**Ovarian transcriptomic signatures of zebrafish females
resistant to different environmental perturbations**

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Keywords:	environmental stress, density, temperature, gonad, molecular markers, masculinization

Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations

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Abbreviated title (running headline): Transcriptomic similarities in fish resistant to masculinization

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Abstract

Sex is remarkably plastic in fish and can be easily influenced by environmental cues, of which temperature has been the most studied abiotic factor. However, although it has been shown that elevated population densities can increase the number of males in several species, little is known about the underlying molecular mechanisms and whether general patterns exist. Here, we studied the long-term effects of population density on the gene expression program in zebrafish gonads. The ovarian transcriptome of females exposed to high vs. low population densities contained 4,341 differentially expressed genes. Among them, a set of pro-male genes (*amh*, *sypc3*, *spata6* and *sox3*) upregulated in the ovaries was identified. Next, we compared the transcriptomes of ovaries of female zebrafish resistant to the masculinizing effects of either high density or elevated temperature. Results showed a set of 131 and 242 common upregulated and downregulated genes, respectively, including the upregulation of known male-related genes (e.g., *amh* and *sypc3*) but also genes involved in other functions (e.g., *faima* *ccm21*, *ankrd6b*) and a downregulation of *cyp19a1a* together with other genes (e.g., *lgals9l1*, *ubxn2a*). We identified common GO-terms involved in reproduction and sexual development that were consistently affected in both environmental factors. These results show that regardless of the environmental perturbator there are common genes and cellular functions involved in the resistance to masculinization. These altered gene expression profiles can be used as robust markers indicative of previous exposure to environmental stress independent of conspicuous alterations in sex ratios or gonadal morphology.

Key words: environmental stress, density, temperature, gonad, molecular markers, masculinization

Research highlights

We identify, in otherwise apparently normal fish ovaries, a series of common persistent gene expression changes that can be used as markers indicative of previous exposure to environmental perturbations of both biotic and abiotic origin.

Graphical Abstract Image

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77 Introduction

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79 In fish, some environmental factors can be perceived as stressors if their magnitude exceeds a
80 certain threshold for a given species at a particular sensitive developmental time. The
81 consequences can be ample, including alterations of physiology, reproduction and sex ratios.

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83 Sex determination in fish can be regulated by genetic factors (genetic sex determination, GSD) by
84 influences of the environment (environmental sex determination, ESD) or a combination of both
85 ([Devlin & Nagahama, 2002](#); [Penman & Piferrer, 2008](#)). In GSD species, sex can be determined
86 by the action of single or master gene, by the action of a master gene plus secundari loci or by the
87 combined action of several autosomal genes, the latter called polygenic sex determination (PSD).

88 In ESD, sex can be influenced by different abiotic and biotic factors, of which temperature and
89 population density are the most common ones ([Penman & Piferrer, 2008](#); [Valenzuela, 2008](#)).

90 Nowadays, GSD and ESD are not considered necessarily mutually exclusive and, hence, are
91 regarded as the two ends of a continuum ([Sarre et al., 2004](#); [Heule et al., 2014](#); [Yamamoto et al.,
92 2014](#)).

93

94 For poikylothermic animals like fish, temperature is perhaps the most important abiotic factor.

95 Abnormally elevated temperatures can masculinize many species if exposure takes place during
96 the process of sex differentiation or earlier ([Ospina-Alvarez & Piferrer, 2008](#)). The masculinizing

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3 97 effect of elevated temperature takes place regardless of the underlying sex determining system
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5 98 and thus have been observed in species with male heterogamety (XX/XY) such as, for example,
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7 99 the rainbow trout (*Oncorhynchus mykiss*) ([Valdivia et al., 2014](#)) or the Nile tilapia (*Oreochromis*
8
9 *niloticus*) ([Baras et al., 2001](#)); and species with polygenic sex determination such as the European
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11 100 sea bass (*Dicentrarchus labrax*) ([Saillant et al., 2002](#); [Diaz & Piferrer, 2015](#)).
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13 101
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15 102
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17 103 Regarding population density, it was found that elevated density in the Paradise fish
18
19 104 (*Macropodus opercularis*) ([Francis, 1984](#)) and in the European eel (*Anguilla anguilla*)
20
21 105 ([Roncarati et al., 1997](#); [Huertas & Cerdà, 2006](#)) was able to skew sex ratio towards males. In
22
23 106 contrast, in the European sea bass no effects were observed when fish were confined during early
24
25 107 stages of development ([Saillant et al., 2003](#)). In zebrafish (*Danio rerio*), elevated density resulted
26
27 108 in male-biased sex ratios ([Ribas et al., 2017b](#)) with inter-family variation ([Liew et al., 2012](#);
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29 109 [Ribas et al., 2017c](#)) due to genetic x environmental interactions.
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35 111 Zebrafish is a small cyprinid widely used as an animal model in many research areas. Wild
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37 112 zebrafish strains exhibit a GSD system of the ZW/ZZ type, with a putative sex master gene
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39 113 located in the telomeric region of chromosome 4 ([Wilson et al., 2014](#)). In contrast, in the two
40
41 114 most used laboratory strains (AB and TŪ) this system was not detected probably due to the loss
42
43 115 of the sex determining region during the domestication process ([Wilson et al., 2014](#)). Because of
44
45 116 this, domesticated zebrafish behave as a PSD system with elevated sensitivity to environmental
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47 117 perturbations ([Liew et al., 2012](#); [Ribas et al., 2017a](#)) which explains why zebrafish facilities
48
49 118 across the world usually encounter skewed sex ratios. Thus, laboratory strains of zebrafish
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51 119 constitute indeed a good model where to study the effects of environmental perturbations on the
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53 120 developing gonads and the resulting sex ratios.
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122 Sex differentiation in zebrafish starts from 10 until 45 days post fertilization (dpf). In all

123 individuals, gonads first develop as an ovary-like immature tissue ([Takashi, 1977](#)). Later, ovarian

124 differentiation continues in about half the individuals whereas in the remaining apoptosis takes

125 place and the ovarian tissue regresses and is progressively substituted by testicular tissue ([Uchida](#)

126 [et al., 2002](#); [von Hofsten & Olsson, 2005](#); [Orban et al., 2009](#)). The stress resulting from exposure

127 to elevated temperature during the period of sex differentiation results in masculinization ([Uchida](#)

128 [et al., 2004](#); [Abozaid et al., 2012](#); [Brown et al., 2015](#); [Ribas et al., 2017a](#)) and thus there are some

129 sex-reversed females (referred to as “neomales”) ([Pandian & Sheela, 1995](#)). In addition to regular

130 males and females, two additional transcriptomic profiles were identified in heat-treated fish: the

131 neomales and a novel type of females. These were phenotypic females but with a male-like

132 transcriptomic profile, where an upregulation of male-related genes (e.g., *amh*, *dmrt1*) and a

133 downregulation of female-related genes (e.g., *cyp19a1a*, *foxl2a*, *vgt2*) was observed ([Ribas et al.,](#)

134 [2017a](#)).

135

136 Further, regardless of type of stressor (temperature or density), a common physiological response

137 is the increase plasma cortisol levels ([Ramsay et al., 2006, 2009](#); [Yeh et al., 2013](#)). This is a

138 protective mechanism in response to stress that helps to regulate basal homeostasis ([Bonga,](#)

139 [1997](#)). It is well established that chronic stress has adverse effects in growth and reproduction

140 ([Schreck, 2010](#)) but recently it has also been demonstrated that elevated cortisol levels due to

141 stress are associated with masculinization ([Hattori et al., 2009](#); [Fernandino et al., 2012](#);

142 [Fernandino et al., 2013](#)). Further, oral administration of cortisol during early development

143 resulted in complete masculinization in zebrafish ([Ribas et al., 2017b](#)).

144

However, the knowledge about the underlying mechanisms responsible for the masculinization of the gonads in response to environmental perturbations is still fragmentary. Further, nothing is known about possible hidden alterations, particularly in resistant females, which exhibit an otherwise apparently normal ovarian morphology. Thus, to date most gonadal transcriptomic studies have been focused on the sex-related differences in adult gonads: Olive flounder (*Paralichthys olivaceus*) (Fan et al., 2014); yellow catfish (*Pelteobagrus fulvidraco*) (Lu et al., 2014); zebrafish (Sreenivasan et al., 2008; Small et al., 2009). Few studies reported the influences of elevated temperature on the gonadal transcriptomes, one in zebrafish (Ribas et al., 2017a) and the other in the Nile tilapia (Sun et al., 2018). However, to our knowledge, there are no transcriptomic studies of the effects of density in fish gonads. For this reason, the first objective of this study was to analyze the gonadal transcriptome of zebrafish exposed to elevated density during sex differentiation. The second objective was to compare this data against transcriptomic data of females exposed to elevated temperature from previous experiments reported in our lab (Ribas et al., 2017a). The ultimate goal was to identify a series of common persistent gene expression changes that can be used as markers indicative of previous exposure to environmental perturbations of both biotic and abiotic origin.

Materials and methods

Gonad samples and gonadal maturation data

Gonad maturation data and RNA samples of zebrafish exposed to different rearing were obtained from fish described in “Experiment 1: effects of stocking density” in Ribas et al. (2017b). Briefly, the zebrafish larvae from four independent families were reared from 6 to 90 dpf at four different densities: 9 (control, low density, LD), 17, 37 and 74 (high density, HD) fish/litre. At 90 dpf

gonads were dissected and classified according to the degree of maturation as immature (type 1), maturing (type 2) or mature gonad (type 3) ([Ribas et al., 2017a](#)).

RNA extraction and cDNA synthesis and primers

RNA was extracted from n = 8 fish per sex and density (n = 16 for each density) for qPCR analysis (**Table S1**) using 400 µl of Trizol reagent (Invitrogen, CA, USA). RNA quality was measured with a NanoDrop 1000 spectrophotometer (260/230 and 260/280 nm absorbance ratios). After removing the genomic DNA with DNase I (Deoxyribonuclease I Amplification Grade, Invitrogen, CA, USA), the cDNA was synthesized from 200 ng of RNA using SuperScript III reverse transcriptase (Invitrogen, CA, USA). Specific primers for target genes were designed using Primer Express® (v3.0) software (Applied Biosystems, USA) and purchased from Invitrogen (Life Technologies, UK). Primers were designed within the inter-exon regions. Primer efficiencies were checked in a pool of male and female cDNA gonad samples by using serial dilutions (1, 1/10, 1/50 1/100 and 1/500). Melting curve analysis was also performed to validate the size of the expected PCR product. The qPCR reactions were performed on a QuantStudio™ 12K Flex System (Applied Biosystems, Life Technologies) using 2 µl of SYBR Green PCR master mix (Applied Biosystems), 0.5 µl for each forward and reverse primers and 2µl cDNA (1/10 dilution) in a total reaction volume of 10 µl per well. The qPCR cycle parameters were an initial denaturing step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min with negative controls. For each sample, the reactions were done in triplicate and the average threshold cycle was calculated using the $2^{-\Delta\Delta C_t}$ method ([Livak & Schmittgen, 2001](#)). Relative gene expression data were normalized against a reference gene, the eukaryotic translation elongation factor 1 alpha 1, like 1 (*eef1a1l1*), previously validated in zebrafish ([McCurley &](#)

[Callard, 2008](#)). The gene symbol, gene name, accession number and forward and reverse sequences for each primer pair used in this study are summarized in the **Table S2**.

Microarray analysis of ovaries obtained from fish exposed to high density

For microarray analysis, the control group (9 fish/liter) and the group exposed to the highest density (37 fish/liter) were used (from now on referred to as the LD and HD groups, respectively). From each group, 6 out of the 8 gonad samples used for qPCR were also used (Table S3) for microarray analysis and hybridized individually in a 4×44 K Agilent platform (G2519F–026437) at the Barcelona Biomedical Research Park (PRBB). Briefly, the cRNA generated from the mRNA of each ovarian sample were prepared for overnight hybridization with the corresponding buffers during 17 h at 65°C and washed on the following day. Hybridized slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies, USA). The results were deposited in Gene Expression Omnibus database (GEO), with the accession number GSE116700.

Common genes and pathways in ovaries of females exposed to either high density or high temperature

Gene expression differences of HD vs. LD females were compared with those of high temperature (HT) vs. low temperature (LT) females, specifically, the females named as FHT2 vs. LT females, described in [Ribas et al. \(2017a\)](#). These FHT2 females were females with apparently normal ovarian morphology but with a male transcriptome. Importantly, the ovarian samples from the density and temperature experiments to be compared had in common: (i) both experiments were performed at the same fish facility; (ii) the laboratory strain used was AB; (iii) the conditions in the control treatments in each experiment (groups LD and LT) were the same:

rearing density of 9 fish/litre and water temperature $28 \pm 1^\circ\text{C}$; (iv) exposure to the environmental perturbations included the sex differentiation period; (v) and all fish were sampled at the same age: 90 dpf.

Statistical analysis of the data

Differences in the proportions of gonad types were analyzed by the Chi-squared test. For the qPCR $\Delta\Delta\text{Ct}$ data, normality was checked with the Kolmogorov-Smirnov test and logarithmic transformations were applied when necessary. The homoscedasticity of variances was checked with the Levene's test. Means were compared by one-way analysis of variance (ANOVA) with a Tukey's post-hoc multiple range test. Significant differences were accepted when $P \leq 0.05$. Data were analyzed by the StatGraphics Centurion (v. 17) software (Open Land, Inc.).

Microarray raw data was processed by Agilent software to avoid saturation and generate feature extraction. The output data were statistically analyzed by R (<http://www.R-project.org/>) language and array normalization was implemented by Quantile method in the Limma package ([Smyth & Speed, 2003](#)). To determine differentially expressed genes (DEGs), an adjusted P -value ≤ 0.05 and fold change (FC) ≥ 1.2 was used as threshold. Microarray validation was carried out by studying the expression of 14 DEGs by qPCR analysis. Gene ontology categories (GO-terms) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from the DEGs were obtained by DAVID software ([Huang Da et al., 2009a, b](#)). Only GO-terms of the Biological processes (BP) category level 2 with an EASE Score (a modified Fisher Exact $P \leq 0.05$) with a minimum of three genes belonging in specific GO-term was considered relevant. The significant GO-terms were plotted by REVIGO software ([Supek et al., 2011](#)). The R software (v 3.4.2) ([Team, 2013](#))

was used with the packages ggplots (v 2.2.1) and gplots, (v 3.0.1) to edit the REVIGO graph and to generate heatmap figures, respectively.

Results

High density affected gonadal maturation in females and males

Elevated rearing density not only masculinized zebrafish in a density-dependent manner ([Ribas et al. \(2017a\)](#)) but in both sexes, there was a progressive increase in the proportion of fish with type 1 (immature) gonads with increasing rearing density and, conversely, a progressive decrease in type 3 (mature) gonads. Significant effects ($P < 0.05$) in the change of proportions with respect to the control group (9 fish/liter) were observed with rearing densities of 37 fish/liter and above (**Fig. S1**).

*High density downregulated gene expression of *cyp19a1a* in ovaries and *nr3c1* in both sexes*

In an exploratory phase of the data, qPCR was used to evaluate how density affected the expression of selected genes related to sexual development and the stress response. In ovaries, the expression of *cyp19a1a* was significantly downregulated at a density of 74 fish/liter (**Fig. 1A**), while the expression of forkhead box L2a (*foxl2a*) was unaffected (**Fig. 1C**). In testes, the expression levels of double sex and mab-3 related transcription factor 1 (*dmrt1*) (**Fig. 1B**) and the anti-Müllerian hormone (*amh*) (**Fig. 1D**) was reduced but differences were not significant. The stress response was assessed by the nuclear receptor subfamily 3 group C member 1 gene (*nr3c1*), also known as the glucocorticoid receptor (GR), which showed a significant downregulation in both ovaries (**Fig. 1E**) and testes (**Fig. 1F**). These results indicate that elevated rearing density affected both sexes.

262 ***Transcriptomic overview and microarray validation***

263 Next, a species-specific and validated microarray was used to compare the transcriptomes of
 264 females of the HD group, i.e., the females that did not become masculinized. These females were
 265 compared with control females. Results showed a total of 4,341 DEGs (adjusted p -value $P \leq$
 266 0.05, $FC > 1$) between the ovaries of fish reared at HD and LD (**Dataset S1**). Microarray data
 267 was fully validated by qPCR in a subset of selected genes covering a ± 4 range of FC values ($R^2 =$
 268 0.83 and $P = 0.0001$) (**Fig. S2**).

270 ***GO-terms associated in ovaries of females exposed to high density***

271 From the up- and downregulated DEGs, a total of 16 and 15 up- and downregulated GO-terms,
 272 respectively, were found associated with the BP category (**Fig. S3**). Among the upregulated GO-
 273 terms (**Fig. S3A**), the most important subcategories were related to reproduction: anatomical
 274 structure morphogenesis (GO:0009653) and developmental process involved in reproduction
 275 (GO:0003006). The former, was represented by 14 genes, of which three are involved in pro-
 276 male pathways (*amh*, *sox3* and *lhb*), two related to meiosis (*mei4* and *sycp3*) and two genes were
 277 ovarian markers (*zp3a.2* and *zp3b*). Of the downregulated GO-terms (**Fig. S3B**), some were
 278 related to reproduction, stress, immunity and growth. For example: anatomical structure
 279 formation involved in morphogenesis (GO:0048646), anatomical structure in morphogenesis
 280 (GO:0009653), immune responses (GO:0006955), responses to external stimulus (GO:0009605),
 281 responses to stress (GO:0006950) and developmental growth (GO:0048589).

283 ***High density promoted the upregulation of pro-male genes in the ovaries***

284 All the DEGs were compared against two independent lists of genes related to sex and stress
 285 responses. One list included a total of 48 genes with proven sex-related functions in zebrafish, as

described in [Ribas et al. \(2017a\)](#), while the other list included genes with proven stress-related responses, as described in [Eissa and Wang \(2016\)](#). The common DEGs obtained for each of the two lists were used for heatmap construction. A total of 24 and 20 genes related to reproduction and stress, respectively, were identified (**Fig. 2**), and allowed the clustering of all but one sample according to rearing density. Regarding sex-related genes (**Fig. 2A**), some male-related genes (i.e. *amh*, *sypc3*, *spata6* and *sox3*) were upregulated in HD ovaries whereas *cyp19a1a* was downregulated, confirming our previous gene expression results obtained by qPCR. Regarding stress-related genes (**Fig. 2B**), 15 out of the 20 genes shown in the heatmap were downregulated in the HD group (e.g., *nudt3b*, *hmox2b*, *b2nl*, *cnrl1*) and five (*nud*, *hsp14*, *duox*, *mstnb*, *hsp90aa.1.1*) were upregulated.

Common genes in ovaries of females subjected to high density or high temperature

Elevated rearing density or temperature during the sensitive period of sexual development result in masculinization of some females (**Fig. S4**), which is easy to quantify by sex ratio analysis. However, while all non-masculinized females have apparently normal ovaries, at least morphologically, at least some females can have an altered transcriptome, sometimes more similar to that of males than to that of unexposed females. Here, we wanted to know what the ovaries of resistant females have in common regardless of whether fish have been exposed to elevated density or temperature. Thus, we compared the reported ovarian transcriptomic results of the HD vs. the LD groups with those obtained comparing the HT vs. LT groups previously described in our laboratory ([Ribas et al., 2017a](#)). We found 1,269 DEGs (**Fig. S5**) commonly regulated and were represented according to their FC (**Fig. 3A**) and to their expression tendency (**Fig. 3B**). We classified them in four sets (**Dataset 2**): set A (n=131) genes that were consistently upregulated by density and temperature; set B (n=550) genes that were upregulated by density

but downregulated by temperature; set C (n=346) genes that were downregulated by density but upregulated by temperature and set D (n=242) genes that were consistently downregulated by both factors. Further analyses were done with the sets A and D and we selected the top ten genes by the highest FC as a result of HD (**Table 1**). In set A, we found, for example the fas apoptotic inhibitory molecule a (*faima*), tyrosine phosphatase containing 1 (*ptpdc1a*) and cerebral cavernous malformation 2-like (*ccm2l*) genes. The set D contained, among others, galectin 9 (*lgals9l1*), deoxyribonuclease II lysosomal (*dnase2*) and the *cyp19a1a* genes.

Common GO-terms in ovaries of females subjected to high density or high temperature

We analyzed separately the genes of the set A and D by DAVID to inquire into the BP GO-terms. There were a total of nine GO-terms in set A (**Fig. 4A**) including cellular component organization (GO:0016043) and negative regulation of response to stimulus (GO: GO:0048583); and eight GO-terms in set D (**Fig. 4B**), including cellular metabolic process (GO:0044237) and methylation (GO:0006306). The three most significant GO-terms in set A were single-organism developmental process (GO:0044767), single-multicellular organism process (GO:0044707) and anatomical structure morphogenesis (GO:0009653) (**Fig. 5A, B and C**), whereas in set D were macromolecule localization (GO:0033036), primary metabolic process (GO:0044238) and cellular metabolic process (GO:0044237) (**Fig. 5D, E and F**). Next, for exploratory analysis, we looked at the top three genes (with the highest FC in the density experiment) in order to further identify those genes commonly altered in both perturbations (**Table S4**). For example, in the single-multicellular organism process, we found kinase D-interacting substrate 220b (*kidins220b*) gene upregulated 1.93 and 3.77 times in the density and temperature experiments, respectively, and in the primary metabolic process GO-term, we found UBX domain protein 2A (*ubxn2a*) gene downregulated -4.3 and -3.98 times in the density and temperature experiments, respectively.

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3 334 Interestingly, FC values of genes altered by temperature were higher those altered by density
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5 335 (Fig. 6A).
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10 337 **KEGG pathways in ovaries of females subjected to high density or high temperature**
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12 338 There were no common KEGG pathways related to the DEGs conforming the A or D sets,
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14 339 probably due to the relatively low number of genes in each set. However, the expression of genes
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16 340 involved in reproduction-related KEGG pathways in zebrafish ([Ribas et al., 2017a](#)), namely, the
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18 341 Wnt signalling (dre04310), homologous recombination (HR, dre03440), transforming growth
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20 342 factor- β (TGF- β , dre04350), oocyte meiosis (OM, dre04114), p53 signalling (dre04115) and
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22 343 steroid hormone biosynthesis (SHP, dre00140) pathways had the same direction of change
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24 344 regardless of perturbation (Fig. 6B). In the Wnt signalling pathway, there were two
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26 345 downregulated genes (axin 2 conductin-axil, *axin2* and protein kinase cAMP-dependent catalytic
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28 346 alpha genome duplicate b, *prkacab*) and one upregulated gene (transforming protein RhoA,
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30 347 *rhoa*). *Rhoa* also belongs to the TGF- β pathway together with *amh*, which was also upregulated.
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32 348 In the HR signalling pathway, *sycp3* was upregulated. The phosphatase and tensin homolog B
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34 349 (*ptenb*), golgin rab6-interacting (*gorab*), the F-box protein 5 (*fbxo5*) and *cyp19a1a* genes
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36 350 belonging to the p53 signalling, OM and SHP pathways, respectively, were downregulated.
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40 352 The KEGG pathways from the 4,341 DEGs found in the density experiment were compared to
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42 353 the KEGG pathways reported in the ovaries of those resistant females treated with elevated
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44 354 temperature ([Ribas et al., 2017a](#)) and no common pathways were found, so we listed in Table 2,
45
46 355 the significant up-and downregulated KEGG pathways found in the density experiment and the
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48 356 four most significant up-and downregulated KEGG pathways found in the temperature
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50 357 experiment. In particular, in the ovaries of fish subjected to density, we found an upregulation of
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the gonadotropin-releasing hormone receptor pathway (dre04080) and a downregulation of five KEGG pathways, for example, the integrin signalling pathway (dre04510). For the temperature factor, the most up-and downregulated pathways were the cardiac muscle contraction (dre04260) and the protein processing in endoplasmic reticulum (dre04141).

Discussion

Fish gonadal development is quite plastic and in many species, the response to environmental perturbations that encompass the period of sex differentiation is masculinization ([Ospina-Alvarez & Piferrer, 2008](#); [Baroiller & D'Cotta, 2016](#); [Piferrer, 2018](#)). The masculinization of genotypic females generates phenotypic males ([Hliwa et al., 2013](#)) that are termed neomales ([Pandian & Sheela, 1995](#)). These neomales are capable of producing viable sperm ([Piferrer, 2001](#)). The remaining females, i.e., non-masculinized females, were thought to be regular females resistant to elevated temperature. However, recently it was found in zebrafish exposed to HT that some of these females in fact have a male-like transcriptome ([Ribas et al., 2017a](#)). In the present study, we showed that these females with apparently normal morphology but with a male-like transcriptome also appear in populations partially masculinized by elevated rearing density and consequently we were interested in finding out what are the common transcriptomic signatures of these resistant fish. Our data, therefore, not only provides the first comprehensive ovarian transcriptomic analysis of the long-term effects of elevated population density, but also identify common markers of previous environmental perturbation regardless of the type of perturbation.

Elevated population densities progressively delayed gonadal development in males and in females, coinciding also with the progressive masculinization observed earlier ([Ribas et al., 2017b](#)). This maturation delay was also observed in the gonads of zebrafish subjected to high

temperatures ([Ribas et al., 2017a](#)), although in that case it was only significant in males, probably due to the shorter duration of the exposure to temperature when compared to density.

A set of canonical sex-related genes were measured in the gonads of fish exposed to elevated density. In the ovaries, the expression of *cyp19a1a*, was downregulated at the highest density while the expression of *foxl2a*, a transcriptional regulator of *cyp19a1a* ([Wang et al., 2007](#)), remained stable, probably due to the fact that *foxl2* has two transcript variants: *foxl2a* and *foxl2b*, and both transcripts cooperate to regulate development and maintenance of the ovary ([Yang et al., 2017](#)) and it is possible that the other variant could be expressed instead. The downregulation of *cyp19a1a* in ovaries was described in zebrafish exposed to elevated temperature ([Ribas et al., 2017a](#)) and to hypoxia conditions ([Shang et al., 2006](#)) as well as in other fish species such as the Japanese flounder ([Kitano et al., 1999](#)) and in the European sea bass ([Navarro-Martín et al., 2011](#); [Díaz & Piferrer, 2015](#)) when subjected to high temperatures. Thus, environmental stress reduces the expression of *cyp19a1a* in the gonads and this is in agreement with the observed masculinization since that *cyp19a1a* is necessary for ovarian development, as demonstrated in loss-of-function experiments ([Lau et al., 2016](#))([de Castro Assis et al., 2018](#)). In testes, *dmrt1*, a transcription factor that plays a key role in male sex determination in zebrafish ([Webster et al., 2017](#)), and *amh*, a gene involved in male sexual differentiation by suppressing estrogen production ([Rodríguez-Mari et al., 2005](#)), did not show differences in gene expression due to high density confinement, suggesting no conspicuous effects of density at least in these two important genes for male sexual differentiation, although this, of course, does not exclude possible effects on genes related to spermatogenesis, which were not the focus of this study.

It is known that abiotic and biotic stressors modulate the synthesis of cortisol altering the expression of genes of the hypothalamic-pituitary-interrenal (HPI) axis ([Alsop & Vijayan, 2008](#); [Alsop et al., 2009](#)). The GR gene, a ligand-activated nuclear receptor, has a high binding affinity to cortisol and acts in inflammatory responses, cellular proliferation and differentiation in target tissues ([Beck et al., 2009](#)). Fish subjected to chronic stress showed lower GR levels in brain ([Piato et al., 2011](#)). Further, changes in rainbow trout GR expression levels in the HPI axis were found after 7 days of confinement ([Kiilerich et al., 2018](#)). Since cortisol exerts different effects on various organ systems, it is likely that the functional targets of GR are different in each tissue ([Le et al., 2005](#)). Cortisol plasma levels increased after 7 and 14 days of chronic confinement in sea bream (*Sparus aurata*) ([Barton et al., 2005](#)) and rainbow trout ([Kiilerich et al., 2017](#)), respectively, but their levels were recovered in longer confinement experiments (e.g., ~200 days in wedge sole, *Dicologlossa cuneate*) ([Herrera et al., 2015](#)). In a similar manner, no difference in cortisol levels were found in juvenile zebrafish subjected to high density during 45 days ([Ribas et al., 2017b](#)) but, when mature, GR was downregulated in the gonads at the highest densities especially in females. This is, to our knowledge, the first time that a downregulation of GR in the gonads due to chronic stress is reported, since it was not observed in reproductive tissues when zebrafish were exposed, for example, to elevated temperature ([Ribas et al., 2017a](#)). The downregulation found of GR in the gonads was probably to attenuate the physiological effects due to the stress.

Prolonged rearing at high population density affected the expression of more than 4,300 genes in the ovaries of non-masculinized females. Further, analysis of the combined expression levels of only 24 genes related to reproduction and 20 to stress was sufficient to clearly distinguish ovaries of fish subjected to elevated density from those of control fish, indicating that these selected

genes could be used as potential molecular markers of elevated population density. Microarray analysis evidenced a downregulation of *cyp19a1a*, as previously found by qPCR analyses, and an upregulation of a set of genes involved in the pro-male pathway. These male genes were *amh*, *sycp3*, a gene that is a spermatocyte marker for meiotic cells ([Ozaki et al., 2011](#); [Saito et al., 2011](#)), *sox3*, a gene associated with male development ([Sutton et al., 2011](#)), and *spata6*, necessary for the correct progression of spermatogenesis ([Yuan et al., 2015](#)). However, other genes were downregulated: the steroidogenic factor 1 (*nr5a1*) required for testis development ([Wilhelm & Englert, 2002](#)) and the androgen receptor (*ar*). The results were in concordance with those previously described in zebrafish females exposed to elevated temperature ([Ribas et al., 2017a](#)). Regarding genes related to the stress response, 14 out of 20 were downregulated, including heme oxygenase enzymes (*hmox1*, *hmox2a* and *hmox2b*) responsible to protect cells from oxidative stress, and usually upregulated by exposure to acute stress ([Poss & Tonegawa, 1997](#)). There were also changes in five genes of the heat-shock protein family, chaperones that play important roles in the cellular stress response ([Parsell & Lindquist, 1993](#)); three were downregulated (*hsp13*, *hsp14* *hspa9*, *hspb8*) and two upregulated (*hsp14* and *hsp90aa1.1*). The *hsp90aa1.1*, is a crucial gene for cortisol activity since it is a GR cofactor ([Pratt, 1997](#)). In the rare minnow (*Gobiocypris rarus*), *hsp90aa1.1* was upregulated in the hepatopancreas, gills and intestine when subjected to high doses of cadmium ([Liu et al., 2017](#)). In European sea bass exposed to chronic confinement stress *hsp90aa1.1* was downregulated in brain but not in liver ([Gornati et al., 2004](#)). This variability in gene expression may reflect not only tissue-specific stress responses but also species-specific differences in responses to acute vs. chronic stress ([Pottinger, 2008](#)).

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3 452 The comparison of the ovarian transcriptomes of females exposed to either elevated population
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5 453 density or temperature allows insights into what are the conserved signatures irrespective of type
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7 454 of perturbation. It is difficult to say whether zebrafish gonads are more sensitive to elevated
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9 455 temperature or elevated rearing density because a proper estimation would require carefully
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11 456 designed dose-response experiments and comparing, for example, the transcriptomes of females
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13 457 when, say, 50% of the initial females become masculinized either after temperature or density,
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15 458 which is not the case in this study. However, at least it is interesting to notice a certain
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17 459 relationship between the degree of masculinization and the average fold change of genes
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19 460 commonly affected by both factors (temperature > density).
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23 462 Of the 1,269 DEGs shared in both type of perturbations, 373 DEGs were affected in the same
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25 463 direction regardless of environmental perturbation. Among these common genes, *faima* was
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27 464 strongly upregulated (FC = 14.07 and 4.78 in density and temperature experiments, respectively).
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29 465 *Faima* is an inhibitor of the apoptotic response and it is expressed in mammalian ovaries during
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31 466 follicular atresia in the granulosa cells ([Matsuda-Minehata et al., 2006](#)) having similar expression
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33 467 pattern to that of *foxl2* gene in the ovaries ([Nikic & Vaiman, 2004](#)). During sex differentiation,
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35 468 juvenile zebrafish ovaries activate apoptotic pathways and, although further research is needed,
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37 469 *faima* could play a role in the resistance to masculinization and, therefore, could be a potential
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39 470 maker for those females that although subjected to environmental perturbations they still manage
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41 471 to develop ovaries. *Ptpdc1*, upregulated in fish ovaries by elevated density and temperature
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43 472 exposure (FC = 8.64 and 4.59, respectively), is a pleiotropic gene that regulates a variety of
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45 473 cellular processes including cellular growth and differentiation ([Bonetti et al., 2014](#)). Other
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47 474 examples of common upregulated genes were: *ccm2l*, involved in malformation in heart and body
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49 475 axis in zebrafish ([Cullere et al., 2015](#)) and *ankrd6b*, which phosphorylates and inhibits the beta-

catenin signal during zebrafish development ([Moeller et al., 2006](#)). Among those downregulated genes, we found *lgals9ll* (FC temperature = -9.21 and density = -2.85), which belongs to the galectin protein family and is implicated in modulating cell-cell and cell-matrix interactions and its associated with immunodeficiency diseases in humans ([Mengshol et al., 2010](#)). In zebrafish, its expression was identified in larva coping with viral RNA infection ([Briolat et al., 2014](#)). We also found *ubxn2a*, which in humans is involved in apoptosis by activating the p53 signaling pathway ([Sane et al., 2014](#)) and interacts with *spata5* gene during spermatogenesis ([Hein et al., 2015](#)). Regarding canonical reproduction-related genes in the common lists, both stressors downregulated the expression of *cyp19a1a* and upregulated *amh* and *sycp3* in the ovaries of non-masculinized females. It is known that during the gonadal development process in zebrafish, testis development requires an upregulation of *amh* together with a downregulation of *cyp19a1a* in the immature ovary ([Wang & Orban, 2007](#)). Thus, regardless of the exposure some females resisted masculinization even though their *amh* levels were upregulated in the mature ovary. The fact that the upregulation of some pro-male genes and pathways was also observed in ovaries of zebrafish treated with a demethylation agent (decitabine) during sex differentiation, which skewed sex ratios towards females ([Ribas et al., 2017d](#)), may indicate that activation of some pro-male genes is consequence of the presence of external disturbances during sex differentiation rather than the masculinization per se brought by these perturbations. The results presented here, open the possibility that these well-known reproduction-related genes, *cyp19a1a*, *amh* and *sycp3*, but also other genes with other functions such as *faima*, *ccm2l*, *ankrd6b*, *lgals9ll* or *ubxn2a*, might be good markers of environmental perturbation independent of conspicuous alterations in sex ratios or gonadal morphology.

The 373 DEGs affected in the same direction regardless of environmental perturbation yield a total of 17 GO terms. Most of these were related to cellular and metabolic processes, suggesting that the biosynthesis of some compounds were affected. In particular, we found a downregulation of the methylation GO-term which is known to be involved in sex differentiation in fish ([Navarro-Martín et al., 2011](#); [Anastasiadi et al., 2017](#)). This GO-included the RNA (guanine-7-) methyltransferase (*rnmt*), histamine N-methyltransferase (*hnmt*) and *suv420h2* genes, which are three methyltransferases that regulate the epigenome of developing organs and of the germline ([Anway et al., 2008](#)) and their dysfunction is involved in multiple human diseases ([García-Martín et al., 2010](#)). It also contained two more genes: the enhancer of zeste 2 polycomb repressive complex 2 subunit gene (*ezh2*), involved in maintaining the transcriptional repressive state of certain genes and the regulation of male germ cell development in mice ([Mu et al., 2017](#)), and the SET domain containing 3 (*setd3*) gene, which acts as a transcriptional activator of histone H3K36 ([Kim et al., 2011](#)).

There were no significant common KEGG pathways from the 373 DEGs after exposure to either elevated temperature or density. However, comparison of the KEGG pathways significantly affected by elevated density with those affected by elevated temperature showed that there were a total of five significant KEGG pathways in which the gonadotropin-releasing hormone receptor (GnRHr) pathway was activated. GnRH stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both acting synergistically in the reproductive system ([Millar, 2005](#)), and in the seabream was observed that GnRH plays an important role in the regulation of gonadal differentiation preventing the regression towards the testis from the ovary ([Soverchia et al., 2007](#)). In contrast, inflammation mediated by chemokine and cytokine, integrin signalling and angiogenesis pathways were inhibited. These pathways are involved in protective

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3 523 response of cells to pathogens, infection or tissue damage, as observed in zebrafish larvae
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5 524 exposed to pathogens ([Díaz-Pascual et al., 2017](#)). Interestingly, these pathways are also activated
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7 525 during ovarian commitment in zebrafish sex differentiation, an inflammatory response process
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10 526 where the activation of NF- κ B and Wnt signalling pathways are required ([Liew & Orbán, 2013](#)).
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15 528 **Conclusion**
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17 529 Previously, we had shown that exposure of zebrafish to constant elevated population density since
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19 530 early life masculinized some females. In the present study, we show that it also delayed gonadal
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21 531 maturation in both sexes and, further, that resistant females had a gonadal transcriptome similar in
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23 532 many ways to the transcriptome of females resistant to the masculinizing effects of elevated
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25 533 temperature. These transcriptomic changes include the upregulation of some known pro-male
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27 534 genes such as *amh*, *sycp3* and *spata6*, but also of other novel genes not previously related to
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29 535 reproduction, such as *faima*, *ccm2l*, *ankrd6b*. Commonly downregulated genes include *lgals9l1*,
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31 536 *ubxn2a* genes in addition to *cyp19a1a*. These persistent gene expression changes can be used as
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33 537 markers indicative of previous exposure to environmental perturbations of both biotic and abiotic
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35 538 origin present in fish with otherwise apparently normal fish ovaries. If conserved in other species,
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37 539 these markers could be used in the assessment of hidden effects of environmental stress, although
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39 540 it remains to be determined the actual consequences in terms of reproduction in affected females.
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Conflict of interest

The authors declare that there is no conflict of interest.

References

- Abozaid, H., Wessels, S., & Horstgen-Schwark, G. (2012). Elevated temperature applied during gonadal transformation leads to male bias in zebrafish (*Danio rerio*). *Sexual Development*, 6, 201-209. doi:10.1159/000336297
- Alsop, D., Ings, J. S., & Vijayan, M. M. (2009). Adrenocorticotrophic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. *PLoS One*, 4, e6463.
- Alsop, D., & Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 294, R711-R719. doi:10.1152/ajpregu.00671.2007
- Anastasiadi, D., Díaz, N., & Piferrer, F. (2017). Small ocean temperature increases elicit stage-dependent changes in DNA methylation and gene expression in a fish, the European sea bass. *Scientific reports*, 7, 12401. doi:10.1038/s41598-017-10861-6
- Anway, M. D., Rekow, S. S., & Skinner, M. K. (2008). Transgenerational epigenetic programming of the embryonic testis transcriptome. *Genomics*, 91, 30-40. doi:10.1016/j.ygeno.2007.10.002
- Baras, E., Jacobs, B., & Mélard, C. (2001). Effect of water temperature on survival, growth and phenotypic sex of mixed (XX-XY) progenies of Nile tilapia *Oreochromis niloticus*. *Aquaculture*, 192, 187-199. doi:10.1016/S0044-8486(00)00452-X
- Baroiller, J. F., & D'Cotta, H. (2016). The reversible sex of Gonochoristic fish: insights and consequences. *Sexual Development*, 10, 242-266. doi:10.1159/000452362
- Barton, B. A., Ribas, L., Acerete, L., & Tort, L. (2005). Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquaculture Research*, 36, 172-179. doi:10.1111/j.1365-2109.2004.01202.x
- Beck, I. M., Vanden Berghe, W., Vermeulen, L., Yamamoto, K. R., Haegeman, G., & De Bosscher, K. (2009). Crosstalk in inflammation: the interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocrine reviews*, 30, 830-882. doi:10.1210/er.2009-0013
- Bonetti, M., Rodriguez-Martinez, V., Overman, J. P., Overvoorde, J., van Eekelen, M., Jopling, C., & den Hertog, J. (2014). Distinct and overlapping functions of ptpn11 genes in zebrafish development. *PLoS One*, 9, e94884. doi:10.1371/journal.pone.0094884
- Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews*, 77, 591-625. doi:10.1152/physrev.1997.77.3.591
- Briolat, V., Jouneau, L., Carvalho, R., Palha, N., Langevin, C., Herbomel, P., . . . Boudinot, P. (2014). Contrasted innate responses to two viruses in zebrafish: insights into the ancestral repertoire of vertebrate IFN-stimulated genes. *The Journal of Immunology*, 1302611. doi:10.4049/jimmunol.1302611
- Brown, A. R., Owen, S. F., Peters, J., Zhang, Y., Soffker, M., Paull, G. C., . . . Tyler, C. R. (2015). Climate change and pollution speed declines in zebrafish populations.

- 588 *Proceedings of the National Academy of Sciences*, 112, E1237-1246.
 589 doi:10.1073/pnas.1416269112
- 590 Cullere, X., Plovie, E., Bennett, P. M., MacRae, C. A., & Mayadas, T. N. (2015). The cerebral
 591 cavernous malformation proteins CCM2L and CCM2 prevent the activation of the MAP
 592 kinase MEKK3. *Proceedings of the National Academy of Sciences*, 112, 14284-14289.
 593 doi:10.1073/pnas.1510495112
- 594 de Castro Assis, L. H., de Nóbrega, R. H., Gómez-González, N. E., Bogerd, J., & Schulz, R. W.
 595 (2018). Estrogen-induced inhibition of spermatogenesis in zebrafish is largely reversed by
 596 androgen. *Journal of Molecular Endocrinology*, 60, 273-284. doi:10.1530/JME-17-0177
- 597 Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an
 598 overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191-
 599 364. doi:10.1016/S0044-8486(02)00057-1
- 600 Díaz-Pascual, F., Ortiz-Severín, J., Varas, M. A., Allende, M. L., & Chávez, F. P. (2017). In vivo
 601 host-pathogen interaction as revealed by global proteomic profiling of zebrafish larvae.
 602 *Frontiers in cellular and infection microbiology*, 7, 334. doi: 10.3389/fcimb.2017.00334
- 603 Diaz, N., & Piferrer, F. (2015). Lasting effects of early exposure to temperature on the gonadal
 604 transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed
 605 genetic and environmental sex determination. *BMC Genomics*, 16, 679.
 606 doi:10.1186/s12864-015-1862-0
- 607 Eissa, N., & Wang, H. P. (2016). Transcriptional stress responses to environmental and
 608 husbandry stressors in aquaculture species. *Reviews in Aquaculture*, 8, 61-88.
 609 doi:10.1111/raq.12081
- 610 Fan, Z., You, F., Wang, L., Weng, S., Wu, Z., Hu, J., . . . Zhang, P. (2014). Gonadal
 611 transcriptome analysis of male and female olive flounder (*Paralichthys olivaceus*).
 612 *BioMed research international*, 2014. doi:10.1155/2014/291067
- 613 Fernandino, J. I., Hattori, R. S., Kishii, A., Strussmann, C. A., & Somoza, G. M. (2012). The
 614 cortisol and androgen pathways cross talk in high temperature-induced masculinization:
 615 the 11beta-hydroxysteroid dehydrogenase as a key enzyme. *Endocrinology*, 153, 6003-
 616 6011. doi:10.1210/en.2012-1517
- 617 Fernandino, J. I., Hattori, R. S., Moreno Acosta, O. D., Strussmann, C. A., & Somoza, G. M.
 618 (2013). Environmental stress-induced testis differentiation: androgen as a by-product of
 619 cortisol inactivation. *General and comparative endocrinology*, 192, 36-44.
 620 doi:10.1016/j.ygcen.2013.05.024
- 621 Francis, R. C. (1984). The effects of bidirectional selection for social dominance on agonistic
 622 behavior and sex ratios in the paradise fish (*Macropodus opercularis*). *Behaviour*, 90, 25-
 623 44. doi:10.1163/156853984X00542
- 624 García-Martín, E., Martínez, C., Benito-León, J., Calleja, P., Díaz-Sánchez, M., Pisa, D., . . .
 625 Agúndez, J. (2010). Histamine-N-methyl transferase polymorphism and risk for multiple
 626 sclerosis. *European journal of neurology*, 17, 335-338. doi:10.1111/j.1468-
 627 1331.2009.02720.x
- 628 Gornati, R., Terova, G., Vigetti, D., Prati, M., Saroglia, M., & Bernardini, G. (2004). Effects of
 629 population density on seabass (*Dicentrarchus labrax*, L.) gene expression. *Aquaculture*,
 630 230, 229-239.
- 631 Hattori, R. S., Fernandino, J. I., Kishii, A., Kimura, H., Kinno, T., Oura, M., . . . Watanabe, S.
 632 (2009). Cortisol-induced masculinization: does thermal stress affect gonadal fate in
 633 pejerrey, a teleost fish with temperature-dependent sex determination? *PLoS One*, 4,
 634 e6548. doi:10.1371/journal.pone.0006548

- Hein, M. Y., Hubner, N. C., Poser, I., Cox, J., Nagaraj, N., Toyoda, Y., . . . Buchholz, F. (2015). A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell*, *163*, 712-723. doi:10.1016/j.cell.2015.09.053
- Herrera, M., Ruiz-Jarabo, I., Vargas-Chacoff, L., la Roca, E., & Mancera, J. M. (2015). Metabolic enzyme activities in relation to crowding stress in the wedge sole (*Dicologlossa cuneata*). *Aquaculture Research*, *46*, 2808-2818. doi:10.1111/are.12440
- Heule, C., Salzburger, W., & Böhne, A. (2014). Genetics of sexual development: an evolutionary playground for fish. *Genetics*, *196*, 579-591. doi:10.1534/genetics.114.161158
- Hliwa, P., Bah, M., Kuźmiński, H., Dobosz, S., & Ciereszko, A. (2013). Ultrasound evaluation of the gonadal structure in sex-reversed rainbow trout females. *Aquaculture International*, *22*, 89-96. doi:10.1007/s10499-013-9646-5
- Huang Da, W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research*, *37*, 1-13. doi:10.1093/nar/gkn923
- Huang Da, W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols*, *4*, 44-57. doi:10.1038/nprot.2008.211
- Huertas, M., & Cerdà, J. (2006). Stocking density at early developmental stages affects growth and sex ratio in the European eel (*Anguilla anguilla*). *The Biological Bulletin*, *211*, 286-296. doi:10.2307/4134550
- Kiilerich, P., Servili, A., Peron, S., Valotaire, C., Goardon, L., Leguen, I., & Prunet, P. (2018). Regulation of the corticosteroid signalling system in rainbow trout HPI axis during confinement stress. *General and comparative endocrinology*, *258*, 184-193. doi:10.1016/j.ygcen.2017.08.013
- Kiilerich, P., Servili, A., Péron, S., Valotaire, C., Goardon, L., Leguen, I., & Prunet, P. (2017). Regulation of the corticosteroid signalling system in rainbow trout HPI axis during confinement stress. *General and comparative endocrinology*. doi:10.1016/j.ygcen.2017.08.013
- Kim, D. W., Kim, K. B., Kim, J. Y., & Seo, S. B. (2011). Characterization of a novel histone H3K36 methyltransferase setd3 in zebrafish. *Bioscience, biotechnology, and biochemistry*, *75*, 289-294. doi:10.1271/bbb.100648
- Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., & Abe, S. (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). *Journal of Molecular Endocrinology*, *23*, 167-176. doi:10.1677/jme.0.0230167
- Lau, E. S., Zhang, Z., Qin, M., & Ge, W. (2016). Knockout of zebrafish ovarian aromatase gene (*cyp19a1a*) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. *Scientific reports*, *6*, 37357. doi:10.1038/srep37357
- Le, P. P., Friedman, J. R., Schug, J., Brestelli, J. E., Parker, J. B., Bochkis, I. M., & Kaestner, K. H. (2005). Glucocorticoid receptor-dependent gene regulatory networks. *PLoS genetics*, *1*, e16. doi:10.1371/journal.pgen.0010016
- Liew, W. C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K. R., & Orban, L. (2012). Polygenic sex determination system in zebrafish. *PLoS One*, *7*, e34397. doi:10.1371/journal.pone.0034397
- Liew, W. C., & Orbán, L. (2013). Zebrafish sex: a complicated affair. *Briefings in functional genomics*, *13*, 172-187. doi:10.1093/bfpg/elt041

- Liu, X., Wang, Z., Jin, L., Huang, J., Pu, D. Y., Wang, D. S., & Zhang, Y. G. (2017). Effects of subchronic exposure to waterborne cadmium on HPI axis hormones and related genes in rare minnows (*Gobiocypris rarus*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 202, 1-11. doi:10.1016/j.cbpc.2017.07.002
- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*, 25, 402-408. doi:10.1006/meth.2001.1262
- Lu, J., Luan, P., Zhang, X., Xue, S., Peng, L., Mahbooband, S., & Sun, X. (2014). Gonadal transcriptomic analysis of yellow catfish (*Pelteobagrus fulvidraco*): identification of sex-related genes and genetic markers. *Physiological genomics*, 46, 798-807. doi:10.1152/physiolgenomics.00088.2014
- Matsuda-Minehata, F., Inoue, N., Goto, Y., & Manabe, N. (2006). The regulation of ovarian granulosa cell death by pro-and anti-apoptotic molecules. *Journal of Reproduction and Development*, 52, 695-705. doi:10.1262/jrd.18069
- McCurley, A. T., & Callard, G. V. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC molecular biology*, 9, 102. doi:10.1186/1471-2199-9-102
- Mengshol, J. A., Golden-Mason, L., Arikawa, T., Smith, M., Niki, T., McWilliams, R., . . . Rangachari, M. (2010). Correction: A Crucial Role for Kupffer Cell-Derived Galectin-9 in Regulation of T Cell Immunity in Hepatitis C Infection. *PLoS One*, 5. doi:10.1371/annotation/d15b793c-85c7-4529-bc80-aabcb088a8cf
- Millar, R. P. (2005). GnRHs and GnRH receptors. *Animal reproduction science*, 88, 5-28. doi:10.1016/j.anireprosci.2005.05.032
- Moeller, H., Jenny, A., Schaeffer, H. J., Schwarz-Romond, T., Mlodzik, M., Hammerschmidt, M., & Birchmeier, W. (2006). Diversin regulates heart formation and gastrulation movements in development. *Proceedings of the National Academy of Sciences*, 103, 15900-15905. doi:10.1073/pnas.0603808103
- Mu, W., Starmer, J., Shibata, Y., Yee, D., & Magnuson, T. (2017). EZH1 in germ cells safeguards the function of PRC2 during spermatogenesis. *Developmental biology*, 424, 198-207. doi:10.1016/j.ydbio.2017.02.017
- Navarro-Martín, L., Viñas, J., Ribas, L., Díaz, N., Gutiérrez, A., Di Croce, L., & Piferrer, F. (2011). DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLoS genetics*, 7, e1002447. doi:doi.org/10.1371/journal.pgen.1002447
- Nikic, S., & Vaiman, D. (2004). Conserved patterns of gene expression in mice and goats in the vicinity of the Polled Intersex Syndrome (PIS) locus. *Chromosome research*, 12, 465-474. doi:10.1023/B:CHRO.0000034746.46789.e0
- Orban, L., Sreenivasan, R., & Olsson, P. E. (2009). Long and winding roads: testis differentiation in zebrafish. *Molecular and cellular endocrinology*, 312, 35-41. doi:10.1016/j.mce.2009.04.014
- Ospina-Alvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLoS One*, 3, e2837. doi:10.1371/journal.pone.0002837
- Ozaki, Y., Saito, K., Shinya, M., Kawasaki, T., & Sakai, N. (2011). Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. *Gene Expression Patterns*, 11, 309-315. doi:10.1016/j.gep.2011.03.002

- Pandian, T., & Sheela, S. (1995). Hormonal induction of sex reversal in fish. *Aquaculture*, 138, 1-22. doi:10.1016/0044-8486(95)01075-0
- Parsell, D. A., & Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics*, 27, 437-496. doi:10.1146/annurev.ge.27.120193.002253
- Penman, D. J., & Piferrer, F. (2008). Fish gonadogenesis. Part I: genetic and environmental mechanisms of sex determination. *Reviews in Fisheries Science*, 16, 16-34. doi:10.1080/10641260802324610
- Piato, Á. L., Capiotti, K. M., Tamborski, A. R., Osés, J. P., Barcellos, L. J., Bogo, M. R., . . . Bonan, C. D. (2011). Unpredictable chronic stress model in zebrafish (*Danio rerio*): behavioral and physiological responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 35, 561-567. doi:10.1016/j.pnpbp.2010.12.018
- Piferrer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. In *Reproductive Biotechnology in Finfish Aquaculture* (pp. 229-281): Elsevier.
- Piferrer, F. (2018). La determinació del sexe. *Atzavara, L'*, 28, 39-60.
- Poss, K. D., & Tonegawa, S. (1997). Reduced stress defense in heme oxygenase 1-deficient cells. *Proceedings of the National Academy of Sciences*, 94, 10925-10930.
- Pottinger, T. G. (2008). The stress response in fish-mechanisms, effects and measurement. *Fish Welfare. Blackwell Publishing Ltd, UK*, 32-48.
- Pratt, W. B. (1997). The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via map kinase. *Annual Review of Pharmacology and Toxicology*, 37, 297-326. doi:10.1146/annurev.pharmtox.37.1.297
- Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2006). Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture*, 258, 565-574. doi:10.1016/j.aquaculture.2006.04.020
- Ramsay, J. M., Feist, G. W., Varga, Z. M., Westerfield, M., Kent, M. L., & Schreck, C. B. (2009). Whole-body cortisol response of zebrafish to acute net handling stress. *Aquaculture*, 297, 157-162. doi:10.1016/j.aquaculture.2009.08.035
- Ribas, L., Liew, W. C., Diaz, N., Sreenivasan, R., Orban, L., & Piferrer, F. (2017a). Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proceedings of the National Academy of Sciences*, 114, E941-E950. doi:10.1073/pnas.1609411114
- Ribas, L., Valdivieso, A., Diaz, N., & Piferrer, F. (2017b). Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology*, 220, 1056-1064. doi:10.1242/jeb.144980
- Ribas, L., Valdivieso, A., Diaz, N., & Piferrer, F. (2017c). Response to "The importance of controlling genetic variation - remarks on 'Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response'". *Journal of Experimental Biology*, 220, 4079-4080. doi:10.1242/jeb.167437
- Ribas, L., Vanezis, K., Imués, M., & Piferrer, F. (2017d). Treatment with a DNA methyltransferase inhibitor feminizes zebrafish and induces long-term expression changes in the gonads. *Epigenetics & chromatin*, 10, 59. doi:10.1186/s13072-017-0168-7
- Rodriguez-Mari, A., Yan, Y. L., Bremiller, R. A., Wilson, C., Canestro, C., & Postlethwait, J. H. (2005). Characterization and expression pattern of zebrafish Anti-Mullerian hormone (Amh) relative to sox9a, sox9b, and cyp19a1a, during gonad development. *Gene Expression Patterns*, 5, 655-667. doi:10.1016/j.modgep.2005.02.008

- Roncarati, A., Melotti, P., Mordenti, O., & Gennari, L. (1997). Influence of stocking density of European eel (*Anguilla anguilla*, L.) elvers on sex differentiation and zootechnical performances. *Journal of Applied Ichthyology*, *13*, 131-136. doi:10.1111/j.1439-0426.1997.tb00112.x
- Saillant, E., Fostier, A., Haffray, P., Menu, B., Laureau, S., Thimonier, J., & Chatain, B. (2003). Effects of rearing density, size grading and parental factors on sex ratios of the sea bass (*Dicentrarchus labrax* L.) in intensive aquaculture. *Aquaculture*, *221*, 183-206. doi:10.1016/s0044-8486(02)00539-2
- Saillant, E., Fostier, A., Haffray, P., Menu, B., Thimonier, J., & Chatain, B. (2002). Temperature effects and genotype-temperature interactions on sex determination in the European sea bass (*Dicentrarchus labrax* L.). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *292*, 494-505. doi:10.1534/genetics.114.161158
- Saito, K., Siegfried, K. R., Nüsslein-Volhard, C., & Sakai, N. (2011). Isolation and cytogenetic characterization of zebrafish meiotic prophase I mutants. *Developmental Dynamics*, *240*, 1779-1792. doi:10.1002/dvdy.22661
- Sane, S., Abdullah, A., Boudreau, D., Autenried, R., Gupta, B., Wang, X., . . . Telleria, C. (2014). Ubiquitin-like (UBX)-domain-containing protein, UBXN2A, promotes cell death by interfering with the p53-Mortalin interactions in colon cancer cells. *Cell death & disease*, *5*, e1118. doi:10.1038/cddis.2014.100
- Sarre, S. D., Georges, A., & Quinn, A. (2004). The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. *Bioessays*, *26*, 639-645. doi:10.1002/bies.20050
- Schartl, M. (2004). Sex chromosome evolution in non-mammalian vertebrates. *Current opinion in genetics & development*, *14*, 634-641. doi:10.1016/j.gde.2004.09.005
- Schreck, C. B. (2010). Stress and fish reproduction: the roles of allostasis and hormesis. *General and comparative endocrinology*, *165*, 549-556. doi:10.1016/j.ygcen.2009.07.004
- Shang, E. H., Yu, R. M., & Wu, R. S. (2006). Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). *Environmental science & technology*, *40*, 3118-3122.
- Small, C. M., Carney, G. E., Mo, Q., Vannucci, M., & Jones, A. G. (2009). A microarray analysis of sex- and gonad-biased gene expression in the zebrafish: evidence for masculinization of the transcriptome. *BMC Genomics*, *10*, 579. doi:10.1186/1471-2164-10-579
- Smyth, G. K., & Speed, T. (2003). Normalization of cDNA microarray data. *Methods*, *31*, 265-273. doi:10.1016/s1046-2023(03)00155-5
- Soverchia, L., Carotti, M., Andreu-Vieyra, C., Mosconi, G., Cannella, N., Habibi, H., & Polzonetti-Magni, A. (2007). Role of gonadotropin-releasing hormone (GnRH) in the regulation of gonadal differentiation in the gilthead seabream (*Sparus aurata*). *Molecular Reproduction and Development: Incorporating Gamete Research*, *74*, 57-67. doi:10.1002/mrd.20484
- Sreenivasan, R., Cai, M., Bartfai, R., Wang, X., Christoffels, A., & Orban, L. (2008). Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain. *PLoS One*, *3*, e1791. doi:10.1371/journal.pone.0001791
- Sun, L. X., Teng, J., Zhao, Y., Li, N., Wang, H., & Ji, X. S. (2018). Gonad Transcriptome Analysis of High-Temperature-Treated Females and High-Temperature-Induced Sex-Reversed Neomales in Nile Tilapia. *International journal of molecular sciences*, *19*, 689. doi:10.3390/ijms19030689

- Supek, F., Bosnjak, M., Skunca, N., & Smuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One*, 6, e21800. doi:10.1371/journal.pone.0021800
- Sutton, E., Hughes, J., White, S., Sekido, R., Tan, J., Arboleda, V., . . . Thomas, P. (2011). Identification of SOX3 as an XX male sex reversal gene in mice and humans. *Journal of clinical investigation*, 121, 328-341. doi:10.1172/JCI42580
- Takashi, H. (1977). Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. 北海道大學水産学部研究彙報= BULLETIN OF THE FACULTY OF FISHERIES HOKKAIDO UNIVERSITY, 28, 57-65.
- Team, R. C. (2013). R: A language and environment for statistical computing.
- Uchida, D., Yamashita, M., Kitano, T., & Iguchi, T. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *Journal of Experimental Biology*, 205, 711-718.
- Uchida, D., Yamashita, M., Kitano, T., & Iguchi, T. (2004). An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 137, 11-20. doi:10.1016/s1095-6433(03)00178-8
- Valdivia, K., Jouanno, E., Volff, J. N., Galiana-Arnoux, D., Guyomard, R., Helary, L., . . . Guiguen, Y. (2014). High temperature increases the masculinization rate of the all-female (XX) rainbow trout "Mal" population. *PLoS One*, 9, e113355. doi:10.1371/journal.pone.0113355
- Valenzuela, N. (2008). Sexual development and the evolution of sex determination. *Sexual Development*, 2, 64-72. doi:10.1159/000129691
- Valenzuela, N., & Lance, V. (2004). *Temperature-dependent sex determination in vertebrates*: Smithsonian Books Washington, DC.
- Vandeputte, M., Dupont-Nivet, M., Chavanne, H., & Chatain, B. (2007). A polygenic hypothesis for sex determination in the European sea bass *Dicentrarchus labrax*. *Genetics*, 176, 1049-1057. doi:10.1534/genetics.107.072140
- von Hofsten, J., & Olsson, P. E. (2005). Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. *Reproductive Biology and Endocrinology*, 3, 63. doi:10.1186/1477-7827-3-63
- Wang, D. S., Kobayashi, T., Zhou, L.-Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., . . . Nagahama, Y. (2007). Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Molecular Endocrinology*, 21, 712-725.
- Wang, X. G., & Orban, L. (2007). Anti-Mullerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. *Developmental Dynamics*, 236, 1329-1338. doi:10.1002/dvdy.21129
- Webster, K. A., Schach, U., Ordaz, A., Steinfeld, J. S., Draper, B. W., & Siegfried, K. R. (2017). Dmrt1 is necessary for male sexual development in zebrafish. *Developmental biology*, 422, 33-46. doi:10.1016/j.ydbio.2016.12.008
- Wilhelm, D., & Englert, C. (2002). The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes & Development*, 16, 1839-1851. doi:10.1101/gad.220102

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Wilson, C. A., High, S. K., McCluskey, B. M., Amores, A., Yan, Y. L., Titus, T. A., . . . Postlethwait, J. H. (2014). Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains. *Genetics*, 198, 1291-1308. doi:10.1534/genetics.114.169284

Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S., & Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLoS One*, 9, e102574. doi: 10.1371/journal.pone.0102574

Yang, Y. J., Wang, Y., Li, Z., Zhou, L., & Gui, J. F. (2017). Sequential, divergent, and cooperative requirements of *foxl2a* and *foxl2b* in ovary development and maintenance of zebrafish. *Genetics*, 205, 1551-1572.

Yeh, C. M., Glöck, M., & Ryu, S. (2013). An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish. *PLoS One*, 8, e79406. doi:10.1371/journal.pone.0079406

Yuan, S., Stratton, C. J., Bao, J., Zheng, H., Bhetwal, B. P., Yanagimachi, R., & Yan, W. (2015). *Spata6* is required for normal assembly of the sperm connecting piece and tight head-tail conjunction. *Proceedings of the National Academy of Sciences*, 112, E430-439. doi:10.1073/pnas.1424648112

Table 1. Top ten up- and downregulated genes in the set A and D of genes commonly expressed in ovaries of females exposed to elevated density or temperature

Set	Gene name	Gene symbol	Ref. seq	Density fold change	Temperature fold change
A	fas apoptotic inhibitory molecule a	<i>faima</i>	NM_001002583	14.07	4.78
	tyrosine phosphatase containing 1	<i>ptpdc1a</i>	ENSDART00000081832	8.64	4.59
	cerebral cavernous malformation 2-like	<i>ccm2l</i>	XM_692004	8.39	3.88
	adenylate kinase 7b	<i>ak7b</i>	NM_001109698	3.87	8.76
	eukaryotic translation elongation factor 1 alpha 1, like 2	<i>eef1a1l2</i>	NM_001039985	2.62	4.51
	IQ motif containing with AAA domain 1	<i>iqca1</i>	NM_001098731	2.52	5.64
	WD repeat domain 66	<i>wdr66</i>	XM_692631	2.42	6.25
	zgc:92052	<i>zgc:92052</i>	BC075870	2.31	2.10
	ankyrin repeat domain 6b	<i>ankrd6b</i>	NM_194423	2.21	2.56
	zgc:101737	<i>zgc:101737</i>	BC085428	2.17	17.75
D	galectin 9	<i>lgals9l1</i>	NM_200072	-9.21	-2.85
	deoxyribonuclease II lysosomal	<i>dnase2</i>	NM_001114738	-4.30	-3.98
	zgc:173693	<i>zgc:173693</i>	NM_001130657	-3.90	-4.70
	si:dkey-78l4.12	<i>si:dkey-78l4.12</i>	NM_001083067	-3.02	-2.40
	zgc:162913	<i>zgc:162913</i>	NM_001099250	-2.67	-1.99
	cytochrome P450 family 19 subfamily A polypeptide 1a	<i>cyp19a1a</i>	NM_131154	-2.52	-11.81
	pannexin	<i>panx1a</i>	NM_200916	-2.46	-7.22
	zgc:173480	<i>zgc:173480</i>	ENSDART00000106432	-2.43	-4.85
	calcyon neuron-specific vesicular protein	<i>caly</i>	ENSDART00000052272	-2.39	-2.60
	UBX domain protein 2A	<i>ubxn2a</i>	ENSDART00000128612	-2.39	-8.52

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Table 2. Significant KEGG pathways found in ovaries subjected to high density and the four most significant KEGG pathways reported in the ovaries subjected to temperature ([Ribas et al., 2017a](#))

Factor	Regulation	KEGG pathway description	KEGG ID	No. genes affected	P-value
Density	Upregulated	Gonadotropin releasing hormone receptor	dre04080	36	0,0490
	Downregulated	Integrin signalling	dre04510	33	3.10E-04
		Ras signalling	map04014	20	3.90E-03
		Inflammation mediated by cytokine-cytokine receptor interaction	dre04060	41	7.80E-03

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Temperature	Upregulated	Angiogenesis	dre04370	29	0,0138
		Cardiac muscle contraction	dre04260	60	1.58E-04
		ECM-receptor interaction	dre04512	60	2.16E-04
		Neuroactive ligand-receptor interaction	dre04080	224	5.37E-04
	Downregulated	Cell adhesion molecules (CAMs)	dre04514	86	1.44E-03
		Protein processing in endoplasmic reticulum	dre04141	140	7.25E-10
		ErbB signalling pathway	dre04012	87	1.99E-09
		Ribosome biogenesis in eukaryotes	dre03008	65	1.11E-08
		Phosphatidylinositol signalling system	dre04070	63	1.52E-08

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For Peer Review

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3 916 **Figure legends**
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8 918 **Fig. 1.** Expression of sex-related genes: *cyp19a1a* and *foxl2* in ovaries (**A-C**) and *dmrt1* and *amh*
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10 919 in testes (**B-D**) and of the stress-related gene *nr3c1* in ovaries and testes (**E-F**), respectively.
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12 920 Average values (n=7-8 individuals per treatment) are shown with standard error of the mean
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14 921 (SEM). Fold changes values are shown relative to the 9 fish/litre treatment (control). ANOVA
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16 922 followed by post hoc Tukey test. Statistically significant differences ($P<0.05$) among treatments
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18 923 are indicated by letters.
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24 925 **Fig. 2.** Gene expression represented by heatmaps of (**A**) sex-related and (**B**) stress-related genes
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26 926 in ovaries of females exposed to high density (HD, bright red) and low density (LD, blue). Key
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28 927 color (Row Z-score) represents the level of gene expression for each gene (red: high expression
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30 928 and green: low expression).
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35 930 **Fig. 3.** (**A**) Scatter plot of the fold change (FC) values of the common 1,269 differentially
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37 931 expressed genes (DEGs) found between density (x-axis) and temperature (y-axis). (**B**) Heatmap
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39 932 of the fold change values of the 1,269 DEGs found between density and temperature in sets A, B
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41 933 C and D. The n indicates the number of genes found for each set. Key color (Row Z-score)
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43 934 represents the level of FC for each gene (red: high expression and green: low expression).
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49 936 **Fig. 4.** GO-terms associated to the common genes affected by elevated density or temperature.
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51 937 (**A**) GO-terms associated to set A (red colour), (**B**) GO-terms associated to set D (green colour).
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53 938 For each GO-term graph, the log size indicates the number of genes represented and the colour

saturation indicates the $\log_{10}$ Fisher's P-value along the x-axis. GO-terms are distributed in multidimensional semantic similarities in the y-axis.

Fig. 5. Fold change representation of common upregulated genes of (A) single-organism developmental process, (B) single-multicellular organism process, (C) anatomical structure morphogenesis; and common downregulated genes in (D) macromolecule localization, (E) primary metabolic process, (F) cellular metabolic process of gene ontology terms affected by elevated density and temperature. "n" indicates the number of genes in each case.

Fig. 6. (A) Mean fold change of the total genes found in set A and D groups for density and temperature. (B) Fold change of genes involved in Wnt signalling (dre04310), homologous recombination (HR, dre03440), transforming growth factor- β (TGF- β , dre04350), oocyte meiosis (OM, dre04114), p53 signalling (dre04115) and steroid hormone biosynthesis (SHP, dre00140) pathways for density and temperature factors. Note that *rhoa* belongs to two pathways.

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Low density (LD)
High density (HD)

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