Supporting Information

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SI Materials and Methods

Animal Source. The original source of animals was from a feral stock collected in 1992 from Tecalote Creek in San Diego County, CA (32° 46′ 31.20″ N, 117° 11′ 20.93″ W).

Adult Husbandry. All adults were maintained in fiberglass tanks (79 × 91 × 30 cm) at 22 °C on a 12 h/12 h light cycle (lights on 0700 hours Pacific Coast Time), in 10% (0.1×) Holtfreter's solution (HF) (1) made with UV-treated, carbon-filtered, atrazine-free (2) water and were fed trout chow (Purina Mills) daily. HF was changed and tanks cleaned every 72 h, as described previously (3). Except where indicated, 20 adults were maintained in fiberglass containers (79 × 91 × 30 cm) in 165 L of HF. The captive colony was generated by breeding the wild caught (feral = F0) animals above. For breeding, both males and females were injected with human chorionic gonadotropin (1,000 IU, Sigma-Aldrich), and animals were allowed to pair overnight as described previously (3).

Larval Husbandry. Larvae were reared in static aquaria (29 ×18 ×13 cm) in 4 L of continuously aerated 10% HF at a density of 30 larvae per 4 L. HF was changed every 72 h, and animals were fed rabbit chow (Purina Mills) ad libitum. Animals were removed from the aquaria when they reached Nieuwkoop and Faber stage 66 and then reared to adulthood as described above for 2 years, then apportioned into tanks (46 × 25 × 20 cm) for experiments as described in Fig. S1. Stock females (ZW) were raised similarly.

Generation of the All-Male (ZZ) *Xenopus laevis* Colony. *X. laevis* is a female heterogametic species, even though sex chromosomes are not morphologically distinguishable when karyotyped. ZZ females were generated in 1993 by treating larvae of wild-caught *X. laevis* (F0) with 17β estradiol (E_2; 100 μg /L). E_2-treatment resulted in 100% females (50% ZW and 50% ZZ; F1). The sex-reversed males (ZZ females; F1) were identified by breeding all females back to unexposed (ZZ) males and isolating all females that produced only male (ZZ) offspring. ZZ females were then crossed back to ZZ males, and the resulting offspring (F2) were treated with atrazine (2.5 ppb) for comparison with control (ethanol only) animals (Fig. S2).

Confirmation of Sex-Reversed F1 (ZZ Females). The absence of the W chromosome in the F1 E_2-treated females was confirmed by observing the sex ratio at metamorphosis of samples (n = 45) from each of three clutches produced by the F1 female used in the present study. On the basis of gonadal morphology, 100% of the offspring were male, indicating that the female was ZZ. The absence of the W chromosome was further confirmed by molecular analysis that demonstrated the presence of *DMRT-1* but the absence of *DM-W*.

Morphometric Analyses. For morphometric analyses, body size (weight and snout–vent length were determined) and the presence or absence of nuptial pads and cloacal labia were noted. Tissues of interest were dissected, measured (using calipers), and weighed using a Mettler Toledo AT261 DeltaRange electronic balance.

All dissections were conducted using a Nikon SMZ 10A dissecting scope, fitted with a 0.5× lens (Technical Instruments). Microscopic analyses were conducted using a Nikon Optiphot 2 microscope (Technical Instruments), with photomicrographs captured using a Nikon Digital Sight DS-U (Technical Instruments) and NIS Freeware 2.1 (NIS-Elements; Nikon Instruments). Morphometric analyses were conducted using Scion Image. Histologic sectioning, microscopic analysis, and morphometric analysis have all been described previously (3).

In addition to the statistically significant measurements reported in the main text, we made several other measurements and corrections. These included laryngeal weight (after fixation), laryngeal weight corrected for body weight, laryngeal length, laryngeal length corrected for body size, and maximum cross-sectional area (transverse) of the *dilater laryngis* muscle. For nuptial pads, the number of keratinized hooks, the number of individual breeding glands, and the maximum epithelial cell height of the breeding glands were all analyzed. Gonadal weight, gonadal weight corrected for body weight, and maximum cross-sectional area of the gonads were analyzed, in addition to the number of testicular tubules and maximum testicular tubular cross-sectional area from the largest cross-sectional area of the left testis.

Statistical analyses (ANOVA) for all morphometric measurements were conducted with the aid of SYSTAT software (SPSS), with treatment (control or atrazine-treated) as the independent variable. For analyzing stages of spermatogenesis and the proportion of tubules with and without mature sperm bundles, a G test was used as described in Sokal and Rolf (4). Measurements and corrections that were statistically significant are reported in the main text (*Results*). All other measurements or corrections were not statistically significant (P > 0.05).

Molecular Markers for Sex. Genomic DNA was isolated from toe tips from sexually mature animals prepared by tissue lysis and proteinase k protein digestion. The ZW genotype was determined by multiplex PCR amplification (37 cycles) of *DM-W* (W specific) (5). The following conditions were used for amplifying *DM-W*: denaturing at 95 °C for 30 s, annealing at 53 °C, and extension at 72 °C for 2 min (37 cycles). The primers for DM-W were (forward) 5′-CCACACCCAGCTCATGTAAAG-3′ and (reverse) 5-GGGC-AGAGTCACATATACTG-3′. The DMRT-1 gene (Z specific) was also amplified as a control with the primers (forward) 5′-AACA-GGAGCCCAATTCTGAG-3′ and (reverse) 5′-AACTGCTTGA-CCTCTAATGC-3′. The PCR products were 260 bp (*DM-W*) and 206 bp (*DMRT-1*). The ethidium bromide–labeled PCR products were visualized under UV light after electrophoresis on a 1.2% agarose gel.

Cyp19 Aromatase Expression. For RNA isolation, each 50–100 mg frozen gonad sample (from sexually mature animals) was homogenized using a Power Gen 125 homogenizer (Fisher Scientific) in 1 mL of TRIzol (Invitrogen) and extracted using the manufacturer's protocol. cDNA was produced using a High-Capacity cDNA Reverse Trasncription Kit (Applied Biosystems) with 2 μg total RNA per reaction. PCR was carried out using primers DRB281 5′ TGAAACACAGCCATTATGTCTCACGC and DRB282 5′ CT-TTCATCTAAGGGTATCCTTAGGAAGAG for P450arom and with DRB20b 5′ CGTGGTGCTCCTCTTGCCAAG and DRB21b 5′ GACGACCAGTACGACGAGCAG for rpL8 (control gene) with 2 μL of cDNA per reaction at primer concentrations of 0.2 μM (for P450arom) and 0.02 μM (for rpL8), and PCR conditions were 30 cycles of 94 °C, 30 s; 58 °C, 30 s; and 72 °C, 30 s. PCR products were run on a 2% agarose gel and photographed with a Kodak Gel Logic 100 imaging system.

1. Holtfreter J (1931) Uber die Aufzucht isolierter Teile des Amphibian Keimes II. *Arch F Ent Mech* 124:404–465.
2. Hayes T, et al. (2002) Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. *Environ Health Perspect* 111:568–575.
3. Hayes T, et al. (2002) Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Natl Acad Sci USA* 99:5476–5480.
4. Sokal R, Rohlf F (1981) *Biometry: The Principles and Practice of Statistics in Biological Research* (W.H. Freeman, New York).
5. Yoshimoto S, et al. (2008) A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc Natl Acad Sci USA* 105: 2469–2474.

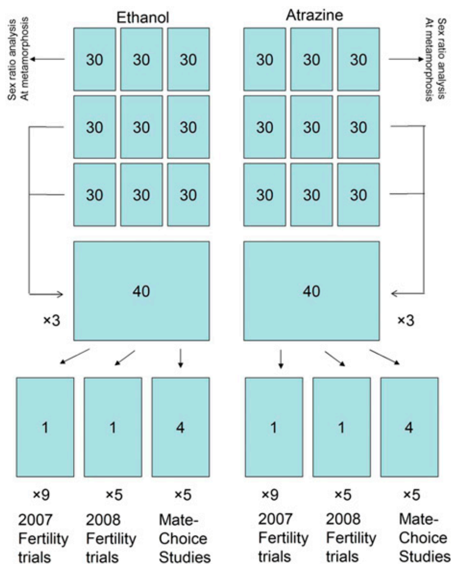

Fig. S1. Diagram illustrating the rearing program for animals involved in the present study. Larvae (n = 540) were reared in 18 tanks (29 × 18 × 13 cm) at 30 tadpoles per tank, with half exposed to atrazine and half to ethanol only. At metamorphosis, three replicates from each treatment were killed for analyses not reported here. The remaining animals from each treatment were mixed and apportioned into three replicates in tanks (79 × 91 × 30 cm). For analyses in the present study, animals were divided into 38 tanks (46 × 25 × 20 cm) and reared singly (for fertilization experiments) or in groups of four (four breeding competitions) as described above. The remaining animals were used for studies not described here.

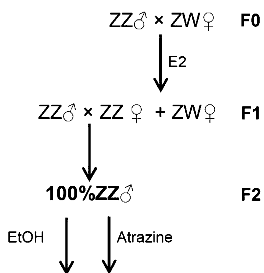

Fig. S2. Generation of the all-male (ZZ) colony. Original colony members (F0) were treated with estradiol to produce ZZ females. ZZ females were identified by examining the progeny when crossed to unexposed males. ZZ females (F1) were then crossed with ZZ males and the resulting larvae (F2) used in the present study.