



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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3 **medaka (*Oryzias latipes*)**

4

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31

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

34

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36 The authors confirm that all data underlying the findings are fully available without
37 restriction.

38

39 **Author Contributions Statement**

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

46

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25 ***Key words:*** androgen, biomarker, estrogen, intersex, medaka, sex reversal

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 17 α -
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 17 β -
35 trenbolone at 0.5–22.1 μ g/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 μ g/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 17 β -
40 estradiol, 17 α -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

47

48 1. Introduction

49 The endocrine system plays an important role in the homeostasis of organisms by
50 overseeing the production and secretion of hormones. Chemicals that interfere with the
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. 17 β -Trenbolone has been detected in the natural
54 environment, especially at beef feedlots. For example, Durhan et al. (2006) reported that
55 17 β -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 17 β -
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 β -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 μ g/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
65 environmental concentrations, suggesting that continued environmental monitoring of
66 17 β -trenbolone levels is needed to protect teleosts from the harmful effects of trenbolone
67 exposure.

68 Japanese medaka (*Oryzias latipes*) are small, freshwater, teleost fish that inhabit the
69 gently flowing rivers and waterways of Japan. The mechanisms underlying sex
70 determination and gonadal sex differentiation are well understood in this fish, thus
71 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 17 α -ethinylestradiol exposure but
85 increased in females after 17 β -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 17 β -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 17 α -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 17 β -trenbolone which has a potent androgenic chemical and still unclear for
98 influence of *gsdf* gene expression in *O. latipes*. Then, we examined the effect of 10 other
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 by the Japanese Ministry of 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 \pm 2 $^{\circ}$ 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,

120 Faculty of Bioresource Sciences, Akita Prefectural University. Our research was also
121 performed in accordance with the ARRIVE guidelines.

122 4-*t*-Octylphenol (CAS no. 140-66-9; purity, >97.0%), *p,p'*-DDE (72-55-9; >99.0%),
123 fenitrothion (122-14-5; >99.0%), flutamide (13311-84-7; >98.0%), tributyltin chloride
124 (1461-22-9; >97.0%), and triphenyltin chloride (639-58-7; >98.0%) were obtained from
125 FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). 17 β -Estradiol (50-28-2;
126 >97.0%), 17 α -ethinylestradiol (57-63-6; >98.0%), amitrole (61-82-5; >98.0%), endrin
127 (72-20-8; >95.0%), and 17 β -trenbolone (10161-33-8; >97.0%) were obtained from
128 Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

129

130 2.2. Trenbolone exposure test

131 17 β -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 μ g/L 17 β -trenbolone were
134 used. To prepare 0.032, 0.1, 0.32, 1, and 3.2 mg/L 17 β -trenbolone aqueous stock solutions,
135 0.144, 0.45, 1.44, 4.5, and 14.4 mg, respectively, of 17 β -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 17 β -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, or 32 μ 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
156 tank were dissected and the abdomen (including gonads) and caudal fins were collected.

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 post-

168 fertilization [developmental stage 38]). Then, 20 fertilized eggs from each treatment
169 group were distributed into 100-mL glass vessels (exposure volume was 60 mL) and
170 cultured until stage 38. The developmental stage was determined by using a previously
171 published atlas (Iwamatsu, 2004). The test solution was refreshed once every 24 h. DNA
172 was obtained from caudal fins (see Section 2.2) and genetic sex was determined (see
173 Section 2.2). The remaining part of the embryos after removal of caudal fins were soaked
174 in RNAlater (Sigma-Aldrich) and stored at 4 °C until RNA isolation (within 7 days).

175

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 μ m, 2.0 mm I.D. \times 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 μ 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

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

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

193

194 2.6. Sex determination by PCR

195 Caudal fins were cut, dissolved in TE/PK solution (200 ng/μL Proteinase K, 10 mM
196 Tris-HCl, 1 mM EDTA, pH 8.0), and incubated for 12 h at 55 °C. Caudal fin lysates were
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 i.e., PG17.5 (5'-CCGGGTGCCCAAGTGCTCCCGCTG-3') and PG17.6 (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 μ L) contained 10 μ L of KAPA SYBR Fast
217 qPCR Master Mix (2 \times), 0.2 μ L of each 20 μ M primer, 1 μ L of 10 ng/ μ L cDNA, and 8.6
218 μ L of PCR-grade water. The reaction profile consisted of 180 s at 95 $^{\circ}$ C followed by 40
219 cycles at 95 $^{\circ}$ C for 10 s, 49 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 1 s. The sequences of the specific
220 primers were as follows: *gsdf* (forward, 50-GGCTGGGACAATTGGGTGATC-30;
221 reverse, 50TTTCATCCATGAAGACGATGG-30) and *ef1 α* (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 *ef1 α* housekeeping gene by using the $2^{-\Delta\Delta C_t}$ method (Livak and
228 Schmittgen, 2001).

229

230 2.8. Statistical analysis

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

237

238 3. Results

239 3.1. Trenbolone exposure test

240 Table 2 shows the nominal and average measured concentrations of 17 β -trenbolone for
241 the whole test period. The measured concentrations of 17 β -trenbolone remained stable
242 during the test period (data not shown), although were lower than the nominal
243 concentrations. 17 β -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 17 β -trenbolone at 0.5, 1.92, 6.32, and 22.1 μ g/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 μ g/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

256 We examined the changes in *gsdf* expression at developmental stage 38 after exposure to
257 chemicals with various activities. First, we examined *gsdf* expression after estrogen and
258 estrogenic chemicals (Fig. 3). In all exposure groups, *gsdf* expression in control XY
259 embryos was significantly higher than that in control XX embryos. After 17 β -estradiol,
260 17 α -ethinylestradiol, 4-*t*-octylphenol, or *p,p'*-DDE exposure in XY embryos no
261 statistically significant difference in *gsdf* expression was observed compared with control
262 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- $\mu\text{g/L}$ 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**

280 Trenbolone is a synthetic androgenic steroid that is used to promote growth or enhance
281 feeding efficiency in beef cattle. Ankley et al. (2018) reviewed the effect of 17 β -
282 trenbolone using *in vivo* and *in vitro* studies and concluded that it is a potent androgen
283 receptor agonist. Here, we show that 17 β -trenbolone exposure induced ovotestis
284 development or female-to-male sex reversal in XX medaka. This finding is consistent
285 with previous reports that 17 β -trenbolone exposure induces ovotestis development and
286 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 17 β -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 β -trenbolone (6.32 μ g/L) that induced
295 increasing of *gsdf* mRNA expression during embryo development (i.e., just before
296 hatching) was not consistent with the 17 β -trenbolone concentration that induced sexual
297 differentiation (0.5 μ g/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 17 β -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 17 β -estradiol and 17 α -ethinylestradiol
304 which is synthetic estrogen. One of the possible differences is the difference in exposure
305 concentration. The previous study with 17 β -estradiol and synthetic estrogen involved a
306 higher concentration; 10 μ g/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 17 α -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 17 α -
315 ethinylestradiol, but increased in females after exposure to 17 β -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 it
335 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 17 β -
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 17 α -ethinylestradiol (Scholz and Guyzeit., 2000), bisphenol A (Horie et al., 2020), and
356 17 α -methyltestosterone (Horie et al., 2016), suggesting that *cyp19a1a* or *dmrt1*

357 expression are also potential biomarkers for predicting the sex-related effects of
358 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 (17 α -
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**

374 Here, we evaluated the potential of using *gsdf* expression as a biomarker for predicting
375 the sex-related toxicological effects of chemicals with androgenic or estrogenic activity
376 in Japanese medaka. Exposure to 17 β -trenbolone, which has androgenic activity, induced
377 ovotestis development and female-to-male sex reversal, as well as increased *gsdf*
378 expression, in XX embryos. Although no statistically significant difference in *gsdf*
379 expression was observed by exposure to four estrogens/chemicals with estrogenic activity
380 in the present study, our previous studies indicate that *gsdf* expression in XY embryos is

381 decreased by exposure to chemicals with estrogenic activity. In contrast, chemicals with
382 anti-androgenic activity or chemicals without hormonal activity did not induce changes
383 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**

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

404

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553

554 **Tables**

555 Table 1. Test chemicals used

Chemical	Concentration used in the present study	Intersex	LOEC for induction of intersex	Reference
4- <i>t</i> -Octylphenol	estrogen receptor agonist 6.2, 12.5, 25, 50, 100 µg/L	○	23.7 µg/L	unpublished data
<i>p,p'</i> -DDE	— 1, 3.2, 10, 32, 100 µg/L	○	32.4 µg/L	unpublished data
17β-Estradiol	estrogen receptor agonist 6.2, 12.5, 25, 50, 100 ng/L	○	23.8 ng/L	unpublished data
17α-Ethinylestradiol	estrogen receptor agonist 6.2, 12.5, 25, 50, 100 ng/L	○	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

Amitrole	—	62.5, 125, 250, 500, 1000 µg/L	—	—	unpublished data
Endrin	—	12.5, 25, 50, 100, 200 ng/L	—	—	unpublished data

556

557

558

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

Nominal concentration ($\mu\text{g/L}$)	n	Average measured concentration ($\mu\text{g/L}$)	Standard deviation ($\mu\text{g/L}$)
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.
563

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

Age	Measured concentration $\mu\text{g/L}$	Genetic sex <u>XX</u>	Gonadal sex (%)		
			Ovary	Ovotestis	Testis
60 dph	control	21	21 (100)	0 (0)	0 (0)
	0.1	23	23 (100)	0 (0)	0 (0)
	0.5	13	0 (0)	7 (54)	6 (46)
	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)

565 dph, days post hatch

566

567 **Figure legends**

568 Figure 1. Histological analysis of gonad after 17 β -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 μ g/L) (d) in XX medaka. Red arrows indicate ovarian tissue. Blue arrows indicate
572 testicular tissue. Scale bars, 100 μ 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 α* 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

584 Figure 3. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time
585 quantitative polymerase chain reaction at stage 38 after exposure to 17 β -estradiol (E2),
586 ethinylestradiol (EE2), 4-*t*-octylphenol (4-*t*-OP), or *p,p'*-DDE. Columns and error bars
587 represent means \pm SEM ($n = 6$ per group). After the expression level of *gsdf* was
588 normalized to that of the *efl α* housekeeping gene, the each data were normalized
589 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
591 test; $P < 0.05$).

592

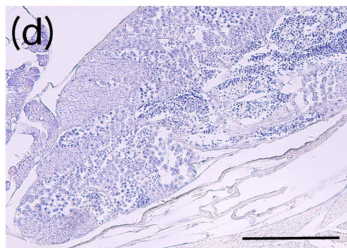
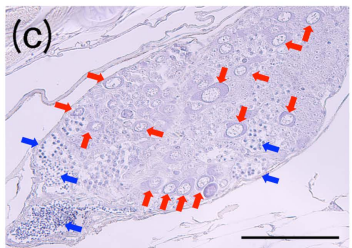
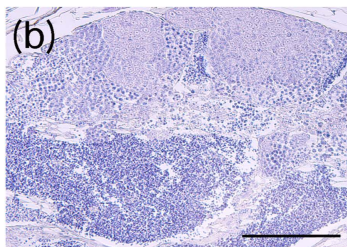
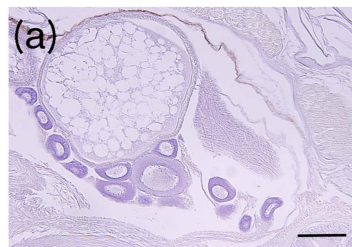
593 Figure 4. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time
594 quantitative polymerase chain reaction at stage 38 after exposure to fenitrothion or
595 flutamide. Columns and error bars represent means \pm SEM ($n = 6$ per group). After the
596 expression level of *gsdf* was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

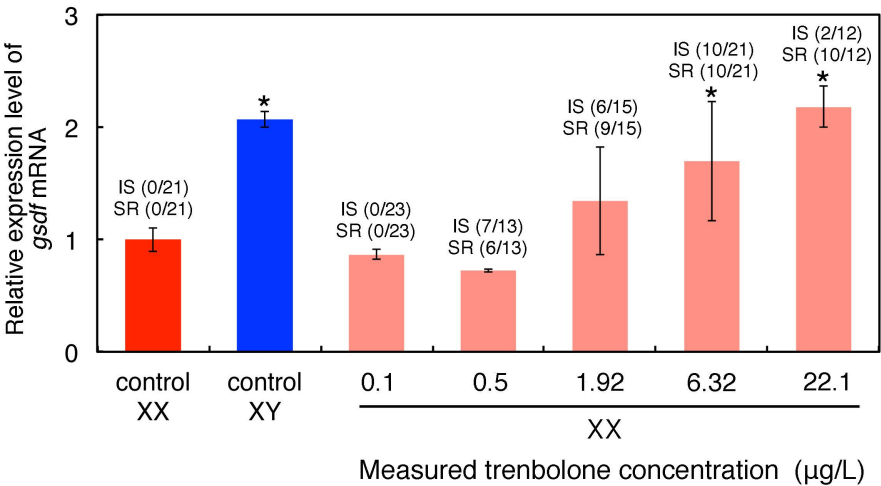
600

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 α* 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
607 statistically significant differences compared with control XX (left figure) or XY (right
608 figure) (Dunnett's test or Steel's test; $P < 0.05$).

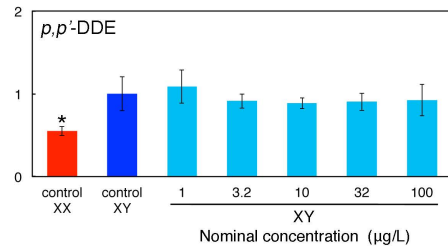
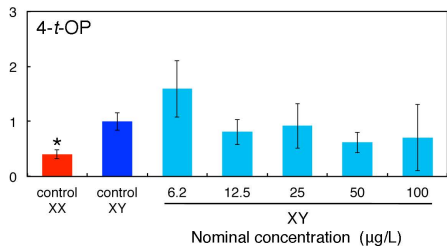
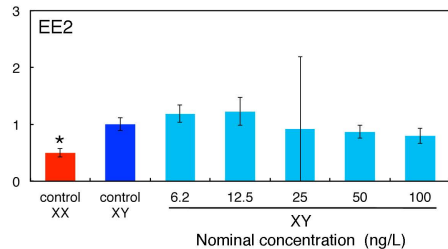
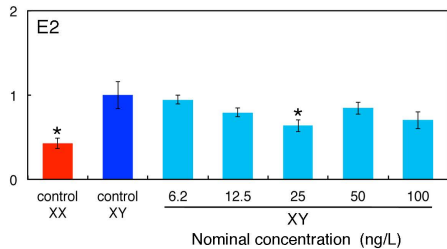
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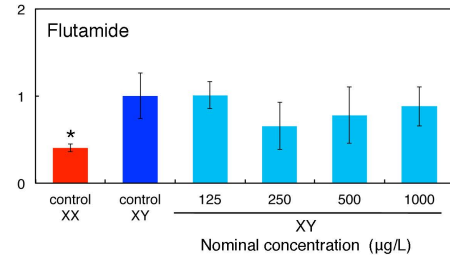
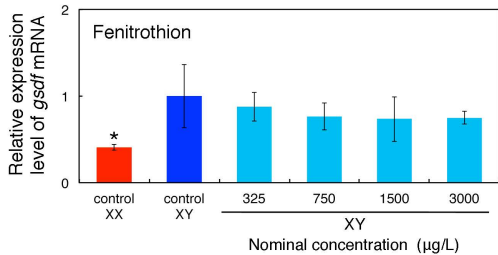
610



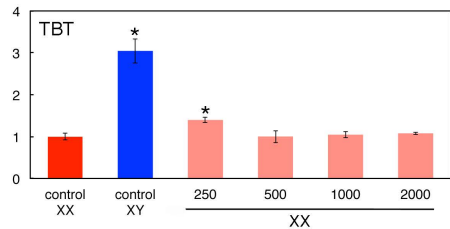


Relative expression level of *gsdf* mRNA

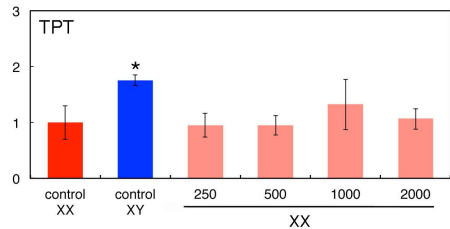
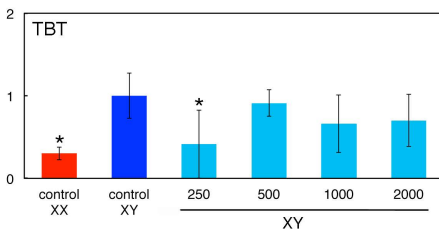




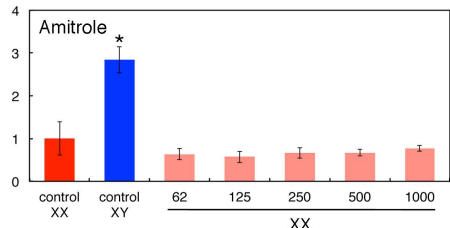
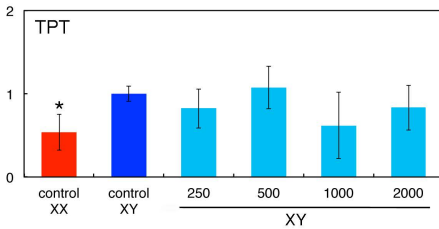
Relative expression level of *gsdf* mRNA



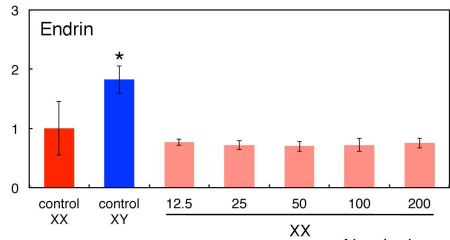
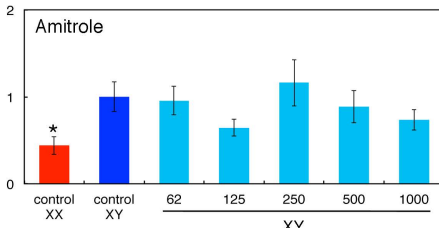
Nominal concentration ($\mu\text{g/L}$)



Nominal concentration ($\mu\text{g/L}$)



Nominal concentration ($\mu\text{g/L}$)



Nominal concentration (ng/L)

