Accumulation, Biotransformation, and Endocrine Disruption Effects of Fluorotelomer Surfactant Mixtures on Zebrafish

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Supporting Information

ABSTRACT: As an alternative to perfluorooctanesulfonate (PFOS) novel fluorotelomer surfactants (6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB) and 6:2 fluorotelomer sulfonamide alkylamine (6:2 FTAA)) are widely used in aqueous film-forming foams and are frequently found to coexist in the environment. However, their potential toxicities remain unknown. Here, we investigated the chronic toxicity of 6:2 FTAB (65%) and 6:2 FTAA (35%) coexposure on adult zebrafish at doses of 0, 5, 50, or 500 μ g/L using a flow-through exposure system for 180 days. Results showed that 6:2 FTAB was undetected in adult tissue and their offspring, while 6:2 FTAA was highly dominant, accounting for ~92% of total quantified poly/perfluoroalkyl substances (PFASs), and their metabolic products (6:2 fluorotelomer sulfonamide and 6:2



fluorotelomer sulfonate) further accounting for 2.8%-8.5%. 6:2 FTAA accumulation exhibited a sex-bias, with higher levels found in male livers than that in female, but in gonad showed an opposite pattern. Co-exposure to 6:2 FTAB and 6:2 FTAA mixture (50 and 500 μ g/L) could decrease the average number of eggs production and increase the malformation and mortality in their offspring. Testosterone (T) and 17 β -estradiol (E2) levels increased in the 50 and 500 μ g/L exposed females, but T level decreased in the 500 μ g/L exposed males. Correspondingly, the transcriptional pattern of hypothalamus-pituitary-gonad axis genes was different between male and female. Increased liver vitellogenin levels in the 50 and 500 μ g/L-exposed males indicated that these compounds might possess estrogen-like activity. Furthermore, 3,5,3'-triiodothyronine (T3) and thyroxine (T4) levels decreased in the 50 and 500 μ g/L females and increased T4 level in 500 μ g/L exposed males. These results suggest that 6:2 FTAB is extensively metabolized in fish, whereas 6:2 FTAB and 6:2 FTAA coexposure disrupted the adult endocrine system and impaired offspring development.

INTRODUCTION

Aqueous film-forming foams (AFFFs), which are mixtures of fluoro- and hydrocarbon-based surfactants and water, are widely applied in military and commercial airports.¹ Since the 1960s, perfluorooctanesulfonate (PFOS) has been widely utilized in AFFFs. However, because of environmental and human health concerns, various regulations have been introduced since 2000 to reduce their usage and release.²⁻⁵ In 2009, PFOS and related substances were added to Annex B of the Stockholm Convention on Persistent Organic Pollutants.⁵ The development of novel surfactants to replace PFOS in AFFFs is therefore an important priority.

Recently, 6:2 fluorotelomer sulfonamide alkylbetaine (6:2 FTAB), a zwitterionic poly/perfluoroalkyl substance (PFAS), has been used in AFFFs and commercial surfactants.⁶⁻⁹ In Canada, for example, 6:2 FTAB was detected in four out of ten fluorinated AFFFs, making it the second most commonly used fluorosurfactant.⁹ Notably, 6:2 FTAB is always found concomitantly with 6:2 fluorotelomer sulfonamide alkylamine (6:2 FTAA).⁸ 6:2 FTAA is not only the synthetic intermediate

but also the primary degradation product of 6:2 FTAB and can account for significant quantities (up to 46%) of 6:2 FTAB in certain AFFFs.⁹⁻¹¹ 6:2 FTAB and 6:2 FTAA are also concomitantly detected in various environmental matrices where AFFFs have been produced or used.¹²⁻¹⁶ In northern France, for example, 6:2 FTAB was reported as the predominant PFAS (4-45.5 mg/L), followed by 6:2 FTAA, in raw effluent wastewater from a wastewater treatment plant (WWTP) that received the wastewater from four plants and one of them produced fluorotelomer-based products (including 6:2 FTAB and 6:2 FTSA for fire-fighting foams), with both of them accounting for over 75% of the total PFAS mass flow.¹⁴ This wastewater was emitted into a major French river, with the 6:2 FTAB concentration decreasing from 968 ng/L near the discharge point to 248 ng/L some 62 km away.¹⁵ In addition, 6:2 FTAB has been found in Canadian soil samples

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around the Lac-Mégantic Railway where 33 000 L AFFFs have been used for firefighting. 16

Although 6:2 FTAB and 6:2 FTAA have been detected in the environment, studies on their potential effects remain limited. Earlier research demonstrated that 6:2 FTAB exposure mainly affects the immune system of turbot, which is inconsistent with the effects of PFOS.⁶ Furthermore, an acute assay on zebrafish embryos reported a median lethal concentration (LC_{50}) for 6:2 FTAB of 43.73 ± 3.24 mg/L (5 d postfertilization (dpf)), which is markedly lower than that of PFOS.¹⁷ Thus, these studies indicate that the toxicity of 6:2 FTAB is lower than that of PFOS, suggesting it may be an environmentally friendly alternative. However, these conclusions have been obtained from acute experiments, with the impacts of long-term exposure, which more closely mimics what occurs in the aquatic environment, still unknown.

As 6:2 FTAB and 6:2 FTAA coexist in AFFFs and contaminated environments, we investigated the potential chronic toxicity of fluorotelomer surfactants on zebrafish using a 6:2 FTAB (65%) and 6:2 FTAA (35%) mixture at doses of 0, 5, 50, and 500 μ g/L for 180 d. We first assessed the bioconcentrations and biodegradations of 6:2 FTAB and 6:2 FTAA in the liver and gonads of F0 adult fish as well in the whole bodies of F1 embryos. Reproductive and developmental toxicological end points of offspring were tested. The levels of sex steroid and thyroid hormones (TH) in adult fish serum were detected to evaluate potential reproductive endocrine-disruption effects.

MATERIALS AND METHODS

Chemicals. The 65:35 ratio mixture of 6:2 FTAB (CAS number: 34455–29–3) and 6:2 FTAA (CAS number: 34455–22–6) was provided by Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. The purity and composition of this mixture were identified by ¹H NMR, ¹⁹F-NMR, and elemental analysis, and the impurity was less than 1% (SI and Figure S1–S4).

Animals and Treatment. In total, 240 male (body weight:, 0.26 \pm 0.01 g; body length, 2.9 \pm 0.007 cm) and 240 female (body weight, 0.34 \pm 0.03 g; body length, 3.1 \pm 0.01 cm) five-month-old zebrafish (wild-type, *Tüebingen* strain) were divided into four different 6:2 FTAB and 6:2 FTAA exposure groups (0, 5, 50, and 500 μ g/L). Considering the similar acute toxicity of 6:2 FTAB and PFOS for zebrafish embryo,¹⁷ the exposure concentration that was used in the present study was according to previous study that focused on the chronic toxicity of PFOS (commonly used 5, 50, and 250 μ g/L). ^{18,19} A flow-through exposure system (ISO7346–3) was used, with an exposure time of 180 d. Each group contained four tanks (two for male and two for female), and each tank (15 mL) contained 30 fish. All zebrafish were maintained following standard zebrafish culture protocols.²⁰

Considering their unusual breeding cycle, ten pairs of male and female fish from each tank were selected randomly each week. Male and female zebrafish (1:1) were paired in spawning boxes overnight before spawning. Spawning was induced when the light was turned on the following morning. After spawning, the fishes were placed back to the corresponding tank. The egg number per female was recorded each time. The F1 embryos obtained from last spawning were examined under an optical microscope to calculate the fertilization percentage, and the fertilized embryos were then maintained in uncontaminated water to further evaluate the potential toxicological effects after parental exposure. Developmental end points, including hatching, malformation, and mortality, were determined following previous study.¹⁷

After the last spawning, F0 fish were anesthetized using an ice-bath. The body weights and lengths were accurately measured. Blood was collected from the tail fin using a glass capillary, after which the liver, brain, and gonads were separated and immediately frozen in liquid nitrogen and stored at $-80\,$ °C.

Quantification of 6:2 FTAB, 6:2 FTAA, and Their Metabolites. Concentrations of 6:2 FTAB and 6:2 FTAA and their potential biodegradation products (including 6:2 fluorotelomer sulfonamide (FTSAm) and 6:2 fluorotelomer sulfonate (FTSA)) were quantified in the tissues of adult fish (gonads and liver) and their embryos. Native standards for 6:2 FTAB and 6:2 FTSA and all mass-labeled standards were purchased from Wellington Laboratories, Inc. (Ontario, Canada), whereas 6:2 FTAA and 6:2 FTSAm were gifted by Dr. Scott A. Mabury from the Department of Chemistry, University of Toronto, Toronto, Ontario, Canada.²¹

For adult fish, the livers and gonads from two fish were separately pooled as one replicate. For the F1 generation, 30 embryos were pooled together as a replicate. Each experiment contained three replicated. All samples were homogenized with ultrapure water (1:10 w/v). The PFASs were then extracted using ion-pair liquid-liquid extraction.²² In brief, 200 μ L of homogenate was spiked with mixed mass-labeled standard (0.5 ng), tetra-n-butylammonium hydrogen sulfate solution (0.5 M, 1 mL), NaHCO₃/Na₂CO₃ buffer solution (2 mL), and methyl tert-butyl ether (MTBE) (4 mL). After vigorous shaking and centrifugation, the organic and aqueous phases were separated, with the organic phase (upper) moved to a new tube. The remaining aqueous phase was extracted with another 4 mL of MTBE twice. All three organic phases were pooled together, evaporated to dryness, and reconstituted with 200 μ L of methanol for instrumental analysis. These compounds were separated and quantified by an Acquity ultra performance liquid chromatograph coupled with a Xevo TQ-S triple quadrupole mass spectrometer (MS, Waters, Milford, MA, USA). Chromatographic separation was accomplished using an Acquity BEH C18 column (Waters, MA, USA) with mobile phases: 2 mM ammonium acetate in water (A) and methanol (B) at a flow rate of 0.3 mL/min. The MS parameters of target analytes are provided in the Supporting Information (Table S1). The limit of quantification (LOQ) was 1 ng/mL.

Serum and Liver Biochemical Assay. Serum 17 β -estradiol (E2) and testosterone (T) levels were detected using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). Serum 3,5,3'-triiodothyronine (T3) and thyroxine (T4) levels were analyzed using commercial ELISA kits (Cloud-Clone Corp., Katy, TX, USA). Hepatic vitellogenin (VTG) content was measured using zebrafish VTG ELISA kits (Biosense Laboratories AS, Bergen, Norway). Serum transthyretin (TTR) protein content was detected using Western blotting. All detailed information is provided in S2–S5.

Quantitative Real-Time PCR (gRT-PCR). For gRT-PCR assay, the adult tissue (brain and gonad) was individually collected as single samples. Total RNA was extracted from brain and gonad samples using Trizol Reagent (Ambion, Life Technologies, USA). The purity and concentration of RNA were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). In each sample, 1 μg of RNA was reverse transcribed to cDNA with an oligo-(dT)₁₅ primer and M-MLV reverse transcriptase. qRT-PCR was performed using SuperReal PreMix Plus (SYBR green) kits (Tiangen, Beijing, China). The primer sequences of selected genes along the HPG axis were based on previous studies.^{23,24} The expressions of house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (gapdh), Actin (β actin), elongation factor 1a (ef1a), and ribosomal protein L13 alpha (rpl13a) were first detected. Rpl13a was the most stable gene and was therefore used as the internal control. The relative expression of selected genes was analyzed using the $2^{-\Delta\Delta Ct}$ method.²⁵ Each group contained six replicates.

Statistical Analysis. Data are shown as means with standard errors (mean \pm SE). Homogeneity was examined by Levene's tests. Differences were analyzed by one-way analysis of variance (ANOVA) and Fisher's least significant differences (LSD) using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P < 0.05 indicated statistical differences between control and exposure groups.



Figure 1. (A) Concentrations of 6:2 FTAA and its metabolites (6:2 FTSAm and 6:2 FTSA); (B) composition profiles of the quantified PFASs (percentage mean mass fraction of individual compounds) in F0 tissues. Tissues from two individual zebrafish were pooled as one replicate and tested with three replicates. Data are means \pm SE (n = 3). *P < 0.05 (LSD) indicates significant differences between males and females derived from the same groups.



Figure 2. (A) Concentrations of 6:2 FTAA and its metabolites (6:2 FTSAm and 6:2 FTSA); (B) composition profiles of the quantified PFASs (percentage mean mass fraction of individual compounds) in F1 embryos (5 dpf). Thirty embryos were pooled as one replicate, and each group contained three replicates. Data are means \pm SE. (C, D) Ratios of PFAS concentrations in F1 embryos to corresponding F0 female tissue. Boxes display the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles min and max values. Each group contained nine replicates.

RESULTS AND DISCUSSION

6:2 FTAB, 6:2 FTAA, and Their Biodegradation Products in F0 Adult Fish and Their Offspring. As the collected serum was used for hormone tests and was insufficient for the analysis of individual distribution of PFASs, we detected PFAS content in F0 liver, gonads, and their embryos (collected on day 180). The concentration of each PFAS in all control samples was lower than the LOQ (data not shown). Interestingly, 6:2 FTAB was not detected in any adult tissue samples, but 6:2 FTAA and their degradation products were present (Figure 1A). 6:2 FTAA was the major compound, accounting for 91.5%–97.2% of total quantified PFASs, with 6:2 fluorotelomer sulfonamide (6:2 FTSAm) and 6:2 fluorotelomer sulfonate (6:2 FTSA) accounting for 2.8%– 8.5% (Figure 1B). Previous studies have demonstrated that 6:2 FTAB can be extensively metabolized in blue mussel and

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Figure 3. Effects of 6:2 FTAB and 6:2 FTAA coexposure on reproductive parameters in zebrafish. (A) Gonadal-somatic index (GSI) of adult fish after 180 d of exposure, GSI = gonad weight (g) × 100/body weight (g) (n = 30). (B) Egg number per female during 180 d of exposure (n = 20). Values are means \pm SE **P* < 0.05 (LSD) indicates significant differences from the control.



Figure 4. Effects of parental 6:2 FTAB and 6:2 FTAA coexposure on development of F1 embryos. (A) Fertilization rate was calculated as the number of viable embryos/number of total embryos produced ×100%. (B) Mortality and (C) malformation percentages were measured using 180 eggs, with six replicates and each replicate containing 30 embryos. Values are means \pm SE. **P* < 0.05 (LSD) indicates significant differences from the control. (D) Typical morphology of zebrafish larvae. The upper picture is normal, and the other picture is the larvae with bent spine, uninflated swim bladder, and pericardial and yolk sac edema.

turbot, with 6:2 FTAA being its major metabolite.⁷ Thus, in the present study, the high concentration of 6:2 FTAA in adult tissue might not only be derived from direct exposure but also from the metabolism of 6:2 FTAB. Because of the presence of 6:2 FTAA in the exposure mixture and the one time-point study design, it was difficult to reveal the degradation rate of

6:2 FTAB in this study. Formation of 6:2 FTSAm and 6:2 FTSA indicated that 6:2 FTAA was degraded in fish, consistent with a previous study on the biodegradation of 6:2 FTAA and 6:2 FTAB in aerobic sludge.²¹ The other biodegradation products, that is, short-chain perfluoroalkyl carboxylic acids (PFCAs), that were present in the sludge sample after aerobic



Figure 5. (A) Serum T, (B) serum E2, and (C) liver VTG levels in adult zebrafish after coexposure to 6:2 FTAB and 6:2 FTAA for 180 d. Serum from eight fish of the same group were pooled as one replicate to detect sex hormone levels, with three replicates for each group. Values are means \pm SE (n = 3). *P < 0.05 indicates significant differences from the control.

biodegradation of 6:2 FTAB and 6:2 FTAA,²¹ were not found in any sample.

Like other PFASs,^{26,27} the accumulation of 6:2 FTAA in tissue was sex dependent (Figure 1A). In the gonads, 6:2 FTAA concentrations were higher in females than in males. In the liver, however, 6:2 FTAA concentrations tended to be considerably higher in males than in females. Previous research has demonstrated that PFAS content in organisms is positively correlated with protein content.²⁸ The higher concentration of 6:2 FTAA in the ovaries may have originated from the sexrelated expression of organic anion transporter proteins. For example, the accumulation of PFOS in liver is associated with very low-density lipoprotein during formation of the egg yolk protein and subsequently is transferred to the eggs as a protein PFOS complex.²⁹ For the liver, the sex-related differences were likely due to higher excretion rates in females via spawning.^{26,18} In the present study, the high concentration of PFASs in F1 embryos provides direct evidence of maternal transfer of 6:2 FTAA, 6:2 FTSAm, and 6:2 FTSA from adult females to their eggs (Figure 2).

The concentrations of 6:2 FTAA in F1 embryos following parental exposure to 5, 50, and 500 μ g/L were 0.17 \pm 0.03, 1.68 \pm 0.17, and 3.84 \pm 0.31 ng/mg, respectively, and the composition profiles of 6:2 FTAA, 6:2 FTSAm, and 6:2 FTSA were similar to those in the ovary (Figure 2). The maternal transfer is an important pathway to eliminate the PFASs, and thus, it is sometimes considered as a protective mechanism for organisms.^{18,30} The maternal transfer ratios were expressed as ratios of compound concentrations in the egg to those in the liver. For F1 embryos, the concentrations of 6:2 FTAA, 6:2 FTSAm, and 6:2 FTSA were more than 25% of that in F0 adult tissues (Figure 2D). Prior research reported a maternal PFOS transfer ratio of 17% for zebrafish after 150 d of exposure.^{18,31} The higher maternal transfer ratio in the current study implies that 6:2 FTAA, 6:2 FTSAm, and 6:2 FTSA may be more easily eliminated from fish than PFOS.

Body Condition and Reproductive Capability of Adult Fish. Mortality in each tank was below 10%, and no abnormal behavior was observed after 180 day of exposure. However, body weight and condition factor (K) exhibited concentration-dependent decreases in both sexes (Figure S5). The gonadal somatic index (GSI) only decreased significantly in the 500 μ g/L-exposed females (Figure 3). This decrease in

GSI is consistent with that observed in female zebrafish after 50 and 250 μ g/L PFOS exposure for 150 days.¹⁸

In the current study, egg number/female was stable in the control and 5 μ g/L exposure groups over the 180 days of exposure (Figure 3). However, egg number/female in the 500 μ g/L-exposed group decreased sharply from 140 days of exposure and was about one-third that of the control at the end. The decreases in egg production and GSI suggest that long-term coexposure to 6:2 FTAA and 6:2 FTAB can have significant inhibitory effects on female fecundity.

Developmental End Point of F1 Generation. As shown in Figure 4, decreased fertilization and increased malformation and mortality were observed in the 50 and 500 μ g/L exposure groups. Malformations, including uninflated swim bladder, pericardial and yolk sac edema, and bent spine, were present in F1 larvae, with uninflated swim bladder predominant (Figure 4D). The uninflated swim bladder was the general malformation in zebrafish after parental exposure to PFASs.¹⁸ In earlier acute experiments, however, rough-edged skin/fins in zebrafish larvae were reported as the dominant malformation after exposure to 20 and 40 mg/L 6:2 FTAB for 5 d.¹⁷ This difference might be due to the different compounds in the embryos. In the present study, 6:2 FTAA was the predominant PFASs in the F1 embryo, which should be different with the PFASs pattern in embryos directly exposed to 6:2 FTAB at high concentration (mg/L).

Sex Hormones and VTG Levels. Many of pollutants can disrupt hormone production, leading to developmental disruption of gonads and gametes.³² Thus, measurement of sex steroid hormones is considered to be one of the most integrative and functional end points for evaluating reproductive toxicity of contaminants.³³ In the present study, the changes in serum T and E2 levels were found to be sex dependent (Figure 5A,B). In male serum, only T levels in the 500 μ g/L-exposed group were markedly decreased. However, serum T and E2 levels were increased in the 50 and 500 μ g/Lexposed females. E2 in females is the major sex steroid responsible for inducing and maintaining ovarian development, while T in males plays a key role in spermatogenesis and the release of spermatozoa.^{34,35} In the present study, the sex hormone imbalance in the fishes after 6:2 FTAB and 6:2 FTAA coexposure may responsible for concentrationdecreased reproduction capability. However, the alternation of sex hormone is inconsistent with the endocrine disruption



Figure 6. Thyroid hormone (A, T3 and B, T4) levels and Western blot analysis of TTR protein expression in adult serum after 6:2 FTAB and 6:2 FTAA coexposure for 180 d. Serum samples from eight individual fish were pooled and tested with three replicates. *P < 0.05 (LSD) indicates significant differences from the control.

effects of other perfluorinated alkyl acids (PFAAs). For example, plasma T and E2 levels were significantly increased in both female and male zebrafish after exposure to 6:2 FTOH for 7 d or after long-term exposure to PFOS.^{36,37}

VTG is a precursor of yolk proteins and is mainly produced by the female liver, with its concentrations being very low or absent in the male liver.^{37,38} Therefore, VTG mRNA and protein levels in male fish are common biomarkers to determine whether tested chemicals possess estrogenic activities.³⁹ In the present study, the VTG protein level in the male liver was significantly increased after 50 and 500 μ g/L 6:2 FTAB and 6:2 FTAA coexposure (Figure 5C). VTG level is dependent on E2 concentration,⁴⁰ and the increase of VTG coincided with the increase trend of E2 in males after 6:2 FTAB and 6:2 FTAA coexposure. An earlier study showed increased expression of vtg in male zebrafish after 70 d of chronic PFOS exposure.¹⁹ In vitro studies have demonstrated that certain PFAAs exhibit estrogen-like activity.^{41,42} The increased VTG protein levels in male liver supported these compounds may exhibit estrogen-like activity. However, considering the degradation of 6:2 FTAB and 6:2 FTAA in fish, whether these compounds themselves or their metabolites possess the estrogen-like activity needs further study.

Transcription of Genes along the HPG Axis. In fish, steroid hormone synthesis is regulated by the HPG axis and thus pollutants acting on any level of this axis may lead to reproductive impairment of fish. Evidence in vertebrates has demonstrated that PFASs exposure can affect the HPG axis.^{36,43} In the present study, we analyzed the transcription profiles of genes along the HPG axis in the brain and gonads using qRT-PCR (SI Figure S6). In the female brain, the transcriptional levels of gnrh2 and gnrh3 were not changed, whereas the transcription of $fsh\beta$ and $lh\beta$ was significantly upregulated in the 50 μ g/L and 500 μ g/L-exposed groups. In the male brain, only gnrh3 was significantly increased in the 500 μ g/L-exposed groups, but the transcriptional levels of *fsh* β and $lh\beta$ were significantly decreased in the male brain. In the fish brain, GnRH stimulates the synthesis and release of gonadotropin hormones (e.g., follicle-stimulating hormone (FSH) and luteinizing hormone (LH), with FSH and LH then binding to their specific receptors (FSHR, LHR) in the gonads, which are the primary mediators of steroidogenesis and gametogenesis.⁴⁴ Here, the changes of *fshr* and *lhr* in gonad are consistent the alternation of $fsh\beta$ and $lh\beta$ in brain. For example, the transcriptional levels of fshr and lhr increased in the 500 μ g/L-exposed ovaries, and fshr decreased in the 50 and 500 μ g/L-exposed testes. The opposite change in the expressions of gonadotropin hormones and their receptors in the present study may explain the increase in T and E2 levels in females as well decrease of T levels in males.

In the ovary, steroidogenic genes, including hmgrb, star, *cyp17*, and *17\betahsd*, were significantly increased in the 50 μ g/Lexposed groups, and hmgra and star were significantly increased in the 500 μ g/L-exposed groups (SI Figure S6), which corresponded well with the obvious increase in T levels in the 50 and 500 μ g/L-exposed groups. In addition, the transcription of cyp19a was markedly up-regulated by 4.7- and 3.5-fold in the 50 and 500 μ g/L-exposed groups, respectively (SI Figure S6). As the terminal enzyme in the steroidogenic pathway, CYP19 controls the formation of estrogens and can catalyze the conversion of androgens to estrogens.⁴⁵ In the present study, the up-regulated transcription of cyp19a may be responsible for the elevated serum E2 levels in female zebrafish after exposure to 6:2 FTAB and 6:2 FTAA mixture. Interestingly, we found mRNA expression of several genes in the 5 μ g/L-exposed groups showed the opposite trend to that in the 50 and 500 μ g/L-exposed groups. The transcription of *cyp11a* and 3β hsd were obviously decreased, which may have led to a slight decrease in T level. This difference implies an adaptation of female fish to low concentration exposure. In the testes, 6:2 FTAB and 6:2 FTAA exposure also up-regulated the transcriptional levels of star and cyp19a, but down-regulated the transcriptional level of cyp17 (SI Figure S6). Cyp17 plays an important role in the conversion of 17a-hydroxyprogesterone to androstenedione.⁴⁶ Previous studies demonstrated the increased cyp19a expression and decreased cyp17 genes in male zebrafish gonads may have contributed to the decreased production of T and increased production of E2.47,48

6:2 FTAB Exposure Disrupted the Thyroid System. Several studies have demonstrated that interference with thyroid hormones (THs) plays an important role in the development of gonads, estrogen synthesis, and spermatogenesis.^{49,50} Our results showed that chronic coexposure to 6:2 FTAB and 6:2 FTAA did not induce obvious changes in T3 levels in male fish, but a remarkable reduction was observed in females (Figure 6A). Furthermore, T4 levels only increased in

males after exposure to 500 μ g/L 6:2 FTAB and 6:2 FTAA but decreased in females after exposure to 5 and 500 μ g/L 6:2 FTAB and 6:2 FTAA (Figure 6B). This sex-dependent alternation in TH levels is consistent with reports on other TH endocrine disruptors (e.g., TDCPP and DE-71).^{51,52} However, the underlying mechanisms of this effect remain to be elucidated. As a TH-binding protein in fish, TTR can bind to THs and transport them to target tissues. However, inconsistent with hormonal fluctuation, the TTR protein levels increased in both sexes (Figure 6C), which is in accordance with previous studies on the effects of other PFAAs on fish.^{53,54} Prior studies have demonstrated that PFASs compete with thyroxine for TTR binding.55,56 Thus, the increased TTR protein levels observed in the present study could also be interpreted as induction caused by 6:2 FTAA or other metabolism competitive binding; however, this needs further study.

In conclusion, this is the first report on the long-term toxicity of 6:2 FTAB and 6:2 FTAA following coexposure. Results indicated that 6:2 FTAB underwent rapid biotransformation and was metabolized into 6:2 FTAA, which was, in turn, the main compound that accumulated in adult zebrafish tissue and decreased reproductive capability of adult fish. In addition, 6:2 FTAA could be passed to offspring via maternal transfer, with this transfer further inducing developmental toxicity in F1 offspring. Thus, environmental assessment should not only focus on the bioaccumulative potential of 6:2 FTAB itself but also on that of 6:2 FTAA, its main downstream metabolite. With their increasing usage and potential toxicity, better aquatic environmental monitoring of these compounds is critical.

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemres-tox.9b00127.

Purity and composition of 6:2 FTAB and 6:2 FTAA mixture; serum 17 β -estradiol (E2) and testosterone (T) levels; hepatic vitellogenin (VTG) content; serum 3,5,3'-triiodothyronine (T3) and thyroxine (T4) levels; Western blot analysis;MS parameters of target analytes; ¹H NMR of 6:2 FTAB; ¹⁹F NMR of 6:2 FTAB; ¹⁰H NMR of 6:2 FTAA; ¹⁹F NMR of 6:2 FTAB; body weight, body length, condition factor in adult zebrafish after exposed to 6:2 FTAB + 6:2 FTAA mixtures for 180 days; transcriptional levels of several genes along HPG axis in adult fish after 6:2 FTAB and 6:2 FTAA mixtures exposure for 180 days (PDF)

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Notes

The authors declare no competing financial interest.

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