Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes

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Supplementary Information Appendix

Supplementary Materials and Methods

5.1. Fish and Husbandry (Ethics Statement)

Fish were housed at the experimental aquarium facilities of the Institute of Marine Sciences (Barcelona) according to the approved institutional guidelines on the use of animals for research purposes and in agreement with the European regulations of animal welfare (ETS N8 123,01/01/91). The *Tg*(*vasa:vasa-egfp*) zebrafish line was maintained at Temasek Life Sciences Laboratory (TLL) Fish Facility according to the approved protocol by TLL Institutional Animal Care and Use Committee (approval ID: TLL (F)-10-001).

The AB zebrafish strain was used in all temperature treatment experiments. Brooders were maintained in 2.8-liter tanks (Aquaneering, mod. ZT280) at 28 ± 0.5ºC. Fish mating took place spontaneously in spawning tanks fitted with a mesh. Fertilized eggs were collected, counted and separated in Petri dishes containing embryo medium with methylene blue and maintained at 26.5ºC. All eggs hatched at 3 days post fertilization (dpf) and larvae were maintained until 6 dpf without feeding to allow complete yolk sac absorption. Fish and larvae were kept under a 12 h light:12 h dark photoperiod and were fed with commercial food for zebrafish. First, larvae were fed three times a day with Sera micron (AquaSchwarz) until 15 dpf and then with a pelleted dried food of the appropriate size (AquaSchwarz) was supplemented with live *Artemia*

nauplii (AF48, INVE Aquaculture). In addition to temperature (to the nearest 0.1°C), pH (7.2), conductivity (750-900 µS) and dissolved oxygen (6.5–7.0 mg/l) were monitored daily, whereas other water quality parameters (ammonium, nitrite and nitrate) were checked periodically to ensure that they were within the appropriate range (1–3). In order to avoid high rearing density-induced masculinization, the number of fish in each 2.8-liter tank was 25–35, as we previously described (4).

The *Tg(vasa:vasa-egfp)* transgenic zebrafish line (AB strain based) was a kind gift from Dr. Lisbeth Olsen (Sars International Centre for Marine Molecular Biology, Bergen, Norway). They were kept in AHAB (Aquatic Habitats) recirculation systems according to standard protocols (1–3) and at ambient temperature of 25–28°C. Breeding was carried out as for the AB zebrafish strain in meshed-bottom spawning cages of one liter volume placed into a second cage containing egg water. Breeding pairs were set up at the previous evening in the presence of artificial plants and fertilized eggs were collected from the bottom of the cage before noon next day, rinsed on a tea filter and transferred into plastic trays with egg water containing methylene blue. Embryos were transferred onto the AHAB system after hatching.

5.2. Temperature Treatments

First, we determined adult sex ratio of the families to be used without the heat treatment. Eleven different pairs of AB strain zebrafish were crossed (five of them between two and six times, the rest once), their offspring grown to maturity at control temperature (28°C) and the sex ratio was determined for each family. Families were randomly picked, numbered and the family numbers were maintained throughout the study. The sex ratios of the different pairs ranged from 13 to 100% males (Fig. 1*A*). Furthermore, for the five pairs that were crossed more than once, brood sex ratios between repeated crosses from the same pair were similar (standard deviations of within-family sex ratios were between 0.35 to 8.6% males; Fig. 1*A*). Both of these results have confirmed earlier findings of Liew and colleagues (5), showing the existence of an interfamily variation. Finally, Chi-squared analysis between the averaged sex ratio combining all families (62.8% males) and the expected Fisherian sex ratio (50% males) showed no significant differences ($P > 0.05$) indicating that the overall sex ratio of all the populations used in our experiments combined had no a sex bias at control conditions.

Two thermal treatments were performed as follows: 1) In the first experiment, a total of ~2,500 larvae were obtained by eight different families (#3, 4, 5, 6, 7, 8, 10 and 11) submitted to three different thermal periods (0–14, 7–21 and 18–32 dpf) and to four different temperatures per period (low "LT" at 22°C, control "CT" at 28°C and high "HT" at 34°C or 36°C; 2) In the second experiment, a total of ~1,050 larvae from six different families (#1, 2, 4, 6, 8 and 9) and one period of thermal treatment (18–32 dpf) and two different thermal treatments per period (control "CT" at 28°C and high "HT" at

36°C) were studied. At the end of each treatment, fish were returned at 28ºC. Temperature changes were carried out at a rate of 1–2°C/day.

Cold water was produced with a Titan 250 (AquaMedic) cooler circulating system, and warm water was generated by Jäger 50W (Eheim) heater. The water temperature was changed gradually at a rate of 2°C per day until the desired temperature was achieved. Individual tank temperatures were measured three times per day to ensure that constant temperatures were maintained throughout the treatment period. At the end of each experimental period, temperature was gradually adjusted to 28ºC and then fish were returned to the racks.

5.3. Sampling Procedures

Regardless of the experimental treatment, tissues were collected at 37 dpf (juveniles) and at 90 dpf (adults). Animals were sacrificed by immersion into ice followed by decapitation. Biometry measurements (length and weight) were performed in all fish and survival was determined for each temperature treatment at 6, 50 and 90 dpf. Tissues were carefully dissected (gonads with adjacent parts of the body trunk for juveniles or completely isolated gonads for adults), flash frozen into liquid nitrogen and kept at −80ºC for further analysis. Fish sex was individually determined after dissection and sex ratio was calculated for each biological replicate.

Trunk samples were dissected from euthanized juvenile zebrafish between the opercula and anal pore. The trunk was gutted carefully using ultra-fine (1 μm tip) tungsten dissecting needle (Roboz Surgical Instrument, Co.) under a stereo-microscope (Leica). The dissecting of *Tg(vasa:vasa-egfp)* juvenile zebrafish was performed under a stereo-microscope equipped with MAA-03 universal light source (BLS Ltd.) to ensure that the gonad (expressing *vasa-egfp*) was still intact in the trunk cavity after gutting.

5.4. Analysis of Dissected Gonads from Adult Fish

Dissected gonads from all adult individuals of all groups at the age of 90 dpf were analyzed. First, they were examined under the dissecting scope to visually assess the level of gonadal maturation based on their overall size, shape, color, and texture. Then a subset of them (13 males and 12 females, Table S6) was also analyzed histologically. Briefly, gonads were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 24 h at room temperature, washed for 24 h in PBS, dehydrated in an ascending series of ethanol concentrations, cleared in xylene and embedded in glycol methacrylate (JB-4; Polysciences, Inc.). Sections (3 μm thick) were cut using a microtome (Reichert-Jung 2040), float mounted on glass slides, stained with haematoxylin-eosin and mounted with DPX. Periodical sections covering the whole length of the gonad (min. 12 sections per gonad) were examined with a light microscope

(Zeiss AxioPlan 2) fitted with a MRc5 Axiocam digital camera. Images were stored and processed using Zeiss Axiovision AC v 4.4.0.0 imaging software.

Visual assessment of the gonadal maturation level under the dissecting scope allowed the classification of gonads into three types (Refs 6, 7; Table S1, Fig. S3). They were as follows:

Type 1: immature gonads. In females, type 1 ovaries had a pale yellow color. Microscopically this type of gonads contained few oocytes at stage I primary growth. In males, type 1 testes were semi-transparent/white and occupied only a small portion of the posterior part of the adipose tissue, in which they appeared imbedded. Microscopically, all germ cells were spermatogonia and spermatocytes, and the testicular lobes were defined.

Type 2: maturing and differentiating gonads. In females, type 2 ovaries contained more oocytes than type 1 but still not enough oocytes to fill the whole ovary and the color was still yellowish. Microscopically, in addition to some stage I oocytes, most of them were at stage II (cortical alveolus stage), but also in stage III (early-vitellogenic stage). In males, type 2 testes were white and surrounded by adipose tissue. Microscopically, spermatogonia, spermatocytes, spermatids and spermatozoa were observed.

Type 3: mature and differentiated gonads. In females, type 3 ovaries were big, filled with oocytes and had a yellow-orange color. Microscopically, most follicles were observed at stages III and stage IV (late-vitellogenic) but also some in stage III, II and few in stage I. In males, type 3 testes were white-beige, wider and partially covered by the adipose tissue. Microscopically, these testes contained less spermatids and more spermatozoa than type 2 testes.

Out of the 25 gonads analyzed histologically, none of them showed any sign of being intersex, i.e., an ovotestis that contained cells characteristic to both female and male gonads. Representative results of each gonadal type are shown in Fig. S3.

5.5. Microarray Analysis

The custom designed zebrafish expression microarray used for this project contained probes for 31,477 unique sequences from NCBI Refseq, Ensembl Genes version e61, UCSC (danRer7 genome assembly) mRNA dataset and a gonadal EST library created previously in the Reproductive Genomics Group at TLL, Singapore (8). During the course of this project there were several updates and thus the probes were re-mapped to Ensembl transcriptome version e70. The microarray was manufactured by NimbleGen (Roche NimbleGen) on a 12x135 array format with each array containing 117,915 probes (length: 60 base pairs) with an average of 3 probes targeting each unique sequence.

We isolated the gonads from four adult females and 10 adult males at 90 dpf of each group (28ºC and 36ºC, Table S6). The maturity of the gonads used for the microarray analysis was the following:

For female gonads: FCT: four type 2 individuals; FHT1: two type 2 individuals; and FHT2: two type 1 individuals.

For male gonads: MCT: eight type 3 and two type 2 individuals; MHT1: six type 3 and two type 2 individuals; and MHT2: one type 3 and one type 2 individual.

Total RNA was extracted by Trizol (Life Technologies) according to the manufacturer's protocol and DNAse treated (Invitrogen). RNA quality was checked by Experion Automated Electrophoresis Station (BioRad). Samples with RNA integrated quality (RIQ) number ranged from 7 to 9.9 (mean RIQ = 9) and were used for the microarray analysis. As the amount of total RNA obtained from the juveniles were not sufficient for microarray and qPCR validation, there was a need to perform whole transcriptome amplification (WTA). For consistency, both juvenile and adult total RNA samples (60 ng) were amplified using Ovation RNA Amplification System V2 (NuGEN). Size distribution of the amplified double stranded cDNA samples were analyzed by Agilent Bioanalyzer with RNA 6000 Nano Chip (Agilent Technologies) to ensure fragment size uniformity among the samples. Following WTA, 1 µg of the amplified cDNA were labeled using NimbleGen One-Color DNA Labelling Kit (Roche NimbleGen,) and hybridization was carried out according to the manufacturer's instructions with MAUI hybridization system (BioMicro Systems). Each sample, corresponding to the gonads of an individual fish, was hybridized separately. After an overnight hybridization (16–20 h) the slides were washed with NimbleGen Wash Buffer Kit (Roche NimbleGen) and scanned at 5 µm resolution with Axon GenePix 4000B Microarray scanner (Molecular Devices).

A total of 48 canonical genes were selected for their importance to reproduction and sex differentiation in fish (9–15) to study their expression among the different transcriptomic groups (Dataset S3).

5.6. Validation of Differential Gene Expression by qPCR Array

A high throughput real-time qPCR array was designed to measure the expression level of 35 genes for validating the microarray result. The primers were designed using online software Primer-BLAST (16) and Real-time PCR Miner (17) was used to estimate the primer efficiency (refer to Table S7 for primer details).

qPCR analysis was performed using BioMark HD System (Fluidigm). All reactions were run in triplicates. The cDNA synthesized during WTA was used for the qPCR. Preparation of the BioMark Dynamic Array reactions was performed according to Fluidigm advance development protocol using EvaGreen DNA Binding Dye with 48.48 and 96.96 Dynamic Array IFCs (PN100-1208A1). Pre-amplification of cDNA

was performed using TaqMan PreAmp Master Mix (Life Technologies) with 50 nM of pooled primers and 1.25 µl of cDNA. Pre-amplification reaction was carried out at 95°C for 10 min followed by 14 cycles of 95°C for 15 s and 60°C for 4 min. The amplified cDNA was diluted 1:5 with low EDTA-TE buffer. For loading the BioMark Dynamic Array, sample and assay mix was prepared according to manufacturer's protocol. Briefly, the sample mix was prepared by combining 2 µl of diluted pre-amplified cDNA, 4.44 µl of TaqMan Gene Expression Master Mix (Life Technologies), 0.443 µl of 20x EvaGreen (Biotum), 0.443 µl of 20x DNA Binding Dye Sample Loading Reagent (Fluidigm) and 0.67 µl of low EDTA-TE buffer. The assay mix was prepared by combining 0.25 µl each of the 100 µM forward and reverse primer, 0.125 µl of 10% Tween 20 (Sigma) and 4.375 µl of low EDTA-TE buffer. After the reaction mixture was loaded into the chip, qPCR cycle was carried out on the BioMark HD System (Fluidigm). The qPCR protocol consisted of a thermal mix (50°C for 2 min, 70°C for 30 min, 25°C for 10 min) followed by uracil-DNA glycosylase (UDG) treatment and hot start (50°C for 2 min, 95°C for 10 min) then quantification (35 cycles of 96°C for 15 s then 60°C for 1 min). Melting curve analysis was performed at the end of the qPCR protocol between 55 to 95°C with 0.5°C increments.

5.7. Statistical Analysis

StatGraphics Centurion XVI.I software (OpenLand, Inc.) was used to analyze biometry results (weight and length), survival, gonad maturity and sex ratio. Prior to sex ratio analysis, the percent males/females for each biological replicate was arcsine transformed. Data were checked for normality using the Kolmogorov-Smirnov test and for homocedasticity of variances by the Levene's test. Means were compared by the Student's-*t* test or by one-way analysis of variance (ANOVA) with a Tukey's *posthoc* multiple range test. The Chi-squared test with arcsine transformation was used to study differences in gonad maturation and sex ratios.

5.8. Microarray Data Analysis

The microarray data were collected and analyzed according to MIAME guidelines (18). Raw fluorescent intensity data was retrieved from the scanned images by NimbleScan version 2.6 (Roche NimbleGen) according to the manufacturer's instructions. Robust multi-array average (19), quantile normalization and background correction were applied as implemented in NimbleScan to generate the Pair files. Then the Pair files were imported into Partek Genomic Suite version 6.6 (Partek Inc.) for analysis. Differentially expressed transcripts (DETs) were identified by ANOVA. The *P* value was adjusted to account for incorrectly rejected null hypothesis in multiple hypotheses testing using False Discovery Rate (FDR) step-up method (20) as implemented in Partek GS. DETs were filtered for significant ($P \leq 0.01$) then by at least

1.5 fold change difference. Results were deposited at Gene Expression Omnibus (GEO) database (Accession number GSE51434).

5.9. qPCR Array Data Analysis

The Fluidigm Real-time PCR analysis software version 3.0.2 (Fluidigm) was used to check for amplification specificity using the melting curve data and to extract quantification cycle (Cq) data. PCR products with unacceptable melting curve were excluded in subsequent analysis. The Cq data were then imported into GenEx Pro version 5.4.2.128 (MultiD Analyses AB) for further analysis. Briefly, the Cq data were corrected for primer efficiency as implemented in GenEx. Outliers were identified by Grubb's test with 0.95 confidence level and 0.25 cutoff SD (cycle). The triplicate Cq data were then averaged and missing data were assigned the highest measured Cq for that gene of interest (GOI) plus one. Average Cq of the reference genes (RG) *eef1a1l1* and *rpl13a* were used for normalization ($Cq_{(GOI)} - Cq_{(Avg\ RG)}$). Finally, relative quantity (RQ; $2^{-\Delta\Delta Cq}$) was calculated using adult females or juveniles (group FCT1 or JCT1 respectively; see results) at control temperature as reference. The qPCR heatmap was generated using hierarchical clustering with normalized Cq data. Average linkage clustering method and Euclidean distance measurement was used as implemented in Partek GS.

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Supplementary Tables

Table S1. Description (macroscopic and microscopic) of the different types of gonads found in adult zebrafish in this study

	Testis		Ovary	
	Macroscopic	**Microscopic (germ cell stage)**	**Macroscopic**	**Microscopic (germ cell stage)**
Type 1	Size: clearly smaller than type 1 ovary Shape: long and thin Color: semi-transparent white Texture: smooth	Spermatogonia Spermatocytes	Size: clearly bigger than type 1 testis Shape: round with protruding oocytes Color: pale yellow Texture: granular	Stage I: primary growth
Type 2	Size: clearly bigger than type 1 testis Shape: long Color: white Texture: smooth	Spermatogonia Spermatocytes Spermatids Spermatozoa	Size: clearly bigger than type 1 ovary Shape: round with protruding oocytes Color: yellow Texture: granular	Stage I: primary growth Stage II: cortical alveolus Stage III: previtellogenic stage
Type 3	Size: bigger than type 2 testis Shape: long and thicker than type 2 testis Color: white-beige Texture: less smooth than type 2 testis	Spermatogonia Spermatocytes Spermatids Spermatozoa	Size: bigger than type 2 ovary Shape: round filled by oocytes Color: yellow-orange Texture: granular	Stage I: primary growth Stage II: cortical alveolus Stage III: early vitellogenic stage Stage IV: late vitellogenic stage

Table S2. Description of gonadal profiles of fish defined by PCA analysis based on expression microarray data

Abbre-viation	Sample group	Description
FCT	Female Control Temperature	90 dpf zebrafish with female phenotype at 28°C.
FHT1	Female High Temperature Group 1	90 dpf zebrafish with female phenotype heat-treated at 36°C between 18–32 dpf and clustered together with FCT on the PCA plot (Fig. 1*E*).
FHT2	Female High Temperature Group 2	90 dpf zebrafish with female phenotype heat-treated at 36°C between 18–32 dpf and formed distinct cluster closer to MCT on the PCA plot (Fig. 1*E*).
MCT	Male Control Temperature	90 dpf zebrafish with male phenotype at 28°C.
MHT1	Male High Temperature Group 1	90 dpf zebrafish with male phenotype heat-treated at 36°C between 18–32 dpf and clustered together with MCT on the PCA plot (Fig. 1*E*).
MHT2	Male High Temperature Group 2	90 dpf zebrafish with male phenotype heat-treated at 36°C between 18–32 dpf and formed distinct cluster on the PCA plot (Fig. 1*E*).
JCT1	Juvenile Control Temperature Group 1	37 dpf zebrafish at 28°C that formed a distinct cluster on the PCA plot (Fig. 4*A*) and in the same clade with future female transgenic fish that displayed strong *vasa-egfp* fluorescent signal (Fig. 4*B*).
JCT2	Juvenile Control Temperature Group 2	37 dpf zebrafish at 28°C that formed a distinct cluster on the PCA plot (Fig 4*A*) and in the same clade with future male transgenic fish that displayed weak *vasa-egfp* fluorescent signal (Fig. 4*B*).
JHT	Juvenile High Temperature	37 dpf zebrafish heat-treated at 36°C between 18–32 dpf that formed a distinct cluster on the PCA plot (Fig. 4*A*) and in the same clade with JCT2 and future male transgenic fish that displayed weak *vasa-egfp* fluorescent signal (Fig. 4*B*).

Table S3. Validation of adult gonad microarray data by qPCR. Fold change values are compared to female control temperature (FCT) group. Significant values ($P < 0.01$) are highlighted in green (in alphabetical order). For full gene names see Table S7

Gene symbol	FHT1 Microarray	qPCR	FHT2 Microarray	qPCR	MHT2 Microarray	qPCR	MHT1 Microarray	qPCR	MCT Microarray	qPCR
amh	1.88	1.01	15.42	327.79	20.07	538.11	31.8	453.59	26.98	804.14
axin1	-1.14	-1.47	-7.09	-12.53	-17.1	-11.83	-8.21	-7.32	-6.84	-7.29
ctnnbip1	1.43	-1.51	-2.85	-10.28	-3.67	-399.89	-4.76	-72.55	-3.12	-16.64
cyp11c1	1.76	-1.6	41.52	504.89	23.81	4.62	54.6	605.6	60.67	1.685.18
cyp19a1a	-1.1	-5.57	-11.81	54.81	-19.11	-24.96	-18.74	-112.32	-18.11	-41.85
cyp19a1b	-1.9	-1.46	-1.62	-1.32	3.2	-132.98	-1.02	-11.53	1.34	-1.19
cyp26b1	1.55	1.44	2.35	1.73	5.47	-11.11	2.78	-1.63	2.59	1.34
dkk3	1	-1.27	7.04	12.77	2.51	-1.97	4.49	4.36	3.88	7.56
dmrt1	1.32	-1.08	3.4	8.79	5.23	7.92	6.02	17.61	6.29	30.61
dnd	-1.14	-1.32	-3.61	-6.03	-5.24	-7.335.78	-3.84	-8.71	-2.82	-2.21
dnmt3bb.2	1.05	1.13	-4.89	-8.58	-4.43	-4.64	-5.3	-17.17	-4.99	-8.71
esr2b	1.21	-1.85	4.44	13.01	7.16	-2.23	6.57	12.37	8.21	24.53
fancl	-1.44	-2.67	-6.75	-55.42	-10.19	-2.578.73	-7.26	-60.19	-6.17	-15.99
foxl2	1.29	-1.02	-8.82	-10.34	-10.91	-36.25	-5.07	-22.95	-4.88	-17.45
fshr	1.04	1.33	1.34	5.98	-1.14	7.69	2.15	12.35	4.63	15.25
ift57	-1.28	-1.48	3.53	4.09	3.39	4.41	4.07	7.87	3.92	11.86
ift81	1.26	-1.37	6.62	16.57	12.22	38.81	14.76	41.77	12.35	36.02
ints3	1.1	1.14	-1.49	-16.4	-2.15	-11.98	-2.27	-13.77	-2.3	-24.01
lhcgr	1.34	-1.19	1.03	2.33	-1.28	-1.43	3.04	1.97	2.05	4.02
naa35	-1.08	-6.38	1.42	-1.37	1.34	-19.83	1.49	-1.07	1.47	-1.31
nabp1a	-1	-1.23	-1.39	-5.38	-1.89	-8.04	-1.83	-5.44	-1.72	-5.59
nr0b1	1.08	-2.38	-1.17	-7.71	-1.84	-12.89	-1.99	-4.29	-1.48	-1.13
odf3b	-1.37	-1.7	18.85	73.27	23.05	95.46	24.44	110.98	19.29	116.24

piwil1	-1.24	-1.49	1.56	1.26	-1.11	-1.06	1.59	2.16	2.14	3.23
pou5f3	-1.07	-1.63	-8.58	-8.76	-10.6	-9.36	-22.28	-28.02	-25.52	-20.74
prkcz	-1.08	1.46	-1.34	-1.78	-1.17	-17.65	-1.8	-4.01	-1.38	-5.35
star	1.29	1.78	14.35	61.13	18.91	2.53	18.53	92.05	19.89	248.51
sycp3	1.49	1.06	16.36	46.68	22.18	42.85	25.97	75.15	26.74	188.31
tbp	1	-1.64	-6.88	-41.39	-5.74	-1.198.54	-7.8	-98.29	-6.42	-31.19
tdrd7	-1.14	-1.88	1.19	-13.8	1.46	-14.52	1.46	-3.35	1.36	4.47
ticrr	-1.06	-1.69	-2.83	-3.51	-18.19	-2.89	-3.37	-2.5	-2.76	-1.68
tnfsf10l	-1.03	2.32	3.3	13.31	2.84	-2.29	4.74	10.1	3.18	24.89
tsc1a	-1.11	-1.16	-2.8	-7.77	-5.32	-31.77	-3.64	-12.38	-3.49	-6.37
vtg5	1.15	2.14	-1.7	-4.98	-5.03	-2.214.27	-6.56	-453.64	-5.39	-120.07
zp2.1	1.23	1.06	-1.08	-1.1	-19.5	-96.9	-18.63	-98.31	-16.25	-52.03

Table S4. Validation of juvenile microarray data by qPCR. Fold change values are compared to JCT1. Significant values (*P* < 0.05 or < 0.01) are highlighted in blue or green, respectively (in alphabetical order). For full gene names see Table S7

Gene symbol	JCT2 Microarray	qPCR	JHT Microarray	qPCR
amh	1.04	1.25	1.04	2.62
axin1	1.05	1.11	-1.38	1.68
cyp11c1	1.17	1.11	1.83	4.13
cyp19a1a	-2.21	-3.39	-2.05	-3.04
cyp19a1b	1.61	-8.23	1.64	-2.61
ddx4	-5.41	-2.47	-23.38	-44.84
dkk3	1.3	1.53	1.59	2.64
dmrt1	-1.13	1.43	-1.46	1.6
dnd	-8.94	-19.27	-35.74	-225.91
dnmt3bb.2	-1.75	-3.27	-1.55	-2.09
esr2b	1.16	2.27	-2.99	-1.28
exosc8	-1.01	-1.54	1.01	-1.14
fancl	-1.53	-1.97	-1.49	-2.28
foxl2	-2.05	-6.53	1.05	1.32
fshr	-1.16	-3.39	-1.57	-3.05
ift57	-1.48	-1.67	1.61	1.83
ift81	1.2	-5.19	-1.01	-2.58
nabp1a	-1.06	-1.32	1	-1.06
odf3b	-2.08	-7.66	1.39	-1.17
pou5f3	-5.72	-2.63	-8.72	-2.55
star	1.01	2.06	1.36	2.69
sycp3	-1.47	-3.27	-1.22	-4.19
tbp	-1.28	-2.69	-1.3	-2.14
tsc1a	-1.04	-1.04	1.06	1.01
vtg5	-1.06	1.01	-2.66	-8.51
zp2.1	-8.67	-12.6	-132.73	-201.91

Table S5. Type of heat effect on genes in juveniles and adults. Juvenile values are fold change of JHT compared to JCT1. Significant values ($P < 0.01$) are highlighted in grey. For full gene names see Table S7

Gene	Accession number	Type of effect	FDR ($P < 0.01$)	FC Juveniles	FDR ($P < 0.01$)	FC Adults
axin1	NM_131503	Permanent	0.011	-1.452	0.000	-4.211
ctnnbip1	NM_131594	Permanent	0.044	-1.290	0.001	-1.829
ddx4	NM_131057	Permanent	0.000	-10.725	0.007	-1.635
dgcr8	NM_001122749	Permanent	0.002	-1.176	0.000	-1.924
dkk3	NM_001089545	Permanent	0.027	1.416	0.009	2.132
dnd	NM_212795	Permanent	0.000	-13.174	0.001	-2.592
ift57	NM_001001832	Permanent	0.000	1.912	0.000	1.747
pou5f3	NM_131112	Permanent	0.001	-3.754	0.017	-1.888
cyp26b1	NM_212666	Transient	0.009	1.356	0.055	2.227
nr0b1	NM_001082947	Transient	0.003	-2.760	0.382	-1.206
piwil1	NM_183338	Transient	0.000	-5.107	0.061	-1.233
piwil2	NM_001080199	Transient	0.000	-3.831	0.088	-1.217
rspo1	NM_001002352	Transient	0.041	-1.272	0.116	1.641
sox9a	NM_131643	Transient	0.004	1.363	0.958	-1.008
tdrd7	NM_001099343	Transient	0.002	-2.797	0.087	1.130
vtg5	NM_001025189	Transient	0.004	-2.519	0.241	-1.260
wnt4b	NM_131500	Transient	0.002	2.661	0.894	1.095
zp2.1	NM_131330	Transient	0.000	-39.002	0.797	-1.136
esr2b	NM_174862	Inconsistent	0.000	-3.428	0.003	1.967
foxl2	NM_001045252	Inconsistent	0.042	1.581	0.002	-4.439
amh	NM_001007779	Delayed	0.898	1.037	0.000	3.387
cyp11c1	NM_001080204	Delayed	0.158	1.529	0.000	4.036
cyp19a1a	NM_131154	Delayed	0.200	-1.433	0.000	-3.530
dmrt1	NM_205628	Delayed	0.070	-1.304	0.000	1.681
dnmt3b	NM_131386	Delayed	0.126	-1.225	0.000	-2.084
fancl	NM_212982	Delayed	0.174	-1.240	0.000	-3.339
ift81	NM_001002313	Delayed	0.797	-1.064	0.000	2.560
ints3	NM_001044932	Delayed	0.850	-1.030	0.033	-1.180
nabp1a	NM_001008643	Delayed	0.745	1.037	0.006	-1.235
odf3b	NM_199958	Delayed	0.069	2.348	0.000	4.746
sox9b	NM_131644	Delayed	0.460	1.291	0.009	-4.114
star	NM_131663	Delayed	0.207	1.463	0.000	3.693
sycp3	NM_001040350	Delayed	0.811	-1.065	0.000	3.684
tbp	NM_200096	Delayed	0.122	-1.168	0.000	-2.480
ticrr	NM_001003887	Delayed	0.185	-1.376	0.000	-4.317
tnfsf10l	NM_131843	Delayed	0.166	1.104	0.024	1.717
tsc1a	NM_200052	Delayed	0.390	1.112	0.043	-2.067
cyp19a1b	NM_131642	None	0.068	1.419	0.560	1.215
exosc8	NM_001002865	None	0.850	1.027	0.277	-1.187
fshr	NM_001001812	None	0.093	-1.517	0.158	-1.985
lhcgr	NM_205625	None	0.719	-1.125	0.130	-1.595
naa35	NM_199550	None	0.296	1.202	0.241	1.137

nr3c1	NM_001020711	None	0.075	1.221	0.737	1.096
nr5a1a	NM_131794	None	0.892	1.021	0.557	-1.182
nr5a1b	NM_212834	None	0.368	-1.268	0.140	-1.722
prkcz	NM_001030091	None	0.279	-1.234	0.771	-1.068
rbm8a	NM_001013345	None	0.346	1.040	0.423	1.068
sox3	NM_001001811	None	0.135	1.445	0.554	-1.360
tp53	NM_001271820	None	0.519	1.131	0.264	-1.119
wnt4a	NM_001040387	None	0.262	1.292	0.558	-1.241

FC = Fold Change
FDR = False Discovery Rate

Table S6. Macroscopic sexing results are confirmed by either histology or gonadal transcriptomics in over 96% of zebrafish adults tested

Fish ID	Macroscopic sex	Macroscopic assigned gonadal type	Histological sex	Sex based on gonadal transcriptome	Group label used in the MS
1	F	Type 1	F	ND	NA
2	F	Type 1	F	ND	NA
3	F	Type 1	F	ND	NA
4	F	Type 1	F	ND	NA
5	F	Type 1	F	ND	NA
6	F	Type 2	F	ND	NA
7	F	Type 2	F	ND	NA
8	F	Type 2	F	ND	NA
9	F	Type 2	F	ND	NA
10	F	Type 3	F	ND	NA
11	F	Type 3	F	ND	NA
12	F	Type 3	F	ND	NA
13	M	Type 1	M	ND	NA
14	M	Type 1	M	ND	NA
15	M	Type 1	M	ND	NA
16	M	Type 1	M	ND	NA
17	M	Type 1	M	ND	NA
18	M	Type 1	M	ND	NA
19	M	Type 2	M	ND	NA
20	M	Type 2	M	ND	NA
21	M	Type 2	M	ND	NA
22	M	Type 2	M	ND	NA
23	M	Type 3	M	ND	NA
24	M	Type 3	M	ND	NA
25	M	Type 3	M	ND	NA
26	F	Type 2	ND	F	FCT
27	F	Type 2	ND	F	FCT
28	F	Type 2	ND	F	FCT
29	F	Type 2	ND	F	FCT
30	F	Type 2	ND	F	FHT1
31	F	Type 2	ND	F	FHT1
32	F	Type 1	ND	M	FHT2
33	F	Type 1	ND	M	FHT2
34	M	Type 3	ND	M	MCT
35	M	Type 3	ND	M	MCT
36	M	Type 3	ND	M	MCT
37	M	Type 3	ND	M	MCT
38	M	Type 3	ND	M	MCT

39	M	Type 3	ND	M	MCT
40	M	Type 3	ND	M	MCT
41	M	Type 3	ND	M	MCT
42	M	Type 2	ND	M	MCT
43	M	Type 2	ND	M	MCT
44	M	Type 3	ND	M	MHT1
45	M	Type 3	ND	M	MHT1
46	M	Type 3	ND	M	MHT1
47	M	Type 3	ND	M	MHT1
48	M	Type 3	ND	M	MHT1
49	M	Type 3	ND	M	MHT1
50	M	Type 2	ND	M	MHT1
51	M	Type 2	ND	M	MHT1
52	M	Type 3	ND	M	MHT2
53	M	Type 2	ND	M	MHT2

F = female

M = male

MS = manuscript

ND = not determined

NA = not applicable

* Yellow shading indicates contrasting results between macroscopic and transcriptomic sexing

Table S7. Gene symbols, names, Refseq IDs and primer sequences for all genes used in qPCR array (in alphabetical order)

Gene symbol	Gene name	Refseq ID	Forward primer (5'-3')	Reverse primer (5'-3')
amh	anti-Müllerian hormone	NM_001007779	ACAACCCGAAGGTCAACCCGC	GTGGCATGTTGGTCAGTTGGCTG
axin1	axin 1	NM_131503	ACCTGCTGACGACATGGAGAGG	AATGCTCCCGTAAGGGCCCC
ctnnbip1	catenin beta interacting protein 1	NM_131594	CTGTCGGGATGTGACCCCGG	CTCCTGACGCACCGCTCTCC
cyp11c1	cytochrome P450, family 11, subfamily C, polypeptide 1	NM_001080204	CCTCGGGCCCATATACAGAGA	CGTCCCGTTCTTGAGGAAGA
cyp19a1a	cytochrome P450, family 19, subfamily A, polypeptide 1a	NM_131154	GATATTTGCTCAGAGCCATGGA	GCTCTGGCCAGCTAAAACACT
cyp19a1b	cytochrome P450, family 19, subfamily A, polypeptide 1b	NM_131642	AAAGAGTTACTAATAAAGATCCACCGGTAT	TCCACAAGCTTTCCCATTTCA
cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	NM_212666	GCAAGCTGCCCATGCCCAAG	GCATGGAAACCTGCACCCTGAAAG
ddx4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	NM_131057	AAGGGCTGCAATGTTCTGTG	CTCAGGCCAATCTTTCCACG
dkk3	dickkopf homolog 3	NM_001089545	GCATGTGGCTCACGGACAAACC	TTCTCCATCTGATGCACAGCCTCC
dmrt1	doublesex and mab-3 related transcription factor 1	NM_205628	TGCCCAGGTGGCGTTACGG	CGGGTGATGGCGGTCCTGAG
dnd	dead end	NM_212795	TCGTGGAAGCTTTTCGGAACCGG	TGTCCTCGACGCGCTTGGAC

dnmt3bb2	DNA (cytosine-5-)-methyltransferase 3 beta, duplicate b.2	NM_131386	CGACTCCCTACGTGATAGCGACC	CCATAACCACCACCGTCCGTCC
eef1a1l1	eukaryotic translation elongation factor 1 alpha 1, like 1	NM_131263	GGAGGCTGCCAACTTCAACGC	GGCGATGTGAGCAGTGTGGC
esr2b	estrogen receptor 2b	NM_174862	CACGTTCACACAGCGCCTGG	GCTCGCGGAGGGATTCAAGC
exosc8	exosome component 8	NM_001002865	CTGTCGTCCAGATGGAAGGG	TCACCAGTGCTGATCCATCG
fancl	Fanconi anemia, complementation group L	NM_212982	ACACTCCAGCTGAAGGCAGAGG	CCAAGGTGCTCTGAGAGGTCCAC
foxl2	forkhead box L2	NM_001045252	AAACACTGGGAAGGTTTGCGTGC	TTTGTCCGGCCCCTTCTCTGG
fshr	follicle stimulating hormone receptor	NM_001001812	TCGTGAGATAGACAGCCATGC	GAGATATCCAGAACGACGGGG
ift57	intraflagellar transport protein 57	NM_001001832	GAGGTGGAGCGAGTTCTTCC	GACAACCCTTGGCATCTTGC
ift81	intraflagellar transport 81 homolog	NM_001002313	AATGGACGTACAGGTGCGG	CTGACGGAGAACCTTGCCC
ints3	integrator complex subunit 3	NM_001044932	TGGACCATGGTACGACAAGC	CCATGAACCTCTCTCGGAGC
lhcgr	luteinizing hormone/choriogonadotropin receptor	NM_205625	AGTTTCGACGGCCTGAAAGG	CTGGATTGAGATCTCGGAGACG
naa35	N(alpha)-acetyltransferase 35, NatC auxiliary subunit	NM_199550	TCTGAGAGTGACGGGAATGC	GTTCCACCTCAGGATCTCGC
nabp1a	nucleic acid binding protein 1a	NM_001008643	GCTGCTAGCACAAGCTAACC	TGGCACAGTTCCATTACCTGC
nr0b1	nuclear receptor subfamily 0, group B, member 1	NM_001082947	TAACGGCGGAGTGGCGTTGAC	GTTGACACTGCAGCCCAGCAAC
odf3b	outer dense fiber of sperm tails 3B	NM_199958	AAACTCCAGGTCCAGCTGCGTAC	ACCGCTGAAGGAGACGTTTGGG
piwil1	piwi-like RNA-mediated	NM_183338	ATCTCAAGAGGCTGCAAAGC	CACCTCTCTCCCCGATCTTC

	gene silencing 1			
pou5f3	POU domain, class 5, transcription factor 3	NM_131112	GGAAGGCACAGTCCGTTCTGC	CGCACTACATCTCTCTCCAGGCC
prkcz	protein kinase C, zeta	NM_001030091	ACTCCTCCTGCACGTATTTCC	CGGTACAGCTTCCTCCATCG
rpl13	ribosomal protein L13	NM_198143	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
star	steroidogenic acute regulatory protein	NM_131663	TGAACAAGCTCTCCGGACCTGG	CAATTGGACTGCTGAGCAAGGAGC
sycp3	synaptonemal complex protein 3	NM_001040350	AGCGGATCTGACGAAGACACGAG	ATGTCCGCACCAAATCTTTCCAGC
tbp	TATA box binding protein	NM_200096	ATGTTTTGCGCGCTCCCTGC	AGCAGTGGTTCAGGGCTTCCC
tdrd7a	tudor domain containing 7a	NM_001099343	TCGCATAACGTCAAACCTCGCTTG	TCTGGAGGAGGGCAGGTCGG
ticrr	TopBP1-interacting, checkpoint, and replication regulator	NM_001003887	AAGACTCAGGGGGACAGTGG	CTGAAGTGCAAGAGGCCAGG
tnfsf10l	tumor necrosis factor (ligand) superfamily, member 10 like	NM_131843	TCGTCCTGCAGATCGCGTCC	AACCCCCTGACTCTTAACCTGCG
tsc1a	tuberous sclerosis 1a	NM_200052	GTACCCAGGCTAAGACGTGC	ACTGGAGTCTTGGCATCACC
vtg5	vitellogenin 5	NM_001025189	AAGGCCCTGCCAACTGACCG	TGGGTCCAGTAACCATCGGTATTGC
zp2.1	zona pellucida glycoprotein 2, tandem	NM_131330	CCCACAGTCTCTGTCTCCTGTGC	TTCCTTGCGCAACTCTGCTCAC

Supplementary Datasets

- **Dataset S1.** Principal Component Analysis (PCA) loading scores of all transcripts, plus those for the FHT2 *vs.* FCT and MHT2 *vs.* MCT DETs. See Table S2 for group abbreviations.

- **Dataset S2.** Differentially expressed transcripts lists from adults and juveniles based on microarray data. All adult groups are compared to FCT: FHT1 *vs.* FCT, FHT2 *vs.* FCT, MHT1 *vs.* FCT, MHT2 *vs.* FCT and MCT *vs.* FCT. All juvenile groups are compared to JCT1: JCT2 *vs.* JCT1, JHT *vs.* JCT1. Fold change: 1.5x; False Discovery Rate, $P \leq 0.01$. See Table S2 for group abbreviations.

- **Dataset S3.** Canonical genes with reproduction-related function differentially expressed in the pairwise comparisons of gonadal gene expression profile groups.

- **Dataset S4.** Gene Ontology term lists of adults and juveniles. All adult groups are compared to FCT: FHT1 *vs.* FCT, FHT2 *vs.* FCT, MHT1 *vs.* FCT, MHT2 *vs.* FCT and MCT *vs.* FCT. All juvenile groups are compared to JCT1: JCT2 *vs.* JCT1, JHT *vs.* JCT1. Fold change: 1.5; False Discovery Rate, $P \leq 0.05$. See Table S2 for group abbreviations.

- **Dataset S5.** Differentially expressed KEGG lists of adults and juveniles comparisons. Adult groups are compared to FCT: FHT1 *vs.* FCT, FHT2 *vs.* FCT, MHT1 *vs.* FCT, MHT2 *vs.* FCT and MCT *vs.* FCT (Fold Change: 1.5; False Discovery Rate, $P \leq 0.05$.). Juvenile groups are compared to JCT1: JCT2 *vs.* JCT1 and JHT *vs.* JCT1 (unadjusted $P \leq 0.05$). See Table S2 for group abbreviations.
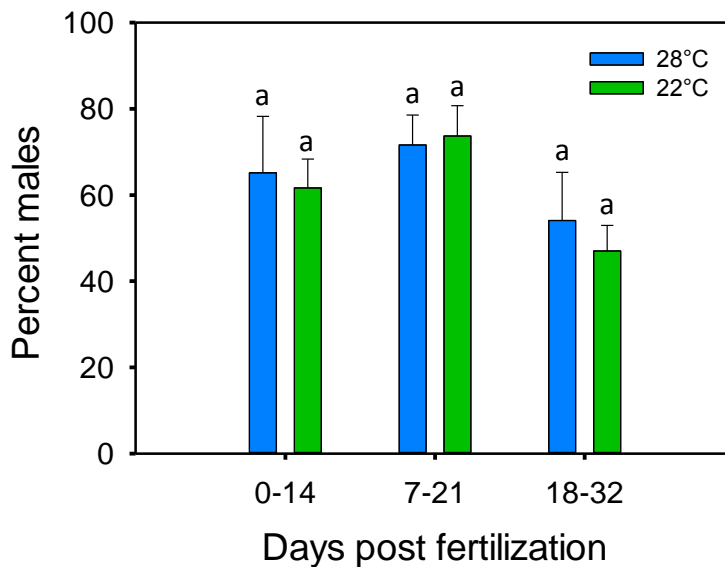
Supplementary Figures

Fig. S1. Sex ratio of zebrafish families subjected to 22°C during three different periods of gonad development. The data of six different families (#3, 5–8 and 10) out of the eight initially tested was used (#4 and 11 did not have enough animals). The mean ± s.e.m. represents data between three to five independent replicates for each temperature and each studied period. Sex ratio data was obtained from a total of 812 fish (454 and 358 fish for 22°C and 28°C, respectively). No statistically significant differences were found between the two temperatures at the three different tested periods ($P < 0.05$; Chi-squared test).
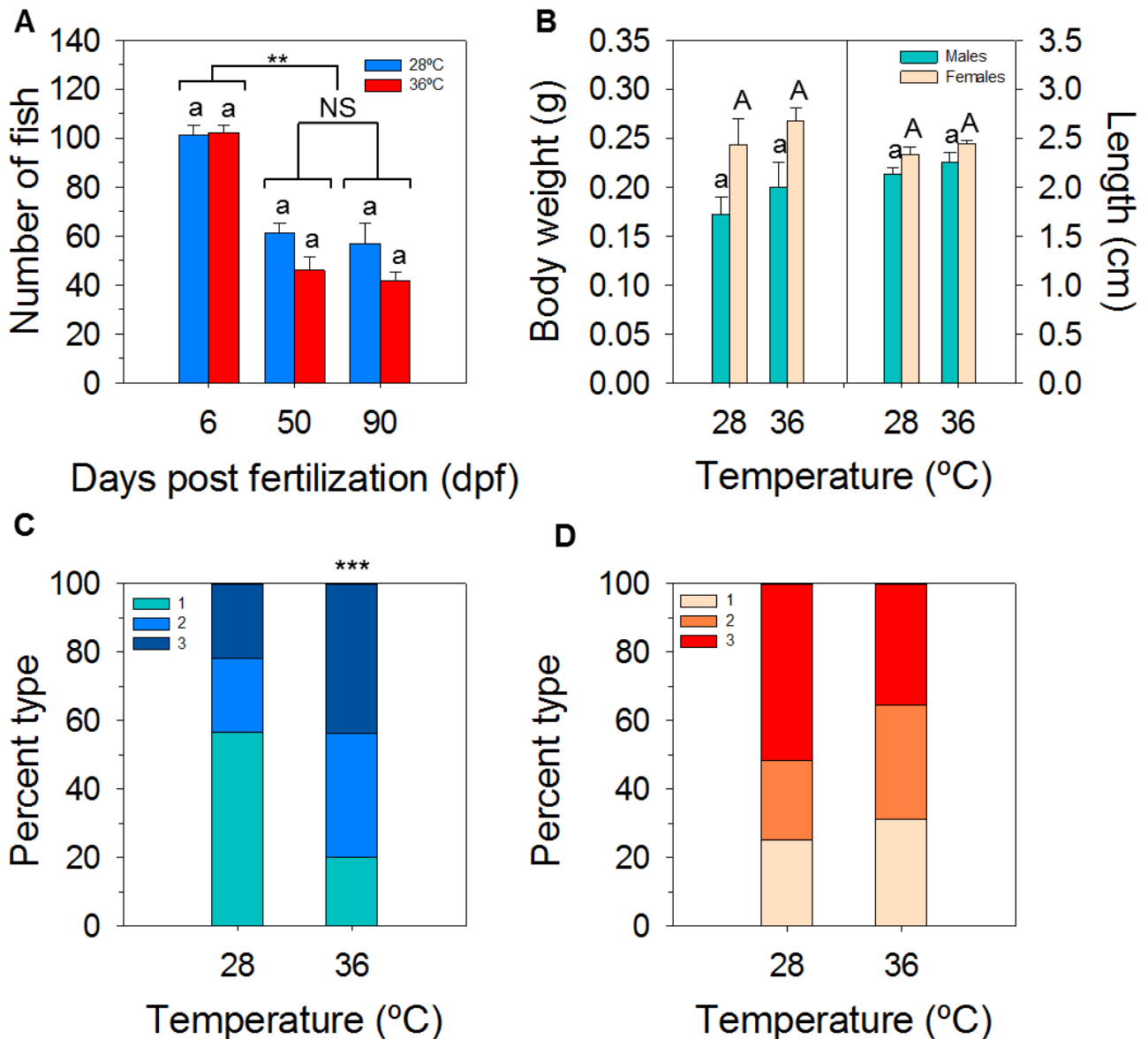
Fig. S2. Survival, growth and degree of gonadal maturation of zebrafish exposed to high temperature. A total of ~2,000 zebrafish larvae originating from 11 different pairs were used for these experiments. (**A**) The number of surviving fish per temperature treatment (28 or 36°C) at three different periods during development: in larvae, after being transferred from the Petri dishes to the rearing tanks at 6 dpf; in juveniles, at completion of sex differentiation (50 dpf); and in adults, at the end of the experiment (90 dpf). (**B**) Body weight (left panel) and standard length (right panel) of males and females at 90 dpf and at two different temperatures (28 and 36°C). (**C**) Male gonad maturation level (types 1, 2 and 3) at 90 dpf at two different temperatures. (**D**) Female gonad maturation level (types 1, 2 and 3) at 90 dpf at two different temperatures. See section 5.4 in SI Appendix and Table S1 for a complete description of the different gonad types. In **A** and **B**, data are shown as mean ± s.e.m., whereas in **C** and **D** as mean. Same letter stands for no significant differences ($P > 0.05$, ANOVA). In panel B, lowercase: males; uppercase: females. Statistically significant differences between sampling points (A) or gonad maturation levels (C and D) are indicated by asterisks: ** = $P < 0.01$; *** = $P < 0.001$; NS, not significant; ANOVA.
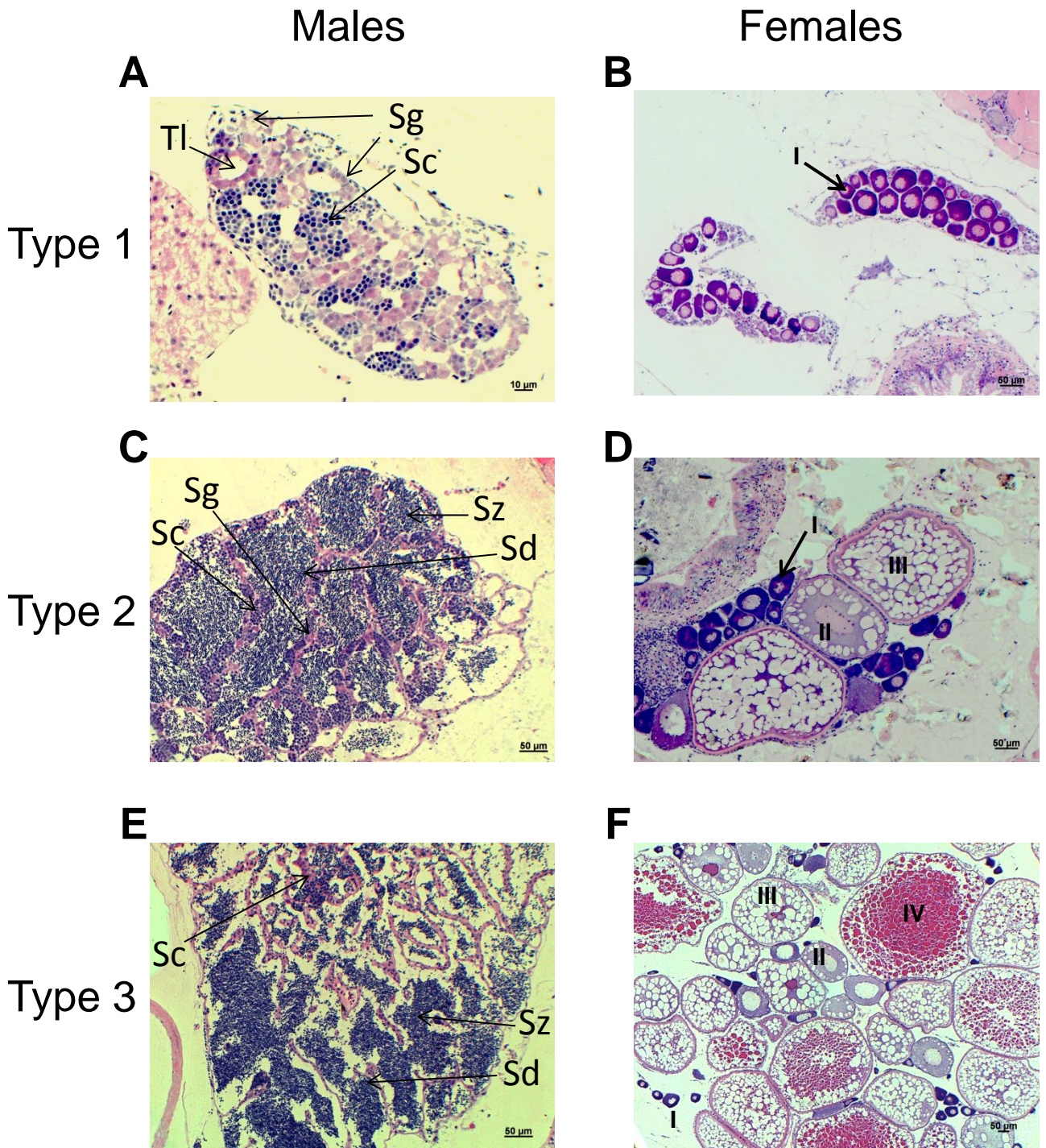
Fig. S3. Histological cross-section of testes (left panels) and ovaries (right panels) showing different degrees of development. (**A**) testis type 1; (**B**) ovary type 1; (**C**) testis type 2; (**D**) ovary type 2; (**E**) testis type 3; (**F**) ovary type 3. See section 5.4 in SI Appendix and Table S1 for a complete description of the different gonad types. Sg: spermatogonia; Sc: spermatocyte; Sd: spermatid; Sz: spermatozoa; Tl: testicular lobe; I, primary growth stage; II, cortical alveolus stage, III, early-vitellogenic stage; IV late-vitellogenic stage.
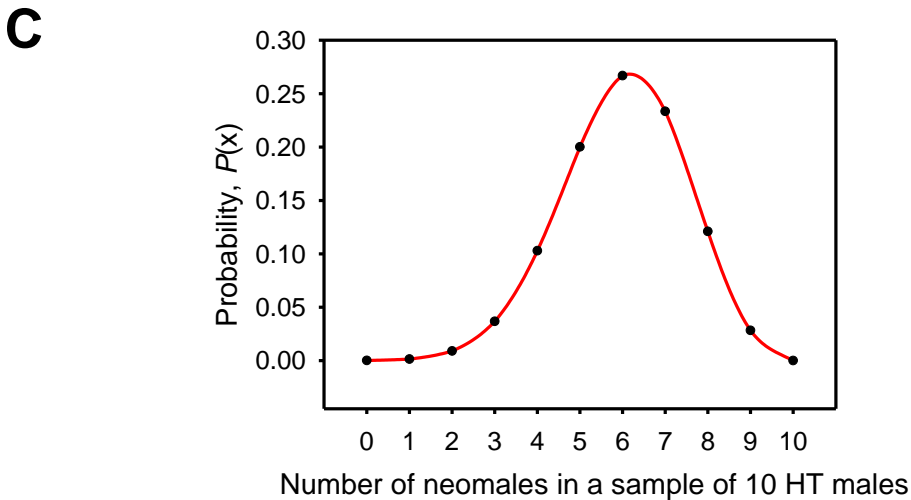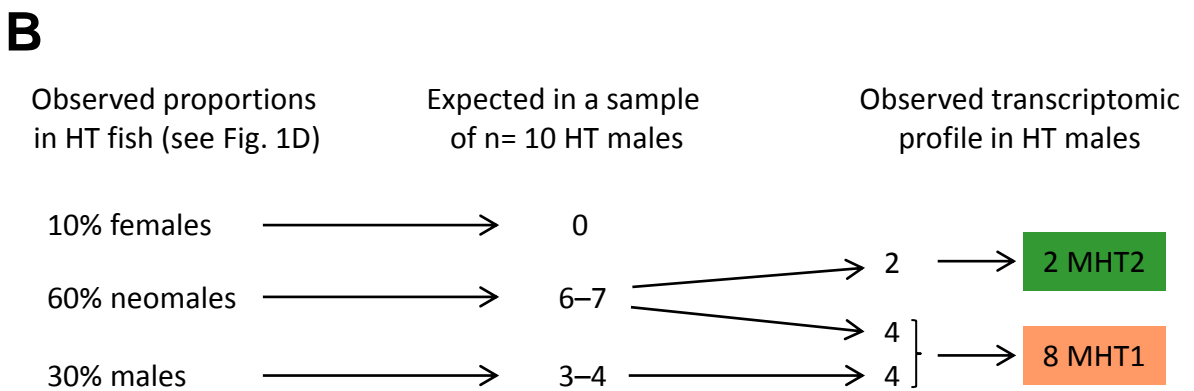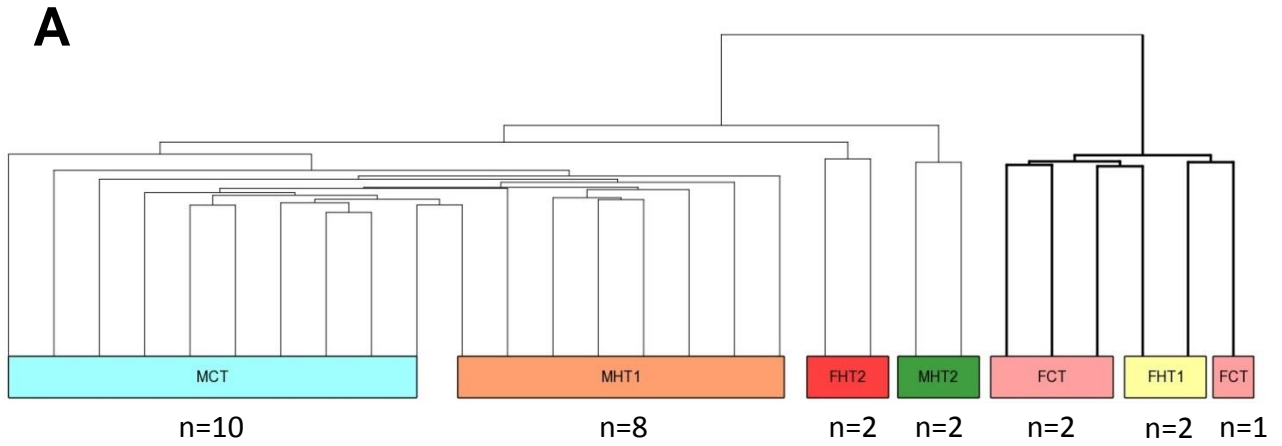
A

MCT	MHT1	FHT2	MHT2	FCT	FHT1	FCT
n=10	n=8	n=2	n=2	n=2	n=2	n=1

B

Observed proportions in HT fish (see Fig. 1D)	Expected in a sample of n= 10 HT males	Observed transcriptomic profile in HT males

10% females $\longrightarrow$ 0

60% neomales $\longrightarrow$ 6–7

30% males $\longrightarrow$ 3–4

2 $\longrightarrow$ 2 MHT2

4

4 $\longrightarrow$ 8 MHT1

C

Probability, $P(x)$

Number of neomales in a sample of 10 HT males

Fig. S4. Zebrafish phenotypes obtained after heat treatment. (**A**) Hierarchical clustering of adult control and heated zebrafish based on sex-related differentially expressed transcripts between MCT vs. FCT. The result supports the PCA plot shown in Fig. 1*E* and further strengthens the observation that the expression profile of FHT2 females is very similar to those of the males (MCT, MHT1 and MHT2). (**B**) Expected number of males and neomales and observed number of male transcriptomes. (**C**) The binomial probability plot corroborates that the number of neomales in a sample of 10 HT males should be between 6 and 7. See Table S2 for group abbreviations.
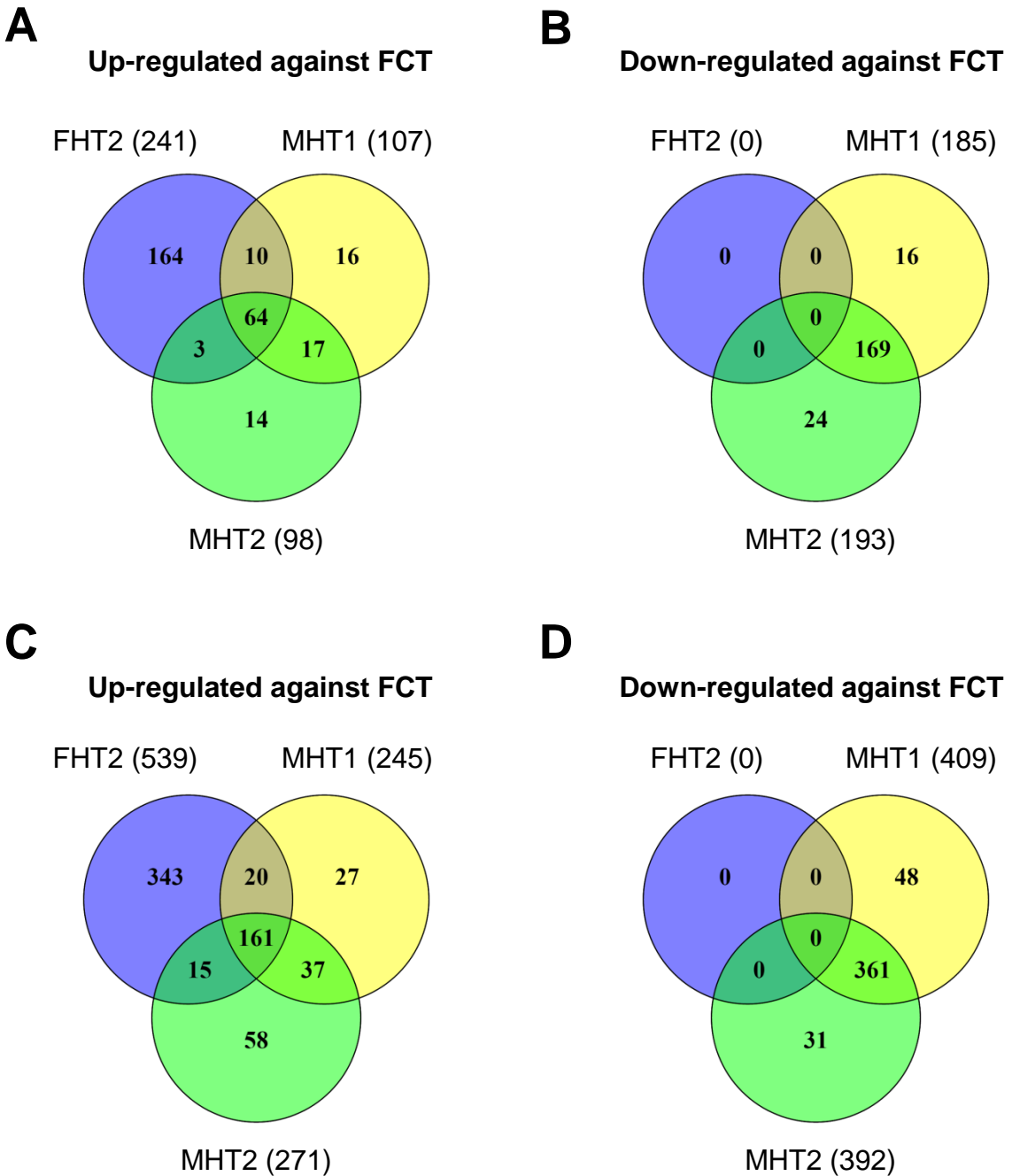
A

Up-regulated against FCT

FHT2 (241)   MHT1 (107)

164   10   16

64

3   17

14

MHT2 (98)

B

Down-regulated against FCT

FHT2 (0)   MHT1 (185)

0   0   16

0

0   169

24

MHT2 (193)

C

Up-regulated against FCT

FHT2 (539)   MHT1 (245)

343   20   27

161

15   37

58

MHT2 (271)

D

Down-regulated against FCT

FHT2 (0)   MHT1 (409)

0   0   48

0

0   361

31

MHT2 (392)

Fig. S5. Venn diagrams showing differentially expressed Cellular Component (**A**, **B**) and Molecular Functions (**C**, **D**) GO terms in heat-exposed zebrafish as determined by the expression microarray (Fold Change, 1.5x ; False Discovery Rate, $P \leq 0.05$). (**A**) Cellular Component GO pathways up-regulated in MHT1, MHT2 or FHT2 in comparison to FCT; (**B**) Cellular Component GO pathways down-regulated in MHT1, MHT2 or FHT2 in comparison to FCT; (**C**) Molecular Functions GO pathways up-regulated in MHT1, MHT2 or FHT2 in comparison to FCT; (**D**) Molecular Functions GO pathways down-regulated in MHT1, MHT2 or FHT2 in comparison to FCT (see Fig. 2*B* and *C* for similar Venn diagrams showing differentially expressed Biological Process GO pathways, as well as Table S5 for the complete list of all GO pathways). See Table S2 for group abbreviations.
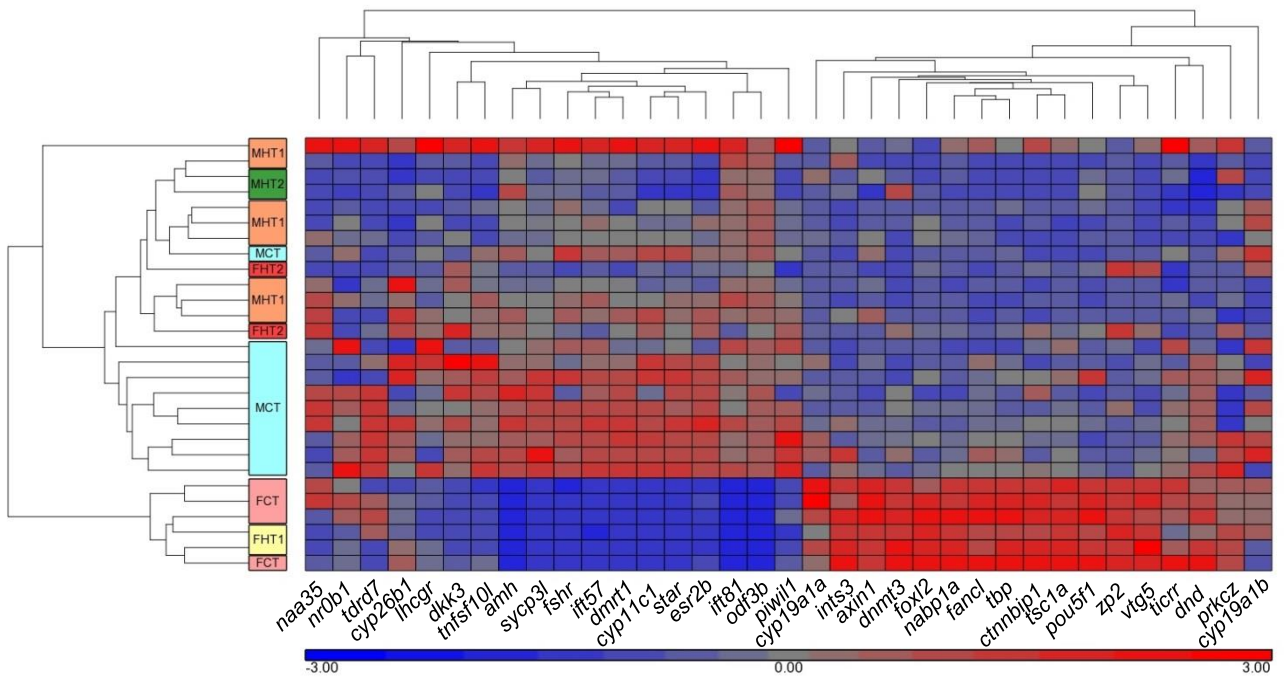
Fig. S6. Heatmap generated using the expression level of 35 genes in 90 dpf adults showing sex- and temperature-dependent transcriptomic profiles. Genes were selected either based on their putative role in reproduction or on their expression profile by qPCR among the different gonadal transcriptomal types. Gene name are listed in Table S7.
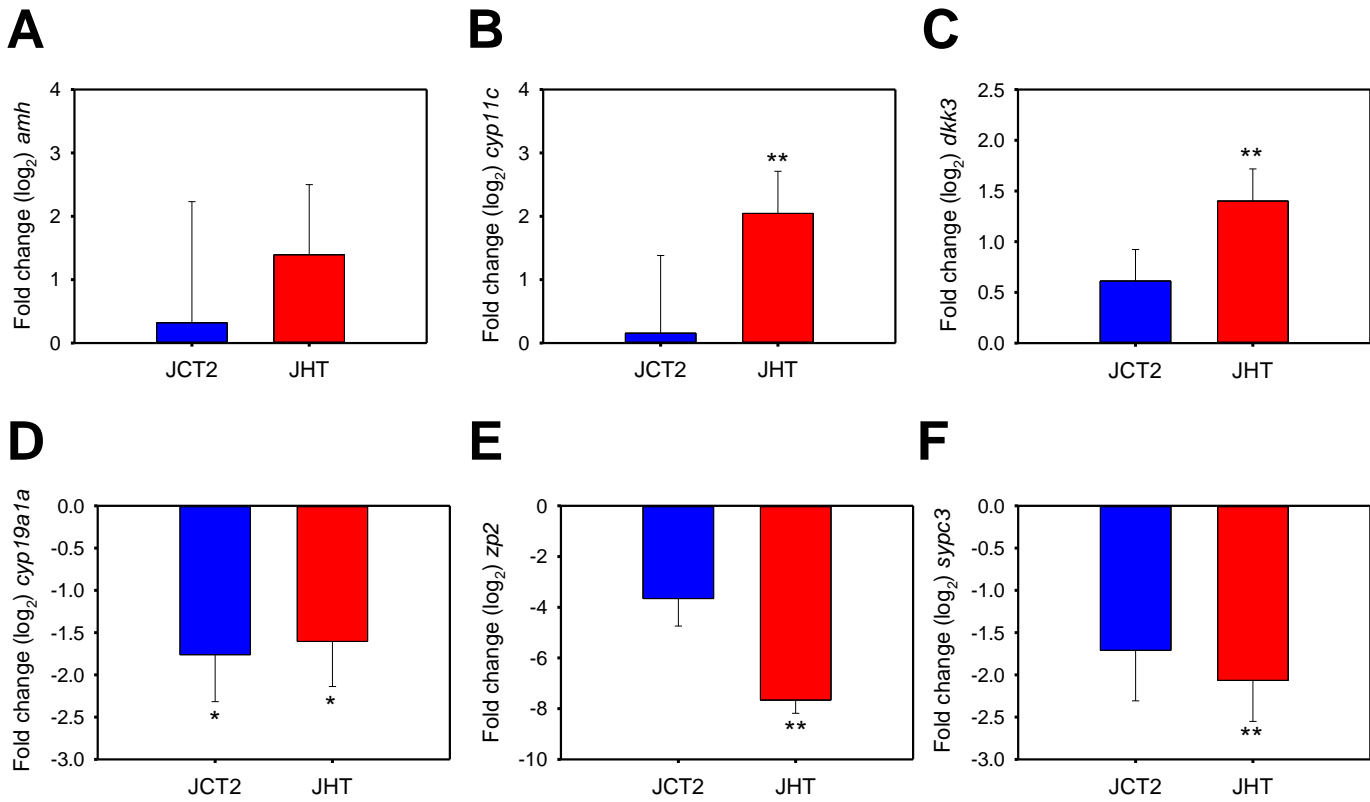
Fig. S7. Gene expression levels of six candidate genes with sex-associated function determined by qPCR at 37 dpf indicated that the juvenile heated group (JHT) transcriptome analysis shown in Fig. 4 corresponds to male gonads. Three genes with a 'pro-male' function were tested: *amh* (**A**), *cyp11c* (**B**), *dkk3* (**C**), and also three with 'pro-female' function: *cyp19a1a* (**D**), *zp2* (**E**) and *sypc3* (**F**). All comparison are against to control females (JCT1). Values that show statistically significant differences (Student's *t*-test) with JCT1 females (n = 5) are labeled with * = $P < 0.05$ or ** = $P < 0.01$. Data as mean ± s.e.m. of three (JCT2) or ten (JHT) individuals.