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

Horie, Yoshifumi
Kanazawa, Nobuhiro
Takahashi, Chiho
Tatarazako, Norihisa
Iguchi, Taisen

(Citation)
Environmental Toxicology and Chemistry,41(8):1875-1884

(Issue Date)
2022-08

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/0100477402

Gonadal soma-derived factor expression is a potential biomarker for predicting the effects of endocrine-disrupting chemicals on gonadal differentiation in Japanese medaka (*Oryzias latipes*)

Yoshifumi Horie[a, b], Nobuhiro Kanazawa[c], Chiho Takahashi[a], Norihisa Tatarazako[d], Taisen Iguchi[e]

[a] Faculty of Bioresource Sciences, Akita Prefectural University, 241-438 Kaidobata-Nishi, Nakano Simoshinjo, Akita 010-0195, Japan

[b] Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan

[c] Faculty of Systems Science and Technology, Akita Prefectural University, 84-4 Ebinokuchi, Tsuchiya, Yurihonjo, Akita 015-0055, Japan

[d] Department of Science and Technology for Biological Resources and Environment, Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan

[e] Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan

Correspondence: Yoshifumi Horie, PhD, Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan
Tel.: +81-78-431-6357
E-mail: horie@people.kobe-u.ac.jp

Acknowledgments

This study was supported in part by the Environment Research and Technology Development Fund (5RF-1951) of the Environmental Restoration and Conservation Agency of Japan, and by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in-Aid for Scientific Research [B] no. 19H04294) to Y.H.

Disclaimer

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

Data Availability Statement

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

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.

Gonadal soma-derived factor expression is a potential biomarker for predicting the effects of endocrine-disrupting chemicals on gonadal differentiation in Japanese medaka (*Oryzias latipes*)

Yoshifumi Horie[a, b], Nobuhiro Kanazawa[c], Chiho Takahashi[a], Norihisa Tatarazako[d], Taisen Iguchi[e]

[a] Faculty of Bioresource Sciences, Akita Prefectural University, 241-438 Kaidobata-Nishi, Nakano Simoshinjo, Akita 010-0195, Japan

[b] Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan

[c] Faculty of Systems Science and Technology, Akita Prefectural University, 84-4 Ebinokuchi, Tsuchiya, Yurihonjo, Akita 015-0055, Japan

[d] Department of Science and Technology for Biological Resources and Environment, Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan

[e] Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan

Correspondence: Yoshifumi Horie, PhD, Research Center for Inland Seas (KURCIS), Kobe University, Fukaeminami-machi, Higashinada-ku, Kobe 658-0022, Japan
Tel.: +81-78-431-6357

E-mail: horie@people.kobe-u.ac.jp

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

Abstract

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

1. Introduction

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 endocrine system by mimicking or blocking hormonal activity (i.e., endocrine-disrupting chemicals, EDCs) can have detrimental effects on the reproductive health of an organism, its offspring, and its population. 17β-Trenbolone has been detected in the natural environment, especially at beef feedlots. For example, Durhan et al. (2006) reported that 17β-trenbolone was detected at 10–20 ng/L in the runoff from a beef feedlot in southwest central Ohio in the United States. Similarly, Gall et al. (2011) have reported that 17β-trenbolone was detected at 3.3–162 ng/L in the discharge from a tile-drained agroecosystem receiving animal wastes in the Midwestern United States. In the present study, ovotestis development or female-to-male sex reversal in XX medaka was induced by 17β-trenbolone exposure at concentrations of 0.5 μg/L or greater. In previous studies, masculinization was induced in zebrafish by exposure to 17β-trenbolone at 10 ng/L (Baumann et al., 2013) or 50 ng/L (Örn et al., 2006), in western mosquitofish by exposure at 1 μg/L (Sone et al., 2005), and in Japanese medaka by exposure at 32 ng/L (*O. latipes*) (Flynn et al., 2017). These toxic concentrations are close to the previously reported environmental concentrations, suggesting that continued environmental monitoring of 17β-trenbolone levels is needed to protect teleosts from the harmful effects of trenbolone 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

dimorphism (Matsuda et al., 2002; Kobayashi et al., 2004). Medaka sex is determined by the presence or absence of a sex-determining gene, *DM-domain gene on the Y chromosome* (*dmy*). When *dmy* is present, the fish will develop to male; if not, the fish will develop to female (Matsuda et al., 2002). When *dmy* is present, it stimulates the expression of gonadal soma-derived factor (Gsdf) in XY gonads at developmental stage 36 (around 6 days after fertilization) in Qurt and HdrR (closed colony) (Shibata et al., 2010), HNI and d-rR strain (Horie et al., 2016), which in turn induces testis differentiation (Shibata et al., 2010). It has also been reported that the *gsdf* gene is involved in sex determination in Luzon medaka (*Oryzias luzonensis*) (Myosho et al., 2012) and in testis differentiation in Nile tilapia (Kaneko et al., 2015).

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

Here, we conducted a series of experiments to confirm the applicability of *gsdf* gene 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 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 17β-estradiol, 17α-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 ± 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,

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). 17β-Estradiol (50-28-2;
126 >97.0%), 17α-ethinylestradiol (57-63-6; >98.0%), amitrole (61-82-5; >98.0%), endrin
127 (72-20-8; >95.0%), and 17β-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 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 17β-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 17β-trenbolone-free water (control) or water containing 17β-
146 trenbolone at one of the predetermined concentrations (0.32, 1, 3.2, 10, or 32 μg/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 17β-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 17β-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 μL ;
183 detector, UV absorbance at 335 nm. Detection limit and quantification limit for the
184 exposure concentration were 0.17 μg/L and 0.58 μg/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 μm thickness) were then prepared and stained with Carazzi's hematoxylin

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

2.6. Sex determination by PCR

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 used for PCR amplification. Genetic sex was determined by PCR analysis with primer sets for the *doublesex and mab-3-related transcription factor 1* (*dmrt1*) and *dmy* genes, i.e., PG17.5 (5′-CCGGGTGCCCAAGTGCTCCCGCTG-3′) and PG17.6 (5′-GATCGTCCCTCCACAGACAAGAGA-3′), as described previously (Kobayashi et al., 2004). The PCR conditions were 5 min at 95 °C, followed by 35 cycles of 20 s at 96 °C, 30 s at 55 °C, 30 s at 72 °C, and then 5 min at 72 °C. The PCR products were electrophoresed in a 1.5% Tris-acetate-EDTA agarose gel for analysis.

2.7. Real-time quantitative PCR

Real-time quantitative PCR was performed as described previously (Horie et al., 2020, 2021). Total RNA was extracted from whole one embryo without caudal fins by using an RNeasy Mini Kit and RNase-Free DNase (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The concentration of RNA in the extracts was measured with a NanoDrop One Microvolume UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was then reverse-transcribed into cDNA by using PrimeScript RT Master Mix (Perfect Real Time, Takara, Shiga, Japan) in accordance with the manufacturer's protocol; the concentration of each cDNA solution was adjusted to 10 ng/μL. Real-time quantitative PCR was performed with a LightCycler 96 System (Roche, Basel, Switzerland) and a Kapa SYBR Fast qPCR Master Mix (2×) Kit (Kapa Biosystems,

Basel, Switzerland). Each reaction mixture (20 μL) contained 10 μL of KAPA SYBR Fast qPCR Master Mix (2×), 0.2 μL of each 20 μM primer, 1 μL of 10 ng/μL cDNA, and 8.6 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 primers were as follows: *gsdf* (forward, 50-GGCTGGGACAATTGGGTGATC-30; reverse, 50TTTCATCCATGAAGACGATGG-30) and *ef1α* (forward, 50-AGTACGCCTGGGTGTTGGAC-30; reverse, 50-AAACGGGCCTGGCTGTAAG-30). All primers and predicted amplicons were checked by using BLAST (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/). Each sample for each target was run in triplicate. The data were analyzed by using LightCycler 96 analysis software (SW 1.1; Roche) and exported to Microsoft Excel (Microsoft, USA). The expression level of *gsdf* (n = 6) was normalized to that of the *ef1α* housekeeping gene by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

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.

3. Results

3.1. Trenbolone exposure test

240 Table 2 shows the nominal and average measured concentrations of 17β-trenbolone for
241 the whole test period. The measured concentrations of 17β-trenbolone remained stable
242 during the test period (data not shown), although were lower than the nominal
243 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 μg/L
246 exposure groups showed abnormal gonadal development (Fig. 1 and Table 3). However,
247 all of the XX medaka exposed to 17β-trenbolone at 0.5, 1.92, 6.32, and 22.1 μg/L except
248 one in the 6.32 μg/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 17β-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
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 17β-estradiol,
260 17α-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.

Next, we examined *gsdf* expression after exposure to chemicals with anti-androgenic activity (Fig. 4). In both exposure groups, *gsdf* expression in control XY embryos was significantly higher than that in control XX embryos. After fenitrothion or flutamide exposure, *gsdf* expression in XY embryos was comparable to that in XY controls.

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

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 17β-trenbolone 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 17β-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 17β-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 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 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 17α-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 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 (17α-
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 17β-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

decreased by exposure to chemicals with estrogenic activity. In contrast, chemicals with anti-androgenic activity or chemicals without hormonal activity did not induce changes in *gsdf* expression in XX or XY embryos.

Acknowledgments

This study was supported in part by the Environment Research and Technology Development Fund (5RF-1951) of the Environmental Restoration and Conservation Agency of Japan, and by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in-Aid for Scientific Research [B] no. 19H04294) to Y.H.

Disclaimer

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

Data Availability Statement

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

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.

405 **References**

406 Ankley GT, Coady KK, Gross M, Holbech H, Levine SL, Maack G, Williams M. 2018.
407 A critical review of the environmental occurrence and potential effects in aquatic
408 vertebrates of the potent androgen receptor agonist 17β-trenbolone. *Environ Toxicol*
409 *Chem* 37(8): 2064-2078.

410 Baron D, Houlgatte R, Fostier A, Guiguen Y. 2008. Expression profiling of candidate
411 genes during ovary-to-testis trans-differentiation in rainbow trout masculinized by
412 androgens. *Gen Comp Endocrinol* 156(2): 369-378.

413 Baumann L, Holbech H, Keiter S, Kinnberg KL, Knörr S, Nagel T, Braunbeck T. 2013.
414 The maturity index as a tool to facilitate the interpretation of changes in vitellogenin
415 production and sex ratio in the Fish Sexual Development Test. *Aquat Toxicol* 128-129:
416 34-42.

417 Durhan EJ, Lambright CS, Makynen EA, Lazorchak J, Hartig PC, Wilson VS, Gray LE,
418 Ankley GT. 2006. Identification of metabolites of trenbolone acetate in androgenic runoff
419 from a beef feedlot. *Environ Health Perspect* 114 (Suppl, 1): 65-68.

420 EU. 2010. Directive 2010/63/EU of the European Parliament and Council of 22
421 September on the protection of animals used for scientific purposes. *Off J Eur Union*
422 L276: 33-79.

423 Flynn K, Lothenbach D, Whiteman F, Hammermeister D, Touart LW, Swintek J,
424 Tatarazako N, Onishi Y, Iguchi T, Johnson R. 2017. Summary of the development the
425 US Environmental Protection Agency's Medaka Extended One Generation Reproduction

426 Test (MEOGRT) using data from 9 multigenerational medaka tests. *Environ Toxicol*
427 *Chem* 36 (12): 3387-3403.

428 Fox J, Bouchet-Valat M. 2018. Rcmdr: R Commander. R package version 2.5-1.

429 Gall HE, Sassman SA, Lee LS, Jafvert CT. 2011. Hormone discharges from a midwest
430 tile-drained agroecosystem receiving animal wastes. *Environ Sci Technol* 45 (20): 8755-
431 8764.

432 Gennotte V, Mélard C, D'Cotta H, Baroiller JF, Rougeot C. 2014. The sensitive period
433 for male-to-female sex reversal begins at the embryonic stage in the Nile tilapia and is
434 associated with the sexual genotype. *Mol Reprod Devel* 81 (12): 1146-1158.

435 Hayman ES, Fairgrieve WT, Luckenbach JA. 2021. Molecular and morphological sex
436 differentiation in sablefish (*Anoplopoma fimbria*), a marine teleost with XX/XY sex
437 determination. *Gene* 764: 145093.

438 He FX, Jiang DN, Huang YQ, Mustapha UF, Yang W, Cui XF, Tian CX, Chen HP, Shi
439 HJ, Deng SP, Li GL, Zhu CH. 2019. Comparative transcriptome analysis of male and
440 female gonads reveals sex-biased genes in spotted scat (*Scatophagus argus*). *Fish Physiol*
441 *Biochem* 45 (6): 1963-1980.

442 Horie Y, Myosho T, Sato T, Sakaizumi M, Hamaguchi S, Kobayashi T. 2016. Androgen
443 induces gonadal soma-derived factor, Gsdf, in XX gonads correlated to sex-reversal but
444 not Dmrt1 directly, in the teleost fish, northern medaka (*Oryzias sakaizumii*). *Mol Cell*
445 *Endocrinol* 436: 141-149.

446 Horie Y, Watanabe H, Takanobu H, Yagi A, Yamagishi T, Iguchi T, Tatarazako N. 2017.
447 Development of an in vivo anti-androgenic activity detection assay using fenitrothion in
448 Japanese medaka (*Oryzias latipes*). *J Appl Toxicol* 37 (3): 339-346.

449 Horie Y, Kanazawa N, Takahashi C, Tatarazako N, Iguchi T. 2020. Bisphenol A induces
450 a shift in sex differentiation gene expression with testis-ova or sex reversal in Japanese
451 medaka (*Oryzias latipes*). *J Appl Toxicol* 40: 804-814.

452 Horie Y, Kanazawa N, Takahashi C, Tatarazako N, Iguchi T. 2021. Exposure to 4-
453 nonylphenol induces a shift in the gene expression of gsdf and testis-ova formation and
454 sex reversal in Japanese medaka (*Oryzias latipes*). *J Appl Toxicol* 41: 399-409.

455 Imai T, Saino K, Matsuda M. 2015. Mutation of Gonadal soma-derived factor induces
456 medaka XY gonads to undergo ovarian development. *Biochem Biophys Res Commun* 467
457 (1): 109-114.

458 Iwamatsu T. 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mech*
459 *Devel* 121: 605–618.

460 Jiang DN, Yang HH, Li MH, Shi HJ, Zhang XB, Wang DS. 2016. gsdf is a downstream
461 gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia.
462 *Mol Reprod Devel* 83 (6): 497-508.

463 Kaneko H, Ijiri S, Kobayashi T, Izumi H, Kuramochi Y, Wang DS, Mizuno S, Nagahama
464 Y. 2015. Gonadal soma-derived factor (gsdf), a TGF-beta superfamily gene, induces
465 testis differentiation in the teleost fish *Oreochromis niloticus*. *Mol Cell Endocrinol* 415:
466 87-99.

467 Kleppe L, Edvardsen RB, Furmanek T, Andersson E, Skaftnesmo KO, Segafredo TF,
468 Wargelius A. 2020. Transcriptomic analysis of dead end knockout testis reveals germ cell
469 and gonadal somatic factors in Atlantic salmon. *BMC Genomics* 21 (1): 99.

470 Kobayashi T, Matsuda M, Kajiura-Kobayashi H, Suzuki A, Saito N, Nakamoto M,
471 Nagahama Y. 2004. Two DM domain genes, DMY and DMRT1, involved in testicular
472 differentiation and development in the medaka, *Oryzias latipes*. *Develop Dynam* 231:
473 518-526.

474 Kobayashi T, Chiba A, Sato T, Myosho T, Yamamoto J, Okamura T, Horie Y. 2017.
475 Estrogen alters gonadal soma-derived factor (Gsdf)/Foxl2 expression levels in the testes
476 associated with testis-ova differentiation in adult medaka, *Oryzias latipes*. *Aquat Toxicol*
477 191: 209-218.

478 Lee YM, Seo JS, Kim IC, Yoon YD, Lee JS. 2006. Endocrine disrupting chemicals
479 (bisphenol A, 4-nonylphenol, 4-*tert*-octylphenol) modulate expression of two distinct
480 cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish
481 *Rivulus marmoratus*. *Biochem Biophys Research Commun* 345 (2): 894-903.

482 Lee SLJ, Horsfield JA, Black MA, Rutherford K, Fisher A, Gemmell NJ. 2017.
483 Histological and transcriptomic effects of 17α-methyltestosterone on zebrafish gonad
484 development. *BMC Genomics* 18 (1): 557.

485 Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time
486 quantitative PCR and the 2(-Delta Delta C(T)) *Method Methods* 25 (4): 402-408.

487 Lyu Q, Hu J, Yang X, Liu X, Chen Y, Xiao L, Liu Y, Wang Q, Chen J, Huang M, Yu Z,
488 Yang H, Shi H, Zhang Y, Zhao H. 2019. Expression profiles of dmrts and foxls during
489 gonadal development and sex reversal induced by 17α-methyltestosterone in the orange-
490 spotted grouper. *Gen Comp Endocrinol* 274: 26-36.

491 Masuyama H, Yamada M, Kamei Y, Fujiwara-Ishikawa T, Todo T, Nagahama Y,
492 Matsuda M. 2012. Dmrt1 mutation causes a male-to-female sex reversal after the sex
493 determination by Dmy in the medaka. *Chromos Res* 20 (1): 163-176.

494 Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Sakaizumi M.
495 2002. DMY is a Y-specific DM-domain gene required for male development in the
496 medaka fish. *Nature* 417: 559-563.

497 Myosho T, Otake H, Masuyama H, Matsuda M, Kuroki Y, Fujiyama A, Sakaizumi M.
498 2012. Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias*
499 *luzonensis*. *Genetics* 191: 163-170.

500 Nakamoto M, Matsuda M, Wang DS, Nagahama Y, Shibata N. 2006. Molecular cloning
501 and analysis of gonadal expression of Foxl2 in the medaka, *Oryzias latipes*. *Biochem*
502 *Biophys Res Commun* 344 (1): 353-361.

503 Örn S, Yamani S, Norrgren L. 2006. Comparison of vitellogenin induction, sex ratio, and
504 gonad morphology between zebrafish and Japanese medaka after exposure to 17alpha-
505 ethinylestradiol and 17beta-trenbolone. *Arch Environ Contam Toxicol* 51 (2): 237-243.

506 Sawatari E, Shikina S, Takeuchi T, Yoshizaki G. 2007. A novel transforming growth
507 factor-beta superfamily member expressed in gonadal somatic cells enhances primordial

508 germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*).
509 *Devel Biol* 301 (1): 266-275.

510 Scholz S, Guyzeit HO. 2000. 17-alpha-ethinylestradiol affects reproduction, sexual
511 differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquat*
512 *Toxicol* 50 (4): 363-373.

513 Shibata Y, Paul-Prasanth B, Suzuki A, Usami T, Nakamoto M, Matsuda M, Nagahama
514 Y. 2010. Expression of gonadal soma derived factor (GSDF) is spatially and temporally
515 correlated with early testicular differentiation in medaka. *Gene Expr Patterns* 10: 283-
516 289.

517 Sone K, Hinago M, Itamoto M, Katsu Y, Watanabe H, Urushitani H, Tooi O, Guillette
518 LJJr, Iguchi T. 2005. Effects of an androgenic growth promoter 17β-trenbolone on
519 masculinization of Mosquitofish (*Gambusia affinis affinis*). *Gen Comp Endocrinol* 143
520 (2): 151-160.

521 Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, Sakai F, Okubo K, Morohashi
522 K, Nagahama Y. 2007. Foxl2 up-regulates aromatase gene transcription in a female-
523 specific manner by binding to the promoter as well as interacting with ad4 binding
524 protein/steroidogenic factor 1. *Mol Endocrinol* 21 (3): 712-725.

525 Wang H, Wu T, Qin F, Wang L, Wang Z. 2012. Molecular cloning of Foxl2 gene and the
526 effects of endocrine-disrupting chemicals on its mRNA level in rare minnow,
527 *Gobiocypris rarus*. *Fish Physiol Biochem* 38 (3): 653-664.

528 Yan H, Shen X, Cui X, Wu Y, Wang L, Zhang L, Liu Q, Jiang Y. 2018. Identification of
529 genes involved in gonadal sex differentiation and the dimorphic expression pattern in
530 *Takifugu rubripes* gonad at the early stage of sex differentiation. *Fish Physiol Biochem*
531 44 (5): 1275-1290.

532 Yan YL, Desvignes T, Bremiller R, Wilson C, Dillon D, High S, Draper B, Buck CL,
533 Postlethwait J. 2017. Gonadal soma controls ovarian follicle proliferation through Gsdf
534 in zebrafish. *Devel Dynam* 246 (11): 925-945.

535 Yang Q, Yang X, Liu J, Chen Y, Shen S. 2018. Effects of exposure to BPF on
536 development and sexual differentiation during early life stages of zebrafish (*Danio rerio*).
537 *Comp Biochem Physiol Part C Toxicol Pharmacol* 210: 44-56.

538 Yang Y, Liu Q, Xiao Y, Xu S, Wang X, Yang J, Song Z, You F, Li J. 2019. High
539 temperature increases the gsdf expression in masculinization of genetically female
540 Japanese flounder (*Paralichthys olivaceus*). *Gen Comp Endocrinol* 274: 17-25.

541 Yuan C, Wu T, Zhang Y, Gao J, Yang Y, Qin F, Liu S, Zheng Y, Wang Z. 2014.
542 Responsiveness of four gender-specific genes, figla, foxl2, scp3 and sox9a to 17α-
543 ethinylestradiol in adult rare minnow *Gobiocypris rarus*. *Gen Comp Endocrinol* 200: 44-
544 53.

545 Zhang X, Guan G, Li M, Zhu F, Liu Q, Naruse K, Herpin A, Nagahama Y, Li J, Hong Y.
546 2016. Autosomal gsdf acts as a male sex initiator in the fish medaka. *Sci Rep* 6: 19738.

547 Zhang Y, Wang J, Lu L, Li Y, Wei Y, Cheng Y, Ru S. 2020. Genotoxic biomarkers and
548 histological changes in marine medaka (*Oryzias melastigma*) exposed to 17α-
549 ethinylestradiol and 17β-trenbolone. *Marine Pollut Bull* 150: 110601.

550 Zhu Y, Meng L, Xu W, Cui Z, Zhang N, Guo H, Wang N, Shao C, Chen S. 2018. The
551 autosomal Gsdf gene plays a role in male gonad development in Chinese tongue sole
552 (*Cynoglossus semilaevis*). *Sci Rep* 8 (1): 17716.
553

554 **Tables**

555 Table 1. Test chemicals used

Chemical		Concentration used in the present study	Intersex	LOEC for induction of intersex	Reference
4-*t*-Octylphenol	estrogen receptor agonist	6.2, 12.5, 25, 50, 100 μg/L	○	23.7 μg/L	unpublished data
p,p′-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

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

Nominal concentration (μg/L)	n	Average measured concentration (μg/L)	Standard deviation (μg/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

* The exposure concentrations of trenbolone were measured by analysis using HPLC-1260 Infinity every week.

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

Age	Measured concentration	Genetic sex	Gonadal sex (%)		
	μg/L	XX	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

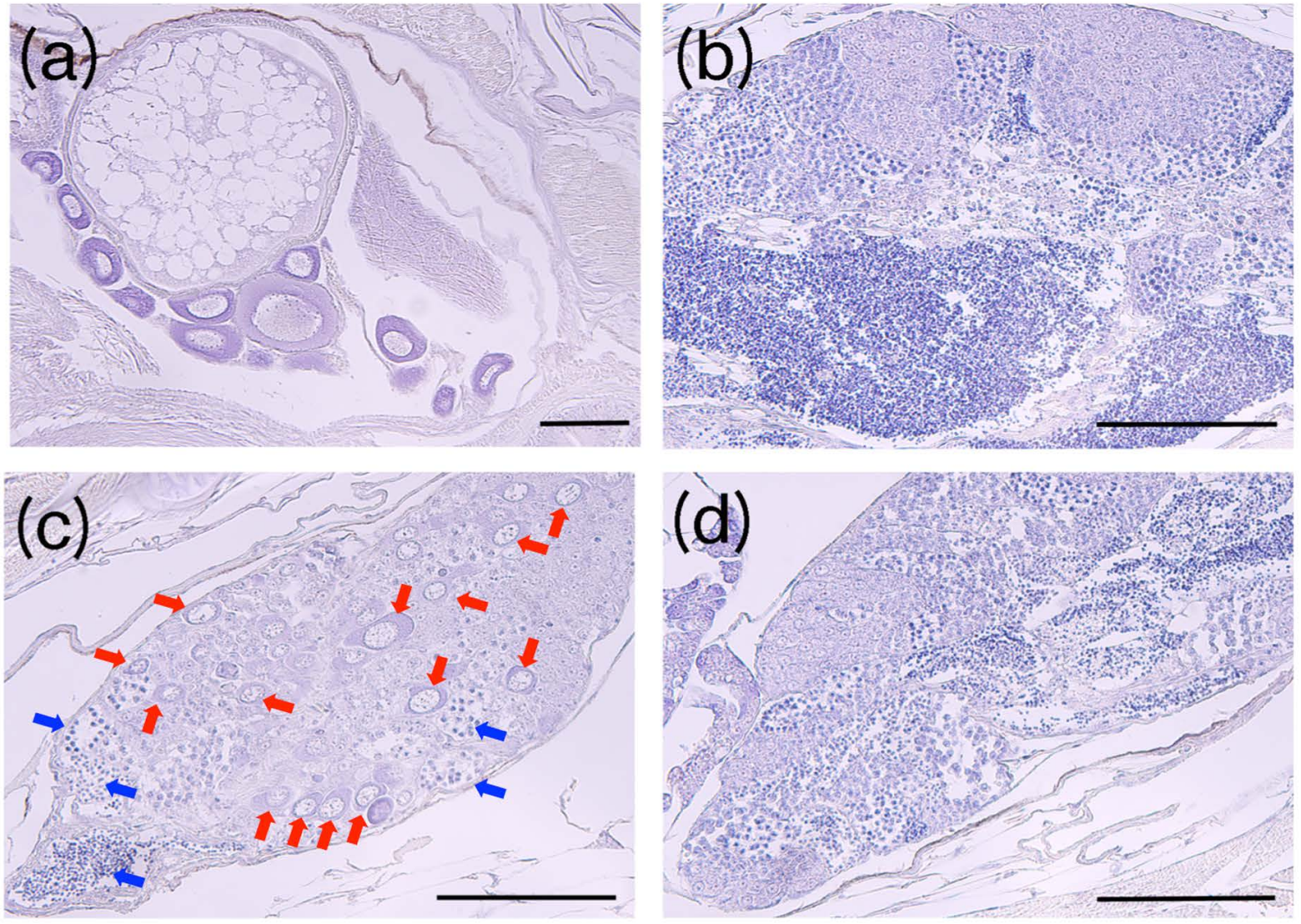
Figure legends

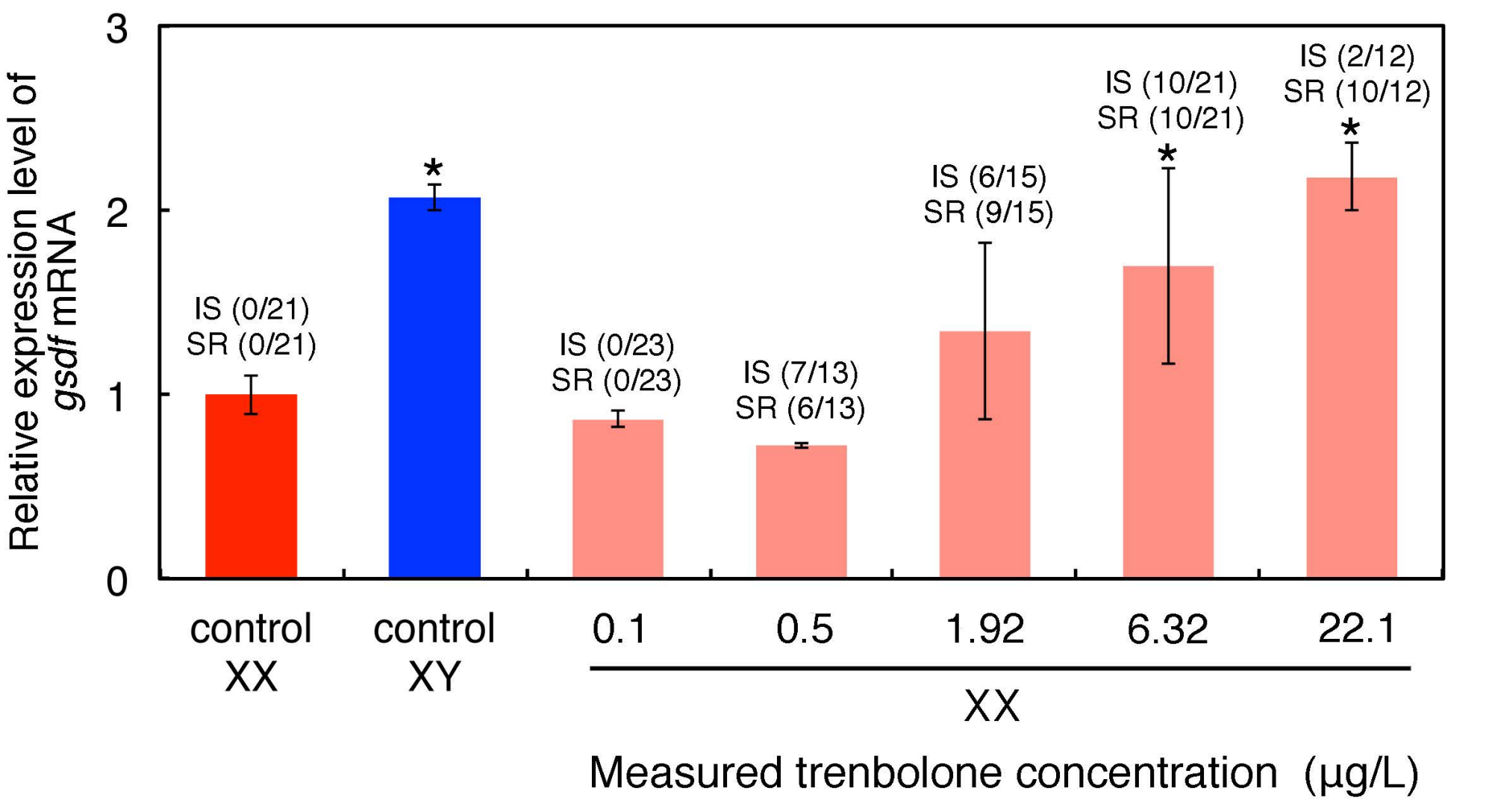
Figure 1. Histological analysis of gonad after 17β-trenbolone exposure at 60 days after hatching. Normal ovary (a) and testis (b) from control XX and XY medaka, respectively. 17β-Trenbolone treatment induced ovotestis development (0.5 μg/L) (c) or sex reversal (0.5 μg/L) (d) in XX medaka. Red arrows indicate ovarian tissue. Blue arrows indicate testicular tissue. Scale bars, 100 μm

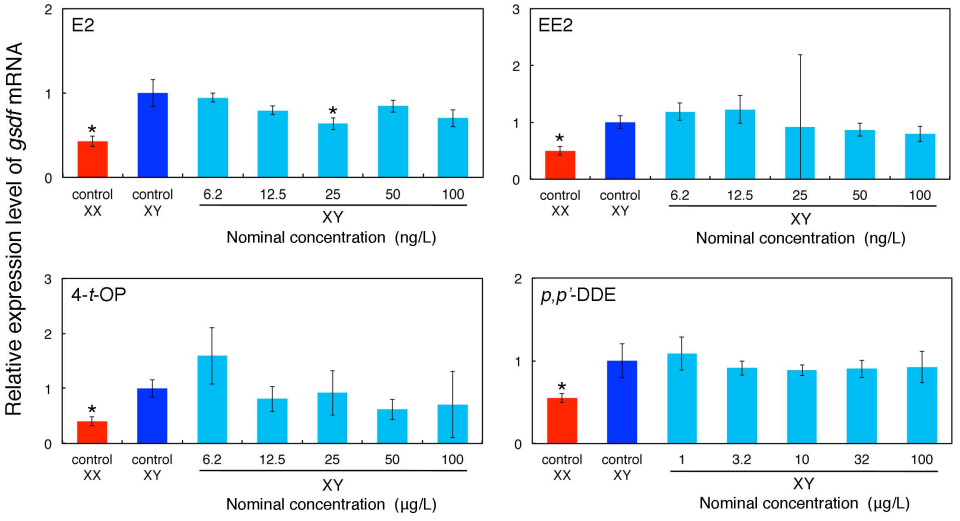
Figure 2. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time quantitative polymerase chain reaction at stage 38 after exposure to trenbolone. Columns and error bars represent means ± SEM (n = 6 per group). After the expression level of *gsdf* was normalized to that of the *ef1α* housekeeping gene, the each data were normalized additionally to a control, in this case to the XX control embryos. Asterisks indicate statistically significant differences compared with control XX (Dunnett's test or Steel's test; $P < 0.05$). Numbers above the columns indicate the number of ovotestis (IS) and sex reversal (SR) individuals at the end of test day (= ovotestis or sex reversal individuals/total number of individuals).

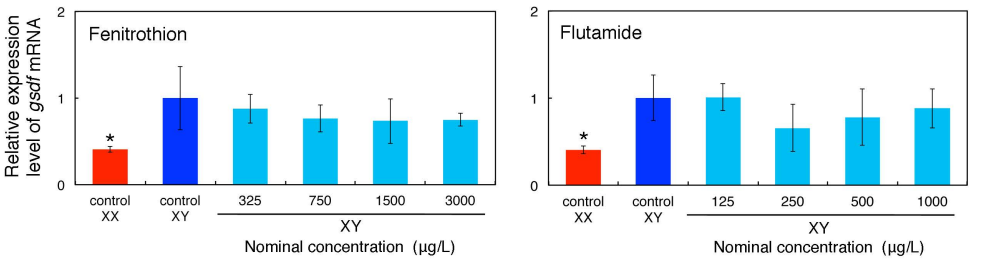
Figure 3. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time quantitative polymerase chain reaction at stage 38 after exposure to 17β-estradiol (E2), ethinylestradiol (EE2), 4-*t*-octylphenol (4-*t*-OP), or *p,p′*-DDE. Columns and error bars represent means ± SEM (n = 6 per group). After the expression level of *gsdf* was normalized to that of the *ef1α* housekeeping gene, the each data were normalized additionally to a control, in this case to the XY control embryos. Asterisks indicate

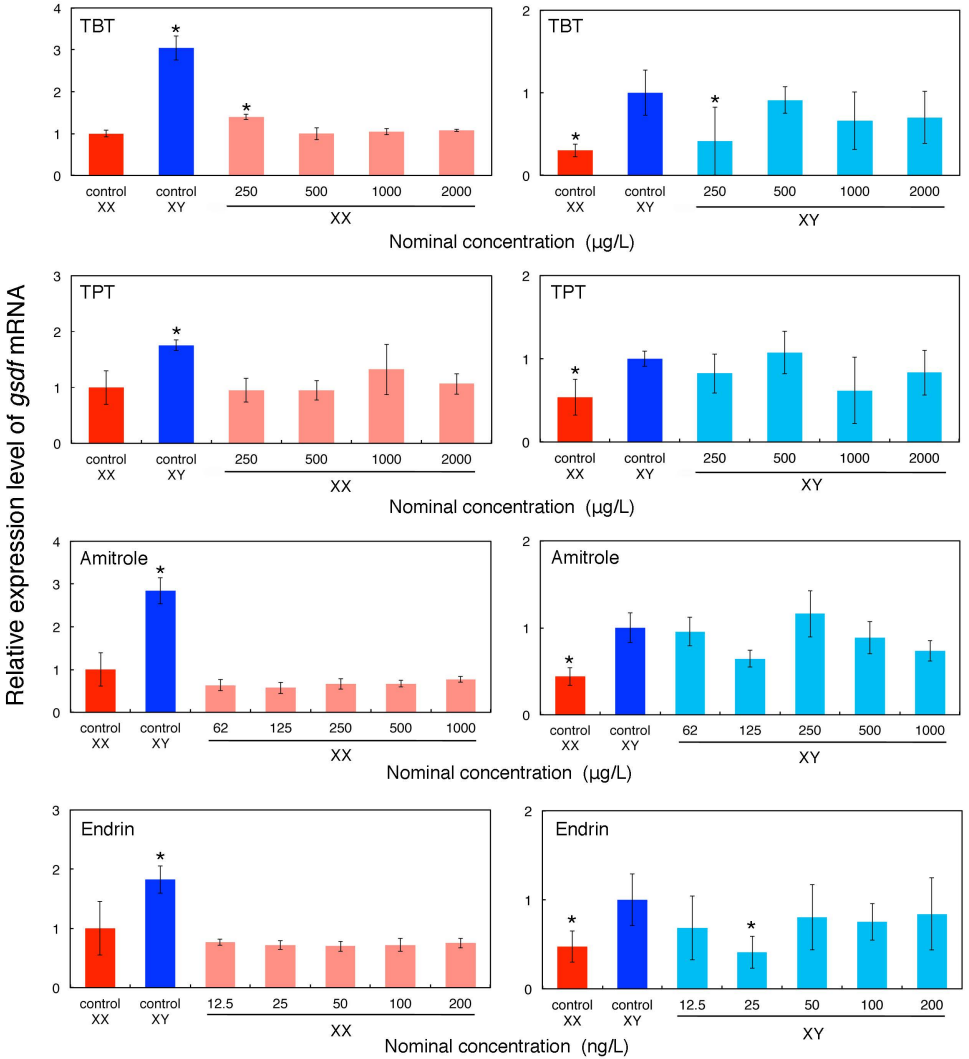
590 statistically significant differences compared with control XY (Dunnett's test or Steel's
591 test; $P < 0.05$).
592
593 Figure 4. Expression of *gsdf* mRNA in Japanese medaka, as measured by real-time
594 quantitative polymerase chain reaction at stage 38 after exposure to fenitrothion or
595 flutamide. Columns and error bars represent means ± SEM ($n = 6$ per group). After the
596 expression level of *gsdf* was normalized to that of the *ef1α* 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 ± SEM ($n = 6$ per group). After the expression level of *gsdf* was normalized to that
605 of the *ef1α* 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; $P < 0.05$).
609
610


(a)
(b)
(c)
(d)


Relative expression level of gsdf mRNA
3
2
1
0
IS (0/21)
SR (0/21)
*
IS (0/23)
SR (0/23)
IS (7/13)
SR (6/13)
IS (6/15)
SR (9/15)
IS (10/21)
SR (10/21)
*
IS (2/12)
SR (10/12)
*
control
XX
control
XY
0.1
0.5
1.92
6.32
22.1
XX
Measured trenbolone concentration (µg/L)


Relative expression level of gsdf mRNA
E2
EE2
4-t-OP
p,p'-DDE
control XX
control XY
6.2
12.5
25
50
100
1
3.2
10
32
XY
Nominal concentration (ng/L)
Nominal concentration (μg/L)


Relative expression level of gsdf mRNA
Fenitrothion
Flutamide
2
1
0
*
control XX
control XY
325
750
1500
3000
125
250
500
1000
XY
Nominal concentration (μg/L)


TBT
TBT
TPT
TPT
Amitrole
Amitrole
Endrin
Endrin
control XX
control XY
250
500
1000
2000
62
125
12.5
25
50
100
200
XX
XY
Relative expression level of gsdf mRNA
Nominal concentration (μg/L)
Nominal concentration (μg/L)
Nominal concentration (μg/L)
Nominal concentration (ng/L)