

1 **Supporting Information**

2 **Equol Induces Gonadal Intersex in Japanese Medaka (*Oryzias latipes*) at**
3 **Environmentally Relevant Concentrations: Comparison with 17 β -Estradiol**

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This supporting information provides text, figures and tables addressing: (1) Detailed methods for determination of actual chemical concentrations in exposure tanks; (2) Dose-response relationships; (3) Detailed methods for evaluation of reproductive success; (4) Detailed methods for yeast assay for evaluating mER α binding activity; (5) Primer sequences of genes used in real-time PCR; (6) Equol and E2 β concentrations in water samples collected during the exposure experiment; (7) Fertilization and hatching of exposed males mated with control females; (8) Intersex testis of transgenic medaka and light micrograph of the same testis by paraffin section with H&E staining; (9) Experimental design for exposures to equol and E2 β and subsequent study of effects of equol on reproduction of medaka; (10) Expression of

VTG-1 mRNA as a function of exposure concentrations (ng/L). (11) Inhibition of 11-KT synthesis as a function of exposure concentrations (ng/L).

18 **Determination of Actual Chemical Concentrations in Exposure Tanks.**

19 Concentrations of equol and E2 β in exposure tanks were determined by use of solid phase
20 extraction (SPE) combined with UPLC-ESI-MS/MS. In brief, water from exposure tanks was
21 collected every 10 days. At collection, equal amounts of water from each replicate were
22 pooled (1 L for vehicle control groups, 500 mL for 2 ng/L equol exposure groups, 250 mL for
23 4 ng/L equol exposure groups, 100 mL for 8 and 16 ng/L equol exposure groups, 50 mL for 40
24 ng/L equol exposure groups, 25 mL for 200 ng/L and 1000 ng/L equol exposure groups; 1 L
25 for vehicle control groups, 2.5 ng/L and 5 ng/L E2 β exposure groups and 500 mL for 10 ng/L,
26 20 ng/L and 40 ng/L E2 β exposure groups) were collected from each exposure tank and then
27 were spiked with 10 ng of equol-d₄ or 10 ng of E2 β -d₃, respectively, as internal standard and
28 were extracted through HLB cartridges (6 mL, 500 mg, Waters) at a flow rate of 5-10 mL/min.
29 Cartridges were preconditioned with 6 mL of methanol, 6 mL of methanol/MTBE (50:50, v/v)
30 and 12 mL of distilled water. Cartridges were rinsed with 10 mL of distilled water and dried
31 under a flow of nitrogen. Target analytes were subsequently eluted with 6 mL of
32 methanol/MTBE (50:50, v/v), and extracts were dried under a gentle nitrogen stream.
33 Samples were redissolved in 100 μ L dansyl chloride (1 mg/mL in acetone). After
34 vortex-mixing for 1 min and incubation at 60 °C for 10 min, 1 mL of distilled water and 3 mL
35 of *n*-hexane were added and shaken mechanically for both derivatization and extraction for 10
36 min. After the organic layer was removed, another 3 mL *n*-hexane was added to extract the
37 analyte. The organic layer was combined with the previous aliquot. The organic fraction,
38 containing dansylated analytes, was dried and redissolved with 200 μ L acetonitrile prior to
39 quantification by use of UPLC-ESI-MS/MS.

40 The LC apparatus was an Acquity Ultra Performance LC (Waters, Milford, MA, USA).
41 Derivatized equol and E2 β were separated using a Waters Acquity UPLC[®] BEH C18 column
42 (100 \times 2.1 mm, 1.7 μ m particle size) (Miford, MA, USA). The column was maintained at
43 40 $^{\circ}$ C at a flow rate of 0.3 mL/min, with an injection volume of 5 μ L. Acetonitrile and water
44 containing 0.1% formic acid were used as the mobile phases. Gradient conditions were
45 initiated with 60% acetonitrile followed by a linear increase to 75% acetonitrile in 0.5 min.
46 After being increased to 80% in 6 min, acetonitrile was increased to 95% in 0.5 min and then
47 to 100% in 2 min and kept isocratic for 2 min.

48 Mass spectrometry was performed using a Quattro Premier[™] XE tandem quadrupole
49 mass spectrometer (Waters, USA) equipped with a Z-Spray ionization (ESI) source.
50 UPLC-ESI-MS-MS detections were performed in positive ion mode for derivatized equol and
51 estrogens. The two most abundant multi-selected reaction monitoring (MRM) transitions,
52 cone voltages and collision energies were optimized for each target chemical by infusing
53 dansyl derivatives of standards, which had been purified by extraction with hexane, into the
54 mass spectrometer. MS parameters were as follows: capillary voltage, 3.2 kV (ESI +); source
55 temperature, 120 $^{\circ}$ C; desolvation temperature, 450 $^{\circ}$ C; source gas flow, 50 L/h; and
56 desolvation gas flow, 800 L/h.

57 **Dose-response Relationship.** BenchMark Dose Software 2.6 (US EPA) was used to
58 establish the relationships between concentrations and incidences of intersex. Following the
59 standard procedure,¹ the Multisage model (Eq. S-1) and LogProbit model (Eq. S-2) were
60 selected as optimal models for E2 β and equol, respectively (Equations S-1 and S-2).

61
$$q = 1 - \exp(0.0012x - 0.0012x^2) \quad (\text{S-1})$$

62 $q = \Phi(0.29 \times \ln(x) - 2)$ (S-2)

63 where: q is the intersex incidence (%), and x is the concentration (ng/L) of E2 β in Equation
64 (S-1) and off equol in Equation (S-2).

65 **Evaluation of Reproductive Success.** Spawned eggs were carefully collected daily at 2-3
66 hour post-fertilization during the final week of exposure and gently separated. A Leica M165
67 FC microscope was used to observe fertilization and eleutheroembryo development. Fertilized
68 eggs were examined at the late morula stage (Stage 9) under a dissecting microscope and
69 fertilization success was calculated by dividing the number of spawned eggs by that of
70 fertilized eggs. Fertilized eggs were collected and cultured in wells of glass plates using
71 carbon-treated water without chemical exposure until hatched. Embryos were observed each
72 day until hatch. Hatching success was calculated by hatched fries/fertilized eggs.

73 **Yeast Assay for Evaluating Binding to ER.** The mER α yeast two-hybrid transactivation
74 assay, was applied to evaluate binding of equol or E2 β to the ER. Both rER α and mER α yeast
75 cells were pre-incubated at 30 °C for 16 h in 5 mL SD medium (6.7 g/L Difco yeast nitrogen
76 base without amino acids, 0.2% glucose, 300 mg/L L-isoleucine, 1500 mg/L L-valine, 200
77 mg/L L-adenine hemisulfate salt, 200 mg/L L-arginine HCl, 200 mg/L L-histidine HCl
78 monohydrate, 300 mg/L L-lysine HCl, 200 mg/L L-methionine, 500 mg/L L-phenylalanine,
79 200 mg/L L-threonine, 300 mg/L L-tyrosine, 200 mg/L L-uracil; Sigma); 50 μ L of overnight
80 culture and 2.5 μ L of DMSO solution diluted to the desired concentrations were then added to
81 200 μ L of fresh medium (2% galactose) in a microtube (Axygen Scientific), respectively.
82 After yeasts were cultured for 4 h at 30 °C, 150 μ L of the above culture was fractionated, and
83 its absorbance at 595 nm was detected. The residual culture (100 μ L) was centrifuged at 4 °C

84 (12,000 g) for 5 min, and the collected cells were resuspended in 200 μ L of Z buffer (0.1 M
85 sodium phosphate, pH 7.0/10 mM KCl/1 mM MgSO₄) containing 1 mg/mL Zymolyase 20T
86 (Seikagaku), and incubated for 20 min at 30 °C. The enzymatic reaction was started by the
87 addition of 40 μ L of 4 mg/mL 2-nitrophenyl- β -D-galactopyranoside (ONPG; Tokyo Kasei),
88 and incubated for 20 min at 30°C. Then the enzymatic reaction was stopped by adding 1 M
89 Na₂CO₃ (100 μ L). After centrifugation, 150 μ L aliquots were placed into 96-wells of
90 microplate. Absorbances at 415 and 570 nm were read on a microplate reader (Bio RAD 550)
91 to estimate the ER-mediated activity, and β -galactosidase activity (U) was calculated
92 according to the following equation: $U=1,000\times([OD_{415}]-[1.75 \times OD_{570}])/([t]\times[v]\times[OD_{595}])$,
93 where t represents the reaction time (min); v is the volume of the culture used in the assay
94 (mL); OD₅₉₅ is the cell density at the start of the assay; OD₄₁₅ is the absorbance by
95 *O*-nitrophenol at the end of the reaction, and OD₅₇₀ is the light scattering at the end of the
96 reaction. The molar concentration for each compound that produces 50% (EC₅₀) of the
97 maximum response of corresponding ER agonistic activity was calculated by the Prism 4 for
98 Windows program (GraphPad).

99 **Reference**

- 100 (1) U.S. Environmental Protection Agency. 2012. Benchmark Dose Technical Guidance.
101 http://www.epa.gov/raf/publications/pdfs/benchmark_dose_guidance.pdf

TABLE S1. Primers Used for RT-PCR Analysis.

Target gene	Primer sequence (5' - 3')	
	Forward	Reverse
17 β -HSD3	TCTTATACAGGCAGTGGCTCCA	GGTAAAAAGGTCACCCTGTTGG
CYP11B	TTGCCGTGAGTCTGCAAAGATA	AAAGTCCCAGCCGGGATGT
11 β -HSD2	CCTCAGAGGTCTGTGGGGTTAG	TGGGCTGGAGATGGTGATAATG
RPL-7	GTCGCCTCCCTCCACAAAG	AACTCAAGCCTGCCAACAAC

Table S2. Equol and E2 β Concentrations in Water Samples Collected During The Exposure Experiment.

Nominal Conc.	0-30 dph			30-100 dph			0-100 dph
	Minimum (ng/L)	Maximum (ng/L)	Mean \pm S.D. (n=4)	Minimum (ng/L)	Maximum (ng/L)	Mean \pm S.D. (n=7)	Mean \pm S.D. (n=11)
equol							
DMSO control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2 ng/L	1.31	1.69	1.5 \pm 0.27	1.07	1.28	1.2 \pm 0.1	1.3 \pm 0.2
4 ng/L	3.29	3.69	3.5 \pm 0.28	1.96	2.92	2.6 \pm 0.52	3.0 \pm 0.6
8 ng/L	6.02	7.41	6.7 \pm 0.98	4.9	5.44	5.1 \pm 0.27	5.8 \pm 1.0
16 ng/L	12.09	14.66	13.4 \pm 1.8	9.5	11.9	10.5 \pm 1.3	11.6 \pm 2.0
40 ng/L	33.63	36.02	34.8 \pm 1.7	24.69	30.91	29.3 \pm 3.2	30.9 \pm 6.8
200 ng/L	152.09	158.06	155.1 \pm 4.2	135.09	144.89	139.5 \pm 5.0	145.7 \pm 9.5
1000 ng/L	776.45	830.6	803.5 \pm 38.3	660.85	776.45	689.1 \pm 43.8	734.9 \pm 72.5
E2β							
DMSO control	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
2.5 ng/L	1.74	1.70	1.72 \pm 0.03	1.50	1.58	1.55 \pm 0.04	1.6 \pm 0.1
5 ng/L	2.52	3.74	3.13 \pm 0.86	2.86	3.56	3.2 \pm 0.35	3.2 \pm 0.5
10 ng/L	6.30	7.21	6.76 \pm 0.64	5.79	6.66	6.32 \pm 0.47	6.4 \pm 0.5
20 ng/L	11.30	15.73	13.72 \pm 3.1	9.61	10.01	9.9 \pm 0.4	11.6 \pm 2.8
40 ng/L	24.2	29.70	26.95 \pm 3.9	18.4	22.11	20.3 \pm 1.9	22.9 \pm 4.3

Table S3. Fertilization and Hatching of Exposed Males Mated with Control Females.

Fertilization (%)					
Control	1.3 ng/L	5.8 ng/L	30.9 ng/L	145.7 ng/L	734.9 ng/L
92.0±4.0	87.3±6.7	65.6±11.4 ^a	89.6±14.4 ^b	90.0±6.3	93.9±6.3 ^b
83.9±7.3	95.0±0.0	70.0±1.1	40.8±19.4 ^c	83.9±20.2 ^b	91.2±4.0
41.1±33.8	55.7±44.6	89.0±6.6 ^a	94.8±5.4 ^a	89.2±2.0	89.7±5.8
89.2±6.4	87.3±4.6	64.9±16.4	85.9±15.7	88.4±6.3 ^a	95.2±1.2
91.9±7.2	93.6±1.4	79.2±3.4 ^a	91.4±3.5	93.7±2.2	90.4±7.9
89.4±12.8	89.7±2.4	84.3±9.6	90.8±3.8	92.0±5.2	92.2±3.9
93.0±3.4	92.4±5.8	80.6±5.7	88.5±4.6 ^d	58.8±24.6 ^c	90.6±7.7
90.1±5.4	93.6±2.4	93.7±1.8	85.1±9.9 ^a	0.0±0.0 ^d	78.2±6.8 ^a
92.4±4.4	91.7±3.5	95.0±0.0	88.6±6.5	50.0±0.0 ^c	30.8±33.3 ^c
89.2±6.4	89.4±8.1	88.2±9.6	95.0±0.0	76.2±29.3 ^a	55.9±54.1
91.9±6.2	95.0±3.2	93.9±1.6	93.7±2.2	95.0±0.0	91.1±6.8
89.4±7.1	91.7±12.1	68.6±7.2	89.9±8.9	94.5±1.5	90.2±8.2
Hatching (%)					
Control	1.3 ng/L	5.8 ng/L	30.9 ng/L	145.7 ng/L	734.9 ng/L
96.1±5.5	84.8±9.5	83.6±9.4 ^a	86.7±13.9 ^b	83.5±7.1	55.6±50.9 ^b
79.9±6.1	90.7±8.2	74.5±33.9	49.5±24.5 ^c	81.3±7.0 ^b	94.6±5.9
95.8±5.9	85.4±2.7	95.8±7.2 ^a	92.0±9.7 ^a	57.2±49.6	86.7±8.6
83.3±8.3	88.9±2.4	92.9±10.1	66.7±24.7	58.2±22.2 ^a	48.4±11.9
82.9±4.4	95.2±3.9	72.3±10.9 ^a	68.4±38.7	80.5±14.1	74.9±1.6
90.9±4.4	83.7±2.5	94.9±4.4	91.8±9.6	95.1±4.8	83.0±9.2
93.5±3.9	91.3±5.9	91.1±9.1	72.8±11.8 ^d	61.5±17.2 ^c	66.7±16.0
86.2±8.9	81.9±2.5	98.7±3.4	72.6±5.2 ^a	0.0±0.0 ^d	73.1±9.0 ^a
95.5±4.2	95.7±1.5	87.1±4.5	76.5±10.9	33.3±0.0 ^c	0.0±0.0 ^c
83.3±7.6	65.6±8.4	66.4±12.1	80.0±9.3	80.2±6.0 ^a	61.9±3.9
82.9±5.4	95.2±6.0	90.7±3.0	75.1±22.6	69.0±16.6	96.8±5.6
90.9±4.4	87.4±6.1	81.2±4.4	90.2±9.2	92.2±9.6	86.1±14.1

Twelve Males were randomly selected from the control and five exposure groups. a, Level 1 of intersex severity (n=8); b, Level 2 of intersex severity (n=3); c, Level 3 of intersex severity (n=4); d, Level 4 of intersex severity (n=2); others (n=55) are all in Level 0 of intersex severity. Data are presented as means ± S.D. (n=3).

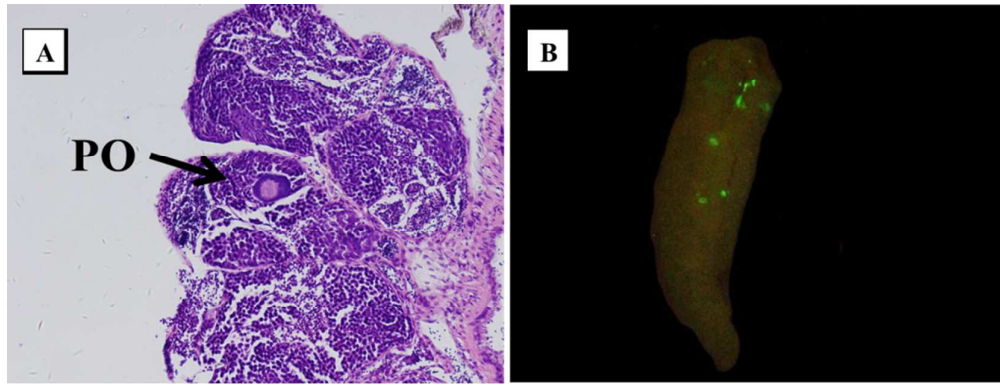


Figure S1. Intersex testis of transgenic medaka (A) and light micrograph of the same testis by paraffin section with H&E staining (B).

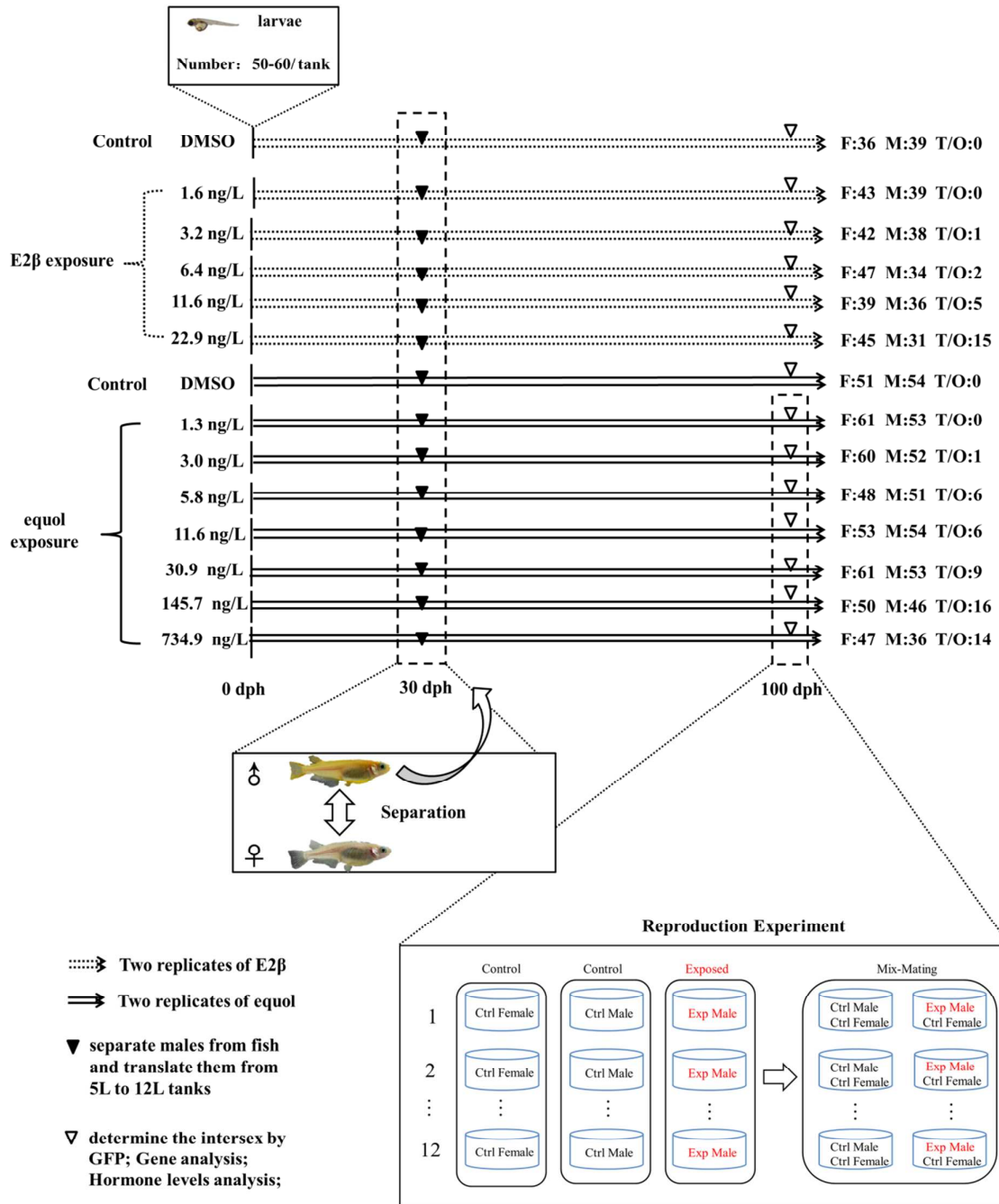


Figure S2. Experimental design for the equol and E2β exposure and subsequent reproduction study with medaka exposed to equol. Medaka were exposed from hatching (0 dph) to 100 dph of age. At 30 dph, both male and female larvae were separated according to sex phenotype (such as body color, dorsal and anal fins), and then transferred into 12 L glass tanks and continued the exposure. A reproduction study was carried out using male medaka which were randomly selected from the control and five equol exposure groups. Gonadal intersex was determined by GFP fluorescence in the testis. F: number of female; M, number of male; T/O: number of intersex.

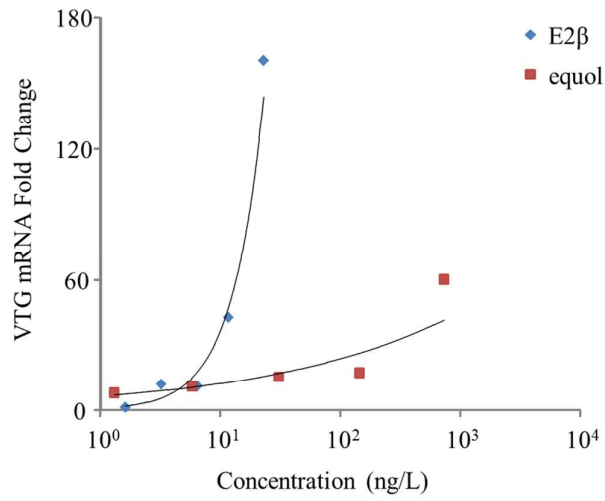


Figure S3. Expression of *VTG-1* mRNA as a function of exposure concentrations (ng/L).

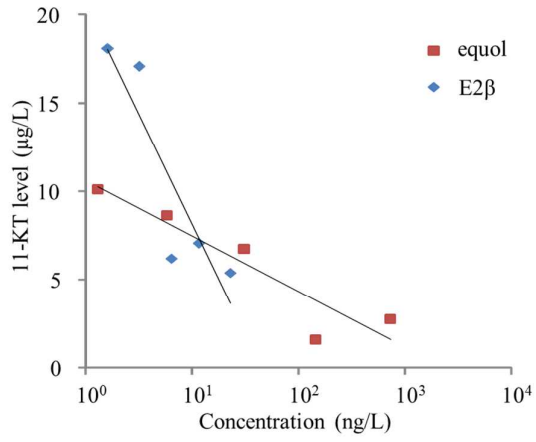


Figure S4. Inhibition of 11-KT synthesis as a function of exposure concentrations (ng/L).