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Gonadal Soma-Derived Factor Expression is a Potential Biomarker for Predicting the Effects of Endocrine-Disrupting Chemicals on Gonadal Differentiation in Japanese Medaka (Oryzias…

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46	

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26 Abstract

27 Chemicals with androgenic or estrogenic activity induce the sex reversal and/or intersex 28 condition in various teleost fish species. Previously, we reported that exposure to 17α -29 methyltestosterone, bisphenol A, or 4-nonylphenol induces changes in expression of 30 gonadal soma-derived factor (gsdf) gene accompanied by disruption of gonadal 31 differentiation in Japanese medaka (Oryzias latipes). These findings suggest that gsdf 32 expression might be a useful biomarker for predicting potential of chemicals on gonadal 33 differentiation. Here, we examined the gsdf expression in Japanese medaka exposed to 34 chemicals with estrogenic or androgenic activity. Exposure to the androgenic steroid 17β-35 trenbolone at 0.5–22.1 µg/L induced ovotestis (presence of ovarian tissue with testicular 36 tissue) development and female-to-male sex reversal in XX embryos, and exposure at 37 6.32 and 22.1 µg/L significantly increased gsdf expression in XX embryos compared with 38 controls at developmental stage 38 (1 day before hatching). In the present study, no 39 statistically significant difference in gsdf mRNA expression was observed by 17β-40 estradiol, 17α-ethinylestradiol, and 4-t-octylphenol exposure, which have estrogenic 41 activity. In addition, anti-androgenic chemicals or chemicals without endocrine-42 disrupting activity did not induce changes in *gsdf* expression in XX nor XY embryos. 43 Thus, an increase of gsdf expression after androgen exposure was observed in XX 44 embryos. Together, these findings indicate that gsdf expression might be useful for 45 predicting the adverse effect of chemicals on gonadal differentiation.

46

48 **1. Introduction**

49 The endocrine system plays an important role in the homeostasis of organisms by overseeing the production and secretion of hormones. Chemicals that interfere with the 50 51 endocrine system by mimicking or blocking hormonal activity (i.e., endocrine-disrupting 52 chemicals, EDCs) can have detrimental effects on the reproductive health of an organism, 53 its offspring, and its population. 17B-Trenbolone has been detected in the natural 54 environment, especially at beef feedlots. For example, Durhan et al. (2006) reported that 55 17β-trenbolone was detected at 10–20 ng/L in the runoff from a beef feedlot in southwest 56 central Ohio in the United States. Similarly, Gall et al. (2011) have reported that 17β-57 trenbolone was detected at 3.3-162 ng/L in the discharge from a tile-drained 58 agroecosystem receiving animal wastes in the Midwestern United States. In the present 59 study, ovotestis development or female-to-male sex reversal in XX medaka was induced 60 by 17 β -trenbolone exposure at concentrations of 0.5 μ g/L or greater. In previous studies, 61 masculinization was induced in zebrafish by exposure to 17\beta-trenbolone at 10 ng/L 62 (Baumann et al., 2013) or 50 ng/L (Örn et al., 2006), in western mosquitofish by exposure 63 at 1 µg/L (Sone et al., 2005), and in Japanese medaka by exposure at 32 ng/L (O. latipes) 64 (Flynn et al., 2017). These toxic concentrations are close to the previously reported environmental concentrations, suggesting that continued environmental monitoring of 65 66 17β-trenbolone levels is needed to protect teleosts from the harmful effects of trenbolone 67 exposure.

Japanese medaka (*Oryzias latipes*) are small, freshwater, teleost fish that inhabit the gently flowing rivers and waterways of Japan. The mechanisms underlying sex determination and gonadal sex differentiation are well understood in this fish, thus making it an ideal model organism for examining the effects of chemicals on sexual 72 dimorphism (Matsuda et al., 2002; Kobayashi et al., 2004). Medaka sex is determined by 73 the presence or absence of a sex-determining gene, DM-domain gene on the Y 74 chromosome (dmy). When dmy is present, the fish will develop to male; if not, the fish 75 will develop to female (Matsuda et al., 2002). When *dmy* is present, it stimulates the 76 expression of gonadal soma-derived factor (Gsdf) in XY gonads at developmental stage 77 36 (around 6 days after fertilization) in Qurt and HdrR (closed colony) (Shibata et al., 78 2010), HNI and d-rR strain (Horie et al., 2016), which in turn induces testis differentiation 79 (Shibata et al., 2010). It has also been reported that the gsdf gene is involved in sex 80 determination in Luzon medaka (Oryzias luzonensis) (Myosho et al., 2012) and in testis 81 differentiation in Nile tilapia (Kaneko et al., 2015).

82 There are several reports showing that exposure to EDCs induces changes in the 83 expression of gsdf in medaka. For example, Zhang et al. (2020) have reported in Indian 84 medaka that gsdf expression is decreased in males after 17a-ethinylestradiol exposure but 85 increased in females after 17β-trenbolone exposure. In Japanese medaka (O. latipes), gsdf 86 expression is reduced in males after exposure to estradiol benzoate (Kobayashi et al., 87 2017) or 17β-estradiol (Shibata et al., 2010). Our group has reported in Japanese medaka 88 that gsdf expression was decreased in males (O. latipes) after exposure to bisphenol A 89 (Horie et al., 2020) or 4-nonylphenol (Horie et al., 2021) but increased in females (O. 90 sakaizumii) after exposure to 17α -methyltestosterone (Horie et al., 2016). These results 91 suggest that gsdf expression may be a useful biomarker for screening the sex-related 92 effects of chemicals using Japanese medaka. However, further studies using a broad range 93 of chemicals are needed to clarify the applicability of this approach.

94 Here, we conducted a series of experiments to confirm the applicability of *gsdf* gene
95 expression in Japanese medaka as a biomarker of chemicals having adverse effects on

96 gonadal development. First, we examined the applicability of gsdf gene expression by 97 using 17β -trenbolone which has a potent and rogenic chemical and still unclear for influence of gsdf gene expression in O. latipes. Then, we examined the effect of 10 other 98 99 chemicals with various activities on gsdf expression at the early stage of gonadal 100 development in Japanese medaka: four estrogen/estrogenic chemicals (4-t-octylphenol, 101 17 β -estradiol, 17 α -ethinylestradiol, and *p*,*p*'-DDE), two anti-androgenic chemicals 102 (fenitrothion and flutamide), two chemicals without hormonal activity (tributyltin 103 chloride and triphenyltin chloride), and two pesticides without endocrine-disrupting 104 activity (amitrole and endrin). These test chemicals were selected by using data from the 105 SPEED (Strategic Programs on Environmental Endocrine Disruptors) '98 medaka test 106 conducted by the Japanese Ministry of the Environment (summary of medaka studies 107 conducted Japanese of by the Ministry the Environment ; 108 http://www.env.go.jp/en/chemi/ed/extend2010 full.pdf), although detailed information 109 has not been published.

110

111 2. Materials and Methods

112 2.1. Test fish and test chemicals

113 The NIES-R strain of Japanese medaka (*O. latipes*), maintained at Akita Prefectural 114 University (Akita, Japan), was used. Medaka fish were bred under an artificial 115 photoperiod of 16-h/8-h light/dark at 25 ± 2 °C. All animal experiments were conducted 116 according to the relevant national guidelines (Act on Welfare and Management of 117 Animals, Ministry of the Environment, Japan) and the fish used in the present study were 118 handled according to the animal care and use guidelines of Akita Prefectural University. 119 All animal experiments were approved by the institutional animal care and use committee, Faculty of Bioresource Sciences, Akita Prefectural University. Our research was alsoperformed in accordance with the ARRIVE guidelines.

- 4-*t*-Octylphenol (CAS no. 140-66-9; purity, >97.0%), *p,p*'-DDE (72-55-9; >99.0%), fenitrothion (122-14-5; >99.0%), flutamide (13311-84-7; >98.0%), tributyltin chloride (1461-22-9; >97.0%), and triphenyltin chloride (639-58-7; >98.0%) were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 17β-Estradiol (50-28-2; >97.0%), 17α-ethinylestradiol (57-63-6; >98.0%), amitrole (61-82-5; >98.0%), endrin (72-20-8; >95.0%), and 17β-trenbolone (10161-33-8; >97.0%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).
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130 2.2. Trenbolone exposure test

131 17β-Trenbolone exposure testing of medaka was conducted by using a flow-through 132 exposure system (SIS-1F; Shibata Scientific Technology, Tokyo, Japan). Exposure 133 nominal concentrations of 0 (control), 0.32, 1, 3.2, 10, or 32 µg/L 17β-trenbolone were 134 used. To prepare 0.032, 0.1, 0.32, 1, and 3.2 mg/L 17B-trenbolone aqueous stock solutions, 135 0.144, 0.45, 1.44, 4.5, and 14.4 mg, respectively, of 17β-trenbolone were placed into 136 separate 5-L glass media bottles (diameter, 182 mm) and dissolved in 4.5 L of Milli-Q 137 water with sonication for 120 min in an ultrasonic bath. The stock solutions were then 138 diluted 1:100 with dechlorinated tap water to obtain the nominal concentrations when 139 using the flow-through exposure system. The water exchange rate was 5 vols per day, and 140 the stock solution was renewed every 4 days by using the same acetone solution, which 141 was kept in a glass reagent bottle.

142 The fish were exposed to 17β-trenbolone by using a method we reported previously
143 (Horie et al., 2020, 2021). Eggs were obtained from natural mating in the early morning,

144 and fertilized eggs were selected under a stereomicroscope. After selection, the fertilized 145 eggs were exposed to 17^β-trenbolone-free water (control) or water containing 17^β-146 trenbolone at one of the predetermined concentrations $(0.32, 1, 3.2, 10, \text{ or } 32 \,\mu\text{g/L})$ within 147 4 h after fertilization until the end of the test (60 days post-hatching [dph]). Then, 20 148 fertilized eggs per vessel from each treatment group were distributed into 100-mL glass 149 vessels (exposure volume, 60 mL) and cultured until hatching. Four replicate 100-mL 150 glass vessels were used for each of the six treatment conditions. During the embryo stage, 151 the test solution was refreshed once every 24 h. After hatching, the fry were pooled, and 152 there were 15 fish per tank in each of 4 replicate tanks for a total of 60 fish per 153 concentration, and cultured until 60 dph by using the flow-through exposure system 154 (water exchange rate, 5 volumes/day). The embryos and fish were exposed to the same 155 concentration of 17^β-trenbolone both before and after hatching. At 60 dph, all fish in each tank were dissected and the abdomen (including gonads) and caudal fins were collected. 156

157

158 2.3. Chemical exposure tests for the 10 test chemicals

159 Table 1 summarizes the concentrations used for the 10 test chemicals examined. The table 160 also shows the four chemicals that are known to induce the intersex condition, as well as 161 the associated lowest observed effect concentrations (LOEC). We selected concentrations 162 of the test substances that were less than the water solubility in all of the substances tested, 163 although we did not conduct chemical analyses for the 10 test chemicals. Eggs were 164 obtained from natural mating in the early morning, and fertilized eggs were selected under 165 a stereomicroscope. After selection, the fertilized eggs were exposed to chemical-free 166 water (control) or water containing one of the test chemicals at one of the predetermined 167 concentrations within 4 h after fertilization until the end of the test (8 days postfertilization [developmental stage 38]). Then, 20 fertilized eggs from each treatment group were distributed into 100-mL glass vessels (exposure volume was 60 mL) and cultured until stage 38. The developmental stage was determined by using a previously published atlas (Iwamatsu, 2004). The test solution was refreshed once every 24 h. DNA was obtained from caudal fins (see Section 2.2) and genetic sex was determined (see Section 2.2). The remaining part of the embryos after removal of caudal fins were soaked in RNAlater (Sigma-Aldrich) and stored at 4 °C until RNA isolation (within 7 days).

176 2.4. Chemical analysis

177 Actual concentrations of the 17^β-trenbolone solutions were measured by analysis using 178 high performance liquid chromatography (HPLC-1260 Infinity; Agilent, CA, USA). 179 HPLC analysis was conducted under the following conditions: LC column, Mightysil RP-180 18 GP (5 μm, 2.0 mm I.D. × 150 mm; Kanto Chemical, Tokyo, Japan); mobile phase A, 181 0.01 mol/L ammonium formate in 0.1% formic acid; mobile phase B, acetonitrile (1:1, 182 v/v); flow rate, 0.2 mL/min; column temperature, 45 °C; injection volume, $5 \mu L$; 183 detector, UV absorbance at 335 nm. Detection limit and quantification limit for the 184 exposure concentration were 0.17 μ g/L and 0.58 μ g/L, respectively.

185

186 2.5. Histopathology

Analysis after exposure was conducted as reported previously (Horie et al., 2020, 2021).
The abdomen (including gonads) were fixed in Bouin's solution for 24 h, dehydrated in
a standard graded series of ethanol, and embedded in Paraplast Plus (McCormick
Scientific, St. Louis, MO, USA). Serial cross-sections of the whole abdomen (including
gonads) (6 µm thickness) were then prepared and stained with Carazzi's hematoxylin

192 193 (Fujifilm Wako Pure Chemical Industries) and used to observe gonadal sex differentiation.

194 2.6. Sex determination by PCR

195 Caudal fins were cut, dissolved in TE/PK solution (200 ng/µL Proteinase K, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and incubated for 12 h at 55 °C. Caudal fin lysates were 196 197 used for PCR amplification. Genetic sex was determined by PCR analysis with primer 198 sets for the doublesex and mab-3-related transcription factor 1 (dmrt1) and dmy genes, 199 (5'-CCGGGTGCCCAAGTGCTCCCGCTG-3') and PG17.6 i.e., PG17.5 (5'-200 GATCGTCCCTCCACAGACAAGAGA-3'), as described previously (Kobayashi et al., 201 2004). The PCR conditions were 5 min at 95 °C, followed by 35 cycles of 20 s at 96 °C, 202 30 s at 55 °C, 30 s at 72 °C, and then 5 min at 72 °C. The PCR products were 203 electrophoresed in a 1.5% Tris-acetate-EDTA agarose gel for analysis.

204

205 2.7. Real-time quantitative PCR

206 Real-time quantitative PCR was performed as described previously (Horie et al., 2020, 207 2021). Total RNA was extracted from whole one embryo without caudal fins by using an 208 RNeasy Mini Kit and RNase-Free DNase (Qiagen, Hilden, Germany) in accordance with 209 the manufacturer's protocol. The concentration of RNA in the extracts was measured with 210 a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, 211 Waltham, MA, USA). RNA was then reverse-transcribed into cDNA by using 212 PrimeScript RT Master Mix (Perfect Real Time, Takara, Shiga, Japan) in accordance with 213 the manufacturer's protocol; the concentration of each cDNA solution was adjusted to 10 214 ng/µL. Real-time quantitative PCR was performed with a LightCycler 96 System (Roche, 215 Basel, Switzerland) and a Kapa SYBR Fast qPCR Master Mix (2×) Kit (Kapa Biosystems, 216 Basel, Switzerland). Each reaction mixture (20 µL) contained 10 µL of KAPA SYBR Fast 217 qPCR Master Mix (2×), 0.2 μL of each 20 μM primer, 1 μL of 10 ng/μL cDNA, and 8.6 218 µL of PCR-grade water. The reaction profile consisted of 180 s at 95 °C followed by 40 cycles at 95 °C for 10 s, 49 °C for 20 s, and 72 °C for 1 s. The sequences of the specific 219 220 primers were as follows: gsdf (forward, 50-GGCTGGGACAATTGGGTGATC-30; 221 reverse. 50TTTCATCCATGAAGACGATGG-30) and eflα (forward, 50-222 AGTACGCCTGGGTGTTGGAC-30; reverse, 50-AAACGGGCCTGGCTGTAAG-30). 223 All primers and predicted amplicons were checked by using BLAST (NCBI; 224 http://www.ncbi.nlm.nih.gov/BLAST/). Each sample for each target was run in triplicate. 225 The data were analyzed by using LightCycler 96 analysis software (SW 1.1; Roche) and 226 exported to Microsoft Excel (Microsoft, USA). The expression level of gsdf (n = 6) was 227 normalized to that of the *efla* housekeeping gene by using the $2^{-\Delta\Delta Ct}$ method (Livak and 228 Schmittgen, 2001).

229

230 2.8. Statistical analysis

Statistical analyses were conducted as we reported previously (Horie et al., 2017). By using the open-source statistical software R (http://www.R-project.org/) and Rcmdr (Fox and Bouchet-Valat, 2018), we first applied Bartlett's test (significance level, 5%) to test for homogeneity of variance. When the criterion for homogeneity of variance was not rejected, we tested for differences in the test results among treatments by using Dunnett's test; otherwise, we used Steel's test.

237

238 **3. Results**

239 3.1. Trenbolone exposure test

Table 2 shows the nominal and average measured concentrations of 17β -trenbolone for the whole test period. The measured concentrations of 17β -trenbolone remained stable during the test period (data not shown), although were lower than the nominal concentrations. 17β -Trenbolone was not detected in the control water.

244

245 At the end of the test period, none of the XX medaka in the control and 0.1 μ g/L 246 exposure groups showed abnormal gonadal development (Fig. 1 and Table 3). However, 247 all of the XX medaka exposed to 17B-trenbolone at 0.5, 1.92, 6.32, and 22.1 ug/L except 248 one in the 6.32 μ g/L exposure group, showed ovotestis (presence of ovarian tissue with 249 testicular tissue) development or female-to-male sex reversal. Also, at the end of the test 250 period, expression of gsdf in control XY embryos was significantly higher than that in the 251 control XX embryos (Fig. 2). After 17^β-trenbolone exposure, gsdf expression in XX 252 embryos in the 6.32 and 22.1 µg/L exposure groups was significantly increased compared 253 with that in the control XX embryos (Fig. 2).

254

255 3.2. Change in *gsdf* expression after chemical exposure

We examined the changes in *gsdf* expression at developmental stage 38 after exposure to chemicals with various activities. First, we examined *gsdf* expression after estrogen and estrogenic chemicals (Fig. 3). In all exposure groups, *gsdf* expression in control XY embryos was significantly higher than that in control XX embryos. After 17 β -estradiol, 17 α -ethinylestradiol, 4-*t*-octylphenol, or *p,p'*-DDE exposure in XY embryos no statistically significant difference in *gsdf* expression was observed compared with control XY embryos in the present study. 263 Next, we examined *gsdf* expression after exposure to chemicals with anti-androgenic
264 activity (Fig. 4). In both exposure groups, *gsdf* expression in control XY embryos was
265 significantly higher than that in control XX embryos. After fenitrothion or flutamide
266 exposure, *gsdf* expression in XY embryos was comparable to that in XY controls.

267 Finally, we examined gsdf expression after exposure to chemical substances with no 268 hormonal activity (Fig. 5). In all exposure groups, gsdf expression in control XY embryos 269 was significantly higher than that in control XX embryos. After tributyltin chloride 270 exposure, gsdf expression was significantly increased in XX embryos and decreased in 271 XY embryos in the 250-µg/L concentration group compared with that in control embryos; 272 there were no other significant differences for the higher exposure groups. After 273 triphenyltin chloride or amitrole exposure, gsdf expression in XX embryos and XY 274 embryos in all exposure groups was comparable to that in the controls. After endrin 275 exposure, gsdf expression was significantly lower in XY embryos at 25 ng/L 276 concentration group than in the control XY group; there were no other significant 277 differences for the other XX and XY embryo exposure groups.

278

279 4. Discussion

Trenbolone is a synthetic androgenic steroid that is used to promote growth or enhance feeding efficiency in beef cattle. Ankley et al. (2018) reviewed the effect of 17βtrenbolone using *in vivo* and *in vitro* studies and concluded that it is a potent androgen receptor agonist. Here, we show that 17β -trenbolone exposure induced ovotestis development or female-to-male sex reversal in XX medaka. This finding is consistent with previous reports that 17β -trenbolone exposure induces ovotestis development and sex reversal in medaka and zebrafish (Baumann et al., 2013; Örn et al., 2006) and in 287 western mosquitofish (*Gambusia affinis*) (Sone et al., 2005). Together, these findings 288 indicate that early life-stage exposure to 17β -trenbolone induces masculinization of 289 female medaka.

290 Here, we also examined the effects of exposure to chemicals with different hormonal 291 activities on gsdf expression in the developing medaka. Together with our previous data 292 (Horie et al., 2016), our findings strongly suggest that gsdf expression in XX medaka is 293 increased by exposure to chemicals with androgenic activity, although the lowest-294 observed-effect concentration value of 17\beta-trenbolone (6.32 µg/L) that induced 295 increasing of gsdf mRNA expression during embryo development (i.e., just before 296 hatching) was not consistent with the 17β-trenbolone concentration that induced sexual 297 differentiation (0.5 µg/L), as determined in the OECD TG234 assay. In addition, findings 298 from previous studies indicate that gsdf expression in XY medaka is decreased by 299 exposure to chemicals with estrogenic activity (Kobayashi et al., 2017; Horie et al., 2020, 300 2021). Although previous reports using 17β -estradiol (Shibata et al., 2010) and estradiol 301 benzoate, which is synthetic estrogen, (Kobayashi et al., 2017) reports that gsdf 302 expression in XY medaka is decreased, in the present study, no statistically significant 303 difference in gsdf expression was observed by 17β -estradiol and 17α -ethinylestradiol 304 which is synthetic estrogen. One of the possible differences is the difference in exposure 305 concentration. The previous study with 17β-estradiol and synthetic estrogen involved a 306 higher concentration; 10 ug/L 17 β -estradiol (Shibata et al., 2010) vs 100 ng/L (this study) 307 and 800 ng/L synthetic estrogen (Kobayashi et al., 2017) vs 100 ng/L (this study). In the 308 present study, we found that gsdf expression was not changed by exposure to anti-309 androgenic chemicals or chemicals without hormonal activity. These findings are 310 consistent with previous reports of an increase or decrease in gsdf expression after

311 exposure to chemicals with androgenic or estrogenic activity. For example, Lee et al. 312 (2017) have reported that exposure to 17α -methyltestosterone induces masculinization of 313 gonads and upregulates *gsdf* expression in zebrafish. Zhang et al. (2020) have reported in 314 Indian medaka that *gsdf* expression is decreased in males after exposure to 17α -315 ethinylestradiol, but increased in females after exposure to 17β -trenbolone. Together, 316 these findings suggest that *gsdf* expression is altered by chemicals having androgenic or 317 estrogenic activity.

318 Gsdf, a member of the transforming growth factor beta superfamily, was first identified 319 in rainbow trout (Oncorhynchus mykiss) (Sawatari et al., 2007). Since then, gsdf 320 expression has been identified in the testes of sablefish (Anoplopoma fimbria) (Hayman 321 et al., 2021), Atlantic salmon (Salmo salar) (Kleppe et al., 2020), spotted scat 322 (Scatophagus argus) (He et al., 2019), Luzon medaka (Myosho et al., 2012), Japanese 323 flounder (Paralichthys olivaceus) (Yang et al., 2019), Chinese tongue sole (Cynoglossus 324 semilaevis) (Zhu et al., 2018), and Japanese pufferfish (Takifugu rubripes) (Yan et al., 325 2018). Jiang et al. (2016) have reported that gsdf-deficient XY Nile tilapia develop 326 ovotestes or ovaries. Similarly, Imai et al. (2015) have reported that XY Japanese medaka 327 (O. latipes) harboring a mutated gsdf develop ovaries. In contrast, Zhang et al. (2016) 328 have reported that gsdf addition induces masculinization in XX Japanese medaka (O. 329 latipes). In addition, Myosho et al. (2012) have reported that gsdf is a sex-determining 330 gene in Luzon medaka. Together, these findings indicate that gsdf is likely a key gene 331 controlling testicular differentiation in medaka and Nile tilapia. It has also been reported 332 that gsdf knockout zebrafish can develop as male or female, and that the mutant males are 333 fertile (Yan et al., 2017), indicating that gsdf expression can probably not be used to

334 predict the effects of chemicals with endocrine disrupting potency in all teleosts, but it335 can be used in medaka.

336 The expression of several teleost genes is altered by exposure to chemicals with 337 androgenic or estrogenic activity, including two genes encoding proteins thought to be 338 involved in ovarian differentiation; forkhead box L2 (Foxl2), a member of the forkhead 339 family of transcription factors, and Cyp19a1a (aromatase), which is involved in 17β-340 estradiol synthesis by catalyzing the conversion of androgens to estrogens (Wang et al., 341 2007), and the gene encoding Dmrt1, which functions in male sex determination and testis 342 development (Masuyama et al., 2012). More specifically, exposure to chemicals with 343 estrogenic activity increases the expression of Foxl2 in zebrafish (Yang et al., 2018) and 344 rare minnow (Yuan et al., 2014; Wang et al., 2012), and of Cyp19a1a in zebrafish (Yang 345 et al., 2018), Nile tilapia (Gennotte et al., 2014), and mangrove rivulus (Lee et al., 2006). 346 In contrast, exposure to chemicals with androgenic activity increases the expression of 347 dmrt1 in orange-spotted grouper (Epinephelus coioides) (Lyu et al., 2019), zebrafish (Lee 348 et al., 2017), and rainbow trout (Baron et al., 2008). In addition, changes in the expression 349 of foxl2, cyp19a1a, and dmrt1 by hormone exposure have been reported in Japanese 350 medaka (O. latipes); for example, Kobayashi et al. (2017) have reported that foxl2 351 expression is increased after exposure to estradiol benzoate. However, we found recently 352 that *foxl2* expression was unchanged after exposure to bisphenol A (Horie et al., 2020), 353 suggesting that *foxl2* expression may not be a good biomarker for predicting the sex-354 related effects of chemicals. Upregulation of cyp19a1a and dmrt1 expression is induced 355 by 17a-ethinylestradiol (Scholz and Guyzeit., 2000), bisphenol A (Horie et al., 2020), and 356 17α -methyltestosterone (Horie et al., 2016), suggesting that *cyp19a1a* or *dmrt1*

357 expression are also potential biomarkers for predicting the sex-related effects of358 chemicals with androgenic or estrogenic activity.

359 In recent years, animal welfare-friendly test methods have been recommended for 360 ecotoxicological investigations, and in the EU, notably, since Directive 2010/63/EU (EU 361 2010) on the protection of animals used for scientific purposes, live non-human vertebrate 362 animals including independently feeding larval forms are covered by its scope. In 363 Japanese medaka, although gsdf expression is detectable in the somatic cells surrounding 364 the primordial germ cells at developmental stage 36 (around 6 days post-fertilization) in 365 XY embryos (Shibata et al., 2010; Horie et al., 2016), dmrt1 and cyp19a1a expression is 366 detectable only after hatching in XY and XX gonads (Kobayashi et al., 2004; Nakamoto 367 et al., 2006). Furthermore, our present and previous findings indicate that whereas gsdf 368 expression in XX embryos is induced during embryogenesis after androgen (17a-369 methyltestosterone) exposure (Horie et al., 2016), gsdf expression in XY embryos is 370 decreased during embryogenesis after estrogen (bisphenol A and 4-nonylphenol) 371 exposure (Horie et al., 2020, 2021).

372

373 5. Conclusions

Here, we evaluated the potential of using *gsdf* expression as a biomarker for predicting the sex-related toxicological effects of chemicals with androgenic or estrogenic activity in Japanese medaka. Exposure to 17β -trenbolone, which has androgenic activity, induced ovotestis development and female-to-male sex reversal, as well as increased *gsdf* expression, in XX embryos. Although no statistically significant difference in *gsdf* expression was observed by exposure to four estrogens/chemicals with estrogenic activity in the present study, our previous studies indicate that *gsdf* expression in XY embryos is decreased by exposure to chemicals with estrogenic activity. In contrast, chemicals withanti-androgenic activity or chemicals without hormonal activity did not induce changes

- in *gsdf* expression in XX or XY embryos.
- 384

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390

391 Disclaimer

392 The authors have no conflicts of interest related to this research.

393

394 Data Availability Statement

395 Data are available from the corresponding author (horie@people.kobe-u.ac.jp).

396

397 Author Contributions Statement

Y. Horie: Conceptualization, Data curation, Formal Analysis, Funding acquisition,
Investigation, Validation, Methodology, and Writing - original draft; N. Kanazawa: Data
curation, Methodology, and Writing - original draft; C. Takahashi: Resources, Data
curation, Visualization, and Methodology; N. Tatarazako: Project administration and
Writing – review & editing; T. Iguchi: Validation, Project administration, Supervision,
and Writing – review & editing.

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554 Tables

555 Table 1. Test chemicals used

Chemical		Concentration word in the	Intersex	LOEC for	
		Concentration used in the		induction of	Reference
		present study		intersex	
4- <i>t</i> -Octylphenol	estrogen receptor agonist	6.2, 12.5, 25, 50, 100 μg/L	0	23.7 µg/L	unpublished data
<i>p,p'-</i> DDE	_	1, 3.2, 10, 32, 100 µg/L	0	32.4 µg/L	unpublished data
17β-Estradiol	estrogen receptor agonist	6.2, 12.5, 25, 50, 100 ng/L	0	23.8 ng/L	unpublished data
17α- Ethinylestradiol	estrogen receptor agonist	6.2, 12.5, 25, 50, 100 ng/L	0	24.5 ng/L	unpublished data
Fenitrothion	androgen receptor antagonist	375, 750, 1500, 3000 μg/L	_	_	Horie et al., 2017
Flutamide	androgen receptor antagonist	125, 250, 500, 1000 μg/L	_	_	Nakamura et al., 2016
Tributyltin chloride	-	250, 500, 1000, 2000 ng/L	_	-	unpublished data
Triphenyltin chloride	_	250, 500, 1000, 2000 ng/L	_	_	unpublished data

A mitrolo	_	62.5, 125, 250, 500, 1000	unpublished
Amitrole		μ g/L	data
En duin	_ 1	12.5.25.50.100.200 mc/L	unpublished
Endim		12.3, 23, 30, 100, 200 lig/L	data
556			
557			

558

559 Table 2. Nominal and average measured concentrations of trenbolone for the whole test

560 period

Nominal concentration	5	Average measured	Standard deviation	
(µg/L)	11	concentration (μ g/L)	$(\mu g/L)$	
Control	10	ND	_	
0.32	10	0.1	0.053	
1	10	0.5	0.11	
3.2	10	1.92	0.35	
10	10	6.32	0.78	
32	10	22.1	0.13	

561 * The exposure concentrations of trenbolone were measured by analysis using HPLC-

562 1260 Infinity every week.

Age	Measured concentration	Genetic sex	Gonadal sex (%)		
	µg/L	XX	Ovary	Ovotestis	Testis
	control	21	21 (100)	0 (0)	0 (0)
	0.1	23	23 (100)	0 (0)	0 (0)
60 dah	0.5	13	0 (0)	7 (54)	6 (46)
60 apri	1.92	15	0 (0)	6 (40)	9 (60)
	6.32	21	1 (4)	10 (48)	10 (48)
	22.1	12	0 (0)	2 (17)	10 (83)

564 Table 3. Effects of trenbolone on gonadal sex differentiation in XX medaka

565 dph, days post hatch

567 Figure legends

568 Figure 1. Histological analysis of gonad after 17β-trenbolone exposure at 60 days after

- 569 hatching. Normal ovary (a) and testis (b) from control XX and XY medaka, respectively.
- 570 17 β -Trenbolone treatment induced ovotestis development (0.5 μ g/L) (c) or sex reversal

571 $(0.5 \ \mu g/L)$ (d) in XX medaka. Red arrows indicate ovarian tissue. Blue arrows indicate

- 572 testicular tissue. Scale bars, $100 \ \mu m$
- 573

574 Figure 2. Expression of gsdf mRNA in Japanese medaka, as measured by real-time 575 quantitative polymerase chain reaction at stage 38 after exposure to trenbolone. Columns 576 and error bars represent means \pm SEM (n = 6 per group). After the expression level of 577 gsdf was normalized to that of the $efl\alpha$ housekeeping gene, the each data were normalized 578 additionally to a control, in this case to the XX control embryos. Asterisks indicate 579 statistically significant differences compared with control XX (Dunnett's test or Steel's 580 test; P < 0.05). Numbers above the columns indicate the number of ovotestis (IS) and sex 581 reversal (SR) individuals at the end of test day (= ovotestis or sex reversal 582 individuals/total number of individuals).

583

Figure 3. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time quantitative polymerase chain reaction at stage 38 after exposure to 17 β -estradiol (E2), ethinylestradiol (EE2), 4-*t*-octylphenol (4-*t*-OP), or *p,p'*-DDE. Columns and error bars represent means \pm SEM (*n* = 6 per group). After the expression level of *gsdf* was normalized to that of the *ef1a* housekeeping gene, the each data were normalized additionally to a control, in this case to the XY control embryos. Asterisks indicate 590

statistically significant differences compared with control XY (Dunnett's test or Steel's test; P < 0.05).

592

591

Figure 4. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time quantitative polymerase chain reaction at stage 38 after exposure to fenitrothion or flutamide. Columns and error bars represent means \pm SEM (n = 6 per group). After the expression level of *gsdf* was normalized to that of the *ef1a* housekeeping gene, the each data were normalized additionally to a control, in this case to the XY control embryos. Asterisks indicate statistically significant differences compared with control XY (Dunnett's test or Steel's test; P < 0.05).

600

601 Figure 5. Expression of gsdf mRNA in Japanese medaka, as measured by real-time 602 quantitative polymerase chain reaction at stage 38 after exposure to tributyltin chloride 603 (TBT), triphenyltin chloride (TPT), amitrole, or endrin. Columns and error bars represent 604 means \pm SEM (n = 6 per group). After the expression level of *gsdf* was normalized to that 605 of the $efl\alpha$ housekeeping gene, the each data were normalized additionally to a control, 606 in this case to the XX (left figure) or XY (right figure) control embryos. Asterisks indicate statistically significant differences compared with control XX (left figure) or XY (right 607 608 figure) (Dunnett's test or Steel's test; P < 0.05). 609





Measured trenbolone concentration (µg/L)







