Gholami, F. Developmental exposure of zebrafish (*Danio rerio*) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults. *Aquat. Toxicol.* **2014**, *148*, 195–203. [[CrossRef](#)] [[PubMed](#)]
33. Xu, Q.; Wu, D.; Dang, Y.; Yu, L.; Liu, C.; Wang, J. Reproduction impairment and endocrine disruption in adult zebrafish (*Danio rerio*) after waterborne exposure to TBOEP. *Aquat. Toxicol.* **2017**, *182*, 163–171. [[CrossRef](#)] [[PubMed](#)]
34. Liu, G.; Ke, M.; Fan, X.; Zhang, M.; Zhu, Y.; Lu, T.; Sun, L.; Qian, H. Reproductive and endocrine-disrupting toxicity of *Microcystis aeruginosa* in female zebrafish. *Chemosphere* **2018**, *192*, 289–296. [[CrossRef](#)]
35. Luzio, A.; Monteiro, S.M.; Rocha, E.; Fontainhas-Fernandes, A.A.; Coimbra, A.M. Development and recovery of histopathological alterations in the gonads of zebrafish (*Danio rerio*) after single and combined exposure to endocrine disruptors (17 α -ethinylestradiol and fadrozole). *Aquat. Toxicol.* **2016**, *175*, 90–105. [[CrossRef](#)] [[PubMed](#)]
36. Carvan, M. Fish Models in Toxicology. *Zebrafish* **2007**, *4*, 9–20. [[CrossRef](#)]
37. Martin, O.V.; Voulvoulis, N. Sustainable risk management of emerging contaminants in municipal wastewaters. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* **2009**, *367*, 3895–3922. [[CrossRef](#)]
38. Glinka, C.O.; Frasca, S.; Provasas, A.A.; Lama, T.; DeGuise, S.; Bosker, T. The effects of model androgen 5 α -dihydrotestosterone on mummichog (*Fundulus heteroclitus*) reproduction under different salinities. *Aquat. Toxicol.* **2015**, *165*, 266–276. [[CrossRef](#)]
39. Watanabe, H.; Horie, Y.; Takanobu, H.; Koshio, M.; Flynn, K.; Iguchi, T.; Tatarazako, N. Medaka extended one-generation reproduction test evaluating 4-nonylphenol. *Environ. Toxicol. Chem.* **2017**, *36*, 3254–3266. [[CrossRef](#)]

40. Crossland, N.O.; Hillaby, J.M. Fate and Effects of 3,4-Dichloroaniline in the Laboratory and in Outdoor Ponds: Ii. Chronic Toxicity to *Daphnia* Spp. and Other Invertebrates. *Environ. Toxicol. Chem.* **1985**, *4*, 489. [CrossRef]
41. Carnevali, O.; Santangeli, S.; Forner-Piquer, I.; Basili, D.; Maradonna, F. Endocrine-disrupting chemicals in aquatic environment: What are the risks for fish gametes? *Fish Physiol. Biochem.* **2018**, *44*, 1561–1576. [CrossRef]
42. Carbajal-Hernández, A.L.; Valerio-García, R.C.; Martínez-Ruíz, E.B.; Jarquín-Díaz, V.H.; Martínez-Jerónimo, F. Maternal-embryonic metabolic and antioxidant response of *Chapalichthys pardalis* (Teleostei: Goodeidae) induced by exposure to 3,4-dichloroaniline. *Environ. Sci. Pollut. Res.* **2017**, *24*, 17534–17546. [CrossRef]
43. Imai, S.; Koyama, J.; Fujii, K. Effects of estrone on full life cycle of Java medaka (*Oryzias javanicus*), a new marine test fish. *Environ. Toxicol. Chem.* **2007**, *26*, 726–731. [CrossRef]
44. Ankley, G.T.; Jensen, K.M. A novel framework for interpretation of data from the fish short-term reproduction assay (FSTRA) for the detection of endocrine-disrupting chemicals. *Environ. Toxicol. Chem.* **2014**, *33*, 2529–2540. [CrossRef] [PubMed]
45. Inoue, K.; Takei, Y. Diverse Adaptability in *Oryzias* Species to High Environmental Salinity. *Zool. Sci.* **2002**, *19*, 727–734. [CrossRef]
46. Cao, Q.; Gu, J.; Wang, D.; Liang, F.; Zhang, H.; Li, X.; Yin, S. Physiological mechanism of osmoregulatory adaptation in anguillid eels. *Fish Physiol. Biochem.* **2018**, *44*, 423–433. [CrossRef] [PubMed]
47. Gilannejad, N.; Dorafshan, S.; Heyrati, F.P.; Soofiani, N.M.; Asadollah, S.; Martos-Sitcha, J.A.; Prat, F.; Yúfera, M.; Martínez-Rodríguez, G. Vitellogenin expression in wild cyprinid *Petroleuciscus esfahani* as a biomarker of endocrine disruption along the Zayandeh Roud River, Iran. *Chemosphere* **2016**, *144*, 1342–1350. [CrossRef]
48. Celino-Brady, F.T.; Petro-Sakuma, C.K.; Breves, J.P.; Lerner, D.T.; Seale, A.P. Early-life exposure to 17 β -estradiol and 4-nonylphenol impacts the growth hormone/insulin-like growth-factor system and estrogen receptors in Mozambique tilapia, *Oreochromis mossambicus*. *Aquat. Toxicol.* **2019**, *217*, 105336. [CrossRef]
49. Araújo, F.G.; Morado, C.N.; Parente, T.T.E.; Paumgarten, F.J.R.; Gomes, I.D. Biomarkers and bioindicators of the environmental condition using a fish species (*Pimelodus maculatus* Lacepède, 1803) in a tropical reservoir in Southeastern Brazil. *Braz. J. Biol.* **2017**, *78*, 351–359. [CrossRef]
50. Pavione, P.D.M.; Da Costa, K.G.; Perônico, C.; McMaster, M.E.; Parrott, J.L.; Hewitt, L.M.; Munkittrick, K.R.; Barreto, F.C.C.; Basilo, T.H.; Gomes, M.P.; et al. Development of environmental effects monitoring protocol in Brazil: A fish guide study of three river estuaries. *Environ. Monit. Assess.* **2019**, *191*, 658. [CrossRef] [PubMed]
51. De Oliveira, E.C.; Auache-Filho, A.A.; Damasio, D.; Ghisi, N.D.C.; Michels-Souza, M.A. Reproductive indicators of the endemic species *Astyanax bifasciatus* (Teleostei: Characidae) in a tributary of the Lower Iguaçú River Basin, Brazil. *Acta Sci. Biol. Sci.* **2019**, *41*, e47720. [CrossRef]
52. Burkina, V.; Zamaratskaia, G.; Sakalli, S.; Giang, P.T.; Kodes, V.; Grabic, R.; Velisek, J.; Turek, J.; Kolarova, J.; Zlabek, V.; et al. Complex effects of pollution on fish in major rivers in the Czech Republic. *Ecotoxicol. Environ. Saf.* **2018**, *164*, 92–99. [CrossRef] [PubMed]
53. Mabansag, C.J.A.; Paraso, M.G.V.; Marcelino, R.T.; Claveillas, A.A.; Lola, M.S.E.G. A Preliminary Survey of Estrogenic Effects in Cultured Adult Male Nile Tilapia (*Oreochromis niloticus*) in the Seven Lakes of San Pablo City, Philippines. *Bull. Environ. Contam. Toxicol.* **2019**, *103*, 400–404. [CrossRef] [PubMed]
54. Rodgers-Gray, T.P.; Jobling, S.; Kelly, C.; Morris, S.; Brighty, G.; Waldock, M.J.; Sumpter, J.P.; Tyler, C.R. Exposure of Juvenile Roach (*Rutilus rutilus*) to Treated Sewage Effluent Induces Dose-Dependent and Persistent Disruption in Gonadal Duct Development. *Environ. Sci. Technol.* **2000**, *35*, 462–470. [CrossRef] [PubMed]
55. Amiard-Triquet, C.; Amiard, J.C.; Rainbow, P.S. (Eds.) *Ecological Biomarkers Indicators of Ecotoxicological Effects*; CRS Press: Boca Raton, FL, USA, 2013; Volume 53, ISBN 9788578110796. Available online: <https://www.routledge.com/> (accessed on 22 March 2013).
56. Chen, T.-H.; Chou, S.-M.; Tang, C.-H.; Chen, C.-Y.; Meng, P.-J.; Ko, F.-C.; Cheng, J.-O. Endocrine disrupting effects of domestic wastewater on reproduction, sexual behavior, and gene expression in the brackish medaka *Oryzias melastigma*. *Chemosphere* **2016**, *150*, 566–575. [CrossRef] [PubMed]