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Hazard/Risk Assessment

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Developmental Polyethylene Microplastic Fiber Exposure Entails Subtle Reproductive Impacts in Juvenile Japanese Medaka (*Oryzias latipes*)

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Abstract: Microplastic pollution has been recognized as a potential threat to environmental and human health. Recent studies have shown that microplastics reside in all ecosystems and contaminate human food/water sources. Microplastic exposure has been shown to result in adverse effects related to endocrine disruption; however, data are limited regarding how exposure to current environmental levels of microplastics during development may impact reproductive health. To determine the impact of environmentally relevant, chronic, low-dose microplastic fibers on fish reproductive health, juvenile Japanese medaka were exposed to five concentrations of polyethylene fibers for 21 days, and reproductive maturity was examined to assess the later life consequences. Fecundity, fertility, and hatching rate were evaluated to determine the organismal level impacts. Gonadal tissue integrity and stage were assessed to provide insights into potential tissue level changes. Expression of key reproductive genes in male and female gonads provided a molecular level assessment. A significant delay in hatching was observed, indicating cross-generational and organismal level impacts. A significant decrease in *11-beta-dehydrogenase isozyme 2* (*HSD11β2*) gene expression in male medaka indicated adverse effects at the molecular level. A decrease in male expression of *HSD11β2* could have an impact on sperm quality because this enzyme is crucial for conversion of testosterone into the androgen 11-ketotestosterone. Our study is one of the first to demonstrate subtle impacts of virgin microplastic exposure during development on later life reproductive health. The results suggest a possible risk of polyethylene fiber exposure for wild fish during reproductive development, and populations should be monitored closely, specifically in spawning and nursery regions. *Environ Toxicol Chem* 2022;41:2848–2858. © 2022 SETAC

Keywords: Aquatic toxicology; developmental toxicity; marine plastics; microplastic; reproductive toxicity

INTRODUCTION

Increasing public concern surrounding aquatic microplastic pollution presents a need for further research investigating the adverse effects of an organism's exposure. Consumption of microplastic particles has been shown in many wild fish populations (Lusher et al., 2017). Specifically, fibers are the most commonly ingested shape of microplastic particles (Cole et al., 2011). Fibers are derived from two main sources: 1) degradation of macroplastics, and 2) shedding from domestic washing of textile fibers (Pirc et al., 2016). The variety of plastic polymers used results in different types of microplastics found in field-sampled biota with polyethylene being the most common type of microplastic (Carlos de Sa et al., 2018).

Although long-term exposure to microplastic pollution has not indicated extreme damage to tissues, present data indicate endocrine system disruption (Chisada et al., 2019; Rochman et al., 2014; Wang et al., 2019). Impacts on reproductive health have been shown after 21-day polystyrene ingestion in zebrafish, with enhanced levels of reactive oxygen species (ROS) in both male and female livers and gonadal tissues, in addition to increased apoptosis levels and histological alterations in the testis (Qiang & Cheng, 2021). Marine medaka (*Oryzias melastigma*) exposed to polystyrene for 60 days displayed a decreased egg production along with postponed hatching (Wang et al., 2019). Similarly, a 12-week exposure to polyethylene beads in Japanese medaka (*Oryzias latipes*) revealed changes in egg production and hatching (Chisada et al., 2019). Other studies have disclosed little to no effect on survival and reproduction post microplastic exposure in mature fish

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(Assas et al., 2020). Moreover, previous research varies in terms of the different polymers, shapes, and concentrations of microplastic examined. Neither of those studies employed polyethylene fibers, which are the most commonly ingested microplastic shape by fish (Beer et al., 2018; Carlos de Sa et al., 2018; Cole et al., 2016; Peters & Bratton, 2016). The available literature is inconclusive about long-term effects on survival and reproduction in fish, with only a little information available about the reproductive effects of environmentally relevant concentrations of commonly ingested polyethylene fibers.

Larval and juvenile stages are generally considered to have a higher susceptibility to environmental pollutants, and size and buoyancy make microplastic particles easily available for intake, particularly in coastal nursery areas (Malinich et al., 2018). Virtually nothing is known about early life-stage microplastic exposure and the potential long-term persistent health impacts. This is critical information for fisheries management, because early life-stage exposure may affect stock recruitment success, an important indicator of fish population size and health.

Using Japanese medaka as a model, the present study aims to determine the impacts of exposure to environmentally relevant polyethylene concentrations during juvenile gonad development on mature male and female reproductive output. The juvenile period between 28- and 50-days post hatching (dph) 1) incorporates major structural changes in the ovary toward adult architecture, and 2) is marked by increased germ cell and somatic cell numbers, endocrine cell proliferation, and tissue maturation toward adult structures in the testis (Kanamori, 2000). To investigate the long-term impacts of polyethylene fiber ingestion during this critical window of gonad maturation, postexposure reproductive health was assessed at multiple biological levels. Fecundity, fertility, and hatching rate measurements can provide vital data on the effect of microplastic exposure at the organismal level. Histological analysis of gonadal tissues can provide information on structural and cellular modifications. Assessment of changes in the expression of key genes in gonadal tissue can provide evidence of microplastic exposure impacts at the molecular level. Previous studies have visualized impairment of reproduction in both sexes on microplastic exposure; thus, understanding the potential underlying molecular mechanisms for polyethylene fiber-induced impairment is crucial. Male medaka and other juvenile male teleost fish sexual development is orchestrated through the androgens, testosterone, and 11-ketotestosterone (11-KT; Harbott et al., 2007; Liu et al., 2015; Ogino et al., 2009; Wang et al., 2019). Thus, we targeted genes involved in androgen synthesis and function to understand how polyethylene fibers influence molecular level changes to male sexual development (Figure 1). The SRY-box transcription factor 9 (*SOX9* [*SOX9a*, *SOX9b*]) is crucial in sex determination during gonad development in general (Gonen & Lovell-Badge, 2019).

FOXL2	Forkhead box L2
OLVAS	*VASA* ortholog gene for *Oryzias latipes*
LHR	Luteinizing hormone receptor
FSHR	Follicle stimulating hormone receptor
CYP19A1	Cytochrome P-450 family 19 subfamily A
GH	Growth hormone
mPRa	G protein coupled progesterone receptor alpha
P	Progesterone
E2	Estradiol
SOX9a/b	SRY-box transcription factor 9 alpha/beta
AMH	Anti-Mullerian hormone
ARa	Androgen receptor alpha
11-KT	11-ketotestosterone
T	Testosterone
HSD11B2	Corticosteroid 11-beta-dehydrogenase isozyme 2
StAR	Steroidogenic acute regulatory protein

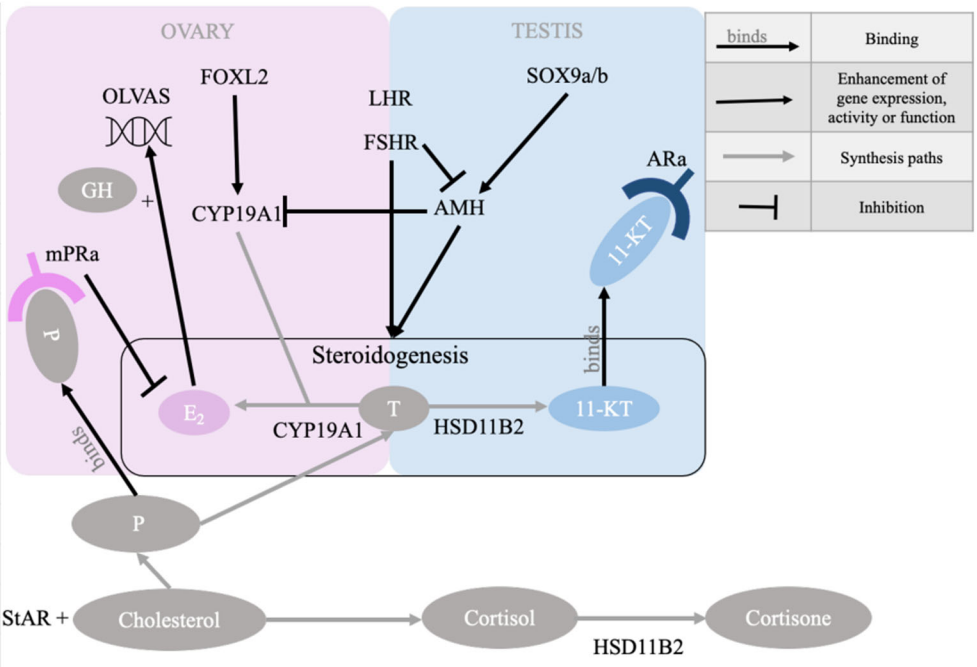

FIGURE 1: Pathway of gonadal development in juvenile medaka. Forkhead box L2 (*FOXL2*) enhances CYP19A1 activity, which is inhibited by anti-Müllerian hormone (*AMH*). CYP19A1 synthesizes testosterone into estradiol (E2). Estradiol in combination with growth hormone promotes *VASA* ortholog gene for *Oryzias latipes* (OLVAS) transcription, leading to female oocyte maturation. G protein–coupled progesterone receptor alpha (*mPRa*) is activated by P binding and inhibits E2 activity. Luteinizing hormone receptor and follicle stimulating hormone receptor promote steroidogenesis. Follicle stimulating hormone receptor also inhibits *AMH* activity. *SOX9a/b* promotes *AMH* activity. The *AMH* promotes steroidogenesis in the male direction. *HSD11β2* synthesizes testosterone into 11-ketotestosterone and cortisol into cortisone. 11-Ketotestosterone binds to androgen receptor alpha, leading to male phenotypic maturation and sperm maturation. Steroidogenic acute regulatory protein brings cholesterol into the mitochondria to begin steroidogenesis (Liu et al., 2015; Todd et al., 2016; Wang et al., 2019; Yan et al., 2020). E2 = estradiol; *LHR* = luteinizing hormone receptor; *FSHR* = follicle stimulating hormone receptor; 11-KT = 11-ketotestosterone; *Ara* = androgen receptor alpha.

Activation of *SOX9* leads to downstream promotion of male development including the release of anti-Müllerian hormone (*AMH*; Liu et al., 2015; Wang et al., 2019). Elevated *AMH* levels inhibit female reproductive tract development and promote male gonad formation (Rajendiran et al., 2021). Steroidogenic acute regulatory protein (*StAR*) is crucial for the initiation of steroid hormone biosynthesis at the mitochondria, leading to androgen production (Yan et al., 2020). Ultimately, the corticosteroid 11-beta-dehydrogenase isozyme 2 (*HSD11β2*) converts testosterone into 11-KT and cortisol into cortisone, both promoting male gonadal development and sperm maturation (Liu et al., 2015; Ozaki et al., 2006; Todd et al., 2019; Wang et al., 2019). Then 11-KT bind and activates androgen receptor alpha (*ARa*), which regulates the expression of genes crucial for the male sexual phenotype (Harbott et al., 2007; Liu et al., 2015; Ogino et al., 2009; Wang et al., 2019). For assessment of female reproductive health at the molecular level, we measured gonadal genes associated with estrogen and progesterone production and oocyte maturation for relative expression (Figure 1). Forkhead box L2 (*FOXL2*) promotes the synthesis of androgen and estrogen via expression of cytochrome P-450 family 19 subfamily A (CYP19A1), triggering the conversion of androgens to estrogen through aromatase (Diotel et al., 2011; Liu et al., 2015; Todd et al., 2016, 2019). The follicle-stimulating hormone receptor (*FSHR*), necessary for follicular development in females, and the luteinizing hormone receptor (*LHR*), regulating reproduction and ovulation, both promote steroidogenesis, leading to estradiol (E2) production (Jozkowiak et al., 2020; Segner, 2011; Todd et al., 2016). Activated by progesterone, the G protein–coupled progesterone receptor alpha (*mPRa*) inhibits E2 binding and activity and orchestrates oocyte development (Thomas, 2008, 2012; Thomas & Pang, 2012). The dead-box helicase 4 or *VASA* ortholog gene for *O. latipes* (*OLVAS*) regulates the migration of primordial germ cells in the ovary. The *OLVAS* gene is crucial for female fertility, and expression is promoted by E2 and growth hormone (Cardinali et al., 2004; Shinomiya et al., 2000). Together, relative expression of these male and female key gonadal genes provide an assessment of reproductive health at the molecular level after polyethylene exposure.

Japanese medaka are an ideal study specimen due to their reliability for toxicological and developmental study. Exposure during a critical reproductive development stage, juvenile (30 days/1 month post hatching), provides a better understanding for the potential exposure consequences experienced by wild populations.

MATERIALS AND METHODS

Exposure experiment

Microplastic fiber preparation. The exposure materials and methods for the present study were previously published in DiBona et al. (2021). Briefly, Lumat USA provided blue multifilament polyethylene yarn, which was confirmed to be an 86% match to low-density polyethylene using a Fourier transform infrared-attenuated total reflectance device (Thermo Fisher Scientific). Fibers were then cut using a paper cutter, ruler, and scalpel to the length of 400 µm, which was determined based on the size of plastics found in wild fish relative to fish size (total length) 4:1 fish:fiber ratio (P. Hajovsky & S. Geist, Texas A&M University-Corpus Christi, personal communication, 2019). Observed juvenile Japanese medaka averaged between 1.3 and 1.9 mm total length at 1 month post hatching.

Model organism. Orange–red inbred *O. latipes* were used as a model (approved by the Texas A&M University-Corpus Christi Institutional Animal Care and Use Committee [IACUC] #19-03). This line of orange-red *O. latipes* was sourced from the late Doris Au Laboratory at the City University of Hong Kong, originally supplied by the David Hinton Laboratory at Duke University (Durham, NC, USA), and has been kept for six generations in the Seemann Laboratory at Texas A&M University-Corpus Christi. Briefly, during the microplastic exposure from 1 to 2 months post hatching, juveniles were randomly allocated to 2-L tanks at a density of 50 individuals/tank ($n = 5$). After exposure, juveniles were then kept at a density of 10 individuals/2-L tank from 2 months post hatching until reproductive maturity at 3 months post hatching. At 3 months, the density of individuals was reduced to 4 (two pairs, male/female)/2-L tank for breeding (Kinoshita et al., 2009). Weekly water changes and water quality measurements were taken, and water was maintained at 25 ± 1 °C, dissolved oxygen at 6 mg/L, nitrates at less than 20 mg/L, nitrites at less than 0.1 mg/L, ammonia at less than 0.01 mg/L, and pH from 7.7 to 8.2 as recommended in *Medaka: Biology, Management, and Experimental Protocols* (Kinoshita et al., 2009). Fish were kept on a 12:12-h light: dark photoperiod and fed 3× times daily, and tanks were aerated to keep microplastic fibers suspended in the water column; a semistatic/closed system was used. Fish were fed 2× daily (9:00 and 17:00) dry feed, OtohimeSD B1 Marine Fish Larval and Weaning Feed (imported by Reed Mariculture; manufactured by Marubeni Nisshin Feed), and 1× daily (1 p.m.) live artemia hatched 48 h prior from artemia cysts provided by INVE Aquaculture Nutrition. All animal experimental procedures were approved by the Texas A&M University-Corpus Christi IACUC (#19-05).

Juvenile exposure. The exposure methods were previously published in DiBona et al. (2021). Briefly, juvenile *O. latipes* were exposed to five concentrations of 400-µm-long polyethylene fibers through 0.1 g of ground dry larval feed once daily for a consecutive 21-day period. Exposure concentrations were 0, 0.5, 1.5, 3, and 6 polyethylene fibers/fish/day. Concentrations of polyethylene fibers were determined based on literature research of field sampling studies indicating an average consumption of 2 microplastic fibers/day/fish (Beer et al., 2018; Lusher et al., 2013; Peters et al., 2017; Phillips & Bonner, 2015). After completion of the exposure experiment, 10 individuals were rinsed, transferred to clean tanks, and raised to reproductive maturity (3-months post hatching, adult), which occurred 40 days after the exposure experiment. Adult medaka were then sexed based on distinct sexual morphological differences (cleft in dorsal fin of males and anal fin size)

and sorted for specific endpoints of breeding, histology, and gonadal gene expression (Kinoshita et al., 2009). A 1 to 1 ratio of males to females was observed in routine medaka husbandry; therefore, the assumption was made for this general sex ratio trend to be true, and in combination with morphological sexing, sex was assumed for breeding. Visualization of successful breeding indicated by at least 1 day of fertile eggs from each replicate verified the reproductive maturity and successful pairing prior to the reproductive output measurements. At the completion of breeding all individuals were euthanized using hypothermic shock and allocated to the necessary endpoints. The polyethylene fiber uptake was validated in a short-term and long-term retention assessment (DiBona et al., 2021).

Reproductive output. Eggs were harvested from 2-L tanks containing two females and two males for two trials of 5 consecutive days. Eggs were collected at the same time daily (10 a.m.) and sorted based on viability. Unfertilized eggs were discarded and recorded as unviable. Fertilized eggs were counted and maintained in glass Petri dishes until hatching. Eggs were reared following the optimal maintenance protocol (Kinoshita et al., 2009). Egg media were changed every other day, and dead or contaminated eggs were discarded daily and recorded. Fecundity was assessed as the average number of eggs laid/females/day using the following equation:

$$\text{Fecundity (egg production)} = \frac{\text{Total number of collected eggs in each tank}}{\text{Number of female fish in the tank}}$$

Fecundity is expected to range between 20 and 50 eggs/week for 1–2 pairs of adult medaka (Kinoshita et al., 2009). This number is an estimated range because other factors such as individual maturity will influence the exact number collected daily. Fertility is measured as the number of fertilized eggs over the number of eggs collected using the following equation:

$$\text{Fertilization success} = \frac{\text{Number of fertilized eggs} \times 100\%}{\text{Total egg number}}$$

Once hatched, larvae were euthanized using hypothermic shock. Eggs that did not hatch within 14 days of fertilization were discarded and recorded as dead. Egg viability was recorded as the total number of dead eggs over the total eggs.

Gonad histology. Adult specimens were euthanized via hypothermic shock and fixed in 4% formalin (five individuals/replicate). Whole fish were dehydrated and embedded in paraffin. Serial cuts of the gonads of the medaka were adhered to microscope slides and left to dry overnight. Slides were stained with hematoxylin and eosin using an automated slide stainer (Thermo Fisher Scientific) and subsequently mounted with a glass coverslip using. Pictures were taken with cellSens Standard software on an Olympus BX53 compound microscope at a magnification of 40×. Image J software (Fiji) was employed for all image analyses (Schindelin et al., 2012). Five males and five females were assessed per concentration. For male specimens, testis stage and Leydig cell count were performed. Testis stage was determined using a ratio of estimated width of the germinal epithelium (EWG) and the estimated width of the testis (EWT). The following scale was used: Stage 1 = EWG > 2/3 EWT, Stage 2 = EWG 2/3 to 1/2 EWT, and Stage 3 = EWG > 1/2 to 1/4 EWT, Stage 4 = EWG < 1/4 EWT (Johnson et al., 2009). For Leydig cell counts, an area of 20 μm^2 was assessed for each individual. For female specimens, oocytes were counted and categorized based on stage of development. An area of 25 μm^2 was assessed for oocytes. The following descriptions were used to determine the stage for each oocyte: In Stage 1, *perinucleolar oocytes* are seen; these are any oocytes in which the nucleus has increased in size and multiple nucleoli have appeared. In Stage 2, *cortical alveolar oocytes* are larger than perinucleolar oocytes and are characterized by the appearance of cortical alveoli. In Stage 3, *early vitellogenic oocytes* are larger than cortical alveolar oocytes and have a centralized appearance of spherical eosinophilii along with vitellogenic yolk granules. In Stage 4, *mature/spawning oocytes* are oocytes in which vitellogenesis has reached its peak, the cell has become larger and more hydrated, and the nucleus has migrated toward the periphery of the cell (Johnson et al., 2009). Finally, in Stage 5, *postovulatory follicles* are identified by a collapsed perifollicular sheath after the release of an oocyte.

Gonadal gene expression. Gonadal tissue from adult medaka ($n = 5$ individuals/concentration) was isolated during dissection and stored at −80 °C. Then messenger (m)RNA was obtained from tissue samples with the TRI-Reagent (500 µl) extraction protocol. Concentration and quality of RNA were determined through gel electrophoresis and a BioSpectrometer (Eppendorf). Reverse transcription was performed to obtain 1 µg of complementary (c)DNA for real-time-quantitative polymerase chain reactiom (RT-qPCR) using the Promega Reverse Transcriptase kit following the manufacturer's instructions. The cDNA samples were stored at −20 °C.

Specific primers were designed for RT-qPCR using the National Center for Biotechnology Information Primer-BLAST tool and confirmed using Primer3Plus (Untergasser et al., 2007).

The RT-qPCR was performed with male *O. latipes* gonadal gene primers (*StAR*, *ARa*, *HSD11β2*, *AMH*, *SOX9a*, and *SOX9b*) and two reference genes (eukaryotic translation factor 1 alpha 1 [*EF1a*] and ribosomal protein L7 [*RPL7*]) using a 1:2 dilution of template cDNA (Supporting Information, Table 1). In addition, RT-qPCR was performed with female *O. latipes* gonadal gene primers (*OLVAS*, *mPRa*, *FOXL2*, *FSHR*, *LHR*, and *CYP19A1*) and two reference genes (*EF1a* and *RPL7*) using a 1:2 dilution of template cDNA (Supporting Information, Table 1). Relative gene expression was performed using RT-qPCR in a 384-well plate with 10 µl of master mix and 2.5 µl of template/well for a total volume of 12.5 µl/well. The 2(-Delta C(testosterone)) method was used to determine the relative expression of genes (Livak & Schmittgen, 2001).

Statistical analysis. Both R Studio and R Ver 4.0.3 were used to analyze all data unless otherwise stated (R Core Team, 2020). Data are presented as means ± standard deviation; homogeneity of variances and normality were tested using the Shapiro–Wilk method and qqnorm plots. All comparisons were made between the control group and the experimental groups, and only $p < 0.05$ values were considered significant. A survival analysis was done to determine any significance in the hatching rate, and survival curves were drawn with the "survminer" package 2 (Kassambara et al., 2021; Therneau, 2021). A one-way analysis of variance (ANOVA) was used for analysis of histological and RT-qPCR data. If significant differences were found, a Tukey's Honestly Significant Difference post hoc test was performed to identify significant differences.

RESULTS

Reproductive output

Whereas polyethylene fiber exposure did not induce significant differences in fecundity (3–4 eggs/female) or fertility (85%–95% fertilization success; Figure 2A and B), a significant difference was seen in the overall hatching rate for groups after chronic exposure to 0, 0.5, 1.5, 3, and 6 polyethylene fibers/fish/day. The polyethylene fiber exposure during juvenile development delayed the hatching peak (11 dpf) of the offspring cohort for 2 days to 13 dpf when the parent generation was exposed to 3 and 6 polyethylene fibers/fish/day ($p < 0.001$) and 3 days after parental exposure to 1.5 polyethylene fibers/fish/day ($p < 0.001$; Figure 3).

Gonad histology

The polyethylene fiber exposure during juvenile development did not cause a significant change in the histomorphological features of male medaka. There were no significant variations in testis stage (Stages 2–3; Figure 4A) or Leydig cell count (1.5–3.1/20 μm^2; Figure 4B). Similarly, no significant differences were seen in oocyte numbers at different stages (Stage 1: 20–25; Stage 2: 3–5; Stage 3: 5–6; Stage 4: 1–4; Stage 5: 0–2) and overall oocyte numbers/area (25–30 oocytes/25 μm^2) for female medaka exposed to polyethylene fibers during the stage of juvenile development (Figure 5A and B).

Gonadal gene expression

Changes in the expression of the selected molecular markers important for reproduction due to polyethylene fiber ingestion during development may serve as early warning for adverse effects on reproductive success and ultimately population recruitment. In male *O. latipes*, exposure to polyethylene fibers during the juvenile life stage (30 dph) induced no changes to the molecular markers *StAR, ARa, AMH, SOX9a*, or *SOX9b*; however, a slight trend in decreased expression was seen as the concentration of polyethylene fibers increased from 1.5 to 6 (Figure 6A–F). Ingestion of polyethylene fibers did induce a significant decrease in male *O. latipes* expression of *HSD11β2* for treatment groups receiving 1.5 polyethylene fibers/fish/day (−0.5 log fold change; $p = 0.0363$) and 3 polyethylene fibers/fish/day (−0.6 log fold change; $p = 0.0137$) compared with the control (Figure 6F), which was still present at 6 polyethylene fibers/fish/day, although not statistically significant. In female *O. latipes*, no significant changes in the expression of key molecular markers (*OLVAS*, *mPRa*, *FOXL2*, *FSHR*, *LHR*, and *CYP19A1*) for reproduction were observed (Figure 7A–F).

DISCUSSION

The goal of our study was to evaluate whether polyethylene fiber ingestion during late gonad development (30 dph) affects the reproductive health of mature adult male and female fish after a 40-day depuration. Our results reveal minor changes at the organismal and molecular level, indicating that the juvenile life stage (30–51 dph) may be susceptible to reproductive impairments after polyethylene fiber exposure, as demonstrated by the nonmonotonous dose response of delayed offspring hatching in the middle–low (1.5 fibers/fish/day), the middle–high (3 fibers/fish/day), and the high (6 fibers/fish/day) exposure groups in concomitance with reduced *HSD11β2* expression in the male testis. It is noted that a nonmonotonic dose response is

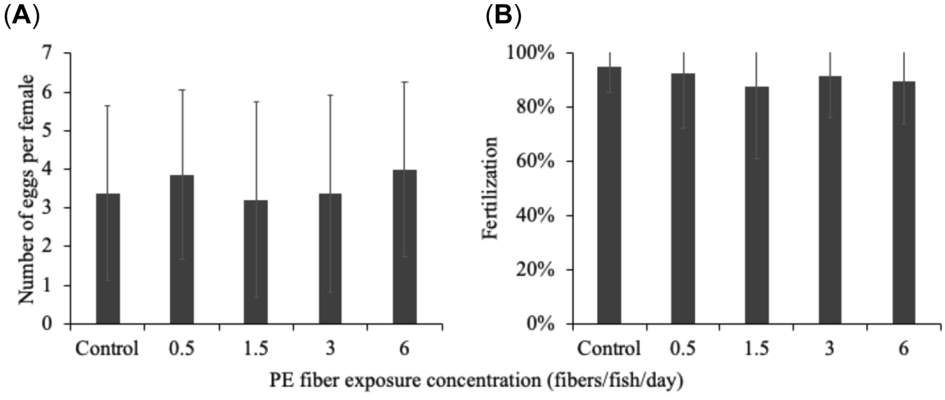

FIGURE 2: Fecundity (**A**) of female *Oryzias latipes* and fertility rate (**B**) of eggs. Comparisons made between the control (0 fibers/fish/day) and the polyethylene (PE) fiber exposure concentrations. No significant differences were seen in fecundity or fertility. Data are displayed as mean ± standard deviation ($n = 5$; analysis of variance, $p < 0.05$).

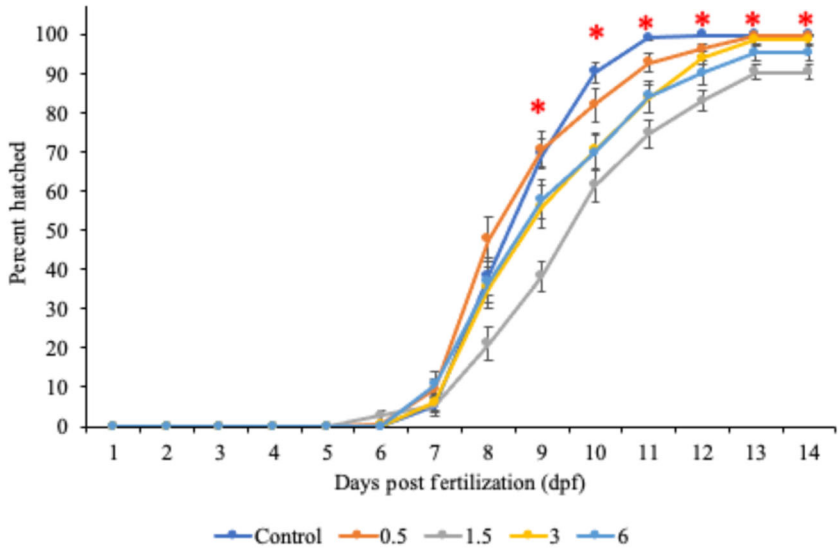

FIGURE 3: *Oryzias latipes* egg hatching rate. Comparisons made between the control (0 fibers/fish/day) and the polyethylene fiber exposure concentrations (0.5, 1.5, 3, and 6 fibers/fish/day; $n = 5$; survival analysis, log-rank test, $p < 0.001$). Eggs from fish exposed to 3 and 6 fibers/fish/day were significantly delayed, reaching hatching peak by 2 days, and eggs from fish exposed to 1.5 fibers/fish/day were significantly delayed, reaching hatching peak by 3 days (Kassambara et al., 2021; Therneau et al., 2021).

typical of a toxicological effect; specifically, endocrine disruptors are known to manifest such a response (Birnbaum, 2012; Vandenberg, 2022; Vandenberg et al., 2012). Microplastic exposures have been shown to have similar nonmonotonic dose responses (Santangeli et al., 2016; Sun et al., 2021). Thus, current environmental polyethylene fiber (400 µm in length) concentrations are having similar effects on offspring development as polystyrene microspheres (10-µm diameter; Wang et al., 2019), indicating that neither plastic type nor shape nor size may play a role impairing the offspring development. A zebrafish (*Danio rerio*) exposure study using polyethylene microspheres at 6.2, 12.5, 25, 50, and 100 mg/L revealed a contrasting premature hatching and decreased survival post hatching (Malafaia et al., 2020), highlighting the possibility of species-specific responses to microplastic exposures. Notwithstanding, both exposures resulted in impacted offspring development and reduced survival.

In agreement with similar studies, a sex-specific deregulation of reproductive gene expression in the gonads was observed. Whereas juvenile medaka exposure (present study) significantly reduced *HSD11β2* expression levels, adult exposure (Wang et al., 2019) and whole-life exposure(Wang et al., 2021) resulted in a significant increase in *HSD11β2* in the testis tissue and a significant decrease in ovary tissue. These variations possibly indicate that certain testis maturation stages may respond differently to microplastic exposure: Wang et al. (2019) exposed sexually mature (more than 3 months old) *O. melastigma*, the present study exposed medaka during reproductive development (1 month old), and Wang et al. (2021) exposed both immature and mature *O. latipes* continuously. Variation in resulting gene dysregulation may also be related to the duration of exposure and/or be species or polymer specific; the present study exposed *O. latipes* to polyethylene fibers for 21 consecutive days with a 40-day depuration to allow fish to

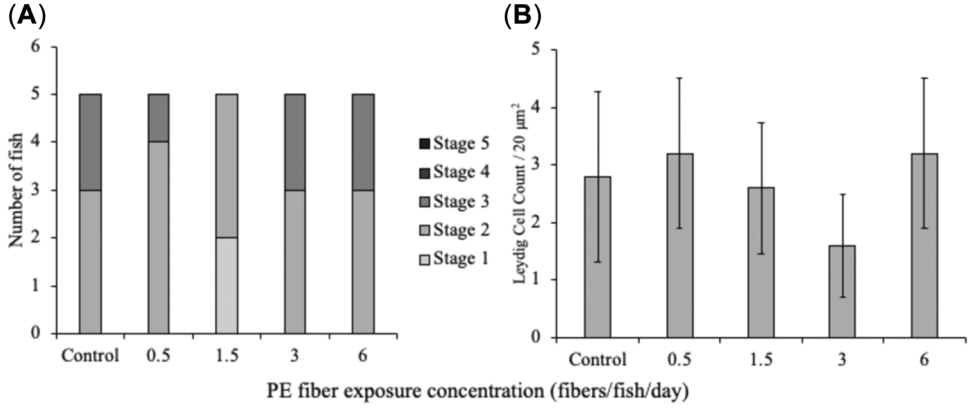

FIGURE 4: Histomorphological measurements of male medaka testis tissue ($n = 5$). No significant differences were found in testis stage (**A**) or Leydig cell count/20 µm^2 (**B**) for comparisons made between the control and other polyethylene (PE) fiber exposure concentrations (analysis of variance, $p < 0.05$).

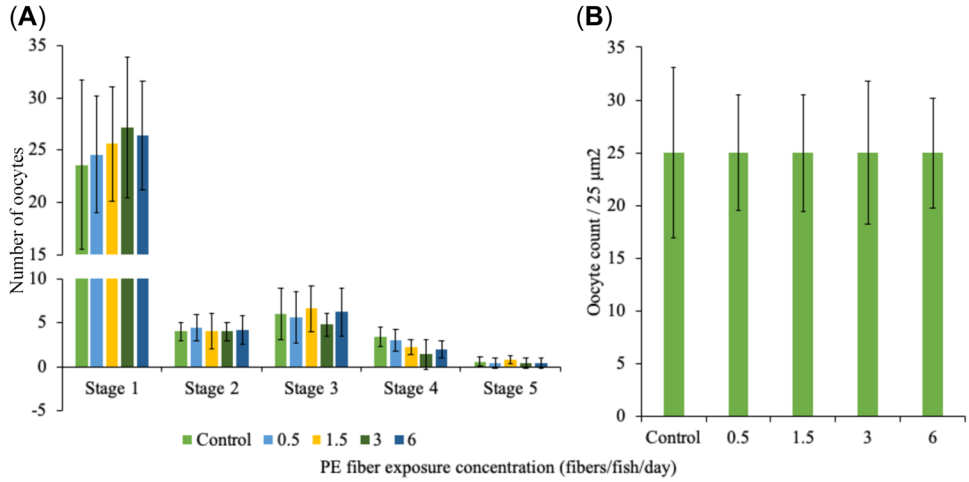

FIGURE 5: Histomorpholgical measurements of female medaka ovary tissue ($n = 5$). No significant differences in number of oocytes/stage (**A**) or number of oocytes/25 μm^2 (**B**). Comparisons were made between the control and different polyethylene (PE) fiber exposure concentrations (analysis of variance, $p < 0.05$).

reach reproductive maturity, whereas Wang et al. (2019) exposed *O. melastigma* to polystyrene spheres for 60 days and did not include a depuration because fish were reproductively mature during exposure. In Wang et al. (2021), *O. melastigma* were exposed to 2 µg/L polystyrene spheres from larvae to adult (150 days), and a significant upregulation in *HSD11β2* was found. In comparison with the present study, *O. latipes* were exposed during development; however, duration, polymer, and concentration again may account for the variation in expression of *HSD11β2*, which is a key enzyme in pathways for sex determination and differentiation in teleosts as well as male reproductive behavior (Rajakumar & Senthilkumaran, 2020). Expression of *HSD11β2* in teleosts has been found to be restricted to somatic cells, presumably Leydig cells (Fernandino et al., 2012); it is required for the conversion of 11β-hydroxytestosterone to 11-KT in fish, which acts as the major androgen (Ozaki et al., 2006). It is also expressed in fish testis tissue during spawning, resulting in 11-KT being produced and released at higher levels during this time (Rajakumar & Senthilkumaran, 2020). A potent androgen, 11-KT has been found to regulate AR-mediated transactivation in mammals (Imamichi et al., 2016). Low levels of 11-KT in fish (zebrafish) implies impairment of hormone production from Leydig cells (Houbrechts et al., 2019).

Moreover, *HSD11β2* has been shown to oxidize cortisol to cortisone, reducing glucocorticoid receptor activation, and thus mediating the stress response in the mammalian testis (Goikoetxea et al., 2017). It is assumed that gonadal *HSD11β2* plays a role in the protection of steroidogenic cells from adverse effects of glucocorticoids in human gonadal tissue

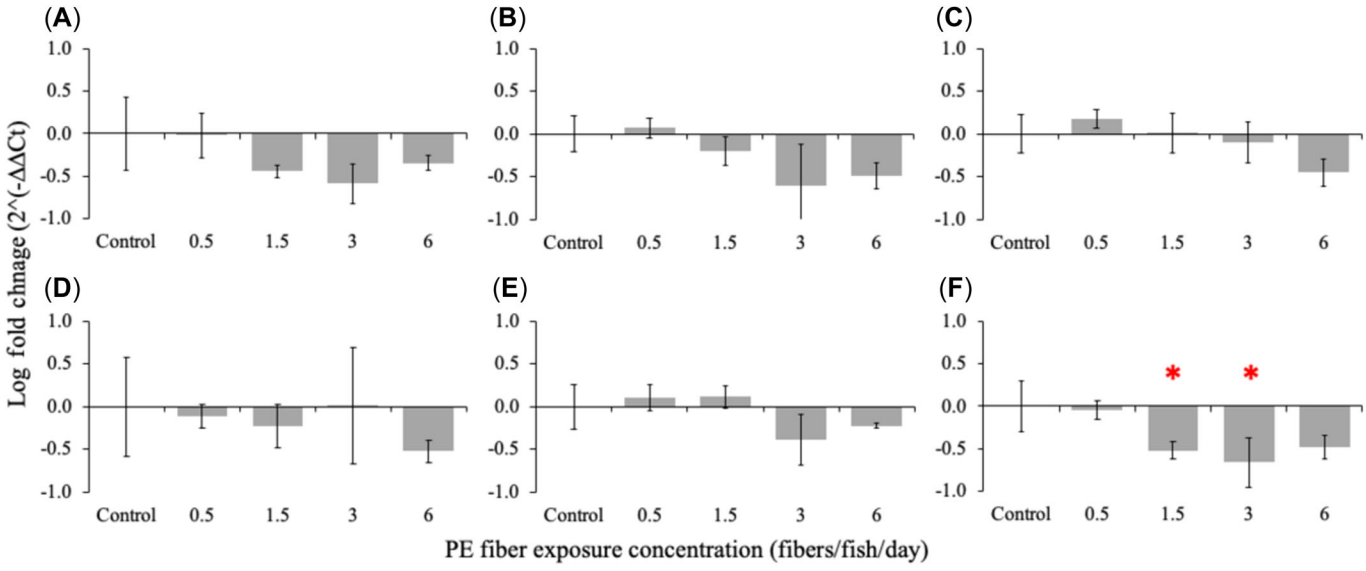

FIGURE 6: Relative expression of steroidogenic acute regulatory protein (*StAR*) (**A**), *AMH* (**B**), *ARa* (**C**), *SOX9a* (**D**), *SOX9b* (**E**), and *HSD11β2* (**F**) in adult male medaka after exposure during juvenile development for 21 days to 0, 0.5, 1.5, 3, and 6 fibers/fish/day. Data are displayed as mean log fold change ± standard deviation ($n = 5$). *$p < 0.05$ (analysis of variance). For abbreviations, see Figure 1.

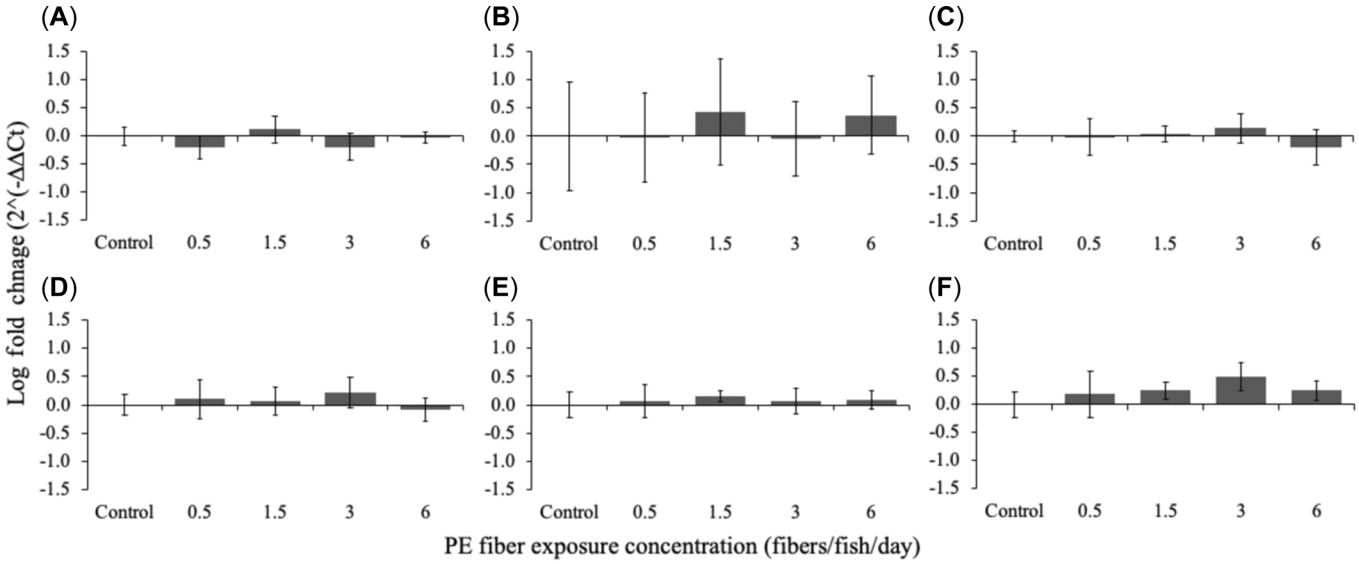

FIGURE 7: Relative Expression of *OLVAS* (**A**), *mPRa* (**B**), *FOXL2* (**C**), *FSHR* (**D**), *LHR* (**E**), and *CYP19A1* (**F**) in adult female medaka after exposure during juvenile development for 21 days to 0, 0.5, 1.5, 3, and 6 fibers/fish/day. Data are displayed as mean log fold change ± standard deviation ($n = 5$). *$p < 0.05$ (analysis of variance). For abbreviations, see Figure 1.

(Imamichi et al., 2016). Increased ROS, indicative of a stress response, has been seen in zebrafish exposure to polystyrene after 21 days at both 100 and 1000 μg/L concentrations (Qiang & Cheng, 2021). A reduction in HSD11β2 could possibly indicate early signs of an impaired stress response. Reduced expression of the *HSD11β2* gene allows for increased cortisol levels, which induce masculinization via regulation of steroidogenic enzymes such as hydroxy-steroid dehydrogenase and result in increased androgen production and decreased aromatase expression in some fish (Baroiller & D'Cotta, 2016; Fernandino et al., 2012). Reduction in *HSD11β2* activities increases the amount of cortisol in Leydig cells, which reduces testosterone production, putatively affecting spermatogenesis as seen in both amphibians and human males (Hu et al., 2008; Vitku et al., 2016). Moreover, *HSD11β2* has been put forward as a target for endocrine disruption in the human testis, and consequently our data demonstrate the potential for *HSD11β2* dysregulation related to current environmentally relevant concentrations of "virgin" polyethylene fibers not contaminated by endocrine-disrupting chemicals (Vitku et al., 2016).

Leaching of endocrine-disrupting chemicals such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and pharmaceutical and personal care products by microplastics may provide a possible explanation for observed reproductive impacts on plastic exposure (Batel et al., 2016; Cousin et al., 2020; Hu et al., 2020; Mato et al., 2001; Teuten et al., 2009; Wardrop et al., 2016; Zhou et al., 2020). In particular, polyethylene plastics have been shown to have a higher affinity for these chemicals in comparison with other plastic polymers (Endo & Koelmans, 2016; Mato et al., 2001; Teuten et al., 2009). Transfer of endocrine-disrupting chemicals via microplastics to aquatic organisms has been demonstrated in previous research (Batel et al., 2016; Cousin et al., 2020; Pittura et al., 2018; Rochman et al., 2014; Teuten et al., 2009; Wardrop et al., 2016). However, the polyethylene fibers used in the present study are considered "virgin" because they were obtained directly from the manufacturer. To ensure that the observed effects seen in our study are indeed associated with polyethylene fibers and not related chemicals, further analysis of any potential leachate from these fibers, such as additives from the manufacturer, would need to be measured. In addition, comparison of these "virgin" polyethylene fibers with other nonplastic polymer "virgin" fibers would allow for clarification as to whether particular microplastic polymers affect reproduction. In general, *HSD11β2* expression may be associated with endocrine disruption whether caused by chemical exposure or "virgin" plastics as our results imply.

Although some studies have indicated histomorphological changes in gonadal tissue due to microplastic exposure, our study observed no histomorphological changes in male or female gonadal tissue, which may be due to the exposure concentration (Jovanovic et al., 2018; Qiang & Cheng, 2021; Wang et al., 2019).

Changes to fecundity and fertility post microplastic exposure have been previously reported; there was no indication of such in the present study (Chisada et al., 2019; Jeong et al., 2017; Wang et al., 2019). However, the significantly delayed and reduced hatching of the F1 generation indicate a persistent impact of polyethylene fiber exposure during the late stage of testis maturation in *O. latipes*. Decreased reproduction output (lower fecundity) has been observed in both zebrafish and marine medaka after chronic (4-month) exposure to either spiked or virgin polyethylene; however, no changes were observed in the spawn quality in the same study (Cormier et al., 2021). A decreased hatching rate was observed in 7-month *O. latipes* exposed to polyethylene microbeads for 12 weeks at both a low (0.065 microbeads-mg/L) and high (0.65 microbeads-mg/L) dose (Chisada et al., 2019). Delayed hatching has also been reported in *O. melastigma* exposed to 2 and 200 μg/L polystyrene microplastics from 0 dpf until

hatching (F0 generation), and an increased hatching rate was observed for the F1 generation in addition to reduced larval growth (Wang et al., 2021). Delayed hatching could be a result of impaired nutrient ingestion because the microplastics "replace" regular food consumption (Cerda et al., 1995; Luquet & Watanabe, 1986). Poor nutrition in the F0 generation could result in decreased fitness of gametes manifested as either lower quality eggs or sperm (Cerda et al., 1995; Luquet & Watanabe, 1986). The impacts of delayed hatching and decreased *HSD11β2* relative expression in males are possibly connected, because decreased *HSD11β2* expression may reduce the conversion of testosterone to 11-KT. Decreased 11-KT levels can disrupt male teleost characteristic development, spermatogenesis, and male reproductive behavior. This disruption could lead to less successful breeding attempts or potentially less vital/healthy sperm, which could manifest as a delay in hatching rate. Decreased *HSD11β2* expression in males could also be a direct connection to polyethylene fiber ingestion as an early sign of a stress response because *HSD11β2* has been associated with mediating stress response, and microplastic ingestion has been found to increase ROS in fish (Goikoetxea et al., 2017; Qiang & Cheng, 2021). Increased oxidative stress is known to impact the function of sperm, which could explain the manifested observations of delayed hatching (Ribas-Maynou & Yeste, 2020). The findings presented in our study indicate that low, chronic levels of common polyethylene fibers can lead to early signs of reproductive impairments for fish exposed during reproductive development.

CONCLUSIONS

There is increasing evidence of impacts from microplastic ingestion in fish species. The present study revealed that ingestion of environmental levels of polyethylene fibers during the critical window of late testis maturation (30–50 dph) is impacting offspring development, as manifested in delayed and reduced hatching. Expression of *HSD11β2* is put forward as a potential indicator of microplastic exposure history in wild fish populations, suggesting a reduction in reproductive health and success specifically in male teleosts. Even though there is still great variation among methodology in microplastic research regarding polymer type, shape, concentration, exposure duration, and method, the vast majority of studies indicate adverse effects on reproduction related to microplastic exposure. Our study is one of the first to show how chronic exposure during a crucial developmental life stage has implications on later life reproductive success. Future research investigating larger scale and longer term impacts, specifically transgenerational implications, would be beneficial in providing more insight and data to support management decisions of wild fish populations.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5456.

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This article has earned an Open Data badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at https://github.com/edibona1/PE_reproductive_impacts_medaka. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

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