Environmental effects during gonadal development in fish: role of epigenetics

Memòria presentada per Alejandro Valdivieso Muñoz
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Preface

This Ph.D. thesis was carried out at the Group of Biology of Reproduction (GBR) of the Department of Renewable Marine Resources, Institute of Marine Sciences (ICM-CSIC) in Barcelona, under the supervision of Dr. Francesc Piferrer and Dr. Laia Ribas, and under the Ph.D. Genetics Program of the Universitat de Barcelona during the years 2015 to 2020. The aim of this thesis was to contribute to our understanding of the genetic and environmental factors on sex differentiation in zebrafish (*Danio rerio*).

The thesis is structured as follows:

Block A: Density effects on zebrafish

Chapter 1.
Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response.

Chapter 2.
Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations.

Block B: Temperature effects on zebrafish

Chapter 3.
Fish reproductive tissues as heat recorders: DNA methylation epigenetic marks in zebrafish gonads correctly recapitulate past thermal history.

Chapter 4.
Hot sex in wild zebrafish: can the natural genetic sex determination mechanism buffer environmental effects on population sex ratios?

Chapter 5.
Family-dependent variation in the multigenerational effects on sex ratios in zebrafish exposed to elevated temperature: changes in the testicular epigenome of unexposed offspring.

Each chapter corresponds to an article either published, to be submitted or soon to be published:

Ribas, L., Valdivieso, A., Díaz, N., Piferrer, F., 2017. Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *Journal of Experimental Biology*, 220: (6), 1056–1064. doi: 10.1242/jeb.144980.

Valdivieso, A., Ribas, L., Piferrer, F., 2019. Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 332: (3–4), 55–68.

Valdivieso A., Anastasiadi, D., Ribas L., Piferrer F. Fish reproductive tissues as heat recorders: DNA methylation epigenetic marks in zebrafish gonads correctly recapitulate past thermal history. *Epigenetics & Chromatin*. *To be submitted.*

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Valdivieso, A., Ribas, L., Monleon, A., Orban, L. Piferrer, F. Family-dependent variation in the multigenerational effects on sex ratios in zebrafish exposed to elevated temperature: changes in the testicular epigenome of unexposed offspring. *In preparation.*

During the realization of this thesis, Alejandro Valdivieso also contributed in the following articles:

Ribas, L., Valdivieso, A., Díaz, N., Piferrer, F., 2017. 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: (6), 4079–4080. doi: 10.1242/jeb.167437.
Note: this paper contains data presented in chapter 3.

Piferrer, F., Anastasiadi, D., Valdivieso, A., Sánchez-Baizán, N., Moraleda-Prados, J., Ribas, L, 2019. The Model of the Conserved Epigenetic Regulation of Sex. *Frontiers in Genetics*, 10, 857. doi:10.3389/fgene.2019.00857.
Note: this paper is related to chapter 2.

The results obtained in this thesis have been presented at the following scientific meetings:

Alejandro Valdivieso, Laia Ribas, Francesc Piferrer. *Gene expression analysis in gonads of zebrafish (Danio rerio) subjected to masculinization treatments.* Avenços en Recerca en Aqüicultura. Societat Catalana de Biologia (SCB), 12 June 2015. Barcelona (Spain).

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Alejandro Valdivieso, Laia Ribas, Francesc Piferrer. *Thermal transgenerational and rearing density effects in laboratory strains of zebrafish (Danio rerio).* Epiconcept Conference. COST European Cooperation in Science and Technology: COST Action FA1201, Epiconcept, 6–7 October 2015. Creta (Greece).

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Alejandro Valdivieso, Javier Moraleda-Prados, Dafni Anastasiadi, Laia Ribas, Francesc Piferrer. *Metilació de cyp19a1a i dmrt1, dos marcadors del desenvolupament gonadal en el peix zebra (Danio rerio) exposats a diferents perturbacions ambientales*. XVI Jornada de Biologia de la Reproducció. Societat Catalana de Biologia (SCB), 19 May 2019. Barcelona (Spain).

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During the course of this research, Alejandro Valdivieso did two stays of three months each during 2017 and 2018 at the Institute of Neuroscience (ION) at the University of Oregon, USA under the supervision of Dr. John Postlethwait. Travel and living expenses were supported by the Spanish grants: EEBB-I-17–12342 and EEBB-I-18–12947.

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Summary

In many organisms, sex is determined by a combination of genetic and environmental influences. Epigenetic regulatory mechanisms such as DNA methylation integrate both types of influences. However, understanding how genetic and environmental changes shape the sexual phenotype and the role of epigenetics in this process is far from clear.

To address these questions that are of major interest in reproductive and evolutionary biology, in this study we used two zebrafish (*Danio rerio*) wild strains, with an intact sex-determining loci (*sar4*), where sex determination is thought to follow a monofactorial system with female heterogamety (ZZ/ZW). We also used the laboratory AB strain, a consolidated model for many research areas, with loss of *sar4* during the process of domestication, and with polygenic sex determination. Genetic variation was accounted for by using different families of each strain, which were exposed to biotic (rearing density) and abiotic (temperature) environmental perturbations during critical stages of sex differentiation.

Elevated rearing density resulted in lower survival and growth, increased masculinization and delayed gonad maturation. Transcriptomic analysis of the adult gonads showed that masculinization was achieved by upregulation of male–related genes and downregulation of female–related genes and, importantly, the participation of the cortisol-mediated stress response. By comparing the gonadal transcriptomes of females resistant to heat- and crowding-induced stress, a common set of differentially expressed genes were identified, constituting novel biomarkers to aid in the identification of hidden effects environmental perturbations. Temperature was able to alter DNA methylation levels of the regulatory regions of sex– and stress–related genes in a clear sex-related fashion. By using machine-learning procedures, we identified specific methylation profiles of some CpG sites in the promoter regions of key genes (*cyp19a1a*, *amh* and *foxl2a*) involved in sex differentiation and in the response to the environment. In wild strains, we discovered elevated rates of spontaneous sex reversal at control temperature, identified novel sexual genotypes and showed genotype-dependent rates of sex reversal under elevated temperature, with possible consequences in sperm production. Contrary to expectations, the presence of *sar4* in wild strains did not confer higher resistance to temperature when compared to the situation in the AB strain. Finally, effects of elevated temperature on sex ratio and/or DNA methylation in the gonads were inherited, at least in males, in the F1 but only in a family-dependent manner while effects were never detected in the F2.

In summary, we developed novel DNA methylation-based biomarkers capable of predicting phenotypic sex and whether fish had been previously exposed to abnormal environmental conditions, paving the way for similar developments in other species. Taken together, these results contribute to our understanding of the role of DNA methylation in shaping the sexual phenotype and can aid towards obtaining a better picture of how environmental changes may affect natural populations in a global warning scenario.

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General introduction

1. Sex determination and sex differentiation

1.1 Definitions

Sex determination

Sex determination is the genetic or environmental process by which the sex of an individual is established (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). Fish have various types of sex determination mechanisms (see below). Once sex is established, it remains the same throughout life, except in sequential hermaphrodite species, where animals change sex at least once during the lifetime and after first maturation (Pla, 2019).

Sex differentiation

Sex differentiation is the process by which an undifferentiated gonad, through molecular, genetic and physiological mechanisms, becomes a testis or an ovary, constituting the male or female sexual phenotype, i.e., the gonadal sex (Takahashi, 1977; Piferrer and Guiguen, 2008). Molecular and physiological mechanisms thus control the process that will lead to morphological changes in the gonad. Morphological and endocrinology aspects are well understood and fairly conserved among fish (Devlin and Nagahama, 2002).

Fish comprise half of extant vertebrate species and have all known reproduction types present in vertebrates, including gonochorism, hermaphroditism and unisexuality (Atz, 1964; Yamamoto, 1969). Among fishes, teleosts are unique in the sense that they also possess all known systems of sex determination known in vertebrates (Barske and Capel, 2008). In addition, fish exhibit the most plastic forms of sex differentiation (Devlin and Nagahama, 2002) and thus they constitute interesting models for the study of the various forms of sex determination and differentiation and their evolution (Mank et al., 2006; Pla, 2019).

1.2 Major types of sex determination

In gonochoristic vertebrates, animals where the two sexes are separate, two major types of sex-determining systems exist and both are responsible for the final proportion of sexes in the population, also often referred to as the sex ratio. In genotypic sex determination (GSD), there are consistent genetic differences between sexes and the future sex of the animal is established already at fertilization, although the actual expression of the sex determining gene(s) may take place much later. The second system is environmental sex determination (ESD), which involves no consistent genetic differences between the two sexes. In ESD, sex is determined by the magnitude of an environmental cue in sensitive windows during early development (Bull, 1983; Sarre et al., 2004; Penman and Piferrer, 2008; Valenzuela, 2008).

GSD is the dominant type of sex determination in fish, although different forms of GSD appear to have repeatedly evolved in different fish species, which makes it difficult to conclusively determine which one was the ancestral GSD system (Mank et al., 2006). Some studies suggest that the ancestral state could be an autosomal sex determination, hence with no sex chromosomes (Ohno et al., 1968; Traut and Winking, 2001).

The classical view on sex determination considered GSD and ESD as being mutually exclusive (Valenzuela et al., 2003) whereas a more modern view sees them as two ends of a continuum, where different combinations between GSD and ESD are possible (Sarre et al., 2004; Beukeboom and Perrin, 2014). Recently, three rather than two sources of phenotypic variance: genes, environment, and random factors, have been postulated to play an important role in sex determination (Perrin, 2016).

Furthermore, the perception of how sex determination actually occurs has changed. Before, it was considered as a sort of hierarchical and lineal system, where a master gene switch or an environmental cue acted at the top of a signaling cascade, triggering the first step for sex determination (Ohno, 1967). Recently, in some fish it has been demonstrated that there might be different initiating switches that act as interconnected factors (Capel, 2017), with networks of genes acting with antagonistic patterns (Ungewitter and Yao, 2013; Herpin and Schartl, 2015; Windley and Wilhelm, 2015). The primary sex is initiated and then is maintained through strong feedback loops that stimulate the expression of genes associated with a particular sex while silencing or repressing the genes associated with the opposite sex (Munger et al., 2009; Matson et al., 2011; Munger et al., 2013; Capel, 2017). Thus, continued action of genetic and or environmental factors tips the scale toward testis or ovarian differentiation. These networks act at the morphological, physiological and behavioural level, and their structure allows rapid transitions (Capel, 2017).

As mentioned above, in GSD species sex depends essentially on the genetic content inherited from the progenitors. Nevertheless, in contrast to mammals (XX/XY) and birds (ZZ/ZW), in which sex is essentially determined by genes in sex chromosomes, in fish the inheritance of genetic sex determination can be based on:

a) Major sex factors or chromosomal sex determination (CSD)

This includes a monofactorial system, in which there is a single pair or multiple pairs of sex chromosomes, or species with a multifactorial system that have a single pair of sex chromosomes with more than two alleles determining sex (reviewed in Piferrer, 2009). Sex chromosomes can be heteromorphic or homomorphic. In heteromorphic sex chromosomes, visual differences in size or banding patters are present. In contrast, in homomorphic sex chromosomes no differences in shape are found, but they can still be identified by molecular markers, if available, targeting sex-linked regions (Penman and Piferrer, 2008; Schartl et al., 2016). Males can be the heterogametic carrier (XY) in the XY/XX system or the homogametic one (ZZ) in the ZZ/ZW system. In multifactorial systems, there can be three or more known major sex-determining

factors and in this system both sexes the sex chromosomes can be heterogametic (Penman and Piferrer, 2008).

b) Minor sex factors
There are no sex chromosomes proper and the final sex ratio is the fruit of a polyfactorial combination of uncharacterized genetic factors located in several autosomes in the genome, each with a minor additive effect (might act epistatically). This system is also referred as to polygenic sex determination (PSD) and sex ratio fluctuations between families due to maternal/paternal effects are typically observed. In this system, genotype x environment (GxE) interactions are frequent (Penman and Piferrer, 2008).

1.3 Genetic sex determination

Despite the diversity of primary sex-determining mechanisms discovered up to date in vertebrates and in invertebrates, the genes at the top of the cascade in the pathway of sex determination have often changed during evolution. Thus, different species, sometimes even closely related, recruited different genes as the sex-determining gene. In contrast, downstream genes in the sex determination pathway appear to be broadly conserved among vertebrates (Devlin and Nagahama, 2002; Guerrero-Estévez and Moreno-Mendoza, 2010; Guiguen et al., 2018). Thus, changes at the top of the sex-determining pathway seems to be better tolerated than changes lower in the pathway because the top ones are less likely to have deleterious effects (Marin and Baker, 1998; Western and Sinclair, 2001). Understanding the genetic basis of sex determination in fish is important not only to understand reproduction and the evolution of these mechanisms but also in the applied aspects for management of fish aquaculture species (Martínez et al., 2014).

1.3.1 Sex determining genes

The most well-known sex determining gene, sex-determining region on the Y chromosome (*Sry*), is found in therian mammalian (placental mammals and marsupials) (Goodfellow and Lovell-Badge, 1993; McElreavey et al., 1993) which is at the top of the sex determination cascade (Gubbay et al., 1990; Koopman et al., 1991; Sekido and Lovell-Badge, 2009). *Sry* appears to be absent in monotremes and is not present in non-mammalian vertebrates (Wallis et al., 2007). The *Sry* gene has been identified as the master inducer of the testis-determining pathway (Schafer, 1995) and is known to inactivate male development repressors (Jordan et al., 2001; Harley et al., 2003).

In general, the upstream signals for sex determination are diverse in animals, but throughout evolution the downstream molecular regulation of sex determination and differentiation are generally conserved across vertebrates (Marin and Baker, 1998; Western and Sinclair, 2001). This is thought to be due to the morphological organization

of the testis and the ovary, where a set of somatic and germ cells participate (DeFalco and Capel, 2009). Importantly, the same genes involved in sex determination and differentiation are found and often have the same function with sexual dimorphic expression between males and females (Cutting et al., 2013). Interestingly, from the amount of genes involved in gonadal development, a current subset of downstream genes of sex determination that has been repeatedly and independently selected throughout evolution to become master sex determining genes (Schartl, 2004; Graves and Peichel, 2010). Most of the sex determining genes in vertebrates have evolved from genes that have an important role function in promoting testicular differentiation. Thus, aside spatial and temporal differences, control over the initiation and rate of cell proliferation appears to be a common feature. Most of the master sex determining genes are derived from transcription factors (e.g., *sox*, *dmrt* and *irf*) or from growth factors (e.g., *amh* and *gsdf*) although genes with different origin can become master sex genes. In teleosts, thirteen master sex genes have been discovered so far and all are male sex determining like is the mammalian *Sry* (**Table 1**).

Briefly, we discuss some of their functions:

Transcriptions factors

Transcription factors participate in regulating the expression of genes. One distinct feature of transcription factors includes DNA-binding domains that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. Regulation of transcription is the most common form of gene expression control. The action of transcription factors allows for unique expression of each gene in different cell types and during development. Some of the transcription factors known to be involved in sex determination and sex differentiation (Penman and Piferrer, 2008; Shen and Wang, 2014; Herpin and Schartl, 2015) are:

a) SRY-related high mobility group (HMG) containing box (SOX) genes

Sox is a large family of genes which encode transcription factors with high-mobility-group DNA binding domain with diverse roles in development (Lefebvre et al., 2007). Few of them are involved in sex determination and differentiation (Denny et al., 1992; Lefebvre et al., 2007). From this family, different genes, such as *sox9* (testis differentiation), *sox3* (male development repressor function), *sox24* (ovary expressed), *sox17* (involved in spermatogenesis) (Kent et al., 1996; Wang et al., 2003) and *sox19* (ovarian differentiation) (Navarro-Martín et al., 2012) are also well characterized in teleost species (Hett and Ludwig, 2005). Thus, for example, *sox3* and *sox2* genes are thought to be responsible to be involved in male sex determination in Indian ricefish (*Oryzias dancena*) (Takehana et al., 2014) and in turbot (*Scophthalmus maximus*) (Martínez et al., 2019), respectively.

b) Doublesex and Mab-3 related transcription factor 1(dmrt1)

Dmrt1 is an important downstream gene in the male sex determination pathway (reviewed in Ferguson-Smith, 2007) and has been categorized as a master sex gene in

several vertebrates. *Dmrt1* is expressed in the fish developing gonads and has been shown in several fish species to be testis-specific (reviewed in Herpin and Schartl, 2011). Given the important function of this gene, several master sex genes homologous to *dmrt1* are found in other vertebrates due to variants or duplications through evolution. This is the case of *DM-W* in *Xenopus laevis* (Yoshimoto et al., 2010) and *dmrt1* in chicken (Smith et al., 2009). In fish we find two examples, *dmrt1* in half-smooth tongue sole (*Cynoglossus semilaevis*) (Cui et al., 2017) and *dmrt1by* or *dmy* in medaka (*Oryzias latipes*) (Matsuda et al., 2002; Nanda et al., 2002).

c) Interferon regulatory factor 9 (irf9)

Interferon regulatory factors (IRFs) are a family of mediators in various biological processes including immune modulation of interferon (IFN) and proinflammatory cytokine expression (Laghari et al., 2018). The *irf9* gene is an immune-related gene and so no gonadal function was previously known (Yano et al., 2013). *Irf9* evolved to become sexually dimorphic on the Y chromosome (*sdY*), first by a duplication event and then a neofunctionalization until it became the master sex-determining gene in rainbow trout (*Oncorhynchus mykiss*) and other salmonids (Yano et al., 2012).

Growth factors

Growth factors are naturally occurring substances, usually protein or steroid hormones, capable of stimulating cellular growth proliferation, healing, and cellular differentiation. Growth factors are important for regulating a variety of cellular processes. Here we briefly discuss the protein-based growth factors related to gonadal development and we further explain some of them in the "Role of steroidogenic enzymes" section 1.5.3, below.

a) Anti-Müllerian hormone (amh)

Amh is a secreted glycoprotein of the TGF-β superfamily best studied in tetrapods, and causes regression of Müllerian ducts during male sexual development (Yoshinaga et al., 2004; Rodríguez-Marí et al., 2005). It has been described that independent male-specific duplication events of the *amh* transcription factor have become the master sex gene in some teleosts. The species concerned are Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al., 2012), Nile tilapia (*Oreochromis niloticus*) (Li et al., 2015), *lingcod* (*Ophiodon elongatus*) (Rondeau et al., 2016) and the Northern pike (*Esox lucius*) (Pan et al., 2019).

In one case, the receptor of the growth factor *amh* has also become a master sex gene (see below in the "Other genes" section).

b) Gonadal soma-derived factor (gsdf)

Gsdf is a cytokine growth factor from the TGF-β superfamily that is closely related to *amh*. It is only found in fish, and its biochemical function is still not well understood. It has been implicated with the proliferation of primordial germ cells (PGCs) and spermatogonia (Sawatari et al., 2007). It is assumed to have a role in male gonad development due to its exclusive expression in the early differentiating testis of all fish

studied so far (Shibata et al., 2010; Gautier et al., 2011; Crespo et al., 2013). Two different genes have appeared as initiators of sex determination in fish, as occurs with the *gsdf-Y* in *Oryzias luzonensis* (Shibata et al., 2010; Myosho et al., 2012) among other species (**Table 1**).

c) Growth differentiation factor 6 (gdf6)

Gdf6 is another gene that belongs to the TGF-β superfamily. *Gdf6* plays a predominant role in regulating the growth and maturation (differentiation) of bone and cartilage (Settle Jr et al., 2003) and also is involved in the development of the eyes (Hanel and Hensey, 2006). *Gdf6* has been proposed as the a master sex gene (*gdf6-Y*) in *Nothobranchius furzeri* but its mechanism of action needs further research (Reichwald et al., 2015).

Other genes

Other genes have been found to have a major role in sex determination in fish. Except for the receptor of *amh*, most of these findings were made in 2019:

a) Anti-Müllerian hormone receptor 2 (amhr2)

The amh signaling pathway functions through its primary receptor, *amhr2*, which, upon *amh*-binding, induces the assembly of a receptor complex activating the expression of different target genes (Josso et al., 2001). In fugu (*Takifugu rubripes*), a*mhr2* acts as the master sex determining gene. Differences between the two sexes involve a single nucleotide polymorphism (SNP) in its kinase domain (Kamiya et al., 2012).

b) Scaffold Protein, Cas family member (bcar1)

Bcar1 is a protein coding gene member of the Crk-associated substrate (CAS) family of scaffold proteins, characterized by the presence of multiple protein-protein interaction domains and many serine and tyrosine phosphorylation sites (Wallez et al., 2012). Recently it has been discovered that *bcar1* acts as a sex determining gene in channel catfish (*Ictalurus punctatus*) (Bao et al., 2019).

c) 17β-hydroxysteroid dehydrogenase (hsd17b1)

The *hsd17b* is a key enzyme in the final steps of steroid hormone synthesis catalysing the reduction of estrone (E_1) to estradiol (E_2) (Mindnich et al., 2004b). Recently, in *Seriola sp*. it has been described that a SNP in the Z-linked allele of the *hsd17b1* attenuates E_1 to E_2 conversion activity and this attenuation is associated to phenotypic male development (Koyama et al., 2019). However, whether this gene is the actual mater sex determining gene in *Seriola* would need further confirmation.

d) Zinc knuckle proteins (zk)

Zinc knuckle proteins are members of the large family of zinc finger proteins possessing a versatility of tetrahedral Cys-and His-containing motifs that bind to DNA and RNA target sites (Laity et al., 2001; Michalek et al., 2011). Other zinc

finger protein include *zfand3*, which is essential for spermatogenesis in mice (de Luis et al., 1999). The polymorphic tilapia *zfand3* was recently mapped in the sex determining locus (Ma et al., 2016b). Importantly, one gene of this *zk* family proteins has become the sex determining gene in Atlantic cod (*Gadus morhua*) (Kirubakaran et al., 2019).

Table 1. Sex determining genes known in teleosts

Type	Subtype	Paralog gene	Mechanism of origin	Determining gene	System	Species and reference
Transcription factor	Sox	*sox3*	Allelic diversification	*sox3-y*	XX/XY	*Oryzias dancena* (Takehana et al., 2014)
		sox2	Allelic diversification	*sox2-W*	ZW/ZZ	*Scophthalmus maximus* (Martínez et al., 2019)
	dmrt		Allelic diversification	*dmrt1*	ZW/ZZ	*Cynoglossus semilaevis* (Cui et al., 2017)
			Duplication	*dmrt1by/DMY*	XX/XY	*Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002)
			Duplication	*dmrt1by/DMY*	XX/XY	*Oryzias curvinotus* (Matsuda et al., 2003)
	IRF	*irf9*	Duplication	*sdY*	XX/XY	*Oncorhynchus mykiss* (Yano et al., 2012)
Growth factor	TGF-β	*gsdf*	Allelic diversification	*gsdf-Y*	XX/XY	*Oryzias luzonensis* (Shibata et al., 2010; Myosho et al., 2012)
			Allelic diversification	*gsdf-Y*	XX/XY	*Anoplopoma fimbria* (Rondeau et al., 2013)
			Allelic diversification	*gsdf-Y*	XX/XY	*Sebastes carnatus & S. chrysomelas* (Fowler and Buonaccorsi, 2016)
			Allelic diversification	*gsdf-Y*	XX/XY	*Sebastes schegeli* (Xuemei et al., 2019)
		gdf6	Allelic diversification	*gdf6-Y*	XX/XY	*Nothobranchius furzeri* (Reichwald et al., 2015)
		amh	Duplication	*amhbY*	XX/XY	*Esox lucius* (Pan et al., 2019)
			Duplication	*amhY*	?	*Ophiodon elongatus* (Rondeau et al., 2016)
			Duplication	*amhY*	XX/XY	*Oreochromis niloticus* (Li et al., 2015)
			Duplication	*amhY*	XX/XY	*Odontesthes hatcheri* (Hattori et al., 2012)
Receptor	serine/threonine-kinase	*amhr2*	Allelic diversification	*amhr2-Y*	XX/XY	*Takifugu rubripes* (Kamiya et al., 2012)
Adaptor protein	Cas	*bcar1*	Allelic diversification	*bcar1-Y*	XX/XY	*Ictalurus punctatus* (Bao et al., 2019)
Enzyme	Steroidogenic enzyme	*hsd17b1*	?	*hsd17b1*	ZW/ZZ	*Seriola sp.* (Koyama et al., 2019)
Zinc finger protein	Zinc knuckle proteins	*zk*	Allelic diversification	*zkY*	XX/XY	*Gadus morhua* (Kirubakaran et al., 2019)

1.3.2 Polygenic sex determination (PSD)

PSD was discovered nearly fifty years ago in platyfishes (*Xiphophorus* spp.) (Kosswig, 1964). In *X. maculatus* three alleles (X, Y and W) in the same sex determination locus interact to determine sex (Volff and Schartl, 2001; Schultheis et al., 2009). In some East African cichlids (*Metriaclima* spp.), XY and ZW sex determination systems reside on distinct chromosomes and dominances and interactions between genotypes at the two loci determine the sex (Roberts et al., 2009; Ser et al., 2010). Another species with PSD is the European sea bass (*Dicentrarchus labrax*) (Vandeputte et al., 2007; Palaiokostas et al., 2015). It is estimated that with new sequencing strategies that PSD will be easier to identify and so more representatives among fish species will appear (Moore and Roberts, 2013), as recently occurred in *Astatotilapia burtoni* (Roberts et al., 2016).

PSD has likely played an important intermediate role in the evolution of other genetic sex-determination systems (Moore and Roberts, 2013). PSD can arise through modifications of autosomal loci elsewhere in the genome that create new inputs for regulation of gonad development (Kirpichnikov, 1981). PSD may be inherently unstable if they can easily revert to single factor systems by loss of sex determination loci via drift or selection (Rice, 1986). In contrast, PSD can remain a stable mechanism if different polymorphisms result in alternative fitness benefits according to the environmental context; then selection may maintain them in the population (Moore and Roberts, 2013). Whether or not PSD systems are stable, they provide a model for evolutionary transitions from one genetic sex determination system to another (e.g. XY to ZW or vice versa), where presumably a novel sex determination gene would coexist and interact with an ancestral sex determination gene within a population (Ezaz et al., 2006).

1.3.3 Frequency-dependent selection sex ratio

Sex ratio is defined as the number of males to females in a population (Hardy, 2002). Balanced (1:1) sex ratios are thought to evolve from a process known as frequency-dependent selection of the minority sex. GSD species tend to produce balanced sex ratios due to natural selection, although in some circumstances the sex ratio may vary due to environmental factors that above a certain threshold can override the genetic component. In the case of ESD species, the fluctuating environment may cause a highly unbalanced sex ratio in the population (Conover and Kynard, 1981; Conover et al., 1992). In PSD species, depending on the segregation of sexual factors during meiosis, each gamete will carry a particular endowment, so these populations can show fluctuations of sex proportions in different families (Liew et al., 2012; Ribas et al., 2017a).

1.4 Environmental sex determination

The second major type of sex determination is environmental sex determination (ESD), where sex is determined after fertilization during early sensitive periods (Penman and Piferrer, 2008), influenced by different abiotic and biotic factors (Bull, 1983). ESD is expected to be favored when the offspring lives in fluctuating environments, which may confer advantages to one sex or the other (Charnov and Bull, 1977). In vertebrates, this sex determination mechanism only occurs in fish and reptiles (Valenzuela and Lance, 2004) and it has been stated that several environmental factors in fish are capable of determining sex (Ospina-Alvarez and Piferrer, 2008; Bachtrog et al., 2014).

1.4.1 Temperature-dependent sex determination

The most common form of ESD is temperature-dependent sex determination (TSD), where the early ambient temperature triggers the first ontogenetic differences between sexes and then fixes the final phenotypic sex of the individual. Many examples of TSD occur in reptiles, such as the crocodiles and turtles (and all sea turtles), where egg incubation temperature is critical for sexual fate (Deeming and Ferguson, 1988). In reptiles, there are three patterns of sex ratio response to temperature during the thermosensitive period: species where low temperatures produce female-biased sex ratios and high temperatures produce male-biased sex ratios (Valenzuela and Lance, 2004). Other species experience the opposite pattern: low temperatures produce male-biased sex ratios and high temperatures produce female-biased sex ratios. The third pattern, females are produced at extreme temperatures and balanced sex ratios at intermediate ones (Valenzuela and Lance, 2004).

In fish, the most common environmental cue affecting sex determination and differentiation is temperature, but there is only one pattern of response to temperature: more males with increasing temperatures (Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009). This is the case of ESD species like the Atlantic silverside (*Menidia menidia*) (Conover and Van Voorhees, 1990) and the pejerrey (*Odontesthes bonariensis*), which also has *amhY* as a sex determining gene (Strüssmann et al., 1997; Ito et al., 2005). Further, temperature is able to skew sex ratios in PSD species such as the European bass (Piferrer et al., 2005) or GSD species such as the Nile tilapia (Bezault et al., 2007). The distinction between species with TSD and species with GSD plus temperature effects (GSD+TE) in fish need critical consideration because most of studies have been performed under laboratory conditions and thus the ecological or adaptive importance of temperature is not necessarily proved (Ospina-Alvarez and Piferrer, 2008).

1.4.2 Other environmental factors

Aside from temperature, there are a number of other environmental factors that can also affect sex determination and, consequently, sex ratios in fish, although they have been

less studied (Eddy and Handy, 2012). These factors can have a combined effect on resulting sex ratios, and the most important are listed below:

a) Density. The number of fish in a given space or volume of water is referred to as the rearing, crowding or population density. Most studies concerned with density in fish focus on the effects of density on the reproductive success and not on the effects on sex differentiation. Nevertheless, elevated density, particularly during larval stages, can affect the sex ratio in some species. This is the case of the European eel (*Anguilla Anguilla*) (Roncarati et al., 1997; Huertas and Cerdà, 2006) and the American eel (*Anguilla rostrata*) (Krueger and Oliveira, 1999), where crowding is associated with higher male proportions (Davey and Jellyman, 2005).

Sometimes density effects can be misled with the social control of sex determination in hermaphrodite species. For instance, in the paradise fish (*Macropodus opercularis*) individuals totally isolated from their conspecifics became males whereas the highest number of females were found with high density (Francis, 1984).

b) pH. In some ESD species, water pH can affect the sex ratio. Some examples are documented in which acidic water during development in West African cichlid (*Pelvicachromis pulcher*) produces an increase in percent males (Reddon and Hurd, 2013). In the swordtail (*Xiphophorus helleri*), acidic water has been shown to induce male monosex population, whereas almost total female monosex populations (> 98% females) were obtained at higher pH (Rubin, 1985). However, there are also species in where acidic waters produce balanced populations (Römer and Beisenherz, 1996).

c) Photoperiod. The ratio of the number of hours of light and darkness can affect sex ratio in some fish, although more research is needed. A recent study in California grunion (*Leuresthes tenuis*) showed that a long photoperiod promotes female-biased sex ratios (Brown et al., 2014). In pike silverside (*Chirostoma estor*), continuous illumination produced male-skewed populations compared to control fish reared at 12L: 12D (Corona-Herrera et al., 2018).

d) Salinity. An osmotic stress due to abrupt changes in salinity concentrations, e.g., transfer from low to high salinity during the labile period of gonad development can affect the sex ratio. This was the case in the European sea bass, where high salinity resulted in masculinization (Saillant et al., 2003a).

e) Colour of the tank. The color of the tank in which fish are reared can sometimes affect sex ratios. In southern flounder (*Paralichthys lethostigma*), more males were produced when fish were reared in light blue tanks than in black or gray tanks. It was suggested that the color of the tank provoked a stress situation in which cortisol seemed to be involved in masculinization (Mankiewicz et al., 2013).

1.4.3 Cortisol as the link mediator to masculinization

As mentioned above, environmental factors can override the primary sex in many species, leading to sex reversal. Sex reversal has been defined as the mismatch between genotypic and phenotypic sex. Sex reversed females into males are called neomales or pseudomales (Piferrer, 2001; Baroiller and D'Cotta, 2016). Sex reversal can be considered as a consequence of a change in the threshold trait for sex susceptible to environmental modifiers (Heule et al., 2014). The change of sex induced by elevated temperatures, hypoxia, pH, or density might be explained by stressful conditions. Thus, fish exposed to these environmental factors trigger stress responses with increasing the plasma level of cortisol, an hormone considered as the main glucocorticoid hormone in fish (Barton and Iwama, 1991; Barton, 2002). High cortisol levels have thus been associated with male differentiation in some fish species after being exposed to stressful conditions, as described in medaka (Hayashi et al., 2010), in pejerrey (*Odontesthes bonariensis*) (Hattori et al., 2009; Fernandino et al., 2012) and the Southern flounder (Mankiewicz et al., 2013). In one study, cortisol has been observed as the direct mediator linking the effects of elevated temperature with the increase expression of *dmrt1a* during sexual development of medaka, leading to sex reversal (Adolfi et al., 2019). In addition, it has been observed that cortisol can affect the expression of many steroidogenic genes in the male pathway (Yamaguchi et al., 2010; Fernandino et al., 2012; Fernandino et al., 2013) leading sex to change (Solomon-Lane et al., 2013; Nozu and Nakamura, 2015). The effect of cortisol on sex determination is reviewed in Goikoetxea et al. (2017).

1.5 Sex differentiation

In gonochoristic species, once the bipotential gonad has developed into an ovary or testis it remains of the same sex throughout life, so gonadal sex does not change. As mentioned previously, the morphological and endocrinological aspects of this process are fairly well understood and conserved among fish (Guerrero-Estévez and Moreno-Mendoza, 2010; Guiguen et al., 2018). Nevertheless, differences in the pattern of early gonadal development can be found among gonochoristic species (Yamamoto, 1969).

1.5.1 Patterns of sex differentiation

There are two patterns (Yamamoto, 1969). In gonochoristic differentiated species, undifferentiated gonads differentiate directly into an ovary or a testis regardless of whether sex is determined genetically or environmentally (**Figure 1A**). In undifferentiated species, independent of their genetic sex, all individuals initially develop an ovarian tissue. Then, approximately half of the individuals continue with ovarian differentiation, while the remaining other half experience a reorganization of the gonads consisting of ovarian tissue degeneration through apoptosis followed by testicular differentiation (**Figure 1B**) (Yamamoto, 1969; Takahashi, 1977; Takahashi and Shimizu, 1983).

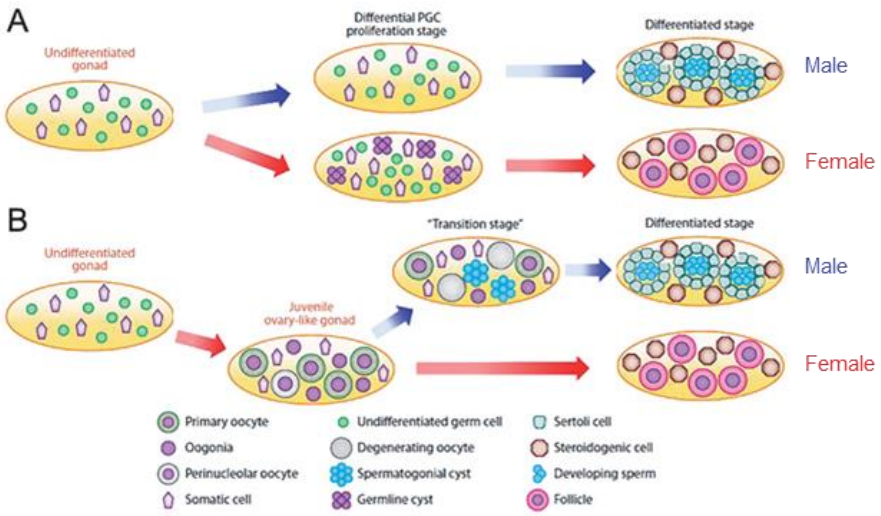

Figure 1. Classification of patterns of sexual differentiation in gonochoristic fish. (**A**) Differentiated species (**B**) Undifferentiated species. Figure modified from (DeFalco and Capel, 2009) and adapted from (Piferrer, 2019b) with permission.

1.5.2 Morphological aspects

Gonadal development comprises gonadogenesis and gametogenesis. Gonadogenesis includes gonad formation and differentiation into testes or ovaries. Gametogenesis is the formation of the gametes (eggs or sperm) in the differentiated gonads (testes or ovaries) (Takashima and Hibiya, 1995; Strüssmann and Nakamura, 2002; Nishimura and Tanaka, 2014). Gonadogenesis occurs after fertilization. In the zygote, the first cell line to differentiate is the germ cell line consisting of primordial germ cells (PGC) (Yoshizaki et al., 2002). These cells migrate to the gonadal ridge to give rise to the future gonads. Once sexual differentiation occurs, the germ cells along with interstitial and supporting cells start male or female differentiation based on the genetic and environmental inputs (DeFalco and Capel, 2009). The ovary comprises three main cell types: supporting cells, which are granulosa and theca cells, and germ cells: oogonia that become oocytes until they are released as eggs. The oocytes are surrounded by granulosa and theca cells in follicles that grow and differentiate during the reproductive cycles (Nagahama, 1983; Lubzens et al., 2010). The testis comprises three main cell types: Sertoli, Leydig cells and germ cells at different stages of maturation, from spermatogonia to spermatozoa. The Sertoli cells and spermatogonia reside in seminiferous tubules where spermatogenesis occurs (Schulz et al., 2010) **(Figure 2)**.

The reproductive system in fish is anatomically and histologically different in each sex. The gonads, usually elongated and paired, are contained laterally in the abdominal cavity, ventral to the air bladder suspended from the dorsal wall and surrounded by peritoneum (Wootton and Smith, 2014). Most males have two similar testes wrapped in a tunica albuginea and inside there are seminiferous tubules with germ cells which give rise to

sperm that are released in the tubules (Grier, 1981). Most female teleosts have two ovaries with some exceptions (Sherwood and Parsons, 1977). The ovary often contains a hollow space called the ovarian cavity which opens into the oviduct, and into which the eggs are released (Sherwood and Parsons, 1977; Patiño and Sullivan, 2002; Nishimura and Tanaka, 2014).

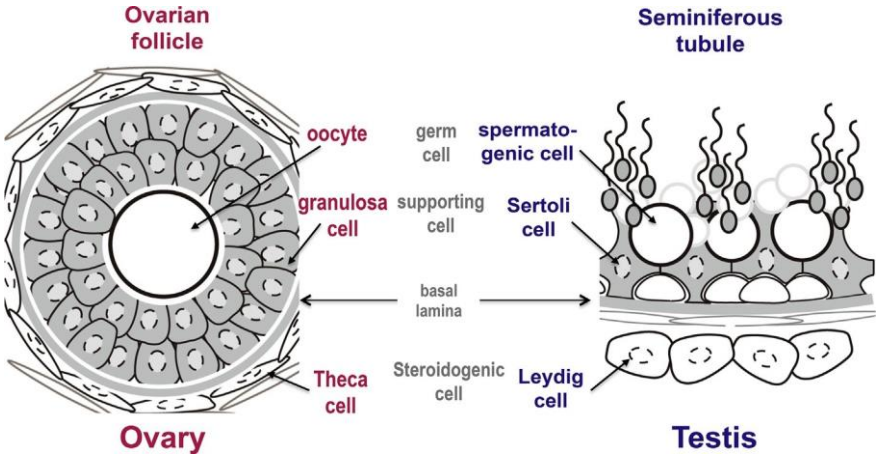

Figure 2. Comparative structures of the ovarian follicles and the seminiferous tubules in the testis at the adult stage, showing male and female counterparts of the germ cells, supporting cells, and interstitial steroidogenic cells. Figure taken from Smith et al., (2014).

1.5.3 Role of steroidogenic enzymes

Gonads have two main functions; to produce gametes and to produce sexual steroids (including progestogens, androgens and estrogens) for sex differentiation and later to sex maintenance (Lubzens et al., 2010; Schulz et al., 2010; Nishimura and Tanaka, 2014; Tokarz et al., 2015). Gonadal sex differentiation in teleosts depends on the relative ratio/abundance between androgens and estrogens, which is determined by the activity of the steroidogenic enzymes (**Figure 3**). Sexual steroids are synthetized by steroidogenic enzymes, a process that starts with cholesterol being converted to biologically active steroid hormones, with the action of acute regulatory protein (StAR) and trough a complex of various proteins involved to the inner membrane of the mitochondria. The catabolism of cholesterol into pregnenolone is mediated by the cytochrome P450 family 11 subfamily A member 1 (*cyp11a*) enzyme, and this is considered the first rate-limiting step for the biosynthesis of all steroid hormones (Arakane et al., 1997; Payne and Hales, 2004).

One of the key downstream target genes during gonad differentiation is aromatase, an enzyme encoded by the cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a)* gene, responsible for the irreversible conversion of androgens into estrogens (Simpson et al., 1994). Teleosts aromatase is coded by two separate genes, the gonad-specific *cyp19a1a* and brain-specific *cyp19a1b* isoforms (Chiang et al., 2001a; Chiang et al., 2001b; Zhang et al., 2014). The gonad-specific *cyp19a1a* is primarily expressed in

the ovary, mostly in the granulosa cells (Nagahama, 1997; Goto-Kazeto et al., 2004; Piferrer and Blázquez, 2005) although estrogens are also implicated in male gonad functions (Carreau and Hess, 2010; Delalande et al., 2015). More precisely, the aromatase has high affinity to testosterone (T) and rapidly converts into 17β-estradiol (E_2) (Miller, 1988; Hong et al., 2009). The distribution and regulation of the aromatase in fish has been reviewed by Piferrer and Blázquez (2005). High levels of E_2 is essential for ovarian development in teleosts and their suppression or down-regulation either by aromatase inhibitors or environmental factors (e.g., temperature) leads to testis development (Guiguen et al., 2010). Currently, estrogens are well known to be important not only for promoting ovarian development but also for maintaining femaleness (Baroiller and Guiguen, 2001; Guiguen et al., 2010).

On the other hand, there are other important enzymes, the cytochrome P450 11β-hydroxylase (*cyp11c1*) enzyme converts androstenedione (Δ^4) and T into 11β-hydroxyandrostenedione (11β-OHΔ^4) and 11β-hydroxytestosterone (11β-OH-T), respectively. Another enzyme in the pathway, the hydroxysteroid 11β- dehydrogenase 2 (*hsd11b2*) converts 11β-OHΔ^4 and 11β-OH-T into 11-Ketoandrosterone (11-KΔ^4) and 11-Ketotestosterone (11-KT), respectively, (reviewed in Tokarz et al., 2015).11-KT is the major androgen hormone in fish and is involved in testis development (Piferrer et al., 1993; Borg, 1994; Baroiller and Guiguen, 2001) and in spermatogenesis (Miura et al., 1991; Amer et al., 2001; Leal et al., 2009b).

In the steroidogenic pathway (**Figure 3**), transcription factors are important to regulate gene expression of some steroidogenic enzymes. This is the case of helix/forkhead group (*foxl2*). Foxl2 has been demonstrated to be a crucial transcription factor involved in gonadal development and is detected as an early dimorphic marker of ovarian differentiation (Wang et al., 2004). Two foxl2 paralogs (*foxl2a* and *foxl2b*) have been identified in several teleost species. *Foxl2a* is a crucial transcription factor shown to be involved in the transcriptional regulation of *cyp19a1a* expression (Schmidt et al., 2004; Bertho et al., 2016) while the function of *foxl2b* remains unclear (Baron et al., 2004). The steroidogenic factor 1 (*nr5a1*), is a nuclear receptor that act as the main regulator of cholesterol metabolism in steroidogenic cells, promoting the expression of transcription factors involved in the mobilization of cholesterol and steroid hormone biosynthesis, (Luo et al., 1994; Parker and Schimmer, 1997). In teleosts, transcriptional activation of *cyp19a1a* by *nr5a1* has been reported (Yoshiura et al., 2003; Kobayashi et al., 2005; Cheshenko et al., 2008).

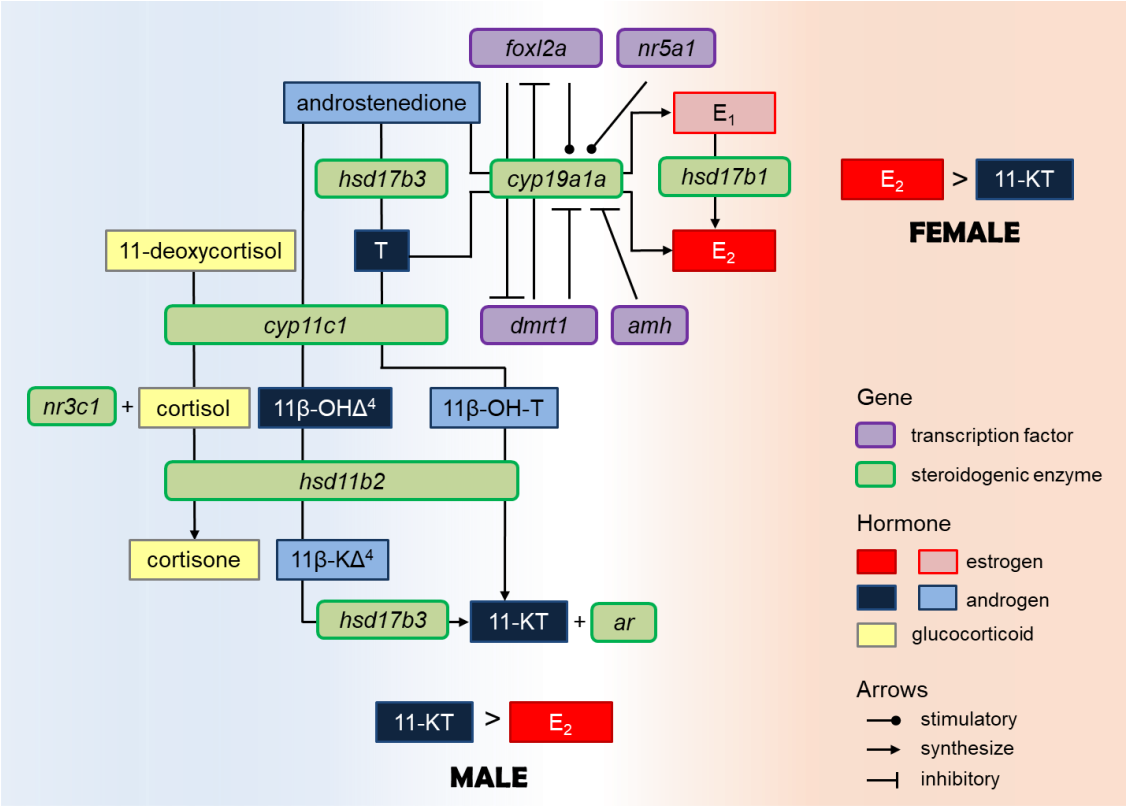

Figure 3. Simplified steroidogenic route. In females, the *cyp19a1a* (aromatase) enzyme converts testosterone (T) to estradiol (E_2) required for ovarian function (Guiguen et al., 2010). The *cyp19a1a* expression is stimulated by transcription factors *foxl2a* and *nr5a1* that interact with promoter region to upregulate *cyp19a1a* expression (Wang et al., 2007a). The E_2 can also be synthetized from estrone (E_1) by *hsd17b1* enzyme. In males, the aromatase expression is suppressed by *dmrt1* and *amh* and then *cyp11c1* and *hsd11b2* promote the production of 11-Ketotestosterone (11-KT) from T and androstenedione (Δ^4). Inhibitory effects of *amh* (Pfennig et al., 2015) and *dmrt1* that suppresses *cyp19a1a* activity directly (Wang et al., 2010) and indirectly via its antagonistic relationship with *foxl2a* transcription factor. Notice the relationship between the steroidogenic enzymes responsible for the synthesis of corticosteroids such as cortisol hormone and androgen synthesis from *cyp11c1* and *hsd11b2* enzymes.

2. Epigenetics

Conrad Waddington coined the term "epigenetics" in the 1940s (Waddington, 1942). Since then, there has been almost continuous refinement of this definition (Russo et al., 1996; Jaenisch and Bird, 2003; Sasaki, 2004; Deans and Maggert, 2015). Here we used the following definition of epigenetics as "the study of phenomena and mechanisms that cause chromosome-bound, heritable changes to gene expression that are not dependent on changes to DNA sequence"(Deans and Maggert, 2015).

2.1 Epigenetic mechanisms

While genetic variation can accumulate changes in a slow fashion, epigenetic changes, such as methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation, can

occur much more rapidly (Kilvitis et al., 2014). Epigenetic processes are essential to many organismal functions. One of the main major differences between epigenetic and genetic processes is that the latter can be directly modified in response to environmental signals (Dowen et al., 2012), which adds an additional level of adaptation for the species. There are three major epigenetic mechanisms: DNA methylation, histone modifications and regulatory non-coding RNAs (Goldberg et al., 2007) (**Figure 4**). Here, we briefly present these epigenetic mechanisms, although this thesis focuses on DNA methylation.

2.1.1 DNA methylation

DNA was the first epigenetic mark to be discovered and has become one of the most widely studied of the epigenetic mechanisms (Deans and Maggert, 2015). Methylation is the process by which a methyl-group (CH_3) is transferred from a methyl donor, S-adenosyl-L-methionine (SAM), to the sixth position of an adenine, converting it to N6-methyladenine (6mA) (Ratel et al., 2006), or to the fifth position of a cytosine, converting it to 5-methylcytosine (5mC) (Grosjean, 2013). The 6mA is less studied than and 5mC but is considered a true epigenetic mark with regulation on gene expression (Fu et al., 2015; Greer et al., 2015; Heyn and Esteller, 2015; Zhang et al., 2015).

The 5mCs occur almost exclusively in the context of CG dinucleotides (CpG) (Gruenbaum et al., 1981). These CpGs tend to cluster in regions named CpG islands (CGI) (Gardiner-Garden and Frommer, 1987; Deaton and Bird, 2011). The enzymes responsible to transfer a CH_3 are the DNA (cytosine-5) methyltransferases (*dnmt*) (Okano et al., 1998), and in eukaryotes there are multiple families of DNA methyltransferases (Goll and Bestor, 2005). In mammals, dnmts include four members in two families that are structurally and functionally distinct (Cheng and Blumenthal, 2008). The dnmt3 family (*dnmt3a*, *dnmt3b* and *dnmt3L*) establishes the initial CpG methylation pattern de novo, whereas *dnmt1* maintains this pattern during chromosome replication (Chen and Li, 2006) and repair (Mortusewicz et al., 2005). In addition *dnmt1* activity is required for de *novo* methylation at non-CpG cytosines (Grandjean et al., 2007) and perhaps to an extent even in CpG islands (Feltus et al., 2003; Jair et al., 2006). Demethylation, the process that oxidize 5mC to the form 5-hydroxymethylcytosine (5hmC) and other derivatives, are carried out by enzymes called ten eleven translocation (TET) (Ito et al., 2011; Pastor et al., 2013). The conversion of 5mC back to the unmodified methylation state (non-5mC) can occur by either active or passive process. Passive DNA demethylation refers to the absent of *dnmt* activity to maintain DNA methylation patterns across cell divisions and is believed to result in replication-dependent dilution of 5mC (Ji et al., 2014). On the other hand, active DNA demethylation occurs via direct removal of a methyl group independently of DNA replication through TET enzymes (Sadakierska-Chudy et al., 2015). The active process of genome-wide DNA demethylation is well studied in zygotes (Mayer et al., 2000; Oswald et al., 2000) and in primordial germ cells in mammals (Reik et al., 2001; Hajkova et al., 2002; Morgan et al., 2005).

2.1.2 Histone modifications

The nucleosome is the smallest unit of chromatin organization and can be divided into core canonical histones (H), H2A, H2B, H3 and H4, which are clustered into one H3-H4 tetramer with two H2A-H2B dimers. This complex of histones wraps 147 base pairs (bp) of DNA sequence with a linker histone H1 (Luger et al., 1997; Cutter and Hayes, 2015). Core histones are globular structures with unstructured N-terminal tails from several histone variants (Talbert and Henikoff, 2010) where post-transcriptional modifications such as acetylation, methylation or phosphorylation occur (Turner, 2005; Kouzarides, 2007). These histone modifications are able to alter gene expression, either by changing the chromatin structure or by recruiting enzymes with specific binding domains (Bannister and Kouzarides, 2011).

2.1.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) are transcribed from genomic sequences and do not encode proteins (Mercer et al., 2009). ncRNAs can be divided into two main groups. The first group consists of infrastructural ncRNAs that have a role in translation and splicing, e.g., ribosomal (ribRNA), transfer (tRNA) and small nuclear RNAs (snRNA) (Collins, 2011). The second group includes regulatory ncRNAs that are involved in diverse biological processes with specific functional roles of developmental regulation such as reproduction (Robles et al., 2019). These ncRNAs that are involved in epigenetic processes are categorized based on their length and biological function, and divided into two main groups: the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts) (Li and Tollefsbol, 2011). The three major classes of short non-coding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). Both short and long ncRNAs play a role in heterochromatin formation, histone modification, DNA methylation targeting, and gene silencing (Mattick and Makunin, 2006; Taft et al., 2007; Keller and Bühler, 2013).

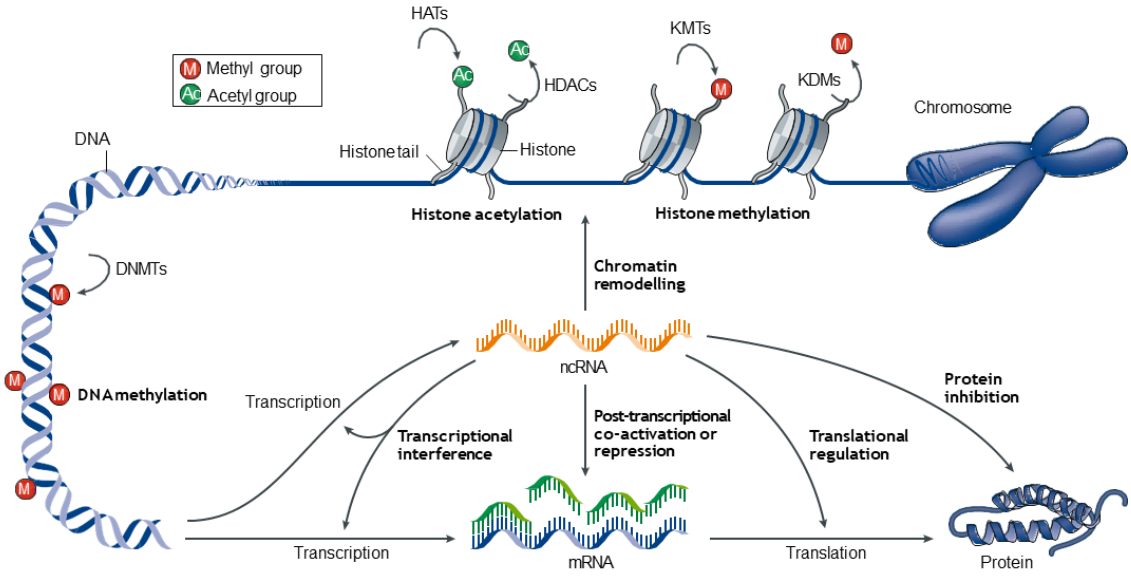

Figure 4. Overview of the different types of epigenetic modifications. Histones can undergo methylation and acetylation among other chemical modifications. Acetylation of histone tails is done by the histone acetyltransferases (HATs). Deacetylation is achieved by the histone deacetylases (HDACs) that compact the chromatin. Methylation and demethylation of histone tails are mediated by lysine methyltransferases (KMTs) and removed by histone-lysine demethylases (KDMs) that can lead to either transcriptional activation or transcriptional repression. DNA methylation by the addition of a methyl group to carbon position 5 on cytosine bases, a reaction catalysed by DNA methyltransferase enzymes (DNMTs), which maintains repressed gene activity. mRNA is translated into a protein product, but this process can be repressed by binding of microRNAs (miRNA), a class of noncoding RNA (ncRNA) acting at different levels. Figure taken from Joosten et al. (2018).

2.2 Methods to evaluate DNA methylation

There are different methods available to determine the methylation status of the DNA (Fraga and Esteller, 2002; Dahl and Guldberg, 2003) and a substantial number of reviews summarizing the major techniques (Shen and Waterland, 2007; Plongthongkum et al., 2014; Metzger and Schulte, 2016). Some of these techniques are better when assessing unknown epigenetic changes (nonspecific) and others are more suitable when high accuracy information is required (specific) (Saluz and Jost, 1993; Kurdyukov and Bullock, 2016). Selecting the method most suitable for a particular study is generally determined by a balance of the weighs, the cost, the resolution of the assay and the time and the length of the genome. DNA methylation techniques are categorized based on three levels: the level of the methylation resolution provided, how this methylation state is detected and the informative range of the genome that can be analysed.

a) Level methylation resolution. The methylation profiling approaches vary in the extent of the genome that can be interrogated. There are variable degrees on the resolution of the methylation profiling, which can be globally grouped into:

1) Low: methods that can only detect major 5mCs changes ignoring the precise state (for example, number or location) of 5mCs in CpGs.
2) Medium: methods where the status of the 5mCs can be studied in an approximate context but some regions are not evaluated.
3) High: these methods give the exact status and genomic location of both 5mCs and Cs in the genome sequence.

b) Level of detection of the 5mCs. The 5mCs can be identified and distinguished from the unmethylated Cs. There are three general approaches used for the identification of 5mCs and they can be used isolated or in combination:

1) Methylation-sensitive restriction enzymes: they are based on differential cleavage of methylated DNA. Some restriction enzymes have differential sensitivity to 5mCs and can be used to distinguish 5mCs from Cs. For instance, the most common restriction enzymes are *MspI* and *HpaII* which are isoschizomers enzymes. Both of these restriction enzymes cut on the CpG dinucleotide of the CCGG sequence (Waalwijk and Flavell, 1978).

2) Affinity-capture: specific antibodies against 5mCs to immunoprecipitate methylated DNA can be used as in ELISA-type assay or methyl capture assay by using recombinant proteins that contain a methyl-CpG binding domain (MBD) to enrich the methylated fraction of chromatin (Nair et al., 2011).

3) Sodium bisulfite conversion and sequencing: sodium bisulfite treatment converts an unmethylated cytosine into uracil, which is replaced by thymine after a PCR amplification, while the 5mCs remain unchanged. Differential methylated and unmethylated residues are calculated after sequencing using bioinformatic tools (Li and Tollefsbol, 2011).

c) Level of the information of DNA methylation extracted. The methylation profiling approaches may vary in the extent of the genome, and the information can range:

1) Whole-genome: the 5mC status of the entire genome are identified.
2) Genome-wide: the 5mC status only from a representative portion of the genome.
3) Locus-specific: the 5mCs are measured only in a specific target regions of interest.

From all the techniques available, the most comprehensive method for assessing patterns of DNA methylation at single base-pair resolution is bisulfite conversion of genomic DNA followed by whole-genome shotgun sequencing (Whole-Genome Bisulfite Sequencing, WGBS) (Laird, 2010). Although costs of sequencing techniques have drastically reduced along the years, the cost of comprehensive sequencing still represents a major part of the budget for experiments, in particular for those species with large

genomes, or those which genome is not sequenced yet. To reduce the expensive cost of WGBS, a variety of methods have been derived. In general, these methods were designed to reduce costs and time consuming of bioinformatics by focusing sequencing efforts on the informative methylated portions of the genome. For instance, Reduced Representation Bisulfite Sequencing (RRBS) (Jeddeloh et al., 2008; Gu et al., 2011) is a restriction-enzyme based approach where genomic DNA is first digested using a methylation-sensitive restriction enzyme and second these fragments are sequenced. This approach allows that sequencing efforts be concentrated primarily on regions of the genome that have the potential to be methylated (Jeddeloh et al., 2008; Gu et al., 2011).

2.3 Epigenetic regulation of gene expression

Evidence confirms that DNA methylation plays a crucial role in repressing gene expression (Cedar, 1988; Razin and Cedar, 1991; Jones, 2012). There are genomic regions of variable length (**Figure 5A**) showing low and high density of CpG (Deaton and Bird, 2011). The position and distribution of these elements across the genome seems to have specific roles for gene regulation (Suzuki and Bird, 2008). In enhancers (regions about 50–1500 base pairs (bp) of DNA that can be bound by proteins such transcription factors) having low CpG density, methylation has been suggested to reduce enhancer activity (Lister et al., 2009; Schmidl et al., 2009) while high levels of 5hmC leads enhancer activation (Serandour et al., 2012). In contrast, methylation near gene promoters varies considerably depending on the cell type. The CGI tend to be close to transcription start sites of the promoter and so evidence shows that methylation in these regions correlate with low or no transcription (Suzuki and Bird, 2008; Deaton and Bird, 2011). Nevertheless, it seems that methylation levels in the gene body (the region of a gene between the start and stop transcription sites) are tightly linked to transcriptional silencing (Brenet et al., 2011) and also it can be related to the control of gene splicing (Jones, 2012). Recently, it has been shown that DNA methylation of the first intron possess even a stronger inverse relationship between the methylation and gene expression levels (Anastasiadi et al., 2018a; Jo and Choi, 2019).

There are two mechanisms by which DNA methylation influence gene expression and are not necessarily exclusive (**Figure 5B**). The first is the methylation levels of the CGI in the promoter regions that overlaps with sequences consensus of binding transcription factors. The presence of methylation is important for preventing the transcription factors to have access to binding sites and thus activate the gene (Larsen et al., 1992; Weber et al., 2007; Bell et al., 2011). In the second, regulation is carried out by proteins with specific domains that recognize and bind to the methylated CpGs (methyl-CpG binding proteins, MBPs) and recruit transcriptional co-repressor molecules to silence the synthesis of the gene (Nan et al., 1993). The complex of recruited co-repressors inhibit transcription through the stabilization of the nucleosomes in a repressive state remodelling chromatin and consequently not allowing an open accession (Bird and Wolffe, 1999; Cedar and Bergman, 2009; Moore et al., 2013). Furthermore, is important

to mention that DNA methylation and histone modifications act together to regulate gene expression (Cedar and Bergman, 2009).

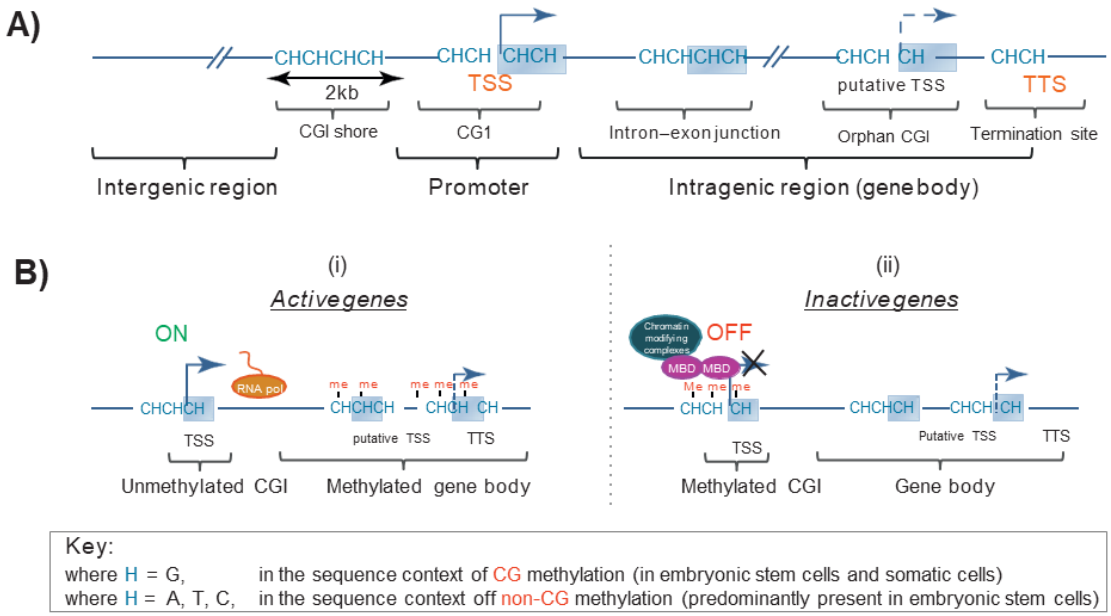

Figure 5. Overview of the regulation of gene expression by DNA methylation. Important gene and genomic features associated with this regulation are shown (**A**) as well as the relationship between DNA methylation in genomic features and the expression state of the gene (**B**). Abbreviations: CGI, CpG islands; TSS, Transcription Start Sites; MBD, Methyl-CpG-Binding Domain. Figure adapted from (Ndlovu et al., 2011).

2.4 Environmental integration and epigenetic changes

The environment can influence the genome through epigenetic modifications including DNA methylation, histone modifications and non-coding RNAs. External cues can be biotic and abiotic (Jaenisch and Bird, 2003; Guerrero-Bosagna and Skinner, 2012; Skinner, 2015). The external stimulus triggers a cascade of events into the nucleus. Subsequently, a molecular agent, such as ncRNA, a DNA-binding protein or even an epigenetic enzyme induced by the stimulus, can establish new chromatin context in precise genes or regions. For instance, in Atlantic cod a continuous thermal stress during different stages of embryo development decreased the gene expression levels of epigenetic-related genes *dnmt1*, *dnmt4* and *dnmt3ab* (Skjærven et al., 2014).

These new epigenetic changes may be transient or may be integrated (not erased) and become permanent due to methylation process of CpG sites or histone modifications and then transmitted during the subsequent cell divisions (Berger et al., 2009). Thus, epigenetic mechanisms provide organisms with the ability to modify their gene expression patterns in response to internal and external environments, actively contributing to GxE interactions (Turner, 2009) as an adaptation to new environmental conditions (Burggren, 2016; Vogt, 2017).

2.5 Epigenetics in fish reproduction

During development, cells differentiate and acquire new identity in response to interactions between internal signals and environment (Senner et al., 2012). Changes in gene expression patterns are an essential feature of cell differentiation, and the conservation of these patterns is a key component to maintaining cell identity (Brock and Fisher, 2005; Kiefer, 2007). Epigenetic modifications are now known to be involved in sex determination and differentiation across a wide range of taxa (Piferrer, 2013). To date, a clear relationship between the role of epigenetic and the regulation of sex has been documented in gonochoristic (Navarro-Martín et al., 2012; Piferrer, 2013; Shao et al., 2014; Wen et al., 2014) and in sequential hermaphrodite species (Todd et al., 2019). Fish show extraordinary sexual plasticity, some changing sex naturally as part of their life cycle; others reversing sex because of environmental stressors (Liu et al., 2017a). A clear example where epigenetics is involved in sex is in hermaphrodite fish. In these species, individuals possess the same genome but according to different cues, such as age, growth, or social factors can develop one sex and then change to the functional opposite sex (Warner, 1984; Godwin, 2009; Kobayashi et al., 2013). Sexual fate is determined by activating male or female pathways and inhibiting the alternative pathway with many genes being expressed in a sexually dimorphic manner (Kim and Capel, 2006; Munger and Capel, 2012). The epigenetic process and molecular events that underpin those expression changes remain poorly understood and thus more research is needed.

DNA methylation and temperature have been linked to sex differentiation in fish. This is the case of the European sea bass (PSD system) (Vandeputte et al., 2007) in which high DNA methylation levels of *cyp19a1a* promoter in the ovaries of females exposed to high temperature during early stages was responsible for aromatase silencing and subsequent masculinization (Navarro-Martín et al., 2012). In the half tongue sole (ZZ/ZW system), by using a WGBS approach it was discovered that *dmrt1* had higher levels of DNA methylation in females, whereas males and pseudomales showed similar hypomethylated pattern at the *dmrt1* locus, suggesting that demethylation of *dmrt1* is necessary for male gonad development (Chen et al., 2014; Shao et al., 2014). In Japanese flounder (*Paralichthys olivaceus*, XX/XY system) high levels of *dmrt1* expression in testis were correlated with low levels of DNA methylation in the *dmrt1* promoter, whereas in ovaries was just the contrary: high levels of *cyp19a1a* expression were correlated with low levels of DNA methylation (Wen et al., 2014). Based on the accumulative knowledge of the methylation levels of genes involved in sex determination and differentiation in fish gonads, a pattern of two of the most important reproduction-related genes have been found so far. The *dmrt1* and *cyp19a1a*, two key genes for testis and ovary development, respectively, consistently showed an inverse relationship between their DNA methylation and gene expression levels across fish species involving both gonochoristic and different forms of hermaphroditism (Piferrer et al., 2019). This has led to the model of the Conserved Epigenetic Regulation of Sex or CERS (Piferrer et al., 2019).

2.6 Epigenetic inheritance

The term epigenetic inheritance refers to the transmission of heritable epigenetic alterations (Richards, 2006). There are two types of epigenetic inheritance. The first occurs when undifferentiated cells acquire a lineage-specific epigenetic pattern (cell identity) and this is transmitted through mitosis to daughter cells (Probst et al., 2009). The second, is called meiotic inheritance or 'gametic epigenetic inheritance' (Youngson and Whitelaw, 2008). It occurs in many eukaryotes, where the parent-to-offspring transmission involves at least one reprogramming event of epigenetic marks, so that the zygote cells remain totipotent (Messerschmidt et al., 2014). However, there are specific loci with specific epigenetic marks that are able to escape this reprogramming event, consequently passing these marks to the next offspring generation and hence the epigenetic inheritance (Bond and Finnegan, 2007).

Studies in mammals have shown that in the lifespan of an individual, there are two critical windows in which environmental factors can affect germ cell lineages and can impact epigenetic plasticity with potential long-lasting effects of various different nature, including alterations on metabolic pathways and disease susceptibilities. One occurs in cells of the preimplantation embryo, from where later a small set of cells become the primordial germs cells (PGCs) that will migrate into the genital ridges and eventually will develop the future gonads. The second window occurs in adult stages during gametogenesis affecting the germ cells (Jammes et al., 2010; Hales et al., 2011; Seisenberger et al., 2013a; Seisenberger et al., 2013b; Marsit, 2015). The typology of the environmental exposures that directly influence/promote epigenetic changes in germ cells can range from temperature, nutrition and stress to a large number of endocrine disruptors (Jirtle and Skinner, 2007). Consequently, these induced-epigenetic marks in the PGCs and/or in gametes can skip reprogramming and be inherited by the offspring across generations either via the parental, the maternal or both lines (Heard and Martienssen, 2014). In this way, descendants can manifest the same altered phenotypes as the exposed ancestors, even when the environmental perturbation is no longer present (Feil and Fraga, 2012). Multigenerational effects of nutritional induced DNA methylation changes in the liver epigenome was reported in the untreated progeny derived from zebrafish parents reared with a high intake of arachidonic acid during their larval stages (Adam et al., 2019).

It is important to distinguish between mutigenerational and transgenerational inheritance (**Figure 6)**. In mammals, if the exposed individual is a male or a non-pregnant female, direct environmental exposures can have effects on them (F_0) as well as their germ cells that will form the next generation (F_1). Therefore, F_1 derived from these germ cells is still considered exposed, and so is not truly a transgenerational but a multigenerational effect. In this case, a true transgenerational effect can only be proved if the effect of exposure is transmitted to the F_2 generation and/or beyond in which those individuals never were under direct exposure. The situation is different when the female is pregnant, because direct exposures affect three generations: the female F_0, the fetus F_1 and the germline of this fetus that will form the F_2. Therefore, the F_3 would be the first unexposed generation

and the effects observed in F_3 are called transgenerational (Skinner, 2008; Skinner et al., 2010). In species with external fertilization, i.e., most fish, exposed parental individuals (F_0) also involves the exposure of the F_1 germline (multigenerational), but in this case the F_2 generation is the first unexposed generation (transgenerational) (Baker et al., 2014; Corrales et al., 2014; Bhandari, 2016; Labbé et al., 2017). Thus, fish are a good model to study transgenerational epigenetic inheritance.

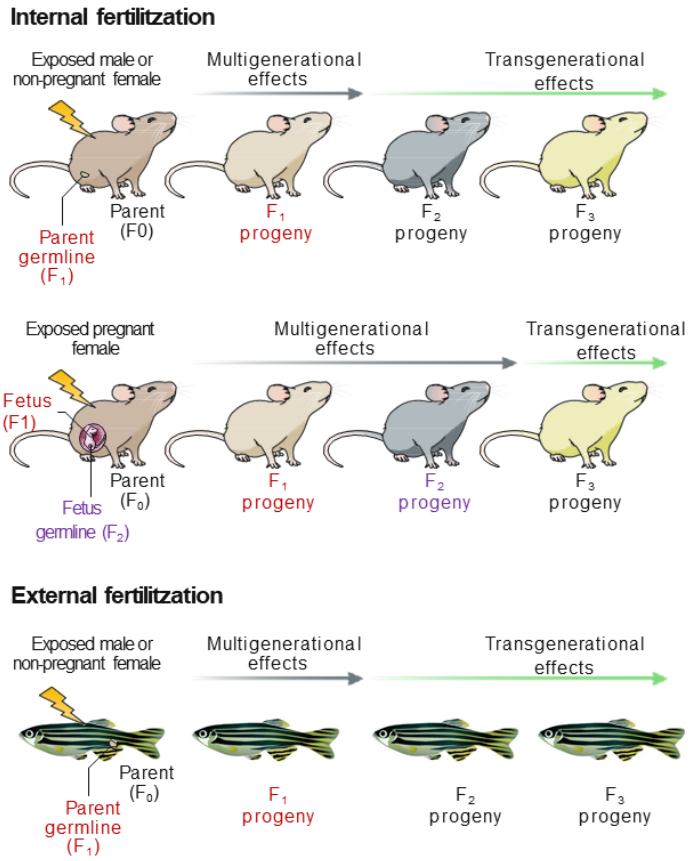

Figure 6. Consideration of epigenetics inheritance in the context of embryonic and germ line exposure to a perturbation (either environmental or chemical compound) in mammals and fish. In mammals with internal fertilization, if male and/or non-pregnant female (F_0) are exposed to a perturbation, their germ lines are also affected that will form the first generation (F_1). The first generation not exposed to the perturbation is the F_2 generation and beyond. If the perturbation is applied to a pregnant female (F_0), then the fetus (F_1) as well as its primordial germ cells, which have the potential to form the F_2 generation, would be affected. Therefore, the transgenerational inheritance would be visible in F_3 generation and beyond. In fish with external fertilization, the first transgenerational effects appear in F_2 generation. Figure adapted from Skvortsova et al. (2018).

2.7 Epigenetic biomarkers

A biological marker or biomarker is a measurable indicator of some biological state or condition that is easily identified and quantified for a particular disease, disorder or state (Group et al., 2001). Part of the methylation marks in the epigenome can change with age, lifestyle or can be modulated by environmental exposures (Marsit, 2015), as discussed above. Consequently, they can be used as an epi-(bio)marker (Laird, 2003) able to predict future diseases or biological events (Godfrey et al., 2015).

The development of new technologies to measure DNA methylation is enabling the discovery of epigenetic biomarkers (epimarkers) on a truly genome-wide scale (Dirks et al., 2016). In the last few years, DNA methylation changes, aside their importance in the regulation of gene expression (Ren et al., 2018) are being implemented as epi-biomarkers (Mikeska and Craig, 2014). In clinical studies, the methylation differences in particular genes or specific methylated regions have been associated with specific illnesses (Jones and Baylin, 2007). Therefore, after discovering significant and specific epimarks (mostly CpG sites), this information has been used to develop tools (Moran et al., 2016), supported with bioinformatic steps and machine learnings procedures (Kourou et al., 2015), to have the most effective and accurate decision for the diagnosis and prognosis of diseases (Costa-Pinheiro et al., 2015).

Furthemore, epimarkers have been used to design an epigenetic clock in humans, which can be useful to predict the biological age of an individual based on the methylation patterns in the genome (Horvath, 2013). In addition, DNA methylation marks have been used to predict the age in other vertebrates, such as humpback whales (*Megaptera novaeangliae*) (Polanowski et al., 2014), a long-lived seabird (*Ardenna tenuirostris*), (De Paoli-Iseppi et al., 2019) and for canids (Thompson, 2017). Recently, in the European sea bass it has been described an epigenetic clock (age predictor), the first one in fish and a cold-blooded vertebrate, and a tool to predict sex, using DNA methylation differences from four genes (48 CpGs) in muscle and three (23 CpGs) reproduction-related genes in gonad tissues, respectively (Anastasiadi et al., 2018b; Anastasiadi and Piferrer, 2019).

3. The zebrafish model

3.1 Zebrafish biology

Zebrafish is a tropical freshwater fish native to southeast Asia belonging to the Cyprinidae family (Laale, 1977). Possibly due to the fact that this region has monsoon seasons, zebrafish have adapted to tolerate a broad range of temperatures (6–38°C) and different environmental conditions. Their habitat are typically slow-moving waterways ponds, shallow lakes and waterlogged rice fields (Boisen et al., 2003; Engeszer et al., 2007; Spence et al., 2008). Zebrafish are omnivores, consuming small insects and crustaceans

and algae (McClure et al., 2006; Spence et al., 2007; Arunachalam et al., 2013). They are small fish (2.5–4 cm length) with a minimal degree of sexual dimorphism and where females achieve larger sizes than males (Santos et al., 2008). This species has external fertilization producing many eggs (~100–300) per mating event. Embryos are transparent, most hatch between 48 and 72 hours after fertilization, and provide excellent optical clarity of all stages of their development. Zebrafish have a short life-cycle (**Figure 7**) as sexual maturity takes only ~3 months (Engeszer et al., 2007; Spence et al., 2008).

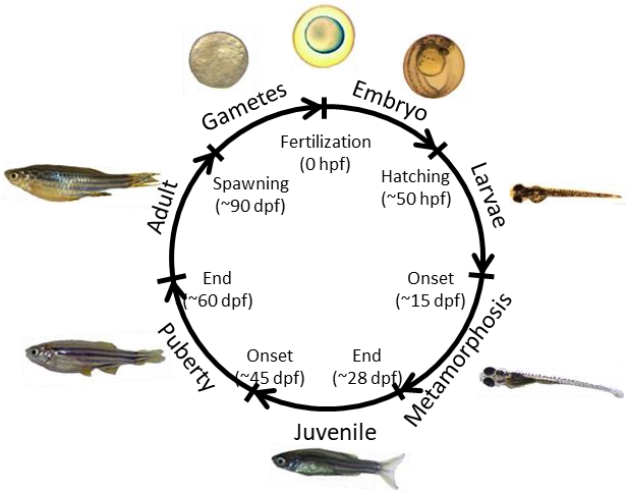

Figure 7. Schematic illustration of the life cycle of the zebrafish. Representative events separating different zebrafish stages are shown. Abbreviation: hours post-fertilization (hpf) and days post-fertilization (dpf). Figure taken from Ribas and Piferrer (2014).

3.2 Zebrafish as a model organism

As a result of these attributes and the intrinsic robustness, zebrafish are relatively easy to raise in captivity. Under laboratory conditions it can be induced to breed the whole year round. First used in the 1960s in biological research by George Streisnsinger, zebrafish have proven to be an excellent model organism to study developmental biology, human disease (~78% genetic homology to humans), genomic research, toxicology and other disciplines (Dooley and Zon, 2000). The zebrafish is also now gaining importance as a model for aquaculture research (Ribas and Piferrer, 2014; Ulloa et al., 2014). Thanks mostly of being an animal model in clinical research, several genetic and genomics tools have been developed. These include from a high-quality assembled genome (Howe et al., 2013), a web based community resource (www.zfin.org) (Sprague et al., 2003), transcriptomic resources such as commercial oligo cDNA microarrays (Ton et al., 2002) and a vast number of transgenic lines (Geisler et al., 2016).

3.3 Reproductive biology of zebrafish

The laboratory or domesticated zebrafish is an undifferentiated gonochoristic species since all individuals first initiate oogenesis by first forming an immature ovary. Later, during sex differentiation, testes develop in about half of the individuals (Takahashi, 1977; Orban et al., 2009). This sexual developmental pattern was confirmed with transgenic zebrafish with fluorescent germ cells (Krøvel and Olsen, 2002; Hsiao and Tsai, 2003; Ye et al., 2019). In fish, and in particular in zebrafish, studies have shown that a minimum threshold number of PGCs are essential prerequisite for female development (Siegfried and Nüsslein Volhard, 2008; Dai et al., 2015; Tzung et al., 2015). Even when female sex is defined, the germ cells are still required to maintain the female phenotype in adults, otherwise this leads to sex reversal (Dranow et al., 2013). Genetic manipulations have shown that ablation of PGCs in early stages only lead to male development (Slanchev et al., 2005; Zhou et al., 2018).

3.4 Zebrafish sex determination

The zebrafish karyotype consists of 25 chromosome pairs and are characterized by metacentric and submetacentric with only few acrocentric chromosomes (Daga et al., 1996). Despite the extensive efforts, no recognizable sex chromosomes (heterochromosomes) have been detected neither the master gene involved in sex determination (Endo and Ingalls, 1968; Schreeb et al., 1993; Pijnacker and Ferwerda, 1995; Daga et al., 1996; Amores and Postlethwait, 1998; Wallace and Wallace, 2003). For decades, the lack of a demonstrated sex-determining system in this model organism has been puzzling researches in laboratories across the world. Genetic analyses with different laboratory strains, identified multiple sex-associated regions spread around the zebrafish genome (Bradley et al., 2011; Anderson et al., 2012; Howe et al., 2013). By using monosex zebrafish populations or zebrafish from nature (Sharma et al., 1998) female zebrafish were found to be the heterogametic sex (ZW). Recently, a conclusive study showed that wild and laboratory zebrafish strains have two different sex determination mechanisms (Wilson et al., 2014). When genomic sequencing results from wild populations and laboratory strains were analyzed (**Figure 8**), a locus of sex determination, a region associated to sex defined as *sar4*, was found on the apical part of chromosome 4, considered a putative sex chromosome and compatible with a ZZ/ZW system in the wild strain (Wilson et al., 2014). In the laboratory strains, inbreeding protocols due to domestication along years of captivity have been responsible to lose *sar4* in the genome due to its telomeric position in the chromosome (Wilson et al., 2014). Consequently, putative loci spread across the genome, without any apparent hierarchy regulation, have adopted the role of sex determination, converting these laboratory strains from a GSD to a PSD system and sex is regulated by different signaling pathways in which environment is a determining factor (Liew et al., 2012).

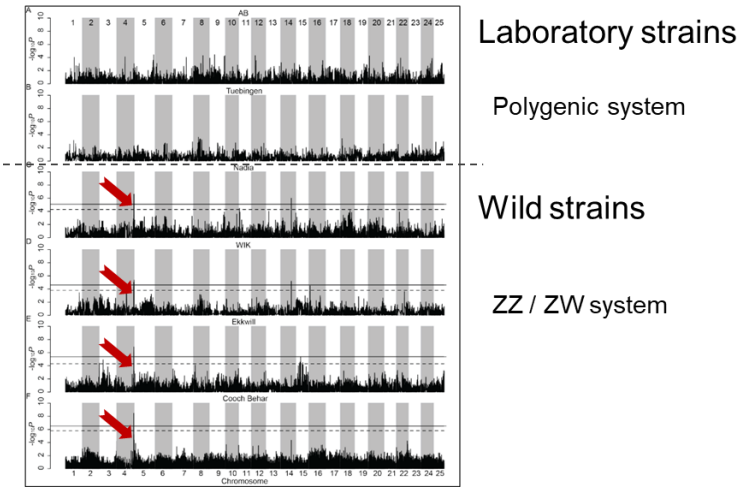

Figure 8. Restriction-site-associated DNA (RAD-sex) results for zebrafish strains show plots of the -log10P of a G-test (likelihood-ratio or maximum likelihood) of genotypes associated with male or female phenotype in zebrafish plotted against the 25 linkage groups of the assembled zebrafish genome. There are single nucleotide polymorphisms significantly associated with sex were identified on Chr4 in Nadia and WIK, EkkWill and Cooch Behar strains (red arrow) whereas the AB or Tuebingen both laboratory strains lack of this region. Adapted from Wilson et al. (2014).

3.5 Environmental factors influencing zebrafish sexual phenotype

Zebrafish sex differentiation is labile and under certain environmental factors and chemical compounds it can be altered, biasing the population to be highly male- (usually) or female-skewed (rarely) (Santos et al., 2017). Especially, sexual plasticity occurs when the exposure of these external perturbations coincide with migration and differentiation both during earlier stages of embryo or gonadal development (Raz, 2003). For example, several environmental factors such as temperature (Uchida et al., 2004; Abozaid et al., 2012; Ribas et al., 2017a), hypoxia (Shang et al., 2006; Lo et al., 2011) and density (Liew et al., 2012) lead to masculinization, whereas plenty of food intake seems to promote the stabilization of the PGCs and more females are developed (Lawrence, 2007). Hence, laboratory zebrafish have a complex sexual system, controlled by a combination of genetic and environmental factors, but still a lot of research is needed to fully understand sex determination and differentiation in this well-known animal research model.

Although several factors mentioned above explain how sex ratio of zebrafish can be affected, there are still many aspects that need to be better characterized. Thus, a broader analysis of the epigenetic mechanisms that govern sex determination and differentiation are needed in order to determine the relevance of epigenetic regulatory mechanisms in fish in general and in this fish species in particular. Finally, whether natural strains, which harbor an intact sex determining mechanism, are also susceptible to external influences as laboratory strain is not known.

Aims

Aims

The **overall objective** of this thesis was to contribute to our understanding of how genetic and environmental changes shape the sexual phenotype and the role of epigenetics in this process. To achieve this, we carried out laboratory experiments under controlled conditions using different strains of the zebrafish (*Danio rerio*), a consolidated animal model.

To achieve the overall objective, this thesis is organized into five research chapters with the following **specific objectives**:

1. To study the effects of high rearing density during gonadal development on the resulting sex ratios. Since high rearing density can be considered a stressful situation, we also studied the possible role of cortisol in the observed masculinization of zebrafish exposed to elevated rearing density.

2. To understand the underlying molecular mechanisms responsible for masculinization in response to density and temperature during sex differentiation. We were particularly interested in identifying persistent gene expression patterns that could be used as biomarkers of previous exposure to environmental perturbations.

3. To study the contribution of epigenetic regulatory mechanisms after exposure to elevated temperature on the regulation of the expression of key genes involved in sexual development. To this end, gonadal DNA methylation profiles were measured using a targeted sequencing approach.

4. To study whether natural populations of zebrafish, which possess an intact genetic sex determination system, can also be masculinized by exposure to elevated temperature during sex differentiation.

5. To determine whether the masculinizing effects of exposure to elevated temperature can be transmitted to the following generations and whether DNA methylation plays any role in such transmission.

Report of the supervisors

Report of the supervisors

Dr. Francesc Piferrer and Dr. Laia Ribas, supervisors of the P.D. thesis entitled "*Environmental effects during gonadal development in fish: role of epigenetics*" by Alejandro Valdivieso, certify that the results obtained have been or will be submitted to peer-reviewed international journals. The entire thesis comprises five papers: two of them already published, one submitted and two in preparation. The details of the papers, journal and their impact factor (Journal Citation Reports) are detailed below:

Chapter 1

"Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response". L. Ribas, A. Valdivieso, N. Díaz, F. Piferrer. *Journal of Experimental Biology* 220 (6), 1056-1064. doi: 10.1242/jeb.144980.

Impact factor (2017): 3.179
Journal Citation Reports:
Area: Biology–Science
Rank:17/85
Quartile: Q1

The Ph.D. candidate conducted one of the two experiments, participated in data analysis, did all the graphs, participated in the interpretation of the results and in writing the manuscript.

Chapter 2

"Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations". A. Valdivieso, L Ribas, F Piferrer. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*. doi:10.1002/jez.b.22848.

Impact factor (2018): 1.716
Journal Citation Reports:
Area: Zoology–Science
Rank: 43/170
Quartile: Q2

The Ph.D. candidate carried out the microarray analysis and the validation of the data by qPCR. He also did all the graphs and the subsequently analysis, participated in the interpretation of results, discussion and writing of the manuscript.

Chapter 3

"Fish reproductive tissues as heat recorders: DNA methylation epigenetic marks in zebrafish gonads correctly recapitulate past thermal history". A. Valdivieso, D. Anastasiadi, L Ribas, F Piferrer. *Epigenetics & Chromatin.* To be submitted.

Impact factor: 4.185
Journal Citation Reports:
Area: Genetics and heredity - Science
Rank: 37/174
Quartile: Q1

The Ph.D. candidate participated in the design of the experiments, set up the experiments, did all thermal exposures and samplings. He also prepared samples and libraries for multiplex bisulfite sequencing and did all gene expression analysis by qPCR. He also did the bioinformatic analysis of the data, did all graphs, participated in the interpretation of the results, discussion and writing of the manuscript.

Chapter 4

"Hot sex in wild zebrafish: can the natural genetic sex determination mechanism buffer environmental effects on population sex ratios?". A. Valdivieso, C. Wilson, A. Amores, H.N., M.D.S. Rodrigues, Rafael, L. Ribas, J. Postlethwait, F. Piferrer. In preparation.

The Ph.D. candidate contributed to the experimental design and was responsible of the sampling and genotyping all fish from wild strains by PCR with the aid of primers he contributed to develop. He did all the analysis of the data and took the photomicrographs and did all the graphs. He participated in the interpretation of the results, discussion and writing the manuscript.

Chapter 5.

"Family-dependent variation in the multigenerational effects on sex ratios in zebrafish exposed to elevated temperature: changes in the testicular epigenome in the unexposed offspring.". A. Valdivieso, L. Ribas, A. Monleon, L. Orban, F. Piferrer. In preparation.

The Ph.D. candidate participated in the experimental design, did all crosses, took care of the fish, performed all sampling. He determined global DNA methylation, analyzed the sex ratio and methylation data, did all the graphs. He also participated in the interpretation of the results, discussion and writing of the manuscript.

The director and co-director also inform, to the best of their knowledge, that colleagues that appear as coauthors in some of the papers included in this have no used, implicitly or explicitly, the data or these articles for the elaboration of other Ph.D. thesis.

Barcelona, January 10, 2020.

Institut de Ciències del Mar (ICM-CSIC)

Dr. Francesc Piferrer	Dr. Laia Ribas
Director	Co-director

Results

Block 1: Density effects

Chapter 1

Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response

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Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response

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Abstract

The zebrafish (*Danio rerio*) has become a well-established experimental model in many research fields but the loss of the primary sex-determining region during the process of domestication renders laboratory strains of zebrafish susceptible to the effects of environmental factors on sex ratios. Further, an essential husbandry aspect – the optimal rearing density to avoid stress-induced masculinization – is not known. We carried out two experiments: the first focusing on the effects of density on survival, growth and sex ratio by rearing zebrafish at different initial densities (9, 19, 37 and 74 fish per litre) for 3 months (6–90 days post-fertilization, dpf), and the second focusing on the effects of cortisol during the sex differentiation period (15–45 dpf) for zebrafish reared at low density. The results showed an increase in the number of males in groups subjected to the two highest initial rearing densities; we also observed a reduction of survival and growth in a density-dependent manner. Furthermore, zebrafish treated with cortisol during the sex differentiation period showed a complete masculinization of the population; treatment with the cortisol synthesis inhibitor metyrapone negated the effects of exogenous cortisol. Our results indicate that the process of sex differentiation in domesticated zebrafish can be perturbed by elevated stocking density and that this effect is likely to be mediated by an increase in cortisol through the stress response. However, the underlying mechanism needs further study.

Key words: sex differentiation, stress, sex ratio, cortisol, *Danio rerio*

1. Introduction

The zebrafish (*Danio rerio*) is a small tropical freshwater fish of the family Cyprinidae (Mayden et al., 2007) and a well-established animal model for many research fields (Streisinger et al., 1981; Chakrabarti et al., 1983; Whitfield et al., 1996; McGonnell and Fowkes, 2006; Ribas and Piferrer, 2014). Studies looking at genetic polymorphisms and using a variety of screening methods on domesticated zebrafish strains have identified putative sex-linked loci in different chromosomes: 3 and 4 (Anderson et al., 2012), 5 (Bradley et al., 2011) and 16 (Howe et al., 2013). Moreover, using different families and several crosses, family-dependent sex ratios were obtained, which led to the proposal that domesticated zebrafish have a polygenetic sex-determining system in which genetic factors and environment determine the sex (Liew et al., 2012; Ribas et al., 2017a). Recently, in wild zebrafish populations it has been found that a locus at the telomeric region of chromosome 4 is strongly linked with sex and compatible with a WZ/ZZ sex determination system (Wilson et al., 2014). Interestingly, this region is not found in most laboratory strains and it has been argued that domestication has caused the loss of the sex-linked region as a result of continuous breeding and mutations (Wilson et al., 2014). Thus, because they lack the master sex-determining gene, laboratory strains of zebrafish probably have several minor sex-linked loci that determine sex, although under strong environmental influence. This would explain why laboratory zebrafish behave as though they have a polygenic sex-determining system (Liew et al., 2012). Environmental influences during early development are able to influence sex ratios in species with polygenic sex determination (Penman and Piferrer, 2008). Surprisingly, and opposite to what happens with rodents, where universal husbandry protocols are available, the zebrafish community lacks universal rearing guidelines. Although many authors have focused on the different aspects of rearing conditions (Westerfield, 1995; Casebolt et al., 1998; Trevarrow, 2004; Lawrence, 2007; Pavlidis et al., 2013; Giacomini et al., 2015), a main variable such as rearing density is still not clearly established. Thus, it is urgent to clarify the influence of rearing density on zebrafish sex differentiation.

Regardless of whether they are a laboratory model such as zebrafish or farmed species, rearing fish at a sufficiently elevated density has obvious advantages regarding space and resource utilization optimization. The influence of stocking density on fish sex ratios has only been documented in a few fish species, e.g. paradise fish, *Macropodus opercularus* (Francis, 1984), some coral reef fish species (Lutnesky, 1994; Kuwamura et al., 2014), European eel, *Anguilla anguilla* (Roncarati et al., 1997; Krueger and Oliveira, 1999; Huertas and Cerdà, 2006), European sea bass, *Dicentrarchus labrax* (Saillant et al., 2003b) and zebrafish (Hazlerigg et al., 2012). However, as for any confined animal, a rearing density beyond a certain threshold has evident detrimental consequences in fish, including lower survival, decreased growth, higher incidence of deformities, increased susceptibility to diseases, and altered reproduction, (e.g., (Iguchi et al., 2003; North et al., 2006). The last of these is manifested in lower fecundity or higher larval mortality (Coman et al., 2007) due to an increase in plasma cortisol levels when fish are reared at

elevated densities (Schreck, 1981; Barton, 2002). Cortisol is a glucocorticoid hormone regulating gluconeogenesis and other metabolic processes where glucose is needed as an energy substrate, and plays a role also in osmoregulation, growth and reproduction (Barton et al., 1987; Mommsen et al., 1999). Cortisol is considered to be a primary stress indicator with detrimental effects in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Thus, elevated cortisol reduced survival in Atlantic cod, *Gadus morhua*, eggs (Kleppe et al., 2013) and inhibited puberty in common carp, *Cyprinus carpio* (Consten et al., 2002).

In recent years, it has become evident that elevated plasma or whole-body cortisol levels during early development can also affect the process of sex differentiation, resulting in an increase in the number of males in different species, including medaka, *Oryzias latipes* (Hayashi et al., 2010), pejerrey, *Odontesthes bonariensis* (Hattori et al., 2009), southern flounder, *Paralichthys lethostigma* (Mankiewicz et al., 2013), and Japanese flounder, *P. olivaceus* (Yamaguchi et al., 2010). However, each one of these studies proposed a distinct, albeit not necessarily mutually exclusive, mechanism on the underlying mechanism responsible for masculinization by cortisol. Furthermore, cortisol synthesis can be inhibited by metyrapone, a chemical compound that blocks the conversion of 11-deoxycortisol to cortisol (Lisansky et al., 1989). Metyrapone has been used in some fish species (e.g. zebrafish or rainbow trout, *Oncorhynchus mykiss*), as a strategy to elucidate cortisol effects (Leach and Taylor, 1980; Miranda et al., 1998; Zanuzzo and Urbinati, 2015). For example, metyrapone treatment through the diet was able to inhibit masculinization induced by high temperature in Japanese flounder (Yamaguchi et al., 2010).

Studies on the effects of rearing density in zebrafish are scarce and sometimes controversial. In a study involving eight major zebrafish facilities across the world, it was found that holding densities as high as 12 fish per litre after 4 months of age did not result in negative effects on clutch size, spawning success or egg viability (Castranova et al., 2011). When mating, changes in egg production, hatching rate or larval length were not observed until a density of 60 fish per litre (Goolish et al., 1998). Cortisol levels increased up to fourfold in adult zebrafish confined at high densities of 40 fish per litre (Ramsay et al., 2006). Differences in the number of reared zebrafish required to observe alterations in behaviour are present in the literature: 0.025 fish per litre in 38 l tanks (Larson et al., 2006) 0.25 fish per litre in 60 l tanks (Spence and Smith, 2005) and 1.4 fish per litre in 21 l tanks (Moretz et al., 2007). In contrast, other authors did not find differences at 1.2 fish per litre using 45 l tanks (Gronquist and Berges, 2013). Despite these studies, the influence of density during sexual development, when fish are more susceptible to the effects of external perturbations, is far from clear in zebrafish. One study found that elevated density caused a decrease in growth and survival rates without a clear link with sex ratios (Hazlerigg et al., 2012). Another study found that high density increased the number of males, although there was a high interfamily variation in the response, suggesting that other factors, both genetic and environmental, could also be affecting sex ratios (Liew et al., 2012).

In this study, we took advantage of the fact that domesticated zebrafish are sensitive to environmental perturbations to address the general question of how environmental factors can influence the process of sex differentiation in fish and, in particular, how stocking density affects the sex ratio. In addition, and in order to study the possible role of cortisol in the masculinization of zebrafish subjected to stress confinement as a result of high density, synthetic cortisol and metyrapone were administered during the sex differentiation period.

2. Materials and Methods

Animal rearing conditions

Domesticated zebrafish (AB strain) were housed in a commercial rack (Aquaneering, San Diego, CA, USA) fitted with a recirculating water system (supplied with a water pump of 6000 l h^{-1}) and placed in an ad hoc chamber facility in our institute subjected to a constant photoperiod (12 h light:12 h dark), air temperature of 26±1°C and humidity of 50±3%. Water quality parameters were monitored daily and included: temperature 28±0.2°C, pH 7.2±0.5, conductivity 750–900 µS and dissolved oxygen 6.5–7.0 mg l^{-1}. Other water quality parameters were checked periodically (2–3 times a month) by the Water Analysis Service of our institute and maintained in the appropriate ranges (Ribas and Piferrer, 2014): ammonium 0.03± 0.00 mg l^{-1}, nitrite 0.25±0.14 mg l^{-1}, nitrate 66.42±8.04 mg l^{-1}, silicate 15.22±2.53 mg l^{-1} and phosphate 32.34±7.43 mg l^{-1}. Breeding was always performed by natural spawning after single- pair crossings. The total number of eggs and fertilized embryos was counted to ensure that fecundity was according to the reference values for this species (Ribas and Piferrer, 2014) and post-hatch viability in accordance with the OECD's guidelines for the Fish Sexual Development Test (OECD, 2011; **Fig. S1**). Eggs were reared in Petri dishes (ThermoFisher Scientific, Waltham, MA, USA) at ~50 eggs per dish filled with embryo medium (pH 7.2±0.5), supplemented with 0.1% Methylene Blue (Sigma-Aldrich, Madrid, Spain) at 26±1°C until 6 dpf. Then, hatched larvae were transferred to tanks at 6 dpf and housed in the commercial rack described above. Fish were fed *ad libitum* 3 times a day with a commercial food according to their developmental stages: 6–15 days post- fertilization (dpf) larvae were fed with Micron (Sera, Heinsberg, Germany), which contains natural plankton (50% spirulina and 16% krill); 15–40, 40–60 and 60–90 dpf fish were fed with pellets of increasing size: ST1, ST2 and ST3, respectively (AquaSchwarz, Göttingen, Germany), containing 54–59% crude protein, 15–16% lipids, 12% crude ash, vitamins A, D3, E, C (C at 100–2000 mg kg^{-1}) and omega-3 (HUFA at 28–30 mg g^{-1}). The commercial feed in all stages was supplemented with live *Artemia nauplii* (AF48, INVE Aquaculture, Dendermonde, Belgium). The debris at the bottom and walls of the tanks was cleaned 3 times a week. Fish were kept in accordance with 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).

Experiment 1: effects of stocking density

Fish were reared in tanks (Aquaneering, model ZT280) of a nominal volume of 2.8 l (the actual capacity was 2.7 l). Different densities were achieved by placing 6-dpf larvae in the following numbers in the 2.7 l actual available volume: 25, 50, 100 and 200 larvae. This gave initial densities of 9.25, 18.51, 37.03 and 74.07 fish per litre. For clarity purposes, the rounded values of 9, 19, 37 and 74 fish per litre will be used from now on. The experiment was repeated 4 times with four different families, and each density treatment was replicated between 3 and 7 times, depending on the fecundity of each family. In total, 1625 fish were used. Survival for each density treatment was recorded at 15, 20, 30, 50, 70 and 90 dpf. At 50 dpf, juvenile fish from the 9 and 74 fish per litre groups were euthanized by immersion into ice for cortisol analysis (see below for further details). At 90 dpf, fish were euthanized on iced water followed by decapitation and total body mass (M_b, precision±0.05 g), standard length (SL, precision±0.01 cm) and sex ratios were recorded. The Fulton's condition factor (*k*) was calculated following the formula: $k=(M_b\times100)/SL^3$ (Fulton, 1902).

Experiment 2: cortisol treatment

The spawn of three different pairs were pooled in each experimental tank (with a total of 36 fish per 2.8 l tank), and received one of the following randomly assigned treatments: control (C), cortisol (F; hydrocortisone; ref. H 0888, Sigma-Aldrich), metyrapone (M; 2-methyl-1,2-di-3-pyridyl-1-propanone; ref. 856525, Sigma- Aldrich), cortisol plus metyrapone (F+M), and the synthetic androgen 17α-methyltestosterone (MT; ref. M7252, Sigma- Aldrich) as a positive control for masculinization. Treated feed was prepared with the following concentrations (μg g^{-1} feed). F group: 50; F+M group: 50+500; M group: 500; MT group: 50. All compounds were diluted with 3 ml of 96% ethanol and sprayed directly on feed. Feed in the control group was also sprayed with 96% ethanol. The treated feed was then air dried in a ventilated hood for 3 h to remove ethanol traces and then stored at −20°C until consumption. For each group, at least two replicates were used, with a total number of 444 fish in the whole experiment. Fish survival calculations and sampling procedures were performed as described above for experiment 1. Coinciding with the sex differentiation period (15–45 dpf), tanks were removed from the recirculating water system housing rack and placed in groups of three inside a large tub filled with water, maintained at a constant water temperature of 27.63±0.11°C with the use of electric waterproof heaters. Tanks were individually oxygenated with an independent air flow source but without altering environmental conditions and natural swimming. Renewal of three-quarters of the tank water was performed 3 times a week, together with removal of the debris at the bottom of the tanks. During this period, fish were fed 3 times a day with the treated feed. Afterwards (at 45 dpf), tanks were moved back to the housing rack.

Whole-body cortisol measurement

Whole-body cortisol levels were measured in juvenile 50 dpf zebrafish samples using a commercial enzyme immunoassay kit (ref. 402710, Neogen, Lansing, MI, USA), following the manufacturer's instructions with slight modifications. The specificity of the test was evaluated by comparing the samples with a standard curve. The linear regression of the standard curve was R^2=0.979. The mean intra-assay coefficient of variation for all tests was always <10% with an assay sensitivity of 0.03 ng ml^{-1}. Frozen fish were homogenized individually in 100 µl cold phosphate-buffered saline (PBS, pH 7.4) using a glass pestle, then suspended in 2 ml of pre-cooled diethyl ether for 15 min at 4°C. Homogenates were centrifuged at 2500 g for 2 min and frozen at −80°C for 30 min to recover the organic phase. This step was repeated 3 times. The diethyl ether from each sample was evaporated using a dry heater at 30°C in a ventilated hood. Samples were immediately resuspended in extraction buffer supplied by the manufacturer and diluted 1:5. Tubes containing samples and standards were measured at 650 nm in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). All samples were measured in duplicate. Cortisol levels of a total of 23 juvenile fish were measured: eight fish in the low-density group (from the 9 fish per litre group) and 15 fish in the high-density group (from the 74 fish per litre group). The mean Mb of the fish at each density was used to calculate whole-body cortisol per gram of fish.

3. Results

Experiment 1

Effect of rearing density on survival

Survival was inversely related to rearing density (**Figure 1**). Fish survival at 90 dpf was 76%, 64%, 45% and 35% relative to the initial number of fish in each case for the 9, 19, 37 and 74 fish per litre groups, respectively. The two highest tested densities significantly decreased (P<0.05) survival when compared with the two lowest densities at all sampling points (**Figure 1A**). The highest mortality was observed between 6 and 15 dpf but survival was density independent after this point (**Figure 1B**).

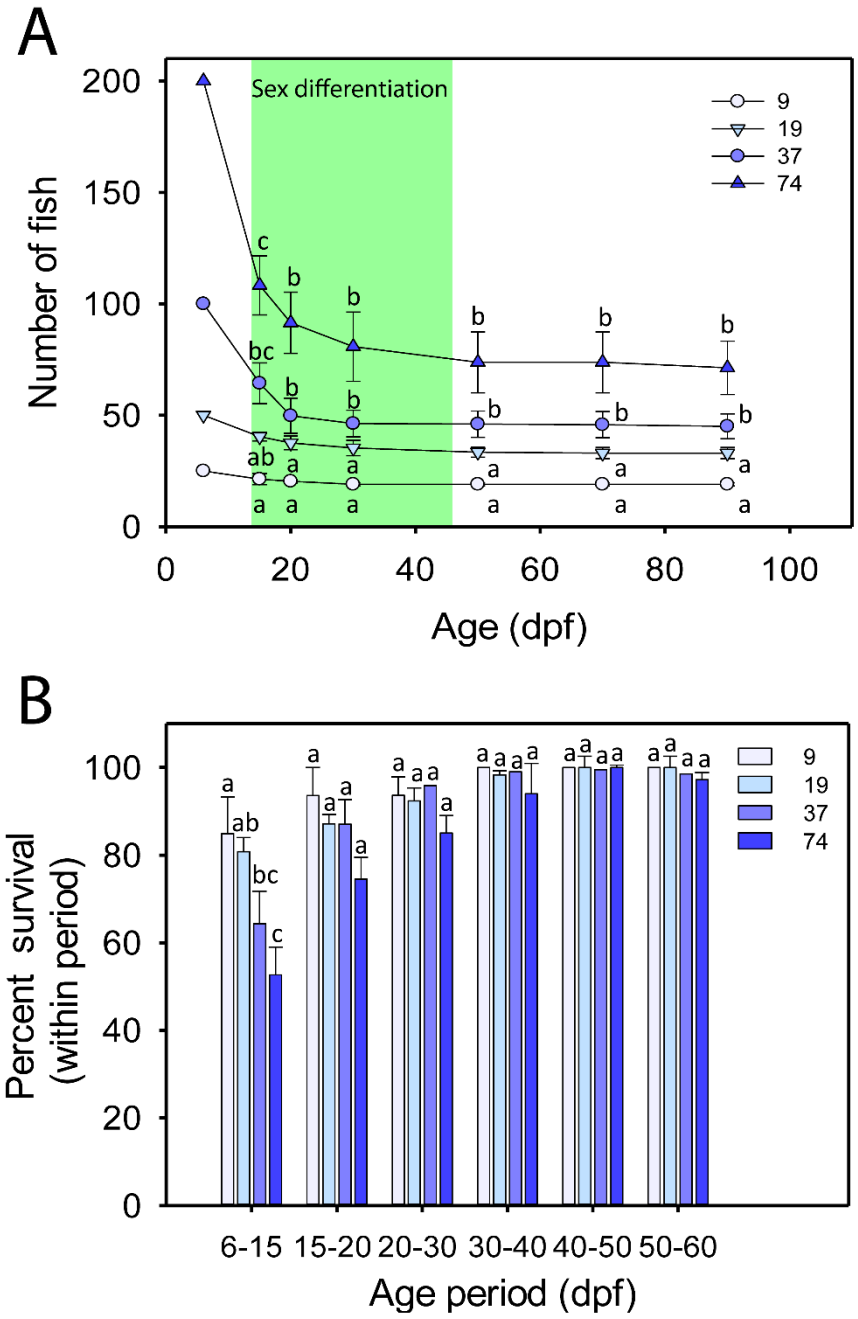

Figure 1. Zebrafish survival as a function of stocking density during the first 3 months of age. Fish were held at densities of 9, 19, 37 and 74 fish per litre. (**A**) Absolute survival from 6 to 90 days post-fertilization (dpf). The sex differentiation period (15–45 dpf) is shaded in green. (**B**) Survival relative to different age periods. Data are presented as means±s.e.m. of 3–6 biological replicates per group. Significant differences (P<0.05) among groups at a given sampling age period were tested by one-way ANOVA and are indicated by different letters.

Effect of rearing density on growth

Growth was also inversely related to rearing density, with sex- related differences **(Figure 2**). M_b was significantly decreased (P<0.05 and P<0.01) in males for groups reared at a density of 19 fish per litre or higher, but in females such an effect was observed only with densities of 37 fish per litre or higher (P<0.05) (**Figure 2A**). SL was significantly (P<0.05) decreased in both sexes at densities of 37 fish per litre and higher (**Figure 2B**). A similar trend of density effects was observed for *k* (**Figure 2C**).

Effect of rearing density on sex ratio

There was a density-dependent effect on zebrafish sex ratio as the number of males observed at 90 dpf increased with rearing density (**Figure 3A**). At a density of 9 and 19 fish per litre, the number of males was 54.4±11.06% and 61.4±6.04%, respectively, a proportion not different from the expected Fisherian sex ratio. Significant differences with respect to the 9 fish per litre group were recorded with densities of 37 fish per litre (71.6±6.5%; P<0.05) and 74 fish per litre (80.1±3.4%; P<0.01). In order to determine density effects when sex differentiation takes place (15–45 dpf), the number of fish alive in the tank during this process was calculated by averaging observed values at 15, 20, 30 and 50 dpf for each replicate treatment (**Table 1**). The mean±s.e.m. number of fish per litre was 7.4±0.4, 13.5±0.3, 18.3±0.7 and 31.9± 1.0 in the 9, 19, 37 and 74 fish per litre groups, respectively. Effect of rearing density on whole-body cortisol levels Whole-body cortisol levels in fish subjected to high density confinement over 45 days (from 6 dpf to 50 dpf) increased at the end of this period by ∼50% in the 74 versus the 9 fish per litre groups (**Figure 3B**). However, these differences were not significant (P=0.49).

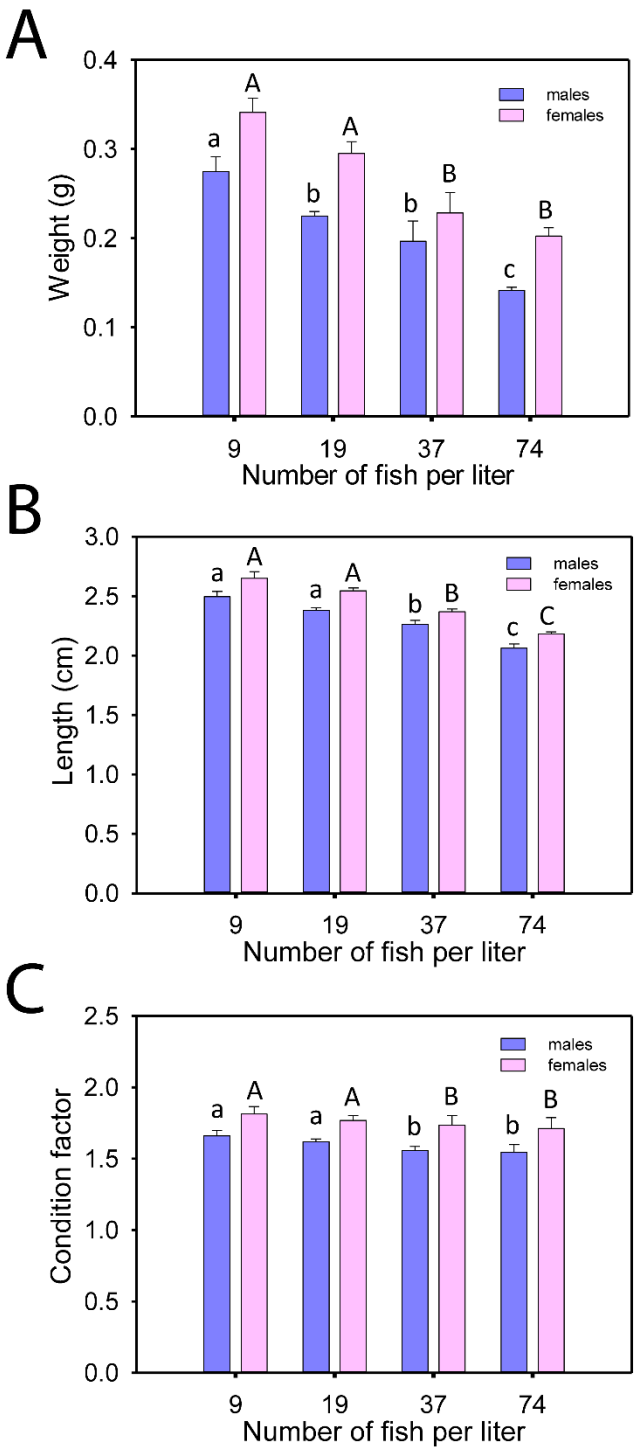

Figure 2. Zebrafish growth as a function of different stocking density during the first 3 months of age and sex. (**A**) Body mass (Mb), (**B**) standard length (SL) and (**C**) condition factor (k). Data are presented as means±s.e.m. of 3–6 biological replicates per group. The number of fish at 90 dpf was 57, 231, 181 and 280 for the 9, 19, 37 and 74 fish per litre groups, respectively. Within the same sex, different letters indicate significant differences (a–b or A–B, $P<0.05$; and a–c or A–C, $P<0.01$) between groups analysed by ANOVA, except for SL and k male data, which were analysed by a Kruskal–Wallis test.

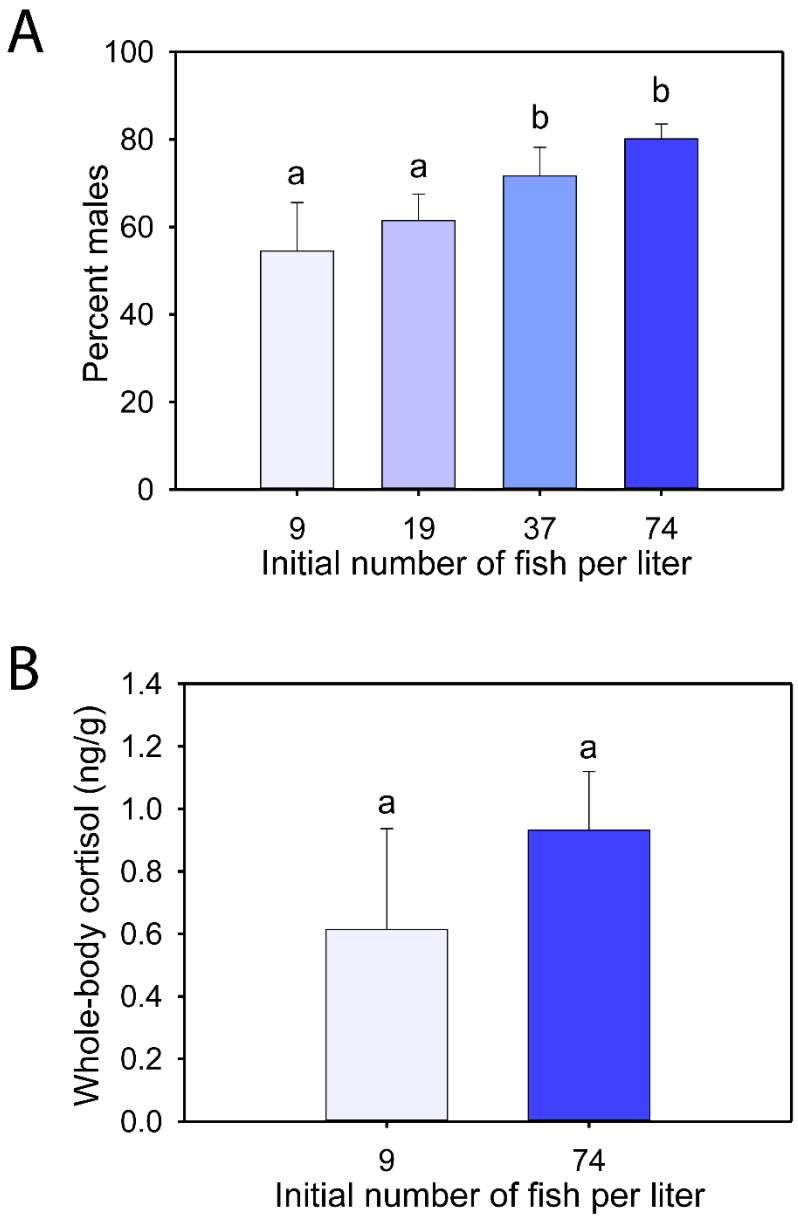

Figure 3. Sex ratio and whole-body cortisol levels in zebrafish reared at different densities. (**A**) Zebrafish sex ratio as a function of stocking density during the first 3 months of age. Data are presented as means±s.e.m. of 3–6 biological replicates per group. The final number of fish per group is as in Fig. 2. Significant differences among groups (indicated by different letters) were analysed by a Chi-squared test with Yate's correction (P<0.05 in 37 fish per litre group and P<0.01 in 74 fish per litre group) with respect to the group with the lowest rearing density. (**B**) Whole-body cortisol levels in juvenile zebrafish at 50 dpf as a function of stocking density. Data are presented as means±s.e.m. of 8 (9 fish per litre) or 15 biological replicates (74 fish per litre). There was no significant difference between groups (Student's t-test, P=0.49).

Table 1. Stocking density effects on zebrafish survival during the period of sex differentiation and on adult sex ratio

		Initial		Sex differentiation period			Final		
Pair	No. of replicates	No. of fish	No. of fish per litre	No. of fish per tank	No. of fish per litre	No. of fish per litre per group	No. of fish per tank	% Males	*P*-value
1	3	25	9	19.0±0.0	7		19	36.8	
2			9	20.5±0.4	7.6		20	60	
3			9	20.5±0.7	7.6	7.4±0.4	18	66.7	ns
1	7	50	19	27.3±0.5	10.1		22	31.8	
2			19	36.8±0.6	13.6		33	75.8	
3			19	37.8±0.0	14		36	63.9	
3			19	46.7±1.2	17.3		40	75	
3			19	31.3±0.8	11.6		30	66.7	
3			19	34.3±0.9	12.7		31	58.1	
4			19	42.0±0.7	15.6	13.5±0.3	39	59	ns
3	4	100	37	31.0±0.0	11.5		31	54.8	
3			37	49.3±0.0	18.2		48	79.2	
3			37	60.0±0.7	22.2		53	79.2	
4			37	57.8±1.3	21.4	18.3±0.7	49	77.6	$P<0.05$
3	4	200	74	121.5±1.6	45		98	74.5	
3			74	74.0±1.7	26.3		69	88.4	
3			74	78.5±1.0	29.1		60	78.3	
3			74	71.0±1.7	24.9	31.9±1.0	53	79.2	$P<0.01$

Experiment 2

Effect of cortisol on survival

At the end of the experiment (90 dpf), survival of the control group in experiment 2 was lower than that of the control group in experiment 1. Treatment with M or MT did not affect survival. However, treatment with F, particularly when it was administered alone, significantly (P<0.05) increased survival (**Figure 4**).

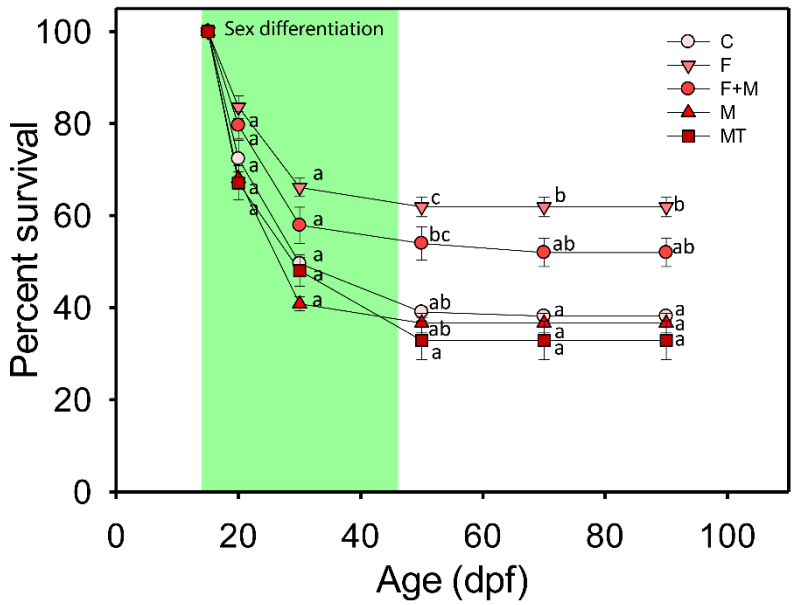

Figure 4. Zebrafish survival during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. The final number of fish was 34, 75, 21, 37 and 26 fish per group, respectively. The sex differentiation period (15–45 dpf) is shaded in green. Data are presented as means±s.e.m. of 2–3 biological replicates per group. Significant differences (P<0.05) among groups at a given sampling age were examined by one-way ANOVA and are indicated by different letters.

Effect of cortisol on growth

Male M_b in the F, F+M and MT groups was significantly reduced (P<0.05) when compared with the control group but not with the M group (**Figure 5A**). Treated females did not show any statistical differences in Mb or SL (the MT group contained only two females; **Figure 5A, B**). Males treated with synthetic cortisol showed a significant decrease in SL (P<0.05; **Figure 5B**). No significant differences were found in k in either males or females when compared with control fish (**Figure 5C**).

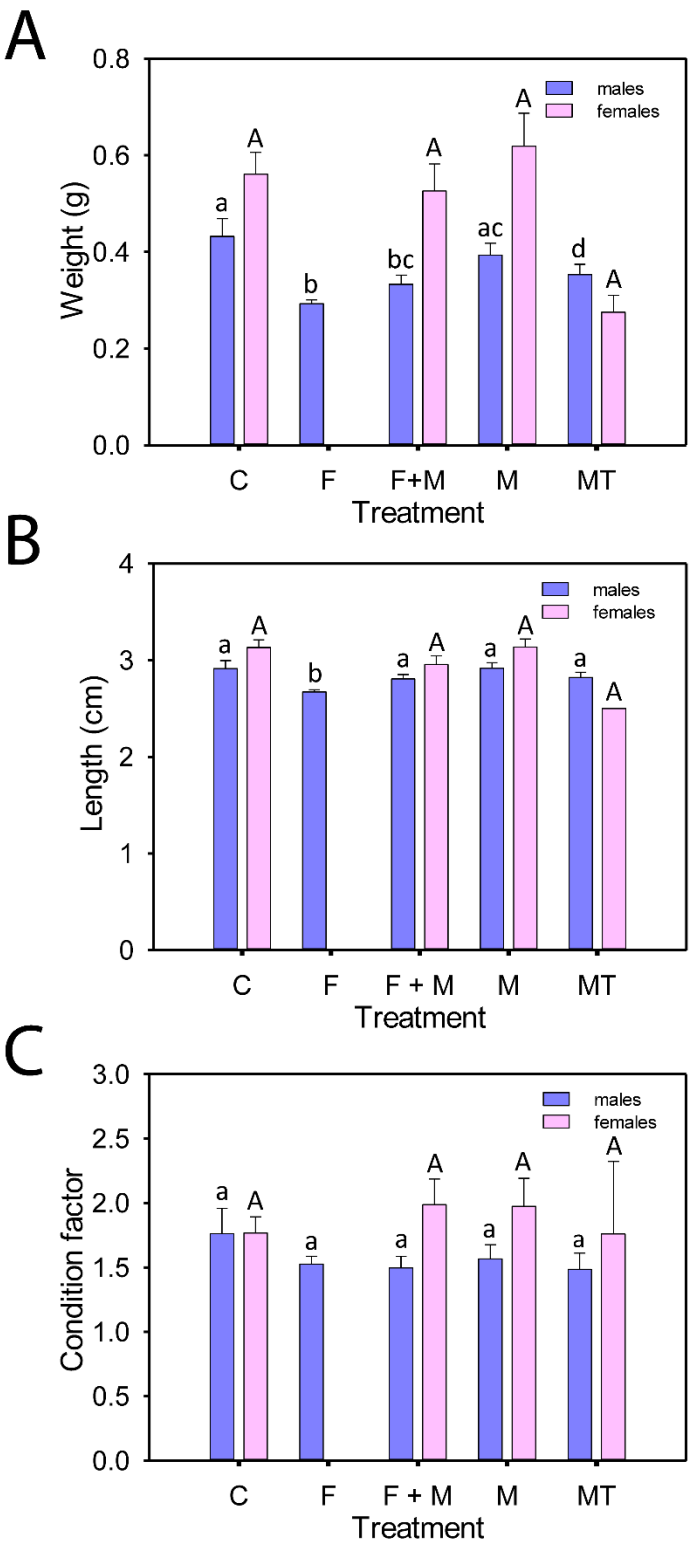

Figure 5. Zebrafish growth during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. (**A**) Mb, (**B**) SL and (**C**) k. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Within the same sex, different letters indicate significant differences (P<0.05) between groups analysed by ANOVA, except for SL and k male data, which were analysed by a Kruskal–Wallis test.

Effect of cortisol on sex ratio

Treatment with cortisol resulted in a complete masculinization of the population ($P<$ 0.001; **Figure 6**). The same effect occurred with the positive control treatment MT, where 92.3±6.7% of the fish were masculinized ($P<0.001$). No differences in sex ratios were found in the M and M+F groups (61.9±0.6% and 48.6±2.7% of males, respectively) with respect to the control group (44.1±3.2%), meaning that metyrapone counteracted cortisol effects, preventing masculinization.

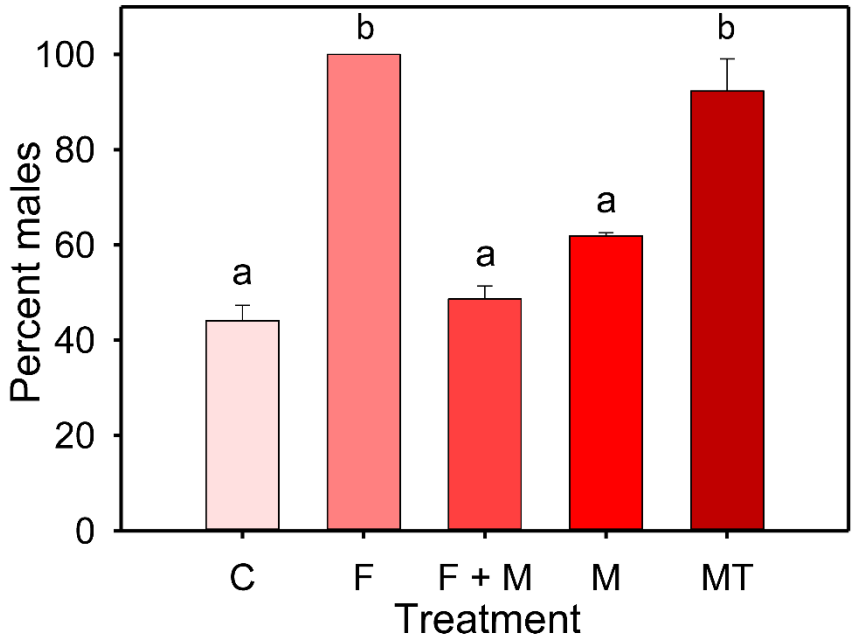

Figure 6. Zebrafish sex ratio at 3 months of age as a function of treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Significant differences among groups were analysed by the Chi-squared test with Yate's correction. Different letters indicate a significant difference ($P<0.001$) with respect to the control group.

4. Discussion

In this study, we tested four stocking densities for their effect on survival, growth and sex ratio of domesticated zebrafish. Consistent with the results of Hazlerigg et al. (2012), stocking zebrafish in high densities was detrimental to their survival, as also observed in other fish species including the Nile tilapia, *Oreochromis niloticus* (Huang and Chiu, 1997), pufferfish, *Takifugu rubripes* (Kotani et al., 2009), vundu catfish, *Heterobranchus longifilis* (Coulibaly et al., 2007), and pigfish, *Orthopristis chrysoptera* (DiMaggio et al., 2014).

The stocking densities used in this study were set up at the time of transfer of the larvae to the rearing tanks but no attempt was made to replace dead fish with new ones to maintain the initial number of fish during the experiment. Thus, effects on growth and

sex ratios determined at 90 dpf have to be seen as the result of the cumulative effects of varying stocking density as some fish died while others grew. Initial rearing densities of 9 and 19 fish per litre did not have any effect on sex ratios at 90 dpf. In contrast, initial densities of 37 and 74 fish per litre significantly increased the proportion of males. It should be noted that rearing fish at 9 and 19 fish per litre did not affect survival during 6–15 dpf, or SL and condition factor *k*, presumably indicating no influence of conditions other than rearing density, although a density of 19 fish per litre decreased M_b. Furthermore, survival of the group reared at 9 fish per litre was around 76%. This value is standard for zebrafish and suggests that rearing conditions other than density were not deleterious. Thus, it is probably safe to state that, based on our results, a stocking density in the range of 13–20 fish per litre in a ∼3 l commercial tank starting at 6 dpf would not cause masculinization. However, with improvements in feeding or diet formulation (e.g. supplemented by rotifers), survival in the initial stages could be higher than the survival recorded in this study.

Thus, based on our data, in order to avoid density-induced masculinization, the stocking density should be taken into account, especially during the sex differentiation period. Ongoing experiments in our lab involving additional families show that elevated rearing density during this period results in a clear sex bias towards males, confirming the present results. Following the above-mentioned guidelines, in recent experiments in our lab, we did not observe masculinization. We do not know for sure whether, taking into account other factors (e.g. social interactions, behaviour, husbandry strategies, etc.) applied in other laboratories, this density range would also work well with tanks of much larger volume, e.g. 45 l tanks, as used by Gronquist and Berges (2013) for behavioural studies. In our experiments with domesticated zebrafish, other than density, environmental conditions (e.g. water quality, feeding regime, etc.) were the same in all tanks. Thus, although the sex ratio of domesticated zebrafish may be influenced by several factors, in this study we focused on rearing density, and aimed to minimize other possible environmental influences.

An interesting aspect is whether the results obtained in this study would apply to other zebrafish strains. We used domesticated zebrafish from the AB strain, but other laboratories have carried out density experiments using other zebrafish strains. Liew et al. (2012), using a Tübingen (TU) strain, found an increase in males of ∼20% with rearing densities of 66.6 fish per litre when compared with 33.3 and 16.6 fish per litre. In contrast, Hazlerigg et al. (2012), using the WIK (Wild Indian Karyotype) strain, found that densities from 2 to 40 fish per litre had no effect on sex ratios when combined with different feeding regimes (constant or limited). Here, it is interesting to note that while the WIK strain has a WZ/ZZ sex determination system with a putative sex-determining gene at the tip of chromosome 4, the TU and AB strains have lost this gene, presumably during the many manipulations in the process of domestication (Wilson et al., 2014). Because of this loss, the TU and AB strain sexual development is more sensitive to environmental cues. This concurs with the fact that domesticated zebrafish show a wide range of interfamily variation in sex ratios and that a polygenic system of sex

determination has been proposed for domesticated zebrafish (Liew et al., 2012; Ribas et al., 2017a) see Table 1). Taken together, it can be concluded that the effects of density found in this study with the AB strain would also apply to the TU strain, while experiments with the WIK strain are needed in order to determine whether the presence of the WZ/ZZ system confers greater resistance to the influence of environmental cues on sex ratios.

We also found that growth is inversely related to stocking density in zebrafish, as also found by Hazlerigg et al. (2012) and conforming to what has been observed in other species (Barton et al., 1987; Holm et al., 1990; Björnsson, 1994). The fish reared at the two lowest densities in this study had a mean M_b of ~0.25±0.01 and ~0.32±0.01 g and a mean fork length of ~2.4±0.04 and ~2.6±0.05 cm at 90 dpf for males and females, respectively, and corresponding with the range for the typical M_b and length of an adult zebrafish at this age reviewed in Ribas and Piferrer (2014). However, the highest stocking density tested produced a decrease in M_b, with mean values of ~0.14±0.003 and ~0.20±0.003 g for M_b and ~2.1±0.03 and ~2.2±0.01 cm for SL in males and females, respectively. Similar results were found in other experiments stocking zebrafish at a density of 60 fish per litre with a constant feeding regime, in which a decrease to ~2.0 cm in length was also observed (Hazlerigg et al., 2012). In addition, in experiment 2, males treated with different compounds showed significant differences in growth rate, whereas in females those differences were not present. Specifically, M_b was reduced in fish from groups treated with cortisol, in agreement with what has previously been described for other fish species, e.g. goldfish (Bernier et al., 2004), largemouth bass, *Micropterus salmoides* (O'Connor et al., 2010), detrimental effects on survival and growth, and will prevent male- biased sex ratios in the subsequent adult population. In addition to providing new information on environmental effects on fish sex ratios, our study thus offers useful information on how to rear zebrafish, filling a gap in an essential husbandry aspect in this important experimental model.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.P. and L.R. designed the study. L.R., A.V. and N.D. conducted the experiments and the subsequent analysis. L.R. drafted the initial manuscript. L.R., A.V., N.D. and F.P. wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary Figures

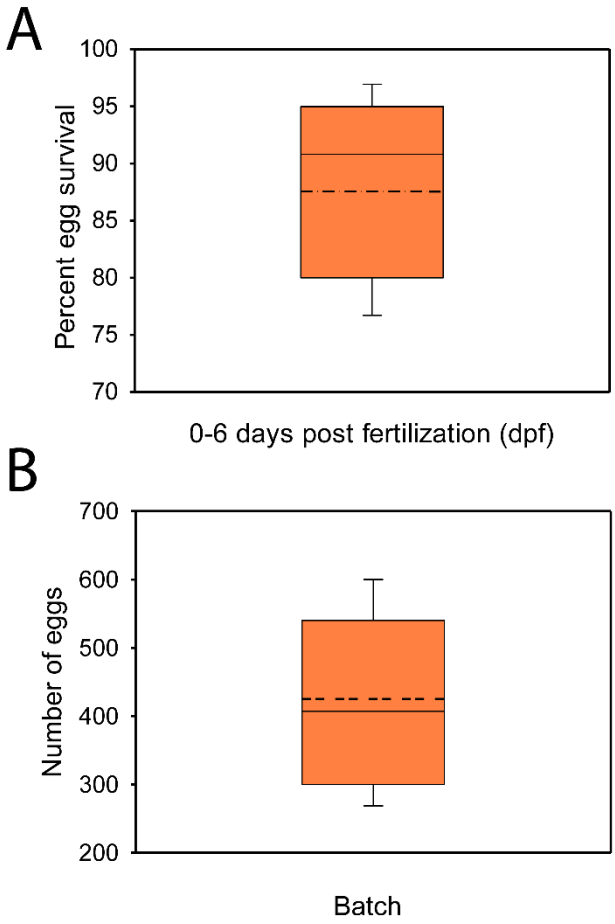

Figure S1. Fecundity of the broodstock and viability of the zebrafish eggs used in this study. **(A)** Number of eggs spawned by the seven pair of breeders used in this study. The dashed line indicates the reference value for zebrafish (Ribas and Piferrer, 2014). (**B)** Egg survival during 0–6 days post fertilization. The dashed line indicates the minimum accepted value in the OCED's Fish Sexual Development Test (OECD, 2011). In the boxplots, the solid and dashed lines indicate the median and mean, respectively; the lower and upper edges indicate the 25th and 75th percentiles, respectively; and the top and the bottom whiskers indicate the 5th and 95th percentiles, respectively.

Chapter 2

Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations

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Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations

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Abstract

Sex is remarkably plastic in fish and can be easily influenced by environmental cues, in which temperature has been the most studied abiotic factor. However, it has been shown that elevated population densities can increase the number of males in several species but 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 versus low population densities contained 4,634 differentially expressed genes. Among them, a set of promale genes (*amh*, *sypc3*, *spata6*, and *sox3*) were upregulated in the high-population density group. 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 *sycp3*) but also genes involved in other functions (e.g., *faima*, *ccm21*, and *ankrd6b*) and a downregulation of *cyp19a1a* together with other genes (e.g., *lgals9l1* and *ubxn2a*). We identified the common Gene Ontology terms involved in the reproduction and sexual development that were consistently affected in both environmental factors. These results show that regardless of the environmental perturbation there are common genes and cellular functions involved in the resistance to masculinization. These altered gene-expression profiles can be used as markers indicative of previous exposure to environmental stress independent of conspicuous alterations in sex ratios or gonadal morphology.

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

1. Introduction

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

Sex determination in fish can be regulated by genetic factors (genetic sex determination, GSD), by influences of the environment (environmental sex determination, ESD), or a combination of both (Devlin and Nagahama, 2002; Penman and Piferrer, 2008; Guiguen et al., 2018). In GSD species, sex can be determined by the action of a single or master gene, by the action of a master gene plus secondary loci or by the combined action of several autosomal genes, the latter called polygenic sex determination (PSD). In ESD, sex can be influenced by different abiotic and biotic factors, of which temperature and population density are the most common ones (Penman and Piferrer, 2008; Valenzuela, 2008; Shen and Wang, 2019). Nowadays, GSD and ESD are not considered necessarily mutually exclusive and, hence, are regarded as the two ends of a continuum (Sarre et al., 2004; Heule et al., 2014; Yamamoto et al., 2014).

For poikilothermic animals like fish, temperature is perhaps the most important abiotic factor. Abnormally elevated temperatures can masculinize many species if exposure takes place during the process of sex differentiation or earlier (Valenzuela and Lance, 2004; Ospina-Alvarez and Piferrer, 2008). The masculinizing effect of elevated temperature takes place regardless of the underlying sex determining system and thus have been observed in species with male heterogamety (XX/XY) such as the rainbow trout (*Oncorhynchus mykiss*) (Valdivia et al., 2014) and the Nile tilapia (*Oreochromis niloticus*) (Baras et al., 2001) and species with PSD such as the European sea bass (*Dicentrarchus labrax*) (Saillant et al., 2002; Díaz and Piferrer, 2015; Shen and Wang, 2019).

Regarding population density, it was found that elevated density in the Paradise fish (*Macropodus opercularis*) (Francis, 1984) and in the European eel (*Anguilla Anguilla*) (Roncarati et al., 1997; Huertas and Cerdà, 2006) was able to skew the sex ratio toward males. In contrast, in the European sea bass, no effects were observed when fish were confined during the early stages of development (Saillant et al., 2003b). In zebrafish (*Danio rerio*), elevated density resulted in male-biased sex ratios (Ribas et al., 2017b) with interfamily variation (Liew et al., 2012; Ribas et al., 2017c) due to genotype–environment interactions. Zebrafish is a small cyprinid widely used as an animal model in many research areas. Wild zebrafish strains exhibit a GSD system of the ZW/ZZ type, with a putative sex master gene located in the telomeric region of chromosome 4 (Wilson et al., 2014). In contrast, in the two most used laboratory strains (AB and TÜ) this system was not detected probably due to the loss of the sex-determining region during the domestication process (Wilson et al., 2014). Due to this, domesticated zebrafish behave

as a PSD system with elevated sensitivity to environmental perturbations (Liew et al., 2012; Ribas et al., 2017a) which explains why zebrafish facilities across the world usually encounter skewed sex ratios. Thus, laboratory strains of zebrafish constitute indeed a good model where to study the effects of environmental perturbations on the developing gonads and the resulting sex ratios.

Sex differentiation in zebrafish takes place from 10 until about 45 days post fertilization (dpf). In all individuals, gonads first develop as an ovary-like immature tissue (Takashi, 1977). Later, ovarian differentiation continues in about half the individuals, whereas in the remaining fish, apoptosis takes place and the ovarian tissue regresses and is progressively substituted by testicular tissue (Uchida et al., 2002; von Hofsten and Olsson, 2005; Orban et al., 2009). The stress resulting from exposure to elevated temperature during sex differentiation causes masculinization (Uchida et al., 2004; Abozaid et al., 2012; Brown et al., 2015; Ribas et al., 2017a), and thus there are some sex-reversed females (referred to as "neomales") (Pandian and Sheela, 1995). In addition to regular males and females, two additional transcriptomic profiles were identified in heat-treated zebrafish: the neomales and a novel type of females. These were phenotypic females but with a male-like transcriptomic profile, where an upregulation of male-related genes (e.g., *amh* and *dmrt1*) and a downregulation of female-related genes (e.g., *cyp19a1a*, *foxl2a*, and *vtg2*) was observed (Ribas et al., 2017a). Further, regardless of the type of stressor (temperature or density), a common physiological response is increased plasma cortisol levels (Ramsay et al., 2006; 2009; Yeh et al., 2013). This is a protective mechanism in response to stress that helps to regulate basal homeostasis (Wendelaar Bonga, 1997). It is well established that chronic stress has adverse effects on growth and reproduction (Schreck, 2010) but recently it has also been demonstrated that elevated cortisol levels due to stress are associated with masculinization (Fernandino et al., 2012; Fernandino et al., 2013). Further, oral administration of cortisol during early development resulted in complete masculinization in zebrafish (Ribas et al., 2017b).

However, 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 the resistant females mentioned above, 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 an 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.

2. Materials and Methods

Gonad samples and gonadal maturation data

Gonad maturation data and RNA samples of zebrafish exposed to different rearing densities 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/liter. 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, cDNA synthesis, and primers

RNA was extracted from eight fish per sex for each density (total $n = 16$ for each density) for quantitative polymerase chain reaction (qPCR) analysis (Table S1) using 400 µl of TRIzol reagent (Invitrogen, CA). 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, Carlsbad, CA), the complementary DNA (cDNA) was synthesized from 200 ng of RNA using SuperScript III reverse transcriptase (Invitrogen). Specific primers for target genes were designed using Primer Express® software (v. 3.0; Applied Biosystems, Foster, CA) and purchased from Invitrogen. Primers were designed within the interexon regions and their efficiencies were checked from 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 check the expected size of the PCR product. The qPCR reactions were carried out in triplicate and performed on a QuantStudio™ 12K Flex System (Applied Biosystems, Foster, CA) using 2 µl of SYBR Green PCR master mix (Applied Biosystems, Foster, CA), 0.5 µl for each forward and reverse primers and 2 µl cDNA (1/10 dilution) in a total reaction volume of 10 µl/well. The qPCR cycle parameters consisted of 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 and gene, the average threshold cycle (Cq) was calculated and normalized against a reference gene, the eukaryotic translation elongation factor 1α 1, like 1 (*eef1α1l1*), previously validated in zebrafish (McCurley and Callard, 2008). Relative gene expression data were obtained using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). The gene symbol, gene name, accession number, and forward and reverse sequences for each primer pair used in this study are summarized in **Table S2**.

Microarray analysis of ovaries obtained from fish exposed to HD

For microarray analysis, the control group (9 fish/liter) and the group exposed to the highest density (74 fish/liter) were used (from now on referred to as the LD and HD groups, respectively). From each group, six of the eight gonad samples used for the qPCR analysis were also used (**Table S3**) for microarray analysis and hybridized individually in a 4×44K Agilent platform (G2519F–026437) at the Barcelona Biomedical Research Park (PRBB). Briefly, the cRNA generated from the messenger RNA of each ovarian sample was prepared for overnight hybridization with the corresponding buffers during 17 hr at 65°C and washed on the following day. Hybridized slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies, Santa Clara, CA). The results were deposited in the Gene Expression Omnibus database with the accession number GSE116700.

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

Gene expression differences of HD versus LD females were compared with those of high temperature (HT) versus low temperature (LT) females, specifically, the "FHT2" females described in Ribas et al. (2017a). The FHT2 were females with an 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: (a) both experiments were performed at the same fish facility, (b) the laboratory strain used was AB, (c) 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±1°C, (d) exposure to the environmental perturbations included the sex differentiation period and (e) 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 $\chi 2$ test. For the qPCR $\Delta\Delta$Cq data, normality was checked with the Kolmogorov–Smirnov test and logarithmic transformations were applied when necessary. The homoscedasticity of variances was checked with Levene's test. Means were compared by one-way analysis of variance 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 software (v. 17; Statgraphics Technologies, Inc. The Plains, VA). Microarray raw data were processed by Agilent software to avoid saturation and generate feature extraction. The output data were statistically analyzed by R software (v. 3.4.2) (Team, 2013) application and the quantile normalization method was implemented (Smyth & Speed, 2003) using the Limma package (v. 3.5). To determine differentially expressed genes (DEGs), an adjusted $P \leq 0.05$ and fold change (FC) ≥ 1.2 was used as a 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 et al., 2008a; 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 ggplots (v. 2.2.1) and gplots (v 3.0.1) packages were used to modify the REVIGO graphs and to generate heatmap figures, respectively.

3. Results

HD-affected gonadal maturation in females and males

Elevated rearing density not only masculinized zebrafish in a density-dependent manner (Ribas et al., 2017b) 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 (**Figure S1**).

HD-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 (**Figure 1A**), whereas the expression of forkhead box L2a (*foxl2a*) was unaffected (**Figure 1C**). In testes, the expression levels of double sex and mab-3 related transcription factor 1 (*dmrt1*) (**Figure 1B**) and the anti-Müllerian hormone (*amh*) (**Figure 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 significant down- regulation in both ovaries (**Figure 1E**) and testes (**Figure 1F**). These results indicated that elevated rearing density affected both sexes.

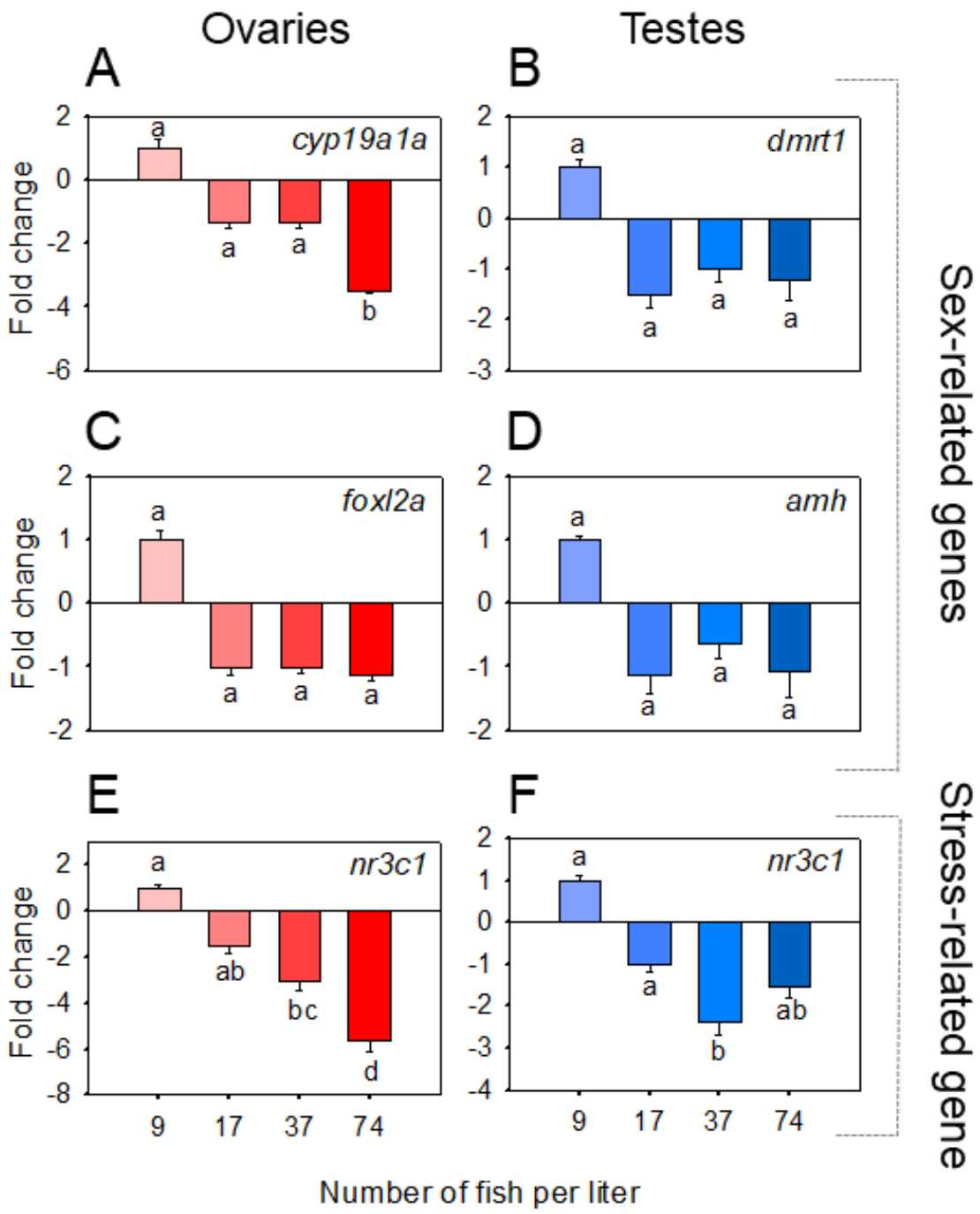

Figure 1. Expression of sex-related genes: *cyp19a1a* and *foxl2a* in ovaries (**A, C**) and *dmrt1* and *amh* in testes (**B, D**) and of the stress-related gene *nr3c1* in ovaries and testes (**E, F**), respectively. Average values (n = 7–8 individuals per treatment) are shown with a standard error of the mean. Fold change values are shown relative to the 9 fish/liter treatment (control). Analysis of variance followed by post hoc Tukey test. Statistically significant differences ($P < 0.05$) among treatments are indicated by letters.

Transcriptomic overview and microarray validation

Next, a species-specific and validated microarray was used to compare the transcriptomes of females of the HD group, that is, the females that did not become masculinized. These females were compared with the control females. Results showed a total of 4,634 DEGs (adjusted $P \leq 0.05$, FC > 1.2) between the ovaries of fish reared at HD versus LD (**Data set S1**). Microarray data was fully validated by qPCR in a subset of selected genes covering a range of ±4 FC values ($R^2 = 0.83$ and P = 0.0001) (**Figure S2**). For a complete list of gene names and abbreviations see **Data set S1**.

GO terms associated in ovaries of females exposed to HD

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

HD promoted the upregulation of promale genes in the ovaries

All the DEGs were compared against two independent lists of genes related to sex and stress responses. One list included a total of 48 genes with proven sex-related functions in zebrafish, as described in Ribas et al. (2017a), whereas 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 (**Figure 2**), and allowed the clustering of all but one sample according to rearing density. Regarding sex-related genes (**Figure 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 (**Figure 2B**), 15 of the 20 genes shown in the heatmap were downregulated in the HD group (e.g., *nudt3b*, *hmox2b*, *b2nl*, and *cnr1*) and five (i.e., *nud*, *hsp14*, *duox*, *mstnb*, and *hsp90aa.1.1*) were upregulated.

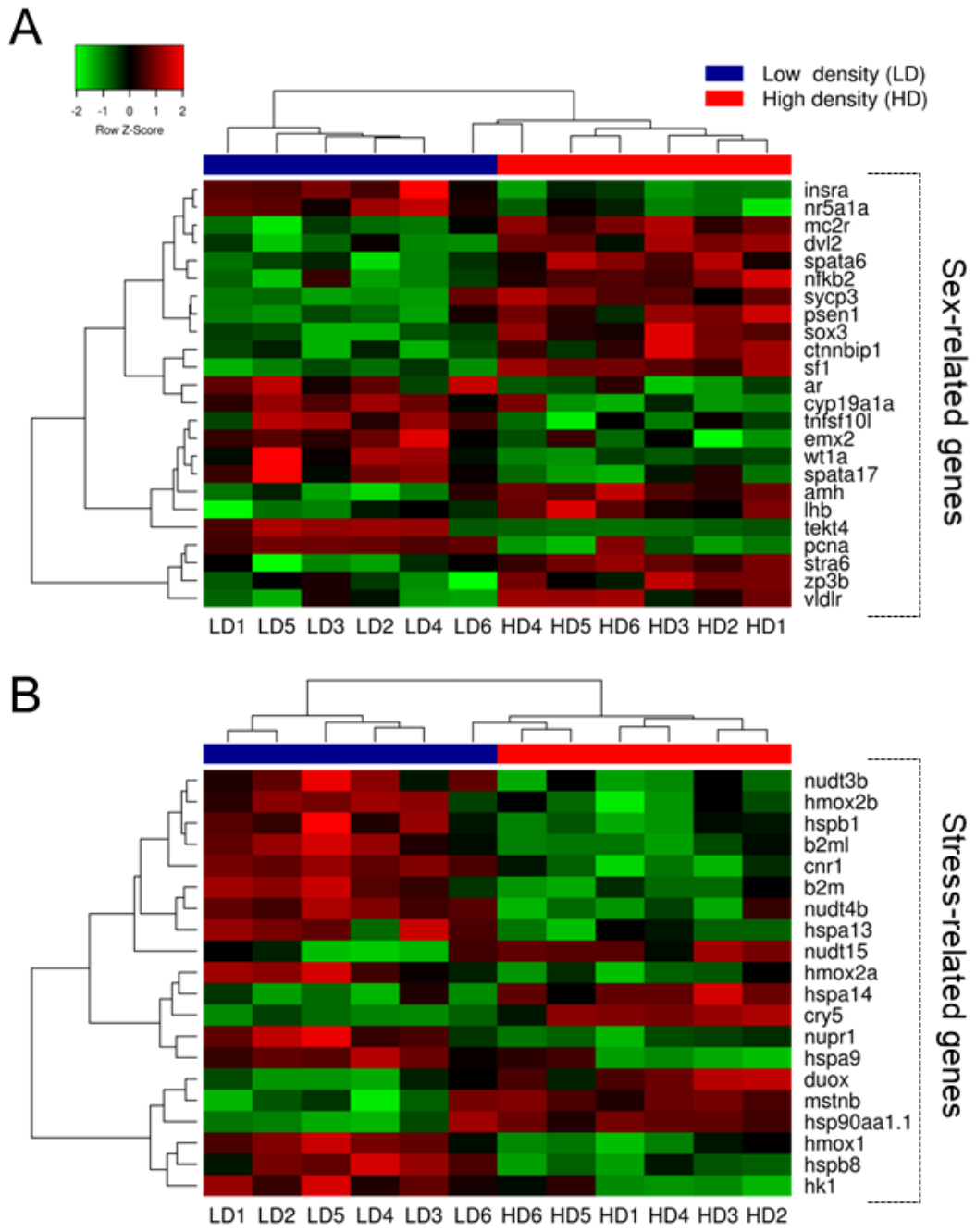

Figure 2. Gene expression represented by heatmaps of (**A**) sex-related and (**B**) stress-related genes in ovaries of females exposed to high density (HD, bright red) and low density (LD, blue). Key color (Row Z-score) represents the level of gene expression for each gene (red: high expression and green: low expression).

Common genes in ovaries of females subjected to HD or HT

Elevated rearing density or temperature during the sensitive period of sexual development result in masculinization of some females (Figure S4), which is easy to quantify by sex ratio analysis. However, though all non-masculinized females have apparently normal ovaries, at least morphologically, 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 the fish have been exposed to elevated density or temperature. Thus, we compared the reported ovarian transcriptomic results of the HD versus the LD groups with those obtained comparing the HT versus LT groups previously described in our laboratory (Ribas et al., 2017a). We found 1,269 DEGs (**Figure S5**) commonly regulated and were represented according to their FC (**Fig. 3**) and their expression tendency (**Figure 3B**). We classified them in four sets (**Data set 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 down- regulated 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 (*ptpdc1*), 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.

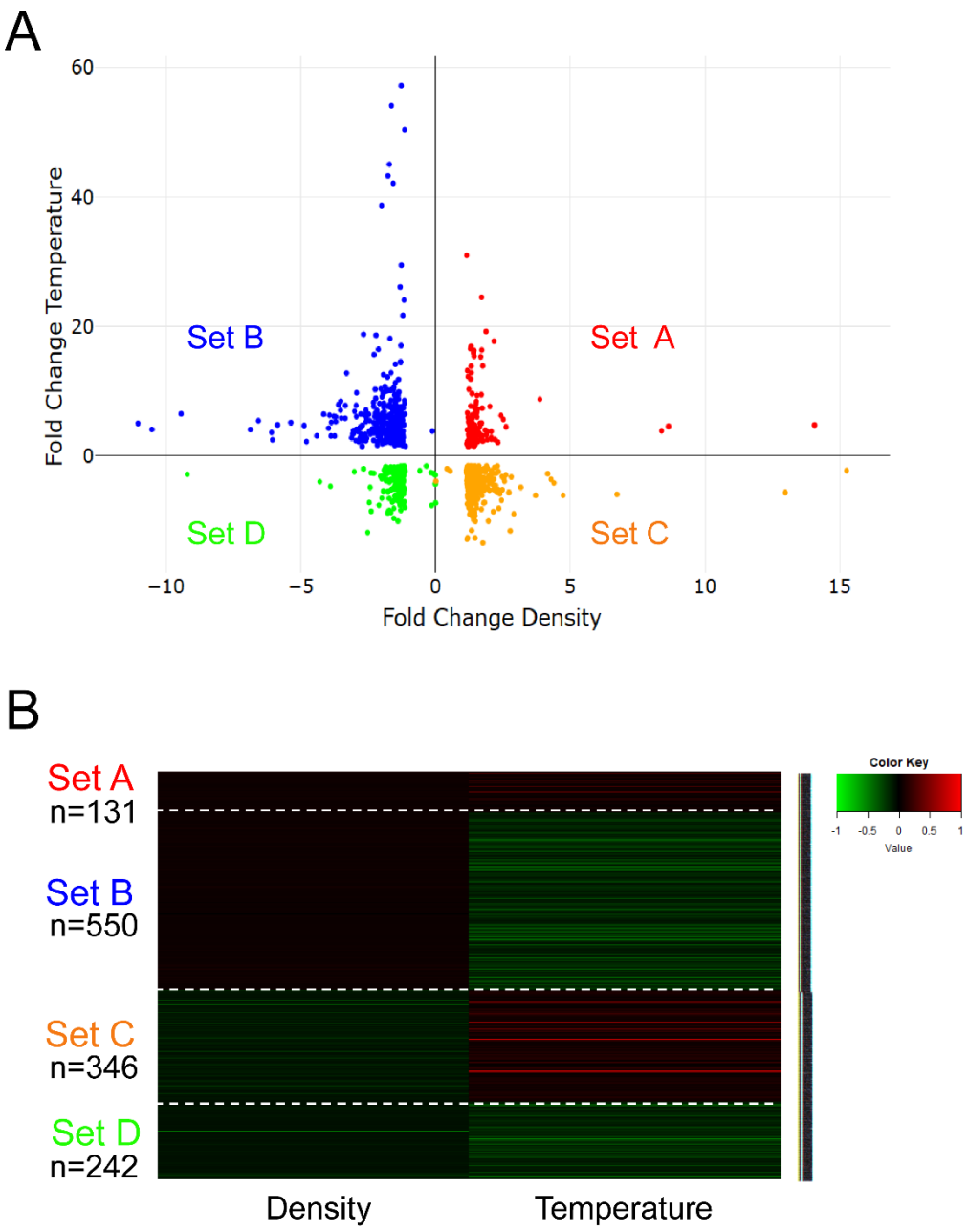

Figure 3. (**A**) Scatterplot of the fold change (FC) values of the common 1,269 differentially expressed genes (DEGs) found between density (X axis) and temperature (Y axis). (**B**) Heatmap of the FC values of the 1,269 DEGs found between density and temperature in Sets A–D. The n indicates the number of genes found for each set. Key color (Row Z-score) represents the level of FC for each gene (red: high expression and green: low expression).

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

Common GO terms in ovaries of females subjected to HD or HT

We analyzed separately the genes of the Sets A and D by DAVID to inquire into the BP-GO terms. There were a total of nine GO terms in Set A (**Figure 4A**) including cellular component organization (GO: 0016043) and negative regulation of response to stimulus (GO: GO:0048583); and eight GO terms in Set D (**Figure 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) (**Figure 5A–C**), whereas in Set D were macromolecule localization (GO: 0033036), primary metabolic process (GO: 0044238), and cellular metabolic process (GO:0044237; Figure 5d–f). Next, for exploratory analysis, we looked at the top three genes (with the highest FC in the density experiment) 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. Interestingly, FC values of genes altered by temperature were higher than those altered by density (**Figure 6A**).

There were no common KEGG pathways related to the DEGs conforming the A or D Sets, probably due to the relatively low number of genes in each set. However, the expression of genes involved in reproduction-related KEGG pathways in zebrafish (Ribas et al., 2017a), namely, the Wnt signaling (dre04310), homologous recombination (HR; dre03440), transforming growth factor-β (TGF-β; dre04350), oocyte meiosis (OM; dre04114), p53 signaling (dre04115), and steroid hormone biosynthesis (SHP; dre00140) pathways had the same direction of change regardless of perturbation (**Figure 6B**). In the Wnt signaling pathway, there were two downregulated genes (axin 2 conductin-axil, axin2, and protein kinase cAMP-dependent catalytic α genome duplicate b, *prkacab*) and one upregulated gene (transforming protein RhoA, *rhoa*). *Rhoa* also belongs to the TGF-β pathway together with amh, which was also upregulated. In the HR signaling pathway, *sycp3* was upregulated. The phosphatase and tensin homolog B (*ptenb*), golgin rab6-interacting (*gorab*), the F-box protein 5 (*fbxo5*), and *cyp19a1a* genes belonging to the p53 signaling, OM, and SHP pathways, respectively, were downregulated.

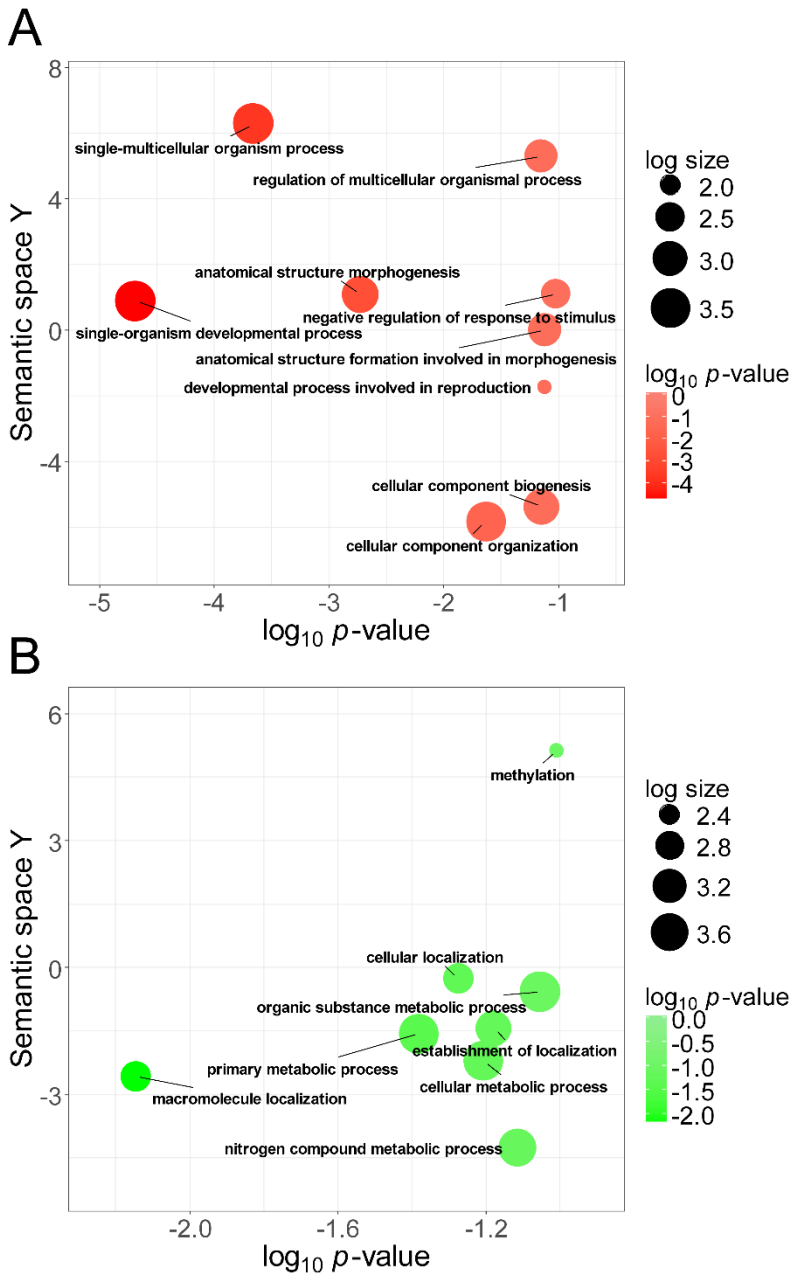

Figure 4. GO terms associated to the common genes affected by elevated density or temperature. (**A**) GO terms associated to Set A (red color), (**B**) GO terms associated to Set D (green color). For each GO term graph, the log size indicates the number of genes represented and the color saturation indicates the log10 Fisher's P value along the X axis. GO terms are distributed in multidimensional semantic similarities in the Y axis KEGG pathways in ovaries of females subjected to HD or HT.

The KEGG pathways from the 4,634 DEGs found in the density experiment were compared to the KEGG pathways reported in the ovaries of those resistant females treated with elevated temperature (Ribas et al., 2017a) and no common pathways were found, so we listed in **Table 2**, the significant upregulated and downregulated KEGG pathways found in the density experiment and the four most significant upregulated and downregulated KEGG pathways found in the temperature experiment. In particular, in the ovaries of fish subjected to density, we found an upregulation of the gonadotropin-releasing hormone receptor (GnRHr) pathway (dre04080) and downregulation of five KEGG pathways, for example, the integrin signaling pathway (dre04510). For the temperature factor, the most upregulated and downregulated pathways were the cardiac muscle contraction (dre04260) and the protein processing in the endoplasmic reticulum (dre04141).

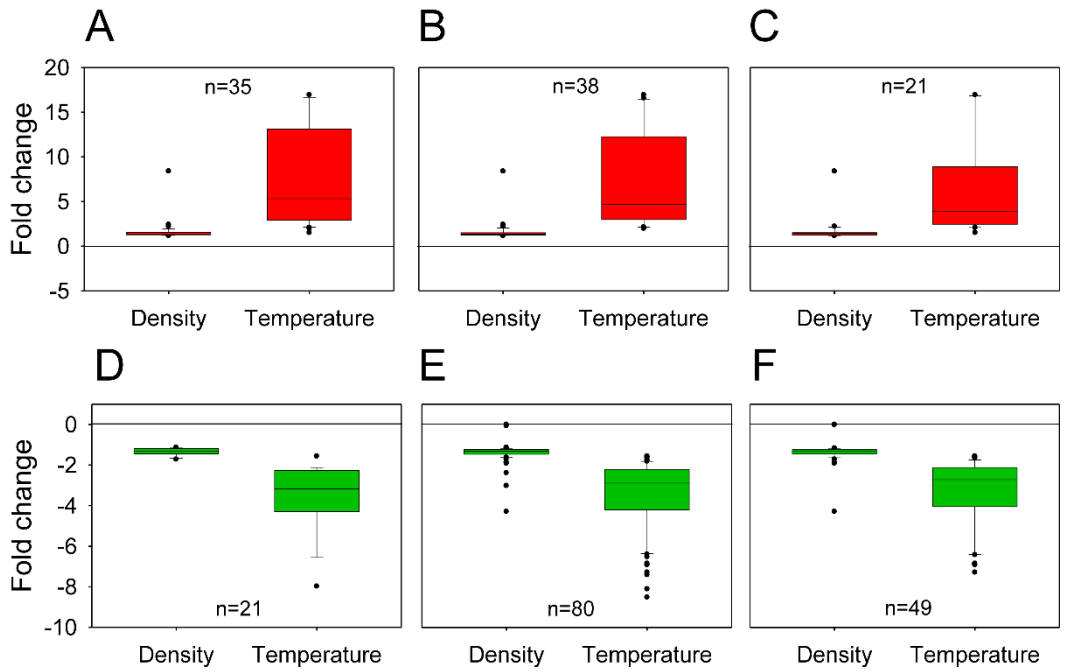

Figure 5. Fold change representation of common upregulated genes of (**A**) single-organism developmental process, (**B**) single-multicellular organism process, (**C**) anatomical structure morphogenesis and common downregulated genes in (**D**) macromolecule localization, (**E**) primary metabolic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

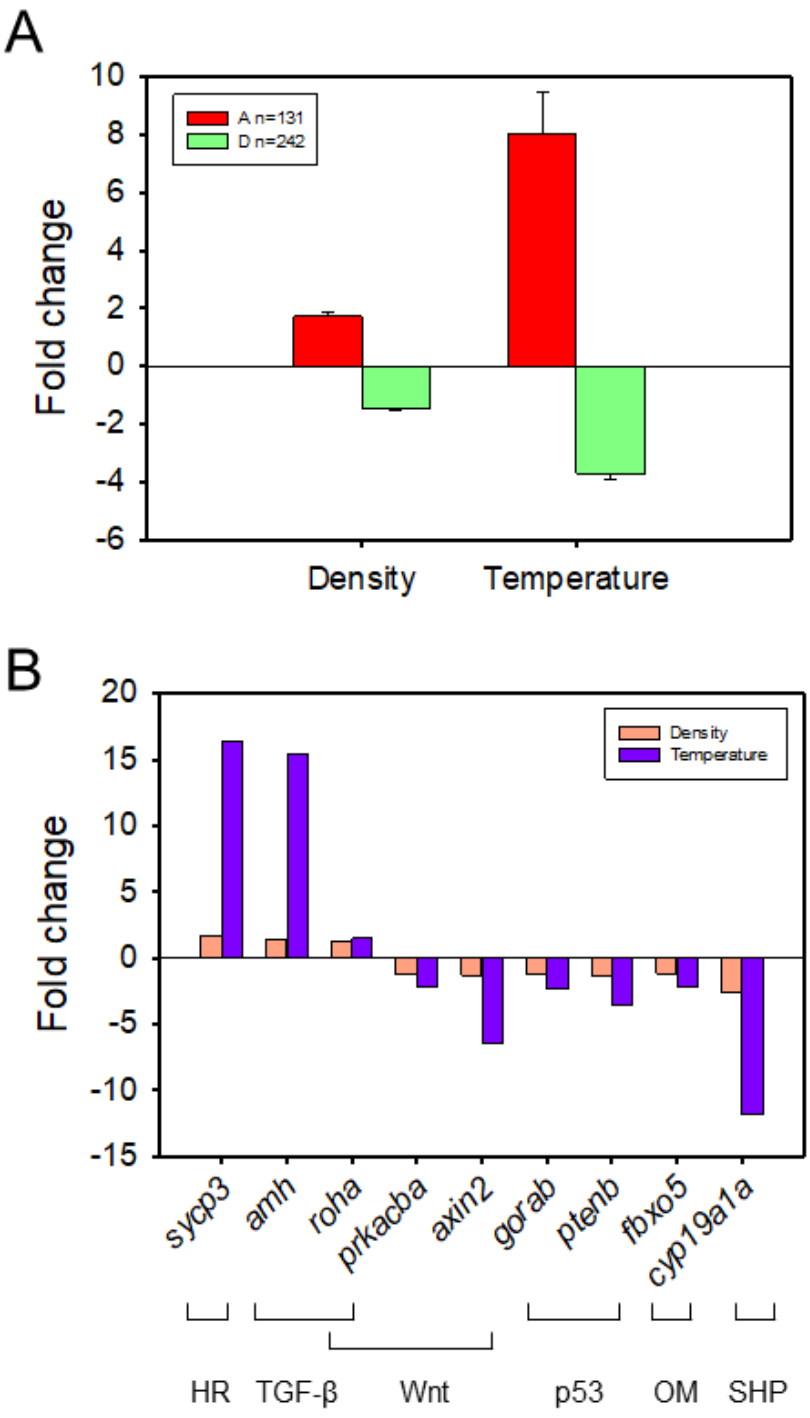

Figure 6. (**A**) Mean fold change for the genes in sets A and D for density and temperature. (**B**) Fold change of genes involved in homologous recombination (HR; dre03440), transforming growth factor-β (TGF-β; dre04350), Wnt signaling (dre04310), p53 signaling (dre04115), oocyte meiosis (OM; dre04114)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

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
		Angiogenesis	dre04370	29	0,0138
Temperature	Upregulated	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
		Cell adhesion molecules (CAMs)	dre04514	86	1.44E-03
	Downregulated	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

4. 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 and Piferrer, 2008; Baroiller and D'Cotta, 2016; Piferrer, 2018). The masculinization of genotypic females generates phenotypic males (Hliwa et al., 2014), that are termed neomales (Pandian and Sheela, 1995). These neomales are capable of producing viable sperm (Piferrer, 2001). The remaining females, that is nonmasculinized 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, had 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 appeared in populations partially masculinized by elevated rearing density and consequently we were interested in finding out what were 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 HT (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, whereas the expression of *foxl2a*, a transcriptional regulator of *cyp19a1a* (Wang et al., 2007a), 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 olive flounder (Kitano et al., 1999) and in the European sea bass (Navarro-Martín et al., 2011; Díaz and Piferrer, 2015) when subjected to HT. Thus, environmental stress reduced the expression of *cyp19a1a* in the gonads and this is in agreement with the observed masculinization since *cyp19a1a* is necessary for ovarian development, as demonstrated in the loss-of- function experiments (Lau et al., 2016; de Castro Assis et al., 2018). In the 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 the estrogen production (Rodríguez-Marí et al., 2005), did not show differences in gene

expression due to HD confinement, suggesting no conspicuous effects of density at least in these two important genes for male sexual differentiation. This, of course, does not exclude the possible effects on genes related to spermatogenesis, which were not the focus of this study.

It is known that the abiotic and biotic stressors modulate the synthesis of cortisol altering the expression of genes of the hypothalamic–pituitary–interrenal (HPI) axis (Alsop and 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 the 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). As cortisol exerts different effects on various organ systems, it is likely that the functional targets of GR were different in each tissue (Le et al., 2005). The 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., 2018), respectively, but they were recovered in longer confinement experiments (~ 200 days) in wedge sole (*Dicologoglossa cuneate*) (Herrera et al., 2015). In a similar manner, no difference in cortisol levels was found in juvenile zebrafish subjected to HD during 45 days (Ribas et al., 2017b) but, when sexually mature, GR was downregulated in the gonads at the highest densities, especially in females. This is, to our knowledge, the first time that downregulation of GR in the gonads due to chronic stress is reported, as it was not observed in the reproductive tissues when zebrafish were exposed to elevated temperature (Ribas et al., 2017a). The downregulation found of GR in the gonads of fish exposed to elevated density was probably to attenuate the physiological long-term effects due to the stress.

Prolonged rearing at high population density affected the expression of more than 4,600 genes in the ovaries of nonmasculinized 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 the 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 promale 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 and Englert, 2002), and the androgen receptor. 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 of 20 were downregulated, including heme oxygenase enzymes (*hmox1*, *hmox2a*, and *hmx2b*), responsible to protect cells from oxidative stress, and usually upregulated by exposure to

acute stress (Poss and 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 and Lindquist, 1993) four were downregulated (*hsp13*, *hsp14*, *hspa9*, and *hspb8*) and two upregulated (*hsp14* and *hsp90aa1.1*). The *hsp90aa1.1* is a crucial gene for cortisol activity as it acts as 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., 2017c). 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 versus chronic stress (Pottinger, 2008).

The comparison of the ovarian transcriptomes of females exposed to either elevated population density or temperature allowed insights into what were the conserved signatures irrespective of the type of perturbation. It is difficult to say whether zebrafish gonads are more sensitive to elevated temperature or elevated rearing density because a proper estimation would require carefully designed dose–response experiments and comparing, for example, the transcriptomes of females when, say, 50% of the initial females become masculinized either after temperature or density, which is not the case in this study. However, at least it is interesting to notice a certain relationship between the degree of masculinization and the average fold change of genes commonly affected by both factors (temperature > density).

Of the 1,269 DEGs shared in both type of perturbations, 373 DEGs were affected in the same direction regardless of the environmental perturbation. Among these common genes, *faima* was strongly upregulated (FC = 14.07 and 4.78 in density and temperature experiments, respectively). *Faima* is an inhibitor of the apoptotic response and it is expressed in mammalian ovaries during follicular atresia in the granulosa cells (Matsuda Minehata et al., 2006) with similar expression pattern than *foxl2* in the ovaries (Nikic and Vaiman, 2004). During sex differentiation, juvenile zebrafish ovaries activate apoptotic pathways and, although further research is needed, *faima* could play a role in the resistance to masculinization and, therefore, could be a potential maker for those females that although subjected to environmental perturbations they still manage to develop ovaries. *Ptpdc1*, upregulated in fish ovaries by elevated density and temperature exposure (FC = 8.64 and 4.59, respectively), is a pleiotropic gene that regulates a variety of cellular processes including cell growth and differentiation (Bonetti et al., 2014). Other examples of common upregulated genes were *ccm2l*, involved in malformation in heart and body axis in zebrafish (Cullere et al., 2015), and *ankrd6b*, which phosphorylates and inhibits the β-catenin signal during zebrafish development (Moeller et al., 2006). Among those downregulated genes, we found lgals9l1 (FC density = −2.85 and temperature = −9.21), 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 larvae 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 nonmasculinized females. It is known that during gonadal development in zebrafish, testis development requires an upregulation of *amh* together with a downregulation of *cyp19a1a* in the immature ovary (Wang and 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 promale genes and pathways was also observed in ovaries of zebrafish treated with a demethylation agent (decitabine) during sex differentiation, which skewed the sex ratios toward females (Ribas et al., 2017d), may indicate that activation of some promale genes is the 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*, *lgals9l1*, 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 yielded 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 term 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) the dysfunction of which 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 GnRHr pathway was activated. GnRH stimulates the secretion of luteinizing hormone and follicle-stimulating hormone, both acting synergistically in the reproductive system (Millar, 2005), and in the seabream it was observed that GnRH plays an important role in the regulation of gonadal differentiation, preventing the regression toward the testis from the ovary (Soverchia et al., 2007). In contrast, inflammation mediated by chemokine and cytokine, integrin signaling, and angiogenesis pathways were inhibited. These pathways

are involved in the protective response of cells to pathogens, infection, or tissue damage, as observed in zebrafish larvae exposed to pathogens (Díaz-Pascual et al., 2017). Interestingly, these pathways are also activated during ovarian commitment in zebrafish sex differentiation, which resembles an inflammatory response process in which the activation of the nuclear factor-κβ and Wnt signaling pathways are required (Liew and Orbán, 2013).

5. Conclusions

Previously, we had shown that exposure of zebrafish to constant, elevated population density since early life masculinized some females. In the present study, we show that it also delayed gonadal maturation in both sexes and, further, that resistant females had a gonadal transcriptome similar in many ways to the transcriptome of females resistant to the masculinizing effects of elevated temperature. These transcriptomic changes included the upregulation of some known promale genes such as *amh*, *sycp3*, and *spata6*, but also of other novel genes not previously related to reproduction, such as *faima*, *ccm2l*, and *ankrd6b*. The commonly downregulated genes included the *lgals9l1* and *ubxn2a* genes, in addition to *cyp19a1a*. These persistent gene expression changes can be used as markers indicative of previous exposure to environmental perturbations of both biotic and abiotic origin present in fish with otherwise apparently normal ovaries. If conserved in other species, these markers could be used in the assessment of the hidden effects of environmental stress. However, it remains to be determined the actual consequences in terms of reproduction in the affected females.

Acknowledgments

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Competing interests

The authors declare that there are no conflicts of interests

Supplementary Figures

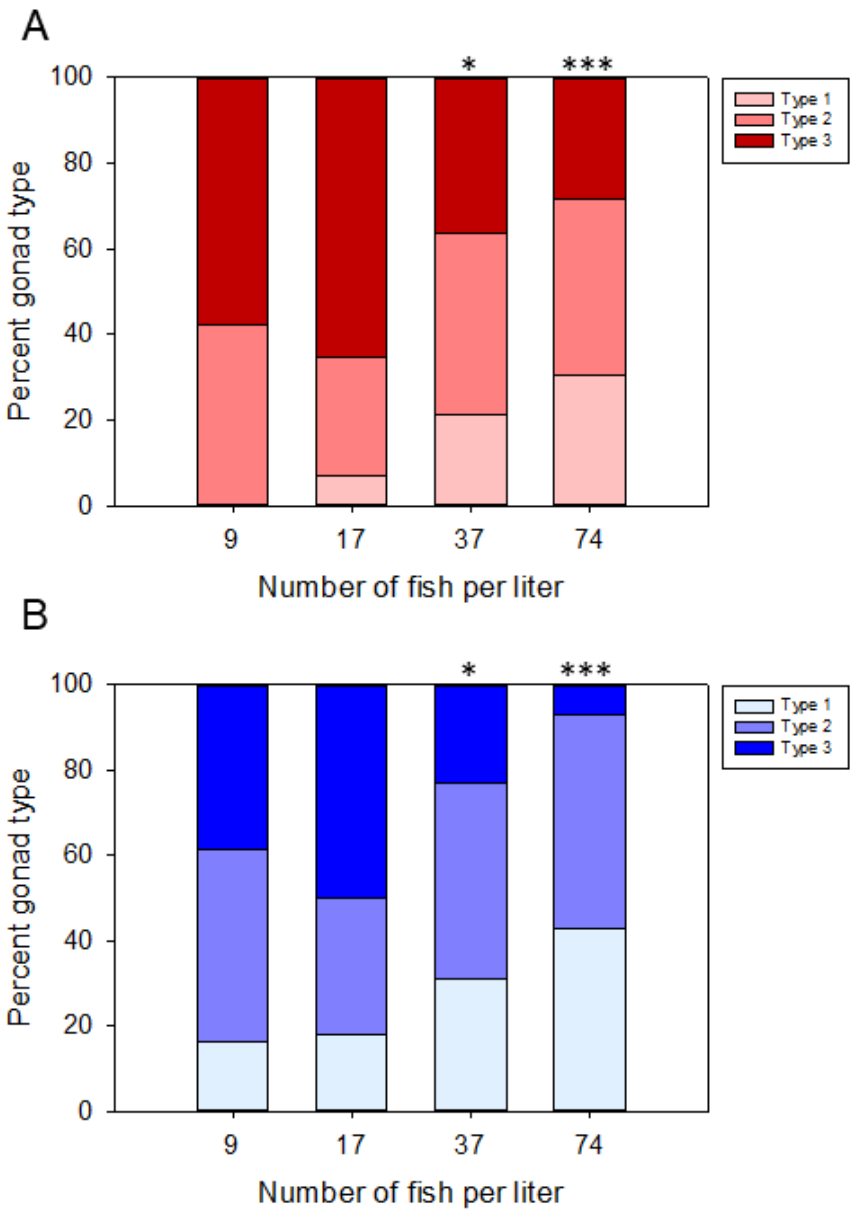

Figure S1. Percent females (**A**) and percent males (**B**) for each type of gonadal maturation level (types 1, 2 and 3) at 90 days post fertilization in four different densities treatments (9, 17, 37 and 74 fish/litre). Statistically significant differences in gonad maturation levels among treatments against the control (9 fish/litre) treatment are indicated by asterisks: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. The chi-square test was applied.

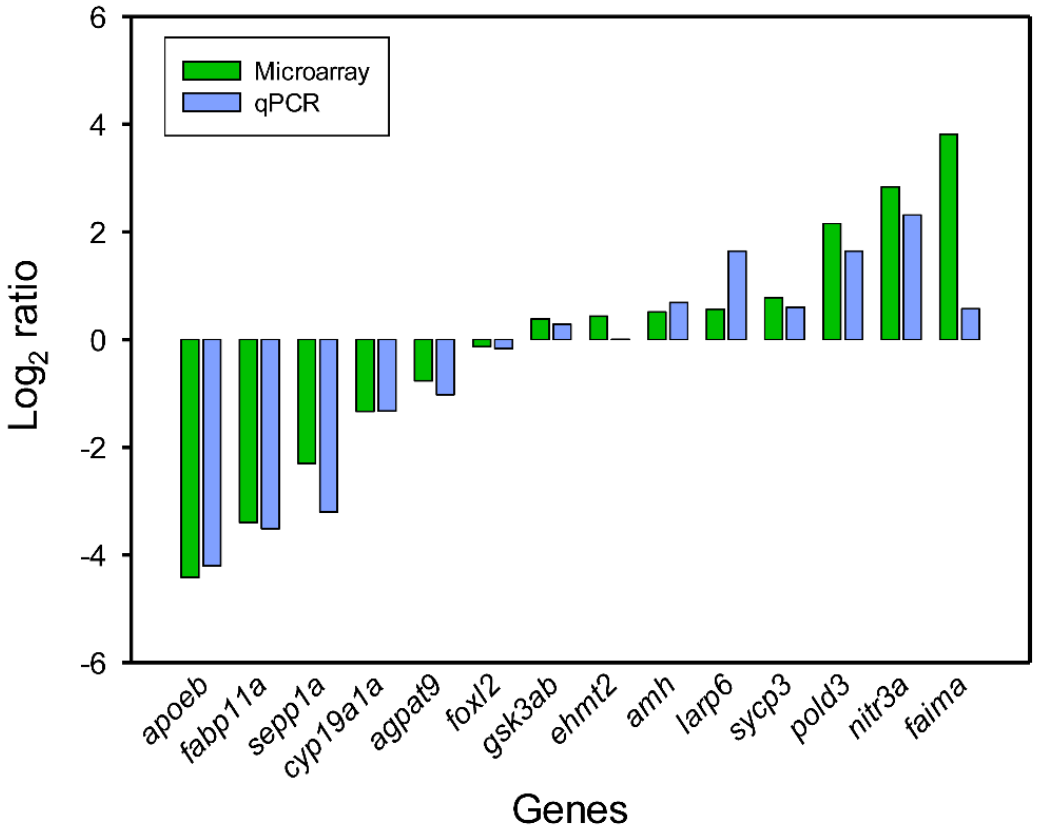

Figure S2. Validation of the microarray results. Representation of the $\log_2$ expression data of 14 differentially expressed genes (six for down- and eight for upregulated) observed by microarray and qPCR results. A Spearman's test was applied to determine the level of correlation between microarray and qPCR expression data (R^2= 0.8263 and *P*= 0.0001).

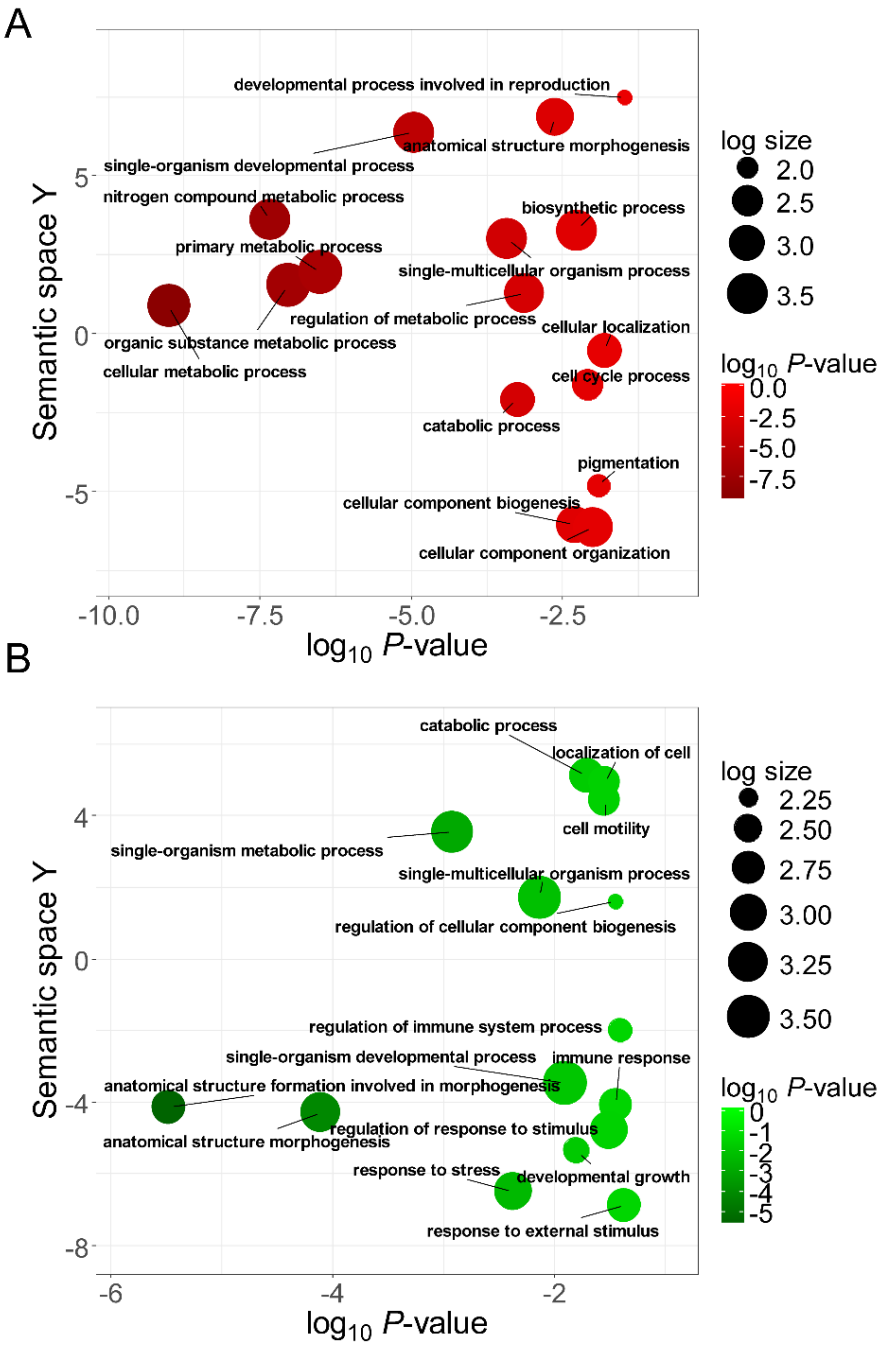

Figure S3. Reduction and visualization of the enrichment of the Biological process GO-terms associated to high density in ovarian tissues. (**A**) GO-terms associated to upregulated genes (red color) and (**B**) GO-terms associated to downregulated genes (green color). For each GO-term the log size indicates the number of genes represented and the color 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.

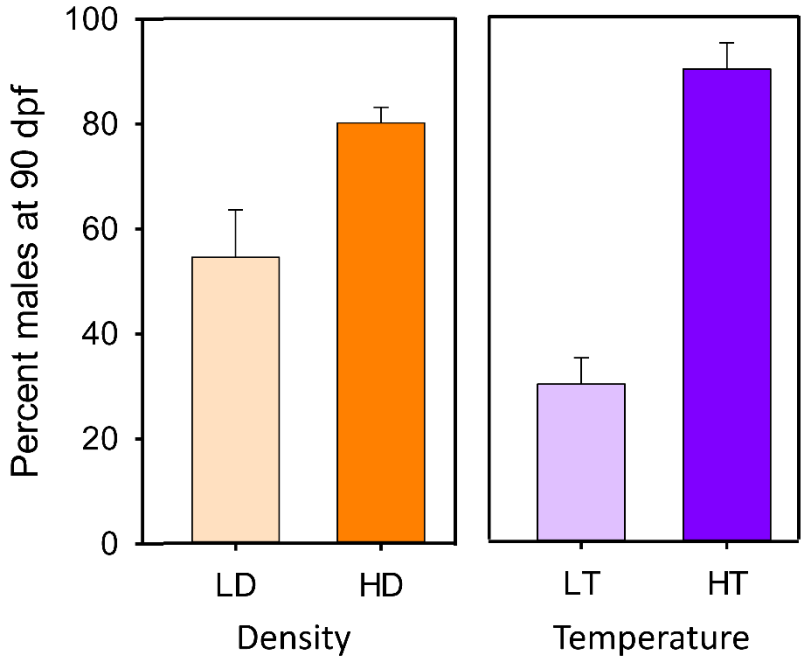

Figure S4. Percent number of males at 90 days post fertilization of zebrafish subjected to low density (LD) or high density (HD), or to low temperature (LT) or high temperature (HT) in (Ribas et al., 2017a) and (Ribas et al., 2017b) experiments, respectively.

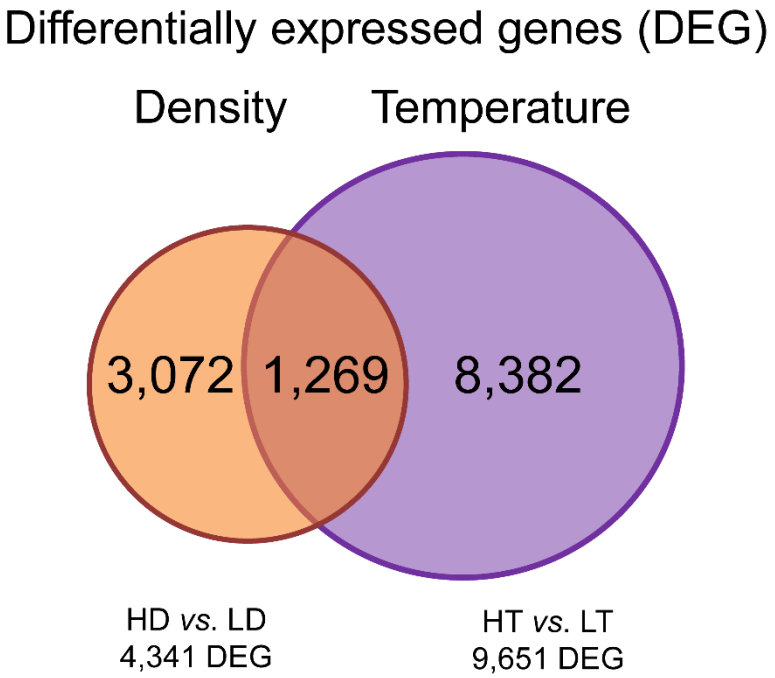

Figure S5. Venn diagram showing the common differentially expressed genes (DEG) in the ovaries of adult zebrafish subjected to two different environmental factors, density or temperature. HD: high density; LD: low density; HT: high temperature. LT: low temperature.

Supplementary Tables

Table S1. Number of gonad samples and the gonad type maturation used for qPCR gene expression analysis. Samples were extracted from Ribas et al. (2017a)

Density treatment (initial number of fish/liter)	**Sex**	**Gonad type**	**Number of gonads**	**Total gonads**
9	Female	1	0	
		2	2	
		3	6	8
	Male	1	0	
		2	8	
		3	0	8
17	Female	1	0	
		2	3	
		3	5	8
	Male	1	0	
		2	6	
		3	2	8
37	Female	1	0	
		2	6	
		3	2	8
	Male	1	0	
		2	5	
		3	3	8
74	Female	1	1	
		2	6	
		3	1	8
	Male	1	0	
		2	7	
		3	1	8

Table S2. Gene symbols, gene names, Refseq IDs and primer sequences for all genes used in qPCR and for microarray validation (in alphabetical order)

Gene symbol	Gene name	Ref. seq. ID	Forward primer (5'-3')	Reverse primer (5'-3')
amh	anti-Müllerian hormone	NM 001007779	ACAACCCGAAGGTCAACCCGC	GTGGCATGTTGGTCAGTTGGCTG
apoeb	apolipoprotein Eb	NM 131098	CGCAACACCGTAGCAACCTA	GGGCCTGGCTCATGTATGG
cyp19a1a	cytochrome P450, family 19, subfamily A, polypeptide 1a	NM 131154	GATATTTGCTCAGAGCCATGGA	GCTCTGGCCAGCTAAAACACT
dmrt1	doublesex and mab-3 related transcription factor 1	NM 205628	TGCCCAGGTGGCGTTACGG	CGGGTGATGGCGGTCCTGAG
eef1a1l1	eukaryotic translation elongation factor 1 alpha 1, like 1	NM 131263	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTACCGCTAGCATTAC
ehmt2	euchromatic histone lysine methyltransferase 2	NM 001113615	ACCATCTGTGTGACCCGAAC	AAGAAAGCGATGCGAGGGAA
fabp11a	fatty acid binding protein 11a	NM 001004682	GGCAAACTTGTGCAGAAACAGA	CATCACCCATCTTGCATTTAGC
faima	fas apoptotic inhibitory molecule a	NM 001002583	TGATATTTTCTGCCGCGTGGA	CTCCAGTACCGGTCCAGATCA
foxl2a	forkhead box L2a	NM 001045252	AAACACTGGGAAGGTTTGCGTGC	TTTGTCCGGCCCCTTCTCTGG
gpat3	glycerol-3-phosphate acyltransferase 3	NM 001002685	GGACATACTGTTCGGGATTTGG	CTGAACAAGTCCTTCACAACACC
gsk3ab	glycogen synthase kinase 3 alpha b	NM 131390	TCTTCAACTTCAGCCCTGTGG	TAAGTGTGATCAGTACCGTGCG
larp6a	la ribonucleoprotein domain family member 6	NM 199903	GAGTCGCTCCCTGAATAGCC	TCGTGGAGATGTCGGATTGC
nitr3a	novel immune-type receptor 3a	NM 131652	CTACAGAACATCTCAGAAGCCAGATAA	CTTCTCGAGGTGGTTGGCTTT
nr3c1	nuclear receptor subfamily 3 group C member 1	NM 001020711	AACTGCCCTGCCTGTCGTTTCC	GAAGGTTGCGCTCCGGAATGG
pold3	polymerase delta 3, accessory subunit	NM 001042769	CCATTAGATACTTGCCTTAGTGCTG	ATGGCCTGAAGGTGAGTGAAA
sepp1a	selenoprotein P, plasma, 1a	NM 178297	ACAAGTGACAGTGGTGGCTTACC	ACCCTGCTTCTCCAGCTTCA
sycp3	synaptonemal complex protein 3	NM 001040350	AGCGGATCTGACGAAGACACGAG	ATGTCCGCACCAAATCTTTCCAGC

Table S3. Number of gonad samples and the gonad type maturation used for microarray gene expression analysis. Samples taken from the table S1

Sex	**Density treatment** (initial number of fish/liter)	**Gonad type**	**Number of gonads**	**Total gonads**
Female	9	1	0	
		2	1	
		3	5	6
	74	1	0	
		2	5	
		3	1	6

Table S4. The most up and downregulated GO-terms commonly expressed in ovaries of female fish subjected to density or temperature and the top three genes found for each GO term

Regulation	GO-term name and ID	GO-term $\log_{10}$ *P*-value	n genes	Gene	Function	Density fold change	Temperature fold change
Upregulated	Single-organism developmental process (GO:0044767)	-4.69	35	*ccm2l*	cerebral cavernous malformation 2-like	8.39	3.88
				wdr66	WD repeat domain	2.42	6.25
				ankrd6b	ankyrin repeat domain 6b	2.21	2.56
	Single-multicellular organism process (GO:0044707)	-3.66	38	*kidins220b*	kinase D-interacting substrate 220b	1.93	3.77
				sema4e	semaphorin 4e	1.75	13.91
				pnpla3	patatin-like phospholipase domain containing 3	1.59	3.21
	Anatomical structure morphogenesis (GO:0009653)	-1.12	21	*vox*	ventral homeobox	1.54	9.36
				ift81	intraflagellar transport 81 homolog	1.53	6.62
				ahi1	Abelson helper integration site	1.51	8.43
Downregulated	Macromolecule localization (GO:0033036)	-2.15	21	*copz2*	coatomer protein complex, subunit zeta 2	-1.72	-4.05
				copz1	coatomer protein complex, subunit zeta 1	-1.65	-3.62
				rp2	retinitis pigmentosa 2 (X-linked recessive)	-1.48	-3.18
	Primary metabolic process (GO:0044238)	-1.38	80	*ubxn2a*	UBX domain protein 2A	-4.30	-3.98
				rnf144aa	ring finger protein 144aa	-3.02	-2.40
				tecrb	trans-2,3-enoyl-CoA reductase b	-2.39	-8.52
	Cellular metabolic process (GO:0044237)	-1.21	49	*acsl4a*	acyl-CoA synthetase long-chain family member 4a	-4.30	-3.98
				sp2	sp2 transcription factor	-1.92	-3.22
				erlin1	ER lipid raft associated 1	-1.86	-2.95

Block 2: Temperature effects

Chapter 3

Fish reproductive tissues as heat recorders: DNA methylation epigenetic marks in zebrafish gonads correctly recapitulate past thermal history

Epigenetics & Chromatin
(To be submitted)

Fish reproductive tissues as heat recorders: DNA methylation epigenetic marks in zebrafish gonads correctly recapitulate past thermal history

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Abstract

Epigenetic regulatory mechanisms contribute to integrate genomic and environmental information that determine the phenotype. In the last years, the search of methylation patterns that might serve as epigenetic biomarkers for the prognosis and diagnosis of specific phenotypes have attracted a lot of interest. Sexual development is a complex process regulated by many interactions between antagonizing signaling pathways that ultimately define the sexual phenotype. The transcriptomic changes involved in the formation of an ovary or a testis have been studied in a number of vertebrate species, from fish to mammals. However, the epigenetic mechanisms regulating gene expression and hence the fate of an undifferentiated gonad remain poorly understood. Using the zebrafish model (*Danio rerio*), we exposed larvae of two independent families to high temperature during the sex differentiation (18–32 days post fertilization). Using a targeted sequencing approach, we analyzed, in adult gonads, the methylation profiles of 18 key genes mainly related to sex development and the stress response after the end of the thermal exposure. Gene expression patterns were measured by qPCR in order to correlate them with the DNA methylation changes. Sex-related differences were observed in the DNA methylation levels of steroidogenic enzymes (e.g., *cyp19a1a*, *hsd17b1* and *hsd11b2*), transcription factors (e.g., *dmrt1* and amh) and epigenetics-related (*dnmt1*) genes. Family-dependent differences were also observed. In testes, exposure to elevated temperature increased the methylation levels of *cyp19a1a*, *cyp11a1* and amh, while it decreased the methylation of *dmrt1*. However, no changes in the methylation level of these genes were observed in the ovaries. We also observed an inverse relationship between DNA methylation and gene expression levels for *cyp19a1a*, *dmrt1* and *hsd17b1*. Finally,

according to sex-specific and temperature responses in methylation changes in the genes studied, we used a machine-learning procedure to select informative CpGs for diagnosis of sex identity and previous temperature exposure. We found that the methylation levels of CpGs of the *cyp19a1a* gene could identify sex while those of amh and *foxl2a* could faithfully inform whether animals had been exposed to high or low temperature while young in males and females, respectively. The epigenetic biomarkers for sex were validated in independent samples with 100% success. This study reports the sex- and temperature-dependent DNA methylation values for a number of steroidogenic enzymes, growth and transcription factors, as well as other genes in zebrafish, some of them (e.g., *cyp11c1*, *hsd11b2*, *dmrt3a* and *ar*) for the first time in fish. Importantly, we were able to identify specific CpG loci located in *cyp19a1a*, *amh* and *foxl2a* that allowed us to accurately identify sex and to tell whether fish had been exposed to abnormally high temperatures when young, i.e., along time after the environmental stressor disappeared, even in the absence of conspicuous morphological alterations of the gonads. These set of CpGs represent epigenetic biomarkers that correctly recapitulate past thermal history and pave the way for similar findings in other species to assess potential effects of environmental disturbances in a context of climate change.

Keywords: sex, gonads, DNA methylation, temperature, epigenetic, predictor, markers.

1. Introduction

Fish sex determining mechanisms can be broadly classified as genetic sex determination (GSD) and environmental sex determination (ESD) (Devlin and Nagahama, 2002; Kobayashi et al., 2013). Traditionally, they were considered mutually exclusive but nowadays these two sex determining systems are seen as the two ends of a continuum, with any combination of genetic *vs.* environmental influences being possible (Sarre et al., 2004; Uller and Helanterä, 2011; Heule et al., 2014). In GSD, there are species where sex depends on a single sex-determining gene (either following a XX/XY or ZW/ZZ systems), defined as chromosomal sex determination (CSD), or species in which sex is determined through a complex action of multiple sex determining loci in different autosomes, defined as polygenic sex determination (PSD) (Kosswig, 1964; Penman and Piferrer, 2008). In ESD species, sex is directly controlled by environmental factors such as temperature (Conover and Kynard, 1981; Luckenbach et al., 2003), pH (Reddon and Hurd, 2013) or social interactions, especially in hermaphrodite species (Warner, 1975; Munday et al., 2006). Temperature is the most important factor influencing the developing gonads and resulting sex ratios in many fish species. Usually, elevated temperature result in an increase in the number of males in fish populations (Ospina-Alvarez and Piferrer, 2008). It has been argued that in a global climate change scenario, ESD species could be the most affected because their sex ratios could become highly male-skewed compromising the viability of populations (Consuegra and Rodríguez López, 2016; Piferrer, 2016) but also in species with PSD or even GSD can be affected (Honeycutt et al., 2019).

The genetic pathways controlling sex differentiation appear to be conserved across species (Western and Sinclair, 2001) and the transcriptomic responses of reproduction-related tissues in response to environmental changes have studied in a variety of fish species (Schunter et al., 2014; Díaz and Piferrer, 2015; Casas et al., 2016; Ribas et al., 2017a; Valdivieso et al., 2019). However, studies on the epigenetic regulation of gonadal development are still scarce among fish.

Epigenetics is defined as "the study of phenomena and mechanisms that cause chromosome-bound, heritable changes to gene expression that are not dependent on changes to DNA sequence" according to Deans and Maggert (2015). The integration of genomic and environmental information through epigenetic regulatory mechanisms produces changes in gene expression that are responsible for the appearance of a given phenotype (Turner, 2009; Vogt, 2017; Piferrer et al., 2019). The three major epigenetic mechanisms for gene expression regulation are DNA methylation, histone modification and non-coding RNA (ncRNA) (Gibney and Nolan, 2010). DNA methylation is particularly important for ensuring long-term changes in gene expression (Bird, 2002; Feng et al., 2010). It is widely accepted that CpG sites (cytosine nucleotide followed by a guanine, CG) conforming CpG islands (CGIs) are located relatively close to the transcriptional start site (TSS) of the gene with an important role for the control of gene

expression (Deaton and Bird, 2011). Thus, when a significant proportion of all CpGs become methylated in the promoter region the associated gene is not expressed (Straussman et al., 2009). However, recently, the methylation status of other genomic regions such as the first exon and first intron have been found to have the similar or even better association with gene regulation (Brenet et al., 2011; Anastasiadi et al., 2018a).

The integration of genomic and environmental influences into methylation patterns are gaining interest, for instance, when seeking to understand how the environment may convey a specific sex phenotype (Navarro-Martín et al., 2011; Anastasiadi et al., 2018b) as an example of phenotypic plasticity (Bradshaw, 2006). In fish, a sensitive period for sex occurs during the early gonadal development and this may be affected by external perturbations that are known to have an influential role on the gene expression of sex-specific genes (Shen and Wang, 2014). These changes can be mediated, at least, in part, by changes in DNA methylation (Navarro-Martín et al., 2011). Due to the sensitive nature of DNA methylation marks (Flores et al., 2013), these changes may remain permanent and detectable in adult gonads, even once the stressor disappeared a long time ago (Anastasiadi et al., 2018b).

Given its chemical nature and the advances in technology (Marzese and Hoon, 2015), DNA methylation variations are relatively easy to measure and recently have become useful as (epi)-biomarkers. Epigenetic biomarkers are extensively used in studies of prediction and diagnosis for cancers and illness (Mikeska and Craig, 2014). The use of DNA methylation as a diagnostic instrument for cancers of unknown primary origin is thus becoming common (Moran et al., 2016) and examples include the Epi proColon® kit designed to detect the aberrant and specifically DNA methylated of the v2 region of the *Septin9* gene (SEPT9) in blood-borne colorectal cancer cells (Model et al., 2007; Devos et al., 2009). DNA methylation changes can also provide reliable information for age estimation in animals, from humans (Horvath, 2013) and other vertebrates (Polanowski et al., 2014; Thompson, 2017; De Paoli-Iseppi et al., 2019) including fish (Anastasiadi and Piferrer, 2019). Prior research has thoroughly investigated DNA methylation changes due to environmental stress conditions such as pollution or temperature, finding good epigenetic biomarkers (Beal et al., 2020). However, no studies have been conducted with epigenetic biomarkers to develop tools to detect past exposures to abnormal environmental conditions.

The knowledge regarding DNA methylation in genes related to gonadal development in fish has been acquired for the most significant sex-related genes, such as *cyp19a1a*, *dmrt1*, *amh* and *foxl2a* genes. Based on this information, a model called "Conserved Epigenetic Regulation of Sex" (CERS) suggests that there is an inverse relationship of DNA methylation levels in the promoter regions between the "pro-male" and "pro-female" genes, associating their expression to a particular gonadal phenotype (Piferrer et al., 2019). However, other genes which also play an important role in the gonad formation such as the steroidogenic genes (Fernandino et al., 2012; Fernandino et al., 2013; Caulier et al., 2015) have not been evaluated. In addition, just few studies have proven the

interactions between thermal effects and DNA methylation marks on the gonadal development, and further, correlated the results with gene expression patterns in fish. This is the case of the European sea bass (*Dicentrarchus labrax*) (Navarro-Martín et al., 2011; Anastasiadi et al., 2018b), the Nile tilapia (*Oreochromis niloticus*) (Wang et al., 2019a) or the half-smooth tongue sole (*Cynoglossus semilaevis*) (Shao et al., 2014). Therefore, more studies should examine the potential effects of environmentally-induced epigenetic changes and its effects on changes in gene expression, particularly among key genes involved in the gonadal development.

In this study, we used the zebrafish, where sex determination depends on genetic and environmental factors and where different families show different genotype x environment interactions (Ribas et al., 2017a; Ribas et al., 2017c). Therefore, taking advantage of its PSD (Liew et al., 2012) zebrafish be used as a model to study temperature influences on DNA methylation in the gonads. To this end, two different families of juvenile zebrafish were exposed to elevated temperatures during sex differentiation to induce masculinization. By using a locus-specific approach, we evaluated the DNA methylation of a set of genes related to reproduction, stress, immunology, and genes encoding the enzymes that mediate epigenetic reactions. In addition, we measured the expression of specific genes to determine the relationship between DNA methylation and expression. Finally, since we observed some methylation marks that were strongly associated to a specific sex phenotype, we used these associations not only to infer the phenotypic sex but also previous exposure to abnormal temperature.

2. Materials and Methods

Animal rearing conditions

Zebrafish (AB strain; ZFIN ID: ZDB-GENO-960809-7) from the European Zebrafish Resource Centre (EZRC, Germany) were housed at the experimental facilities of the Institute of Marine Sciences (ICM-CSIC, Barcelona). The housing conditions, the physicochemical water parameters and the feeding regime for fish were monitored as described elsewhere in Ribas et al. (2017a). Fertilized eggs were obtained after natural spawning and larvae were raised from 6 to 18 days post fertilization (dpf) under regular conditions of temperature (28ºC) and appropriate density levels (8 fish/L) to avoid density-dependent induced masculinization (Ribas et al., 2017a). The experimental procedures were approved by the Bioethical Committee of the Government of Catalonia for animal experimentation with reference code no. 9977

Experimental design

The thermal experiment was carried out according to (Ribas et al., 2017a). Briefly, the larvae from one family (Family 1) were divided into two groups, containing each an equal number of fish, and were exposed from 18 to 32 dpf to two different temperature treatments: 28ºC (control temperature, LT) and 36ºC (high temperature, HT), which was achieved with the aid of an electric resistance and by a gradual increase until 22 dpf. Temperature was maintained stable until 32 dpf, at which point it was gradually decreased until 28ºC by 36 dpf. Thereafter, all groups were reared in a recirculating system at 28°C until sampling. Family 1 gave 100% males in the HT group and thus no females could be obtained. Therefore, the experiment was repeated with a second family (Family 2), but in this case fish were exposed to 34ºC instead of 36°C in order to increase the chances of obtaining females. For both families the experiment finished at 90 dpf and fish were sacrificed by ice-water immersion. Phenotypic sex was visually determined with the aid of a dissecting scope and gonads were classified according to the degree of maturation as immature (type 1), maturing (type 2) or mature gonad(type 3) (Ribas et al., 2017a). In total, 234 fish were sampled. Mature gonads were extracted and kept at −80ºC for molecular analysis. A detailed information of the number of samples per family, per sex and per treatment and the number of samples selected for methylation analysis can be found in **Table S1**.

DNA and RNA extractions

Genomic DNA and total RNA were isolated from the same gonad sample to allow comparisons of DNA methylation and gene expression in the same individual. For DNA extraction, gonads were hemisected and one half was treated overnight at 56ºC with digestion buffer containing 1 μg of proteinase K to eliminate proteins (P2308, Sigma-Aldrich, St. Louis, Missouri). Then, a standard phenol-chloroform-isoamyl alcohol protocol (PCI 25:24:1, v/v/v) with 0.5 μg ribonuclease A (12091021, PureLink RNase A, Life Technologies, Carlsbad, California) to eliminate RNA traces was performed. RNA extraction was carried out in the other half of the gonad by Trizol Reagent (T9424, Sigma-Aldrich, St. Louis, Missouri) according to manufacturer's instructions. Quality and quantity of DNA and RNA was measured by NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts).

Gene selection

A set of 18 genes were selected for DNA methylation analysis based on their functions in reproduction, stress, epigenetics and immune system in zebrafish. Genes were divided in three groups: steroidogenic enzymes (*cyp19a1a*, *cyp11a1*, *cyp11c1*, *hsd11b2*, *hsd3b2*, *hsd17b1* and *hsd17b3*), growth and transcription factors (*amh, dmrt1, dmrt3a* and *foxl2a*) and other genes (*nr3c1, tnfα, mxa*, *fancl, dnmt1, ehmt2* and *ar*) (**Table S2)**.

Primers design for bisulfite converted DNA

The precise genomic regions and sequences were determined using the zebrafish genome (danRer10) obtained from the University of California Santa Cruz (UCSC) genome browser database (Karolchik et al., 2003). Primers were specifically designed for bisulfite converted DNA using the MethPrimer software (Li and Dahiya, 2002). Amplicons targeted the proximal promoter and/or the beginning of the first exon of each gene, encompassing the maximum possible number of CpG sites, given the importance of these elements in the regulation of gene expression (Brenet et al., 2011; Deaton and Bird, 2011). Nextera adapter sequences were then added to the 5' ends of the region-specific primers (**Table S2**) as described in the Illumina's protocol: "16S metagenomic library preparation". The genomic coordinates of the amplicons were checked using the Integrated Genome Browser (IGV) software (v. 2.3) with danRer10 genome (Freese et al., 2016). Detailed information on the sequences of the primers, the corresponding annealing temperature (ATº), their length, the total number of CpG sites and CpG islands within the amplicon gene are listed in **Table S2**.

Polymerase chain reaction (PCR) of amplicons

One microgram of DNA per sample was bisulfite converted using the EZ DNA Methylation-Direct Kit (D5020, Zymo Research, Irvine, California) following the manufacture's protocol. PCR amplifications were performed using 2 µl of bisulfite converted DNA (~26–30 ng) as initial template, 4 µl $MgCl_2$ (25 mM), 2 µl dNTPs (R0193,10 mM, Thermo Fisher Scientific, Waltham, Massachusetts), 0.5 µl forward and reverse primers (10 µM, Life Technologies, Carlsbad, California), 5 µl 5X Green GoTaq Flexi Buffer (M7405, Promega, Madison, Wisconsin), 0.12 µl GoTaq G2 Hot Start polymerase (M7405, Promega) and 10.8 µl distilled water. The PCR conditions of the thermal cycler were: one step of 95ºC for 7 min, followed by 40 cycles of 95ºC for 1 min, then specific ATº for each gene for 2 min (**Table S2**), 65ºC for 2 min and a final step of 65ºC for 10 min. The presence and the size of each amplicon gene were confirmed in all samples using 4 µl of PCR amplification product on a 2% agarose gel. Finally, the primers were validated by Sanger sequencing using amplicons produced from a pool of three males and three females. The primers used for Sanger sequencing were complementary to the Nextera adapters (**Table S2**). The sequences obtained from Sanger sequencing were checked using the BLASTN software (v. 2.7.1) (Altschul et al., 1990) to ensure the identity of the amplicon. In this step, one sample was discarded because no amplifications were observed in the gel.

Size-selection and normalization of amplicons

The working solution of magnetic beads (65152105050250, Sera-mag Speed Beads, GE Healthcare, Chicago, Illinois) was prepared following the protocol described in Anastasiadi et al. (2018b) and adapted from Rohland and Reich (2012). The size-selection

and normalization of DNA quantities across the PCR amplicons were performed following the methodology described in (Anastasiadi et al., 2018b) from a customized version in (Hosomichi et al., 2014). Briefly, for size-selection of the amplicons, 10 μl PCR amplification products from each gene in each sample were transferred in a new tube and 40 μl distilled water and 20 μl 2X beads (0.4X) were added. Samples were incubated at room temperature for 5 min, placed on the magnet for 5 min and supernatant was transferred to a new tube. Then, samples were mixed with 42 μl 2X beads (0.6X), incubated for 5 min and placed on magnet discarding the supernatant. Samples were carefully washed on the magnet with 200 μl of 70% ethanol for 10 s and were dried for 10 min. Samples were rehydrated in 22 μl distilled water out the magnet for 5 min, then placed back on the magnet for 5 additional minutes and finally 20 μl of supernatant was transferred to a new tube. For the normalization step, 40 μl distilled water, 20 μl 20-fold diluted beads (2X) and 20 μl propan-2-ol (I9516, Sigma-Aldrich, St. Louis, Missouri) were added to the 20 μl supernatant from the previous size-selection step. Samples were then incubated for 5 min, placed on the magnet for 5 min and a second ethanol wash was performed exactly as described above. Next, samples were rehydrated in 12 μl distilled water for 10 min and placed back on the magnet for 10 min. Finally, 10 μl of each gene of each sample were transferred into a new tube.

Index PCR

Three microliters from the normalized amplicons of each gene belonging to the same sample were pooled in a new tube. Samples were individually labeled by mixing 5 μl of the pool of each gene per each sample with 5 μl index Nextera primers (Nextera XT Index Kit V2 set A, FC-131–2001, Illumina, San Diego, California), 25 μl 2X KAPA HiFi HotStart Ready Mix PCR kit (07958919001, Kapa Byosystems, Wilmington, Massachusetts) and 10 μl distilled water. The PCR conditions in the thermal cycler were: one step at 95ºC for 3 min, 8 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, with a final step at 72ºC for 5 min, according to the "Illumina's protocol for 16S metagenomic library preparation". Next, an extra clean-up step was carried out to the indexed samples, following the previous customized size-selection and normalization protocol but using 1X beads and eluting the samples in 15 μl distilled water. Finally, 3 μl from each indexed sample were pooled in a tube making a single multiplex bisulfite sequencing library (MBS). DNA concentrations were measured by Qubit fluorimeter (Invitrogen, Carlsbad, California) using Qubit dsDNA HS Assay Kit (Invitrogen) and the sizes of amplicons were checked by High Sensitivity DNA Assay on a Bioanalyzer system (Agilent 2100, Santa Clara, California) before sequencing. The library was sequenced on a HiSeq 2500 sequencing system (Illumina, San Diego, California) using the 250 paired-end (PE) protocol.

Bioinformatics analysis

Samples were demultiplexed based on the index codes by the Illumina software. The Nextera adapters were removed using Trim Galore! software (v. 0.4.5) with parameters: --nextera, --quality 20, and --phred64. After trimming the adapters, the number and quality of the reads were checked using the FastQC software (v. 0.11.6) (Andrews, 2010). In parallel, the whole zebrafish genome (danRer10) was bisulfite converted *in silico* using the function "bismark_genome_preparation". The alignments of the PE reads were performed using the function "bismark": --bowtie2, --non-directional, and --score_min 0 + (−0.6 x read length). The methylation status of all CpG sites was obtained using the function bismark_methylation_extractor": --bedGraph. All three steps were carried out by the Bismark software (v. 16.0) (Krueger and Andrews, 2011). The efficiency of the bisulfite conversion was calculated for each sample as 100% minus the percent of the cytosine (C) methylated in the CHH context (where H indicates a non-G nucleotides). In this step, three samples were removed because they had less than 99.0% bisulfite conversion efficiency. The coordinates of all CpG sites were obtained at the whole-genome level using a specific package for zebrafish "BSgenome.Drerio.UCSC.danRer10" from BSgenome (Pagès, 2018). We intersected the CpG sites of the target gene using the amplicons' boundary coordinates using BED tools (Quinlan and Hall, 2010) with the *bedr* package (v. 1.0.4) (Haider et al., 2016). Finally, the percent methylation of the filtered CpG sites was calculated as: $\frac{C's}{(C's+T's)}100$. From the targeted CpG sites, only those that showed coverage > 5 times were retained for subsequent analysis.

Quantitative real-time PCR (qPCR)

From each sample, 1 µg RNA was treated with DNAse I Amplification Grade (18068-015, Invitrogen) to remove any presence of genomic DNA. Then, 500–600 ng of RNA were reverse transcribed into cDNA using the SuperScript III Reverse Transcriptase (18080093, Invitrogen) for qPCR. The qPCR primers and the two references genes (*eef1a1l1* and *rpl13a*), both previously validated for zebrafish (Tang et al., 2007), are listed in **Table S3**. The qPCR primers targeted regions between at least two exons to avoid amplification of possible genomic DNA traces. The efficiencies of the primers ($E=10^{-1/slope}$) were estimated from the slope value derived from the log-linear regression after performing standard curve of five serial cDNA dilutions (1, 1:5, 1:10, 1:50, 1:100, 1:500) of a pool of 1 µl cDNA of all samples. The qPCR reactions were carried out in triplicate, taking 2 µl of cDNA (1:10), 0.5 µl forward and reverse primer, 2 µl 5X PyroTaq EvaGreen dye (87H24-001, Cultek, Pittsburgh, Pennsylvania) and 5 µl distilled water per well for each gene and sample with their corresponding negative controls. The qPCR conditions in the QuantStudio 12K Flex (Thermo Fisher Scientific, Waltham, Massachusetts) thermal cycler were: 50ºC for 2 min, 95ºC for 10 min and 40 cycles of 95°C for 15 s and 60ºC for 1 min followed by a melting curve to confirm single amplification product.

Statistical analysis

All statistical analyses were carried out using R software (v. 3.0.2) (Team, 2013). Significant results were considered when $P < 0.05$. All graphs were generated using the *ggplot2* package (v. 3.1.0) (Wickham, 2016).

Proportions of males and females

For the analysis of sex ratios, a χ^2 test with Yate's correction (Yates, 1934) was applied to compare the number of males and females between the LT and HT groups in each family and then, between families for the same treatment.

Methylation data analysis

First, we calculated the DNA methylation status of all individual CpG sites by averaging the data of all the reads belonging to same gene and sample. Next, we calculated the mean DNA methylation across all the CpG sites from each gene for each sample. Finally, we averaged the DNA methylation data from all samples, grouped them per sex and temperature for each family and then combining all data of the two families. Data is presented as mean ± s.e.m. The effects of sex and temperature on DNA methylation were evaluated by two-way ANOVA followed by a Tukey's HSD test. Normality of the residuals was checked by the Shapiro-Wilk test and when the assumptions of normality were not accomplished, a logit transformation was applied. In the cases when data transformation failed to follow the assumption of normality of the residuals' distribution, a robust non parametric two-way ANOVA was applied using the *WRS2* package (v. 0.9.2) (Mair and Wilcox, 2016).

Gene expression and correlation analysis

The quantification cycle (Cq) data was averaged, after eliminating samples that had more than 0.30 standard deviation between the three technical replicates. Data was normalized applying the formula: $\Delta Cq = Cq_{\text{(Mean GOI)}} - Cq_{\text{(Geometric mean } \textit{eef1a1l1} \text{ and } \textit{rpl13a}\text{)}}$. Fold change was calculated as $E^{(\Delta Cq)}$, where E is the efficiency of the primer (Livak and Schmittgen, 2001). We selected, among the eighteen genes analyzed at DNA methylation level, only those genes that showed significant methylation differences between males and females and were also influenced by temperature (i.e., *amh*, *cyp19a1a*, *dmrt1*, *cyp11a1*, *cyp11c1*, *hsd11b2* and *hsd17b1*). Data from qPCR were analyzed for normality and homogeneity of variances by the Kolmogorov–Smirnov and Levene's test, respectively. The effects of sex and temperature on gene expression were evaluated for each family separately using two-way ANOVA followed by a Tukey test. To test the correlation between DNA methylation and gene expression for the same gene, a Spearman's rank correlation (ρ) test was applied using the *corrplot* package (v. 0.84) (Wei et al., 2017).

Genes more influenced by sex and elevated temperature in males and females

First, we evaluated those genes that showed differences between sexes and, second, we evaluated for each sex separately those genes that showed differences due to elevated temperature. To do this, we selected those genes that showed significant differences by sex and temperature. We subtracted the mean DNA methylation of males minus the mean DNA methylation of females only from the LT group for each gene selected. Next, we subtracted the mean DNA methylation levels of females of the HT group minus the mean DNA methylation of females of the LT group and we repeated the same procedure separately for the males.

Epigenetic biomarkers: predictors of phenotypic sex and exposure to thermal stress

Based on the observed sex- and temperature-related differences of DNA methylation levels of the genes examined in this study, we reasoned whether these differences could be used as epigenetic biomarkers. Here, a two-step approach involving three separate classifications was implemented. In the first classification, we used all fish to predict their sex regardless of actual sex and the temperature they had been exposed (N = 70 samples). For the second and third classifications, males and females were separated (N males = 37 and N females = 33). In the latter two classifications, the goal was to determine whether the temperature they had been exposed to during the period of development of the gonads while juveniles could be predicted from the analysis of the DNA methylation levels in 90-day-old adults. We only used genes that showed significant differences by sex and temperature. If any sample(s) had missing data, the values were imputed applying the mean value replacement method for the corresponding group in which the sample pertained (Zhang, 2016). For the classification and prediction, we applied a flexible discriminant analysis (FDA) with the repeated K–Fold Cross–Validation (10-fold and 5 repetitions) using the *caret* package (v. 6.0–82) (Kuhn, 2008). The efficiency of the prediction accuracy (comparing the prediction result from the model output with the real data) and the Cohen's kappa coefficient test (κ) values were extracted from the *caret* package. The κ measures inter-rater agreement for qualitative (categorical) items and can take values between 1 (complete agreement) to 0 (no agreement). In addition, the level of the agreement classification was assigned as described in McHugh (2012). First, we evaluated each gene using the mean methylation (mean of all the CpGs of the amplicon) as single variable predictor. Second, for the genes that showed the highest accuracy and κ, we broke down the methylation values to individuals CpGs in order to improve the classification. Third, we combined the best predictors between them (those showed the highest accuracy and κ values) to observe if the addition of more predictors could improve the classification efficiency. We used the ldahist function from the *MASS* package (v. 7.3–51.1) (Venables et al., 2002) to make stacked histograms of the discriminant coefficient.

Independent samples for machine learning validation

To validate the results of the prediction of sex from the machine learning algorithm, we extracted the methylation data (15 CpG of *cyp19a1a* amplicon) of 20 testes and 20 ovaries from an unrelated family (family 3) at the age of 90 dpf (Moraleda-Prados et. al., 2019, unpublished).

3. Results

Sex ratio

Exposure to 36°C resulted in 100% masculinization in family 1 (χ^2 = 38.42; P < 0.001) (**Figure S1**) and thus no females were available in the HT group for further analysis. Therefore, family 2 was exposed to 34°C instead of 36°C. In that case the number of males did not reach 100% at HT (χ^2 = 2.90; P = 0.08) and thus females were available. At control temperature (28°C), percent males were similar (χ^2 = 2.37; P = 0.24) in both families (mean ± s.e.m, 50.6 ± 6.8%). During sampling, visual inspection of the fish subjected to high temperature showed no obvious alterations of the gonads when compared to those of fish exposed to control temperature in both families tested.

DNA methylation in adult zebrafish gonads

The number (mean ± s.e.m) of sequenced reads per sample was 469,779 ± 22,188, alignment efficiency 71.9 ± 1.63% and bisulfite conversion rate 99.6 ± 1.36% (see **Table S4** for details). An overall assessment of DNA methylation of the genes individually analyzed in this study, and regardless of family, showed sex-specific differences (**Figure S2**). The main differences were that in females the DNA methylation median values essentially covered the 0-100% range whereas in males the DNA methylation values were either usually above 75% or below 10% with no intermediate values. Furthermore, in males the DNA methylation at LT showed high variability among individuals but this variation was reduced at HT. Regarding DNA methylation of genes coding for the steroidogenic enzymes we observed variation in males of the LT group between the two families, whereas in females the levels were similar (**Figure 1**). In addition, the DNA methylation levels were predominantly higher in males than in females at LT in family 1 but not in family 2. Temperature significantly affected DNA methylation of all genes except *hsd11b2* and *hsd17b1*. Only *cyp11a1* showed a significant interaction between both factors (**Figure 1**). For growth and transcription factors, family-related differences were observed in males at LT and sex-specific differences were observed for all genes examined. In contrast to *amh*, DNA methylation levels of *foxl2a, dmrt1* and *dmrt3a* were lower in males than in female. Temperature did not affect females (family 2) but decreased and increased DNA methylation levels of *dmrt1* and *amh* in males, respectively, the former being the only gene where the sex-temperature interaction was significant (**Figure 2**).

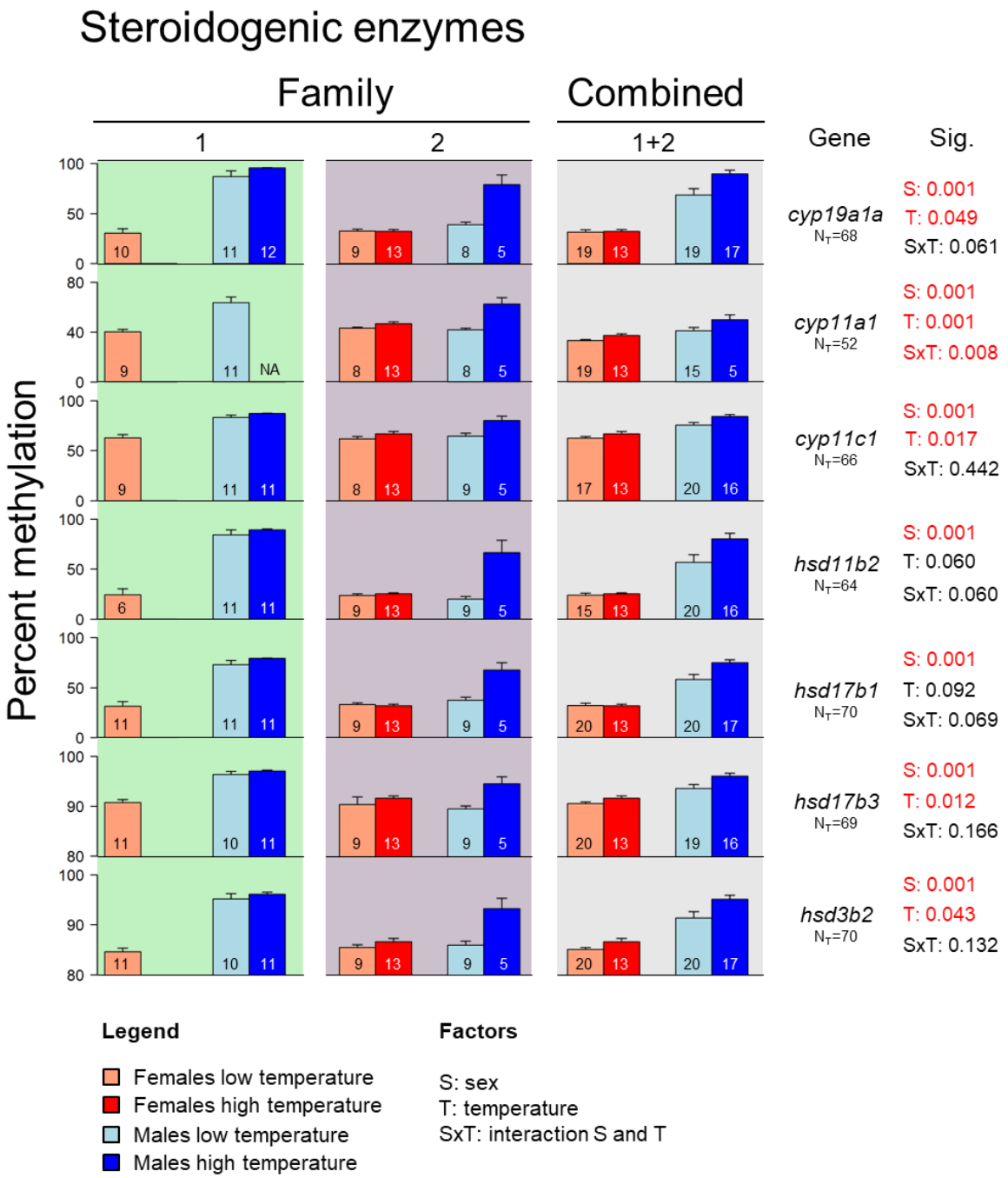

Figure 1. Percent DNA methylation levels of the promoter regions of steroidogenic enzymes in the offspring of family 1 (green background), family 2 (purple background) and the two families combined (grey background). The offspring of each family was divided in groups according to the sex and the temperature treatment experienced during the sex differentiation period. The far-right data indicate *P* values for the effects of sex (S), temperature (T) or the interaction of both factors (SxT). The numbers of fish analyzed in each group are shown inside the bars. A robust no parametric two-way ANOVA with trimmed means was applied. Data is shown as mean ± s.e.m.

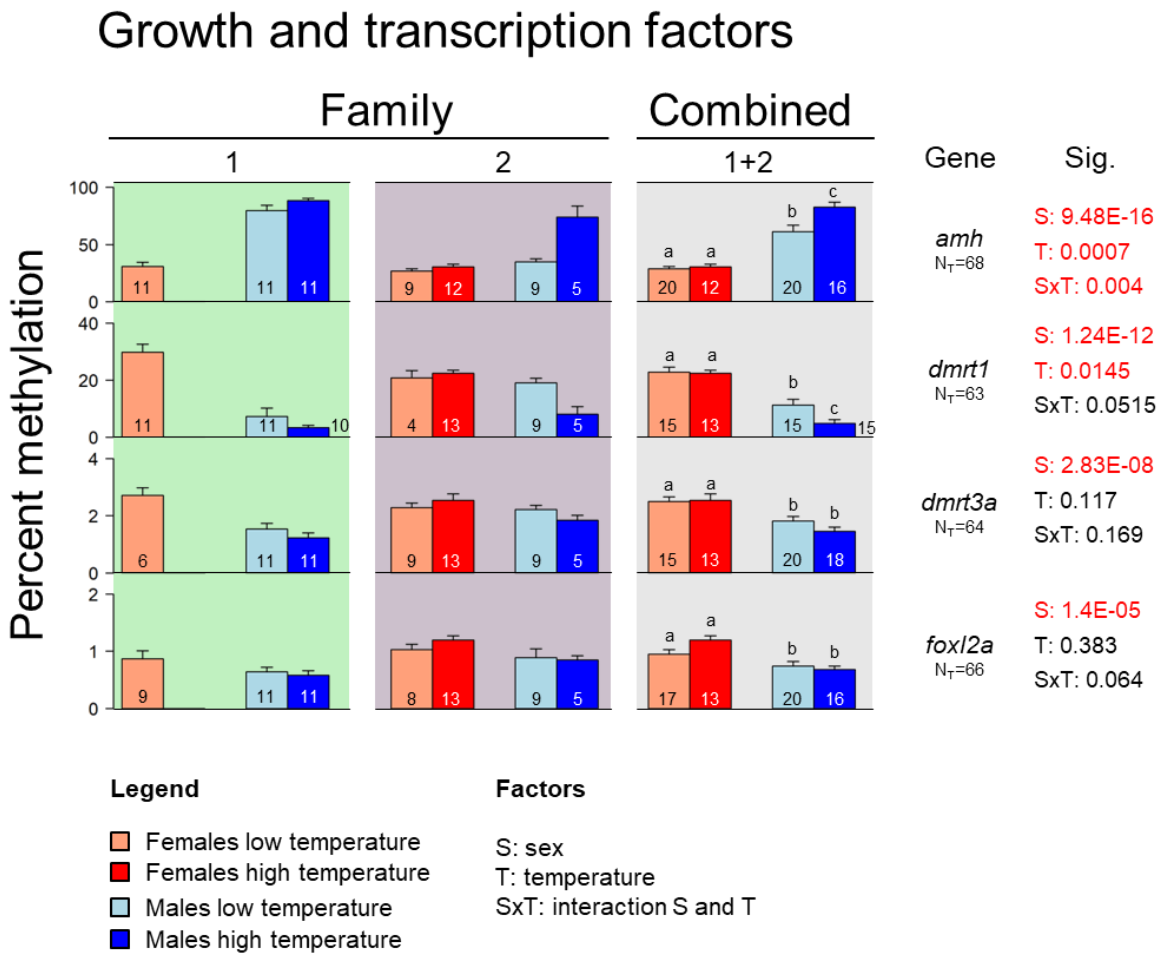

Figure 2. Percent DNA methylation levels of the promoter regions of growth and transcription factors in the offspring of family 1 (green background), family 2 (purple background) and the two families combined (grey background). The offspring of each family was divided in groups according to sex and the temperature treatment experienced during the sex differentiation. The far-right data indicate *P* values for the effects of sex (S), temperature (T) or the interaction of bot factors (SxT). The numbers of fish analyzed in each group are shown inside the bars. Two-way ANOVA followed by post hoc Tukey test was applied. Different letters indicate significant ($P < 0.05$) differences between groups. Data is shown as mean ± s.e.m.

Regarding the other genes examined in this study (**Figure S3**), except *nr3c1* and *tnfa*, who had DNA methylation values > 50% and showed both sex- and temperature-related differences, the rest had DNA methylation values < 2% and only *mxa* and *dnmt1* were influenced by sex. The *mxa* showed significant differences by the interaction of sex and temperature whereas no significant differences in the DNA methylation levels between sex or temperature or interaction of both was observed in *fancl*, *ehmt2* and *ar* genes.

Gene expression and correlation analysis

Genes the methylation of which was affected by sex or temperature also showed different patterns of expression between the two families (**Figure 3**). The expression of *amh*, *dmrt1*, *cyp11a1*, *cyp11c1* and *hsd11b2* (**Figure 3A**, **B, D, E** and **F**, respectively) was significantly different between males and females in family 1 but not in family 2 at LT. In contrast, in *cyp19a1a* (**Figure 3C**), gene expression in males was significantly lower than in females in both families at LT. Temperature was able to increase significantly the gene expression of *amh*, *dmrt1*, *cyp11c1* and *hsd11b2* and decreased in *cyp11a1* in males but not in females (**Figure 3A**, **3B**, **3E**, **3F**, and **3D**, respectively).

Regarding the relationship between DNA methylation and gene expression levels, we found higher methylation and gene expression levels of *amh* in males compared to females in family 1, regardless the treatment, and also between males of HT against the males of LT in family 2 (**Figure 2** and **Figure 3A**). As for *dmrt1*, we found a lower methylation and higher gene expression in males, a difference exacerbated at HT, particularly in family 2, while females were not affected (**Figure 2** and **Figure 3B**). As for *cyp19a1a* in females, lower methylation and higher gene expression levels were observed when compared to males (**Figure 2** and **Figure 3C**). In *cyp11a1*, especially in family 2, a tendency towards an inverse relationship between DNA methylation and gene expression levels was observed (**Figure 2** and **Figure 3D**). As for *cyp11c1*, we observed that males with higher levels of methylation had higher gene expression whereas females and LT males of family 2 showed very low levels of gene expression (**Figure 2** and **Figure 3E**). Finally, *hsd11b2*, showed similar pattern of methylation and gene expression as *cyp11c* (**Figure 2** and **Figure 3F**). However, the only significant negative correlation between DNA methylation and gene expression was observed in males for *dmrt1* but only in family 2 ($r = -0.81$, $P < 0.05$) (**Figure S4**).

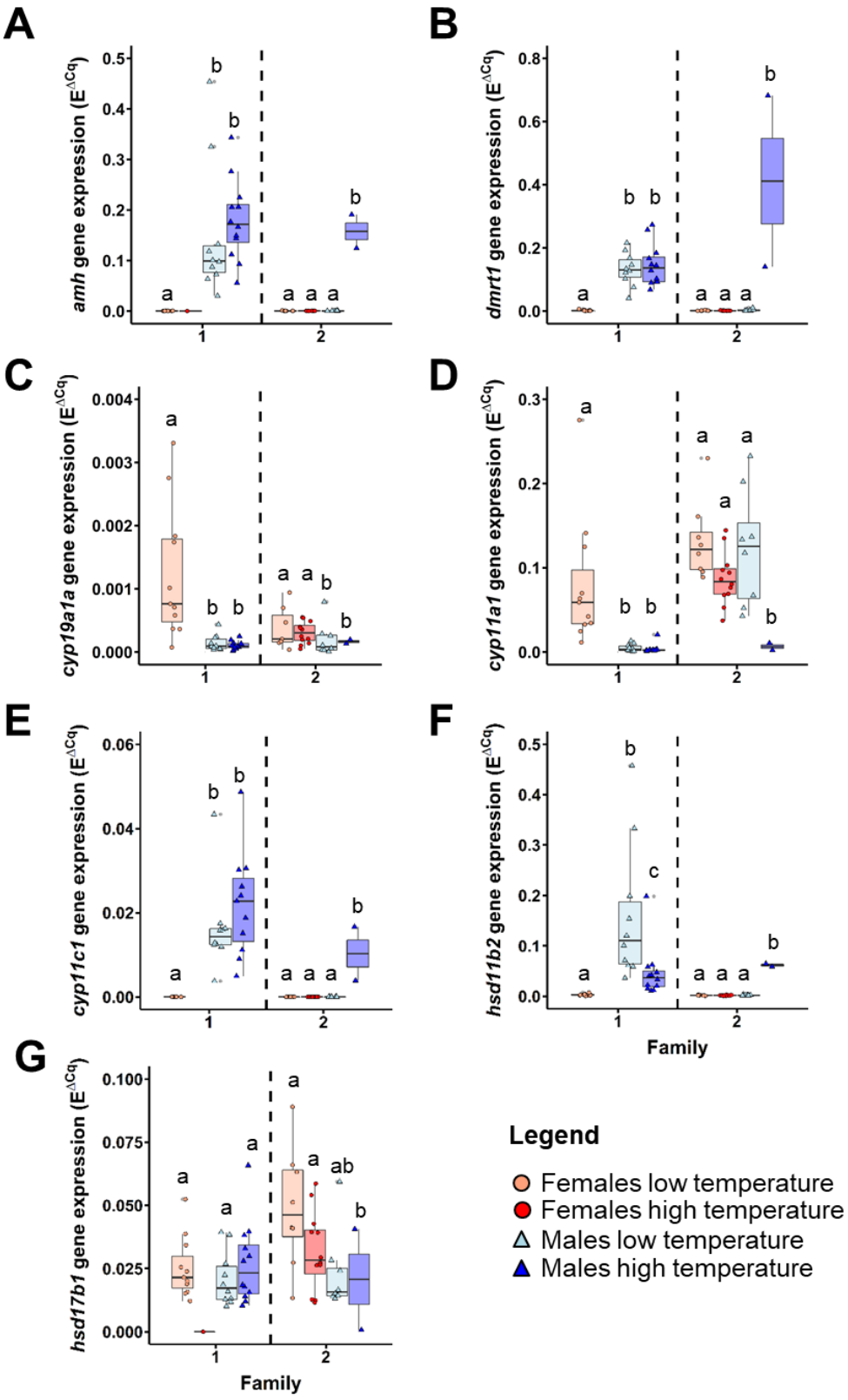

Figure 3. Gene expression of (**A**) *amh*, (**B**) *dmrt1* (**C**) *cyp19a1a*, (**D**) *cyp11a1*, (**E**) *cyp11c1*, (**F**) *hsd11b2* and (**G**) *hsd17b1*. Two-way ANOVA followed by post hoc Tukey test was applied. Within each family, significant differences ($P < 0.05$) among treatments are indicated by letters.

Methylation differences by sex and temperature

We were interested in knowing which genes had the highest sex-related differences in DNA methylation and which ones were the most affected by temperature in each sex. *Cyp19a1a*, *hsd11b2*, *amh* and *hsd17b1* showed the highest hypermethylation (≥ 20% both families combined) in favor of males, whereas *dmrt1* showed the highest hypermethylation (~ 20%) in favor to females (**Figure 4A**). For temperature differences the most affected genes were *amh*, *cyp11a1* and *cyp11c1* in females (**Figure 4B**) and *cyp19a1a*, *hsd11b2*, *amh* and *hsd17b1* with hypermethylation values and *dmrt1* with hypomethylation values (**Figure 4C**) in males.

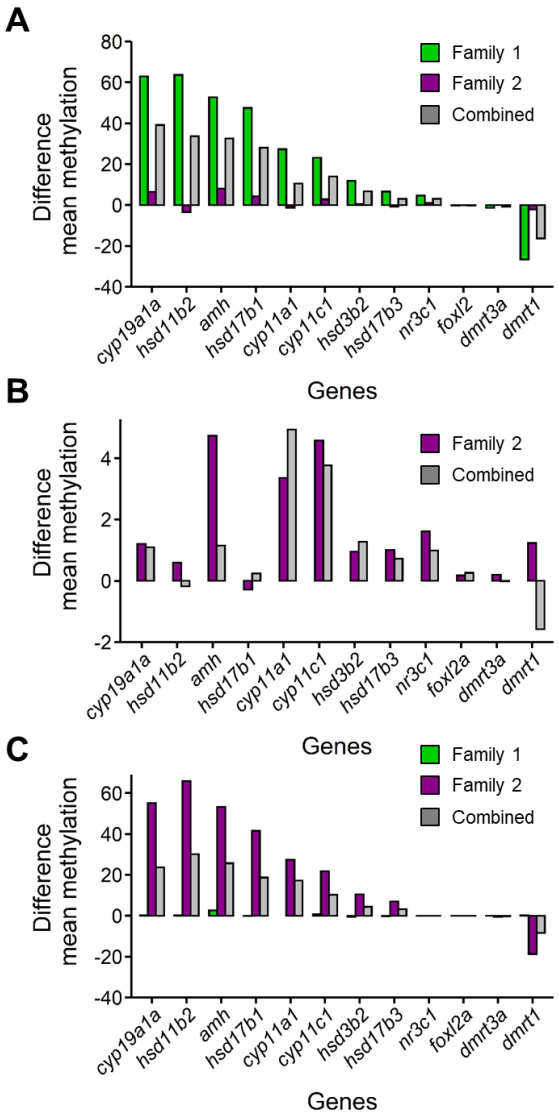

Figure 4. Differences of the percent mean DNA methylation levels in the steroidogenic enzymes and growth and transcription factors in family 1, 2 and the combination of the two families. (**A**) Differences of the percent mean DNA methylation levels between males minus females exposed to control temperature; (**B**) Differences of the percent mean DNA methylation levels between females exposed to high minus control temperature. (**C**) Differences of the percent mean DNA methylation levels between males exposed to high minus control temperature.

Prediction of sex and treatment and validation of the machine learning procedure

Next, we tested whether these sex- and temperature-related differences in DNA methylation levels in specific genes could be used as reliable predictors of: 1) phenotypic sex and 2) whether fish had previously been exposed to high temperatures. To achieve this, we used a k–Fold Cross–validation method to evaluate the predictive model. The best eight predictors (individual genes or a combination of them) based on accuracy and κ values for each purpose are shown in **Table 1**. The classifications gave good accuracies and κ values (between 0.85–0.88 and 0.72–0.77, respectively), to distinguish males from females at control temperature, either using the mean methylation levels of *cyp19a1a*, *dmrt1* and *amh* alone or the combination all three genes. The best sex predictor was *cyp19a1a* using the values of the 15 targeted CpGs of the amplicon. However, using the mean methylation values gave similar accuracy and κ values. Thus, methylation levels of the *cyp19a1a* in zebrafish gonads are enough to predict sex (**Table 1**). Next, we wanted to evaluate if the prediction for the sex classification was reliable and the accuracy of the machine learning obtained from our samples would be repeated with independent samples. First, we extracted the *cyp19a1a* DNA methylation profile of males and females of an unrelated family (family 3) following the same procedure described earlier (**Figure S5A**). Then, we used the algorithm prediction for sex used previously to classify these 40 independent samples. Classification gave 100% accuracy and the predicted samples were classified in two clear distinct groups according to their sex (**Figure S5B**).

To distinguish males previously exposed to high temperature, the best predictors were *amh* and *cyp19a1a* (accuracy = 0.71; 0.69 and κ = 0.44; 0.42, respectively). These values indicated that the power of classification was categorized as "moderate" according to interpretation of Cohen's kappa values (McHugh, 2012). When methylation values of individual CpGs of *amh* (15 CpG) was used, the reliability of the classification (accuracy = 0.64 and κ = 0.27) decreased to "fair". Thus, to predict developmental temperature in males the mean methylation levels of *amh* in male gonads were enough. Finally, to distinguish previous exposure to elevated temperature in adult females, among all predictors and combinations tested, the best were *foxl2a* (accuracy = 0.63 and κ = 0.28) and *foxl2a* + *cyp19a1a* (accuracy = 0.59 and κ = 0.20), achieving "fair" and "none to slight" reliability, respectively. By using the individual CpGs of *foxl2a* as predictors (26 CpG) the classification improved (accuracy = 0.78 and κ = 0.55) and was categorized as "moderate". Thus, to identify females that had experienced high temperature during early development, the methylation levels of *foxl2a* in female gonads can be used. Thus, the identified genes (*cyp19a1a, amh* and *foxl2a*) are good predictors to discriminate between sex or high temperature (**Figure 5**).

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Table 1. Epigenetic biomarkers: predictors of phenotypic sex and previous thermal exposure. Predictors were used either alone or in combination. The kappa values obtained from each predictor follow the classification proposed by (McHugh, 2012). For all predictors, the mean of all CpGs in that predictor were used. Within each purpose, the best predictor was also assayed using the values of all the individual CpGs. Abbreviations: LT = low (control) temperature; HT = high temperature

Purpose	Predictor	Accuracy	Cohen's kappa	Interpretation of Cohen's kappa (*)
To distinguish males from females	*cyp19a1a*	0.88	0.77	substantial
	dmrt1	0.88	0.76	substantial
	amh	0.87	0.75	substantial
	foxl2a	0.73	0.47	substantial
	amh + dmrt1	0.82	0.64	substantial
	cyp19a1a + amh + dmrt1	0.85	0.72	substantial
	cyp19a1a + foxl2a + dmrt1	0.88	0.76	substantial
	cyp19a1a (using 15 individual CpG)	0.88	0.77	substantial
To distinguish males HT from males LT	*amh*	0.71	0.44	moderate
	cyp19a1a	0.69	0.42	moderate
	dmrt1	0.68	0.38	fair
	amh + cyp19a1a	0.63	0.32	fair
	amh + dmrt1	0.68	0.39	fair
	cyp19a1a + dmrt1	0.68	0.41	moderate
	amh + cyp19a1a + dmrt1	0.71	0.40	moderate
	amh (using 18 individual CpG)	0.64	0.27	fair
To distinguish females HT from males LT	*foxl2a*	0.63	0.28	fair
	cyp19a1a	0.27	-0.29	no agreement
	hsd3b2	0.27	-0.29	no agreement
	hsd11b2	0.29	0.52	no agreement
	foxl2a + cyp19a1a	0.59	0.20	none to slight
	foxl2a + hsd3b2 + hsd11b2	0.56	0.13	none to slight
	foxl2a + cyp19a1a + hsd3b2 + hsd11b2	0.57	0.18	none to slight
	foxl2a (using 26 individual CpG)	0.78	0.55	moderate

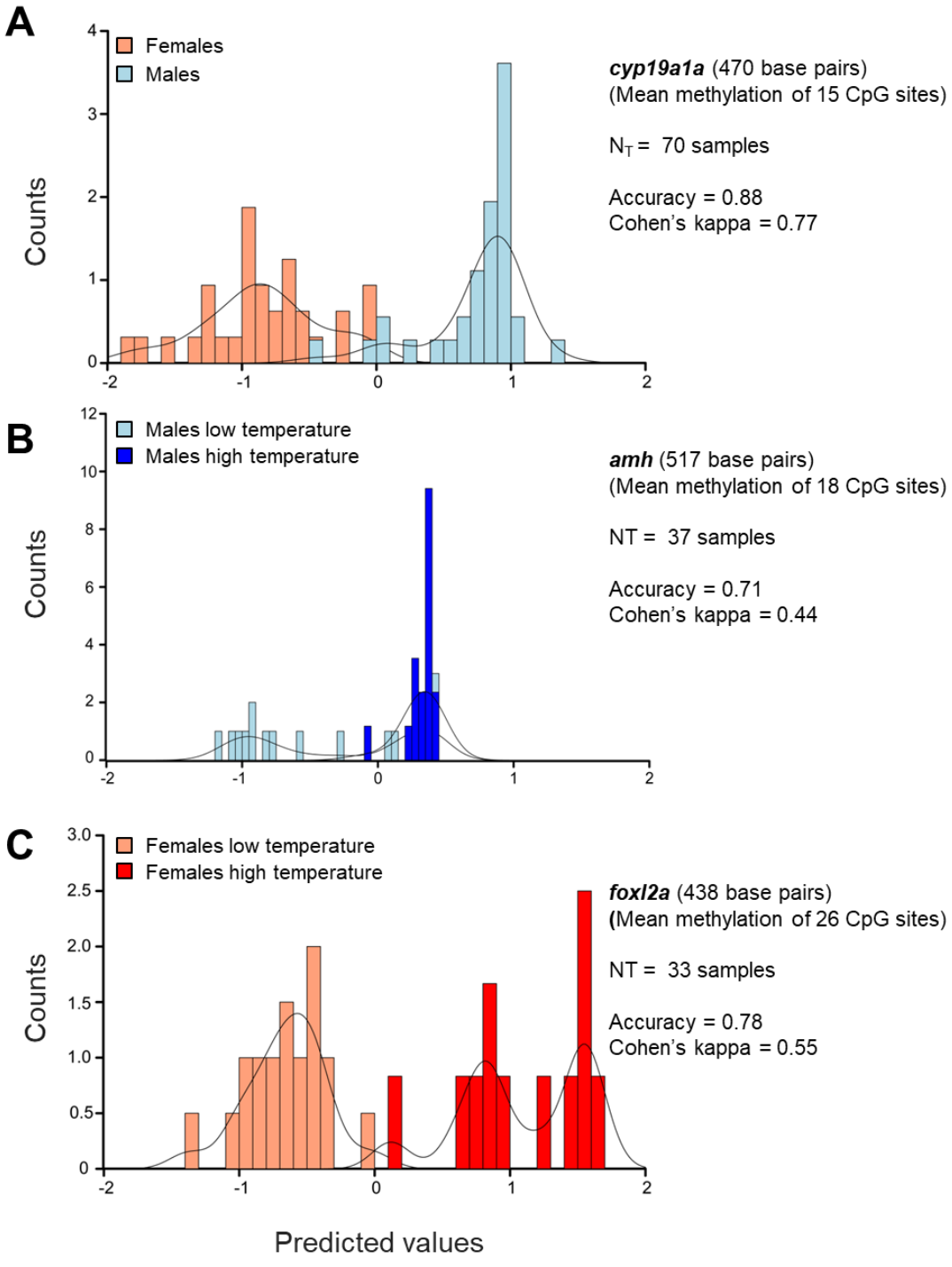

Figure 5. Histogram of prediction values obtained from the first flexible discriminant classification (FDA) using (**A**) *cyp19a1a* methylation values to distinguish the sex of the fish, (**B**) *amh* methylation values to distinguish males at high temperature (HT) from males at control temperature (LT), and (**C**) *foxl2a* methylation values to distinguish females HT from males LT.

4. Discussion

We studied the influence of genetics and environment on the DNA methylation of a series of genes related to sex differentiation, the immune response and epigenetic regulatory mechanisms. In addition, we were interested in knowing whether we could identify epigenetic biomarkers capable of predicting both phenotypic sex and past thermal experiences. To this end, we used two different families of zebrafish subjected to control or high temperature during the critical period of gonadal development and, as expected, differences in temperature response were observed. Thus, although an increase in the number of males occurred in both families, masculinization was significant only in one. This confirms PSD in laboratory strains of zebrafish (Liew et al., 2012; Ribas et al., 2017a). In addition to the sex ratio variation between families, variation was also evident in DNA methylation and, interestingly, more pronounced in males than in females. Inter-family variation in the methylation of sex-related genes in the gonads had been previously observed in other species with PSD such as the European sea bass (Anastasiadi et al., 2018b). This suggests that the allelic components (genetic factors) inherited from the parents can influence DNA methylations patterns in offspring. Our findings highlight the importance of the genetic component and should be taken into account for further studies on DNA methylation and gene expression patterns.

Despite the inter-family variation, we could detect sex- and environment-specific responses of DNA methylation for each group of genes. Steroidogenic enzymes are responsible for the synthesis of sex steroids, which play a key role in gonadal development (Nakamura et al., 1998). All steroidogenic enzymes reported in the present study showed sex-specific methylation differences. *cyp11a1* and *hsd3b2* encode enzymes that participate in the conversion of cholesterol into progesterone, a precursor for the synthesis of testosterone and 17β-estradiol (E_2) (Simpson, 1979; Simard et al., 2005). The methylation levels of these two enzymes were higher in males. *Cyp19a1a* and *hsd17b1* are enzymes that catalyze the conversion of androstenedione, testosterone and estrone (E_1) into E_2, which is required for ovarian development (Mindnich et al., 2004a). In fish, the activity and expression of *cyp19a1a* is essential for female development (Piferrer et al., 1994; Hinfray et al., 2018). Indeed, knock-out experiments of this gene resulted in all male populations (Lau et al., 2016). In the present study, *cyp19a1a* was hypomethylated in the ovaries while its expression was higher when compared to males. The DNA methylation patterns found in the two sexes were in agreement with those reported in other fish species, such as the European sea bass, (Navarro-Martín et al., 2011; Anastasiadi et al., 2018b), the half-smooth tongue sole (Shao et al., 2014) the black porgy (*Acanthopagrus schlegelii*) (Wu and Chang, 2018) and barramundi (*Lates calcarifer*) (Domingos et al., 2018). To our knowledge, a similar inverse relationship between DNA methylation and gene expression patterns in *hsd17b1* is reported here for the first time in fish. Since they are both "pro-female" genes (Payne and Hales, 2004; Liu et al., 2015), they may be regulated in a similar way although further functional studies are needed. On the other hand, the "pro-male" steroidogenic enzymes *cyp11c1*, *hsd11b2* and *hsd17b3*

(Liu et al., 2015) use androstenedione and testosterone as substrates to be converted into 11β-hydroxyandrostenedione and 11β-hydroxytestosterone and finally to 11-ketotestosterone, a potent androgen in teleost fish (Baroiller and Guiguen, 2001; Mindnich et al., 2004a). This is the first time that the methylation levels of *cyp11c1*, *hsd11b2* and *hsd17b3* are reported in fish, with hypermethylation levels found in males. The expression of *cyp11c1* and *hsd11b2* were higher in males confirming similar results found in current literature (Kusakabe et al., 2003; Baker, 2004; Wang and Orban, 2007).

The expression of steroidogenic enzymes is regulated by the action of transcription factors (Manna et al., 2003) and thus they are also important for fish sex differentiation (Yang et al., 2017). All the transcription and growth factors analyzed in this study (*amh*, *dmrt1*, *dmrt3a* and *foxl2a*) showed significant sex-specific differences in methylation. The two genes of the *dmrt* family, both located in the chromosome 5, *dmrt1* and *dmrt3a*, were hypomethylated in testis. While no reports describe the methylation levels of *dmrt3a* in fish, here we found that *dmrt1* was hypomethylated in males, in accordance with previous observations in the European sea bass, half-smooth tongue sole and the Japanese flounder (*Paralichthys olivaceus*) (Fan et al., 2014; Shao et al., 2014; Anastasiadi et al., 2018b), thus suggesting a conserved methylation pattern across species. The *dmrt1* was significantly more expressed in males, and thus an inverse relationship between methylation and gene expression levels was present, which is in accordance with *dmrt1* expression being essential for male development in fish (Webster et al., 2017). Further, *amh* displays a sexual dimorphic expression in teleosts during sex differentiation (Yoshinaga et al., 2004). In this study, *amh* showed higher methylation and gene expression in males. This *amh* methylation pattern is in accordance with other studies in zebrafish (Laing et al., 2018), barramundi (Domingos et al., 2018) and half-smooth tongue sole (Shao et al., 2014), although in the European sea bass the methylation levels were similar between sexes (Anastasiadi and Piferrer, 2019).

Foxl2a is a transcription factor required for ovarian development and regulates the expression of *cyp19a1a* in fish (Wang et al., 2007b; Yang et al., 2017; Zhang et al., 2017). The methylation levels of *foxl2a* were much lower than those of *cyp19a1a,* and were significant hypermethylated in females when compared to males. These results are in accordance with the methylation levels of *foxl2a* found in the ovaries of the European sea bass (Anastasiadi et al., 2018b), barramundi (Domingos et al., 2018) and Japanese flounder (Si et al., 2016). In the latter case, it was observed a strong inverse relationship between methylation levels in *foxl2a* and *cyp19a1a* with its respective gene expression at different ovarian development stages (Si et al., 2016). In the ovaries of different fish species, there is a positive correlation between the transcriptional levels of *foxl2a* and *cyp19a1a* (Baron et al., 2004; Baron et al., 2005). In our case, although we did not specifically study the expression levels of *foxl2a*, our methylation data of *foxl2a* and *cyp19a1a* with gene expression data from *cyp19a1a* in females, and based on previous results in fish (Wang et al., 2007a), suggests a similar and conserved epigenetic pattern specific for both genes in zebrafish that it is essential for female gonadal development (Fan et al., 2017; Fan et al., 2019).

In addition to the sex-specific DNA methylation differences in key genes at control temperature, we also studied the effects of elevated temperature on the methylation patterns of genes involved in sex differentiation. This was done almost two months after the temperature treatment ceased in order to evaluate persistent effects in adult fish. In males, temperature increased the methylation levels of *cyp19a1a, cyp11a1, cyp11c1*, *hsd17b3*, *hsd3b2* and *amh*. In some cases, this increase of methylation in males was associated with an upregulation of gene expression, like in *amh* and in *cyp11c1* or, on the contrary, with a downregulation of gene expression, as in *cyp19a1a* and *hsd17b1*. In males, elevated temperature strongly decreased *dmrt1* methylation levels, while an upregulation of its expression was found, confirming previous results analyzing temperature and gene expression in zebrafish (Ribas et al., 2017a). In females subjected to high temperature, the methylation levels of all studied genes remained similar to the levels methylation of females raised at low temperature. Similar methylation results in the case of *cyp19a1a* in ovaries between females exposed to temperature and control was found in half-smooth tongue sole (Liu et al., 2019). This is likely due to the fact that sex differentiation in zebrafish takes place earlier, as was observed at 14 dpf, a dimorphic proliferation of primordial germ cells are detected in which an elevated threshold of these cells is required for the ovarian development (Tzung et al., 2015). Consequently, this time lapse might have hindered stable epigenetic marks for female differentiation at the time when we subjected fish to high temperatures in our experiment (i.e., 18–32 dpf). Thus, the effects of temperature on DNA methylation in females could have been stronger if applied earlier as it was shown in the European sea bass in which temperature before sex differentiation was able to alter methylation levels of the genes in ovaries (Navarro-Martín et al., 2011; Anastasiadi et al., 2018b). In genotypic female Japanese flounder, elevated temperature during gonad differentiation hypermethylated the promoters of *cyp19a1a* and its transcriptional activator *foxl2a* with a concomitant downregulation of their expression levels, resulting in male development (Fan et al., 2017; Fan et al., 2019). These results open new avenues for studying how methylation marks are established before, during and after the gonadal development in fish when exposed to environmental stressors.

Furthermore, we have studied two epigenetic-related genes, *ehmt2*, in which no differences by sex or treatment were found, and *dnmt1*, which showed sex-specific differences with a hypomethylation in males. Our *dnmt1* results are in agreement with a previous study in zebrafish, where hypomethylation was found in males, although no significant differences between sexes were detected (Laing et al., 2018). DNA methylation levels of two receptors related to sex differentiation in fish are reported. *Ar* did not show any methylation differences neither by sex or temperature but *nr3c1*, the glucocorticoid receptor, showed males with higher methylation levels than females with no significant increase due to temperature. It should be noted that *nr3c1* DNA methylation levels in zebrafish were always >90%, thus in contrast with much lower values (~ 10%) found in both sexes in the European sea bass (Anastasiadi et al., 2018b).

Recently, after analyzing the available data on both gonochoristic and hermaphroditic species, the model of Conserved Epigenetic Regulation of Sex (CERS) was proposed ((Piferrer et al., 2019). From our results, here, *cyp19a1a* in ovaries and *dmrt1* in testes follow this model, as described in the European sea bass (Anastasiadi et al., 2018b), half-smooth tongue sole (Shao et al., 2014; Liu et al., 2019), Japanese flounder (Fan et al., 2014) and barramundi (Domingos et al., 2018). Thus, our results strongly reinforce the CERS model across species. Further, *hsd17b1* may also follow the CERS model, although additional data in other fish species should be gathered to confirm such observation.

When we tested the correlation between DNA methylation and gene expression patterns, with a limited numbers of fish samples per group and gene (n= 4–11), we detected a strong negative correlation only in *dmrt1* in males of family 2. This is consistent with results found in *Culter alburnus* (Jia et al., 2019). However, the lack of significant correlation in the other genes could be due to different reasons. First, the tested methodology, a high resolution technique at locus-specific (MBS), has a physical limitation in which the maximum length is < 600 base pairs. This allowed to study only a limited number of CpG sites (ranging from 4 to 34 in the genes studied), forcibly leaving out other CpG sites that may be relevant in gene regulation. Moreover, although the most studied targeted region for DNA methylation is the promoter region close to the TSS, the methylation levels on distal enhancers and other genomic features like the first exon and the first intron of the gene body have strongly been associated with silencing gene expression (Brenet et al., 2011; Anastasiadi et al., 2018a).

In the last years, the link between DNA methylation, gene expression and certain phenotypes has prompted the identification of specific DNA methylation CpGs as suitable markers of such phenotypes (Jin and Liu, 2018). Part of the DNA methylation marks can change with age, lifestyle or environmental cues (Marsit, 2015). In clinical studies, the methylation differences in particular genes have been associated with specific illnesses (Jones and Baylin, 2007). Therefore, significant specific CpGs have been used to develop tests (Moran et al., 2016) for the diagnosis and prognosis of diseases (Costa-Pinheiro et al., 2015). For instance, DNA methylation of genes involved in lung and colorectal cancer have been used to develop test for their early detection. Nevertheless, there is still a wide range of sensitivity and specificity in such type of tests (20–90% and 65–100%), respectively (Dong and Ren, 2018; Li et al., 2019). However, using the methylation levels of CpGs from different genes combined into a panel of epigenetic biomarkers increased the sensitivity and specificity of diagnosis (Lind et al., 2011). DNA methylation marks have also been used to predict age in humans and other vertebrates (Horvath, 2013; De Paoli-Iseppi et al., 2019) and sex in fish (Anastasiadi et al., 2018b).

Environmental perturbations, specially occurring during early development, can induce lifelong phenotypic changes brought by epigenetic regulation of gene expression. These changes underlie phenotypic plasticity and are thought to contribute to adaptation to new environments (Jaenisch and Bird, 2003; Beal et al., 2020). Thus, epigenetic alterations including changes to the methylome, constitute a sort of “epigenetic memory” which,

potentially, could allow detecting past exposures to environmental stressors such as pollutants (Wang et al., 2009) or increase of temperature (Mirbahai and Chipman, 2014).

Examples of epigenetic biomarkers with thermal-stress have been discovered in marine species. In oyster (*Crasssotreas gigas*), the relation between increase of temperature and histone methyl marks were associated with delayed development and growth (Fellous et al., 2015). The Antarctic polychaete (*Spiophanes tcherniani*) showed an epigenetic adaptation mechanism in which individuals stressed by a slight increase of temperature recovered normal metabolic rates after a month by an increase of DNA methylation (Marsh and Pasqualone, 2014). In four wild populations of the ascidian *Ciona robusta* taken from different habitats around the world, the methylation profiles of CpGs located in the heat-shock protein 90 (*hsp90*) and Na+–K+–2Cl– cotransporter (*nkcc*) genes, with roles in responses to temperature and salinity, allowed the identification of the population of origin, indicating that different geographic locations (in addition to genetics) resulted in characteristic epigenomes (Pu and Zhan, 2017).

Taking into account the possibility of the existence of such an "epigenetic memory" to past environmental stressors, here we used the DNA methylation levels of carefully selected CpGs, not only to predict sex but also to predict what thermal conditions in fish had experienced two months before during larvae stages. Based only on the methylation pattern obtained of the15 CpGs of the *cyp19a1a* amplicon, we were able to classify the sex of fish with 88% accuracy, a value very close to the 89% accuracy of the sex prediction accuracy in the European sea bass (Anastasiadi et al., 2018b). In addition, in the present case, we could validate the results with a set of independent samples with 100% success and using the CpG sites of only this gene. Further, using the DNA methylation levels of *amh* in testes and of *foxl2a* in ovaries, we were able to accurately predict (71% and 78%, respectively) whether fish had been exposed to elevated temperature during early development. To the best of our knowledge, this is the first time that epigenetic marks can be used to predict past environmental conditions in fish. Since the zebrafish is also a well-established model for aquaculture (Ribas and Piferrer, 2014), toxicology (Dai et al., 2014; Tanguay, 2018) and environmental issues (Brown et al., 2015), similar studies as the one presented here can be used for the development of other epigenetic biomarkers of exposure to different environmental disturbances. This will particularly be useful in the current efforts to better understand and detect the effects of climate change on natural populations and in monitoring aquatic production.

5. Conclusions

This study provides novel information on the DNA methylation dynamics of a suite of 18 genes including steroidogenic enzymes, transcription and growth factors, and sex steroid receptors during zebrafish gonadal development. In several cases, they represent the first measurement of the DNA methylation of these genes in fish gonads. We show sex-

specific differences in 15 of these genes as well as the importance of the genetic background and the influence of the environment in shaping DNA methylation and gene expression relationships, which are not always inverse. Interestingly, elevated temperature affected DNA methylation of genes related to sex (*dmrt1* and *dmrt3a*) and the steroidogenic pathway (*hsd11b2* and *hsd17b1*) more in males than in females. We show that analysis of the methylation levels of *cyp19a1a* alone in a DNA sample is capable of correctly identifying the sex with close to 90% accuracy, reinforcing the idea that sex identification in other species with a similar approach is feasible. Further, we show that analysis of the DNA methylation levels of *amh* and *foxl2a* are sufficient to tell whether fish had been exposed to suboptimal environmental conditions (temperature in this case) in absence of conspicuous morphological alterations of the reproductive tissues. Thus, epigenetic biomarkers can be used not only for the identification of key phenotypic traits but also to infer past environmental conditions.

Acknowledgments

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Competing interests

The authors declare that they have no competing interests.

Author contributions

AV performed the temperature experiment, the data analysis, interpreted results and wrote the article. DA performed data analysis and wrote the article. LR conceived the study and wrote the article. FP conceived the study, provided reagents, interpreted results and wrote the article. All authors read and approved the final manuscript.

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Supplementary Figures

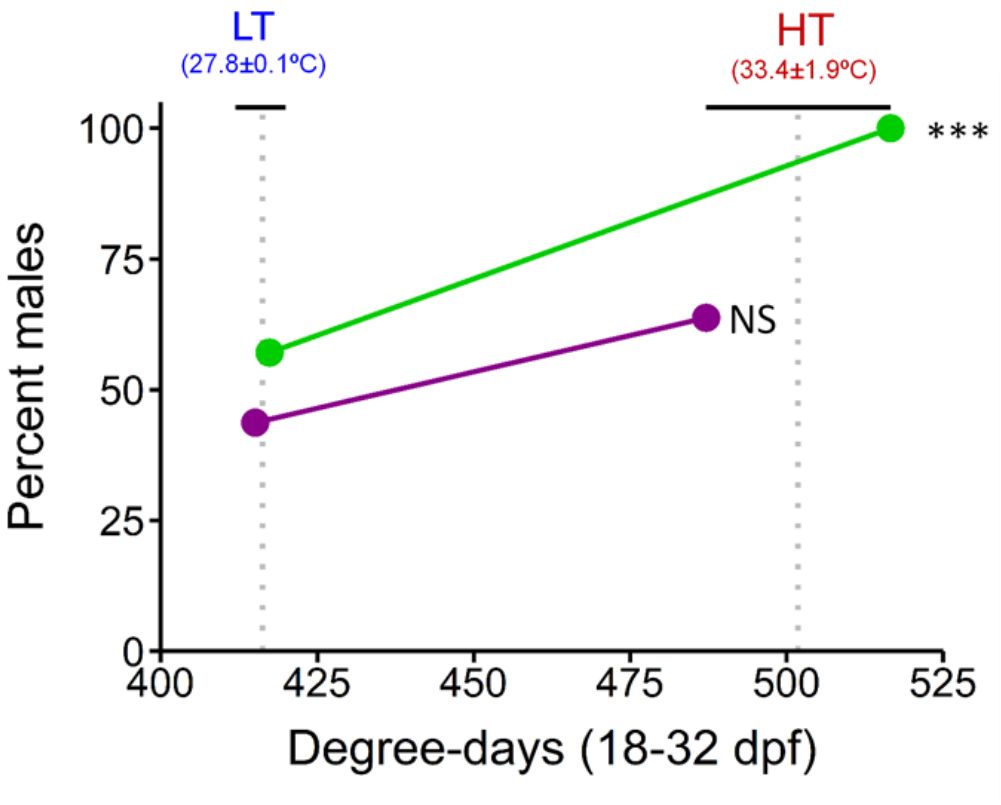

Figure S1. Percent males as a function of temperature (expressed as accumulated degree-days) during the sex differentiation period (18–32 days post fertilization, dpf) in family 1 (green) and 2 (purple). LT, low temperature; HT, high temperature. Numbers below the temperature abbreviations correspond to average experienced temperatures by the two families (mean ± s.d.). Sex ratio was analyzed by the χ^2 test. *** = $P < 0.001$. NS, not significant.

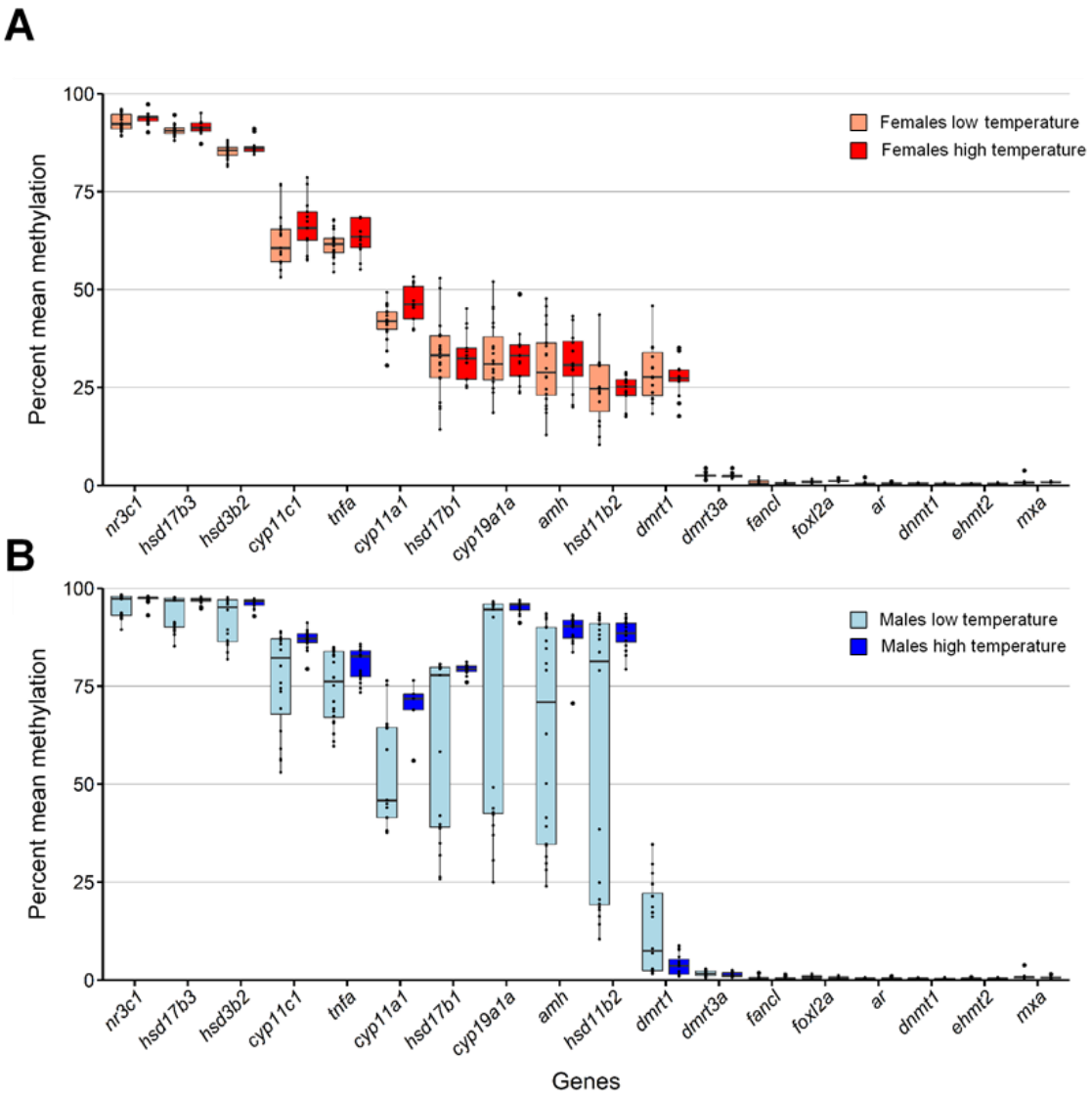

Figure S2. DNA methylation levels per gene in (A) females and (B) males exposed to control (low) and high temperature. The boxes include the values distributed between the lower and upper quartiles. The black dots inside the boxplots correspond to the samples and the black line inside the median.

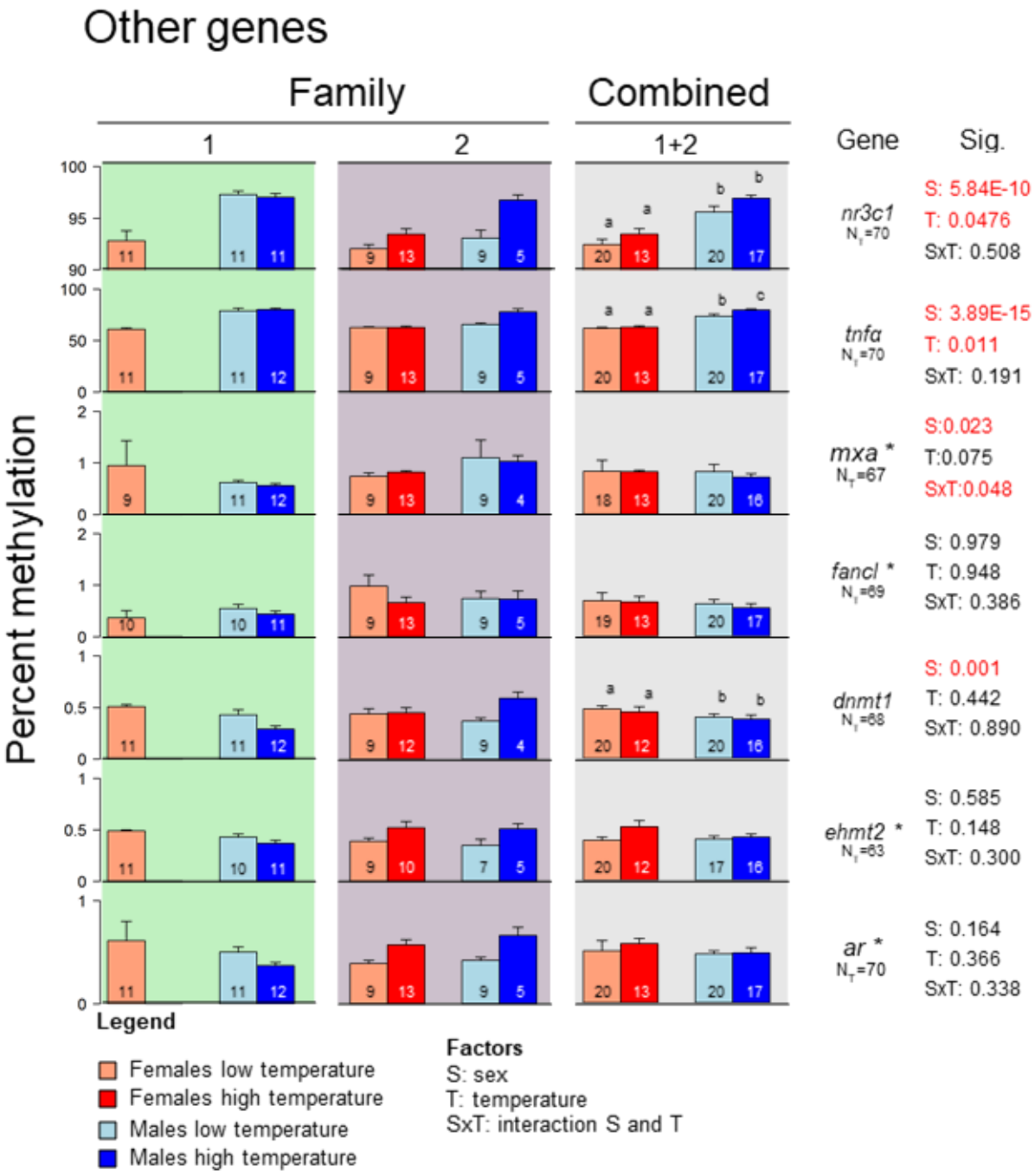

Figure S3. Percent DNA methylation levels of the promoter regions of genes in the offspring of family 1 (green background), family 2 (purple background) and the two families combined (grey background). The offspring of each family was divided in groups according to sex and the temperature treatment experienced during the sex differentiation period. The far-right data indicate *P* values for the effects of sex (S), temperature (T) or the interaction of both factors (SxT). The numbers of fish analyzed in each group are shown inside the bars. Two-way ANOVA followed by post hoc Tukey test was applied Different letters indicate significant ($P < 0.05$) differences between groups. Genes with asterisk, a robust no parametric two-way ANOVA with trimmed means was applied. Data shown as mean ± s.e.m.

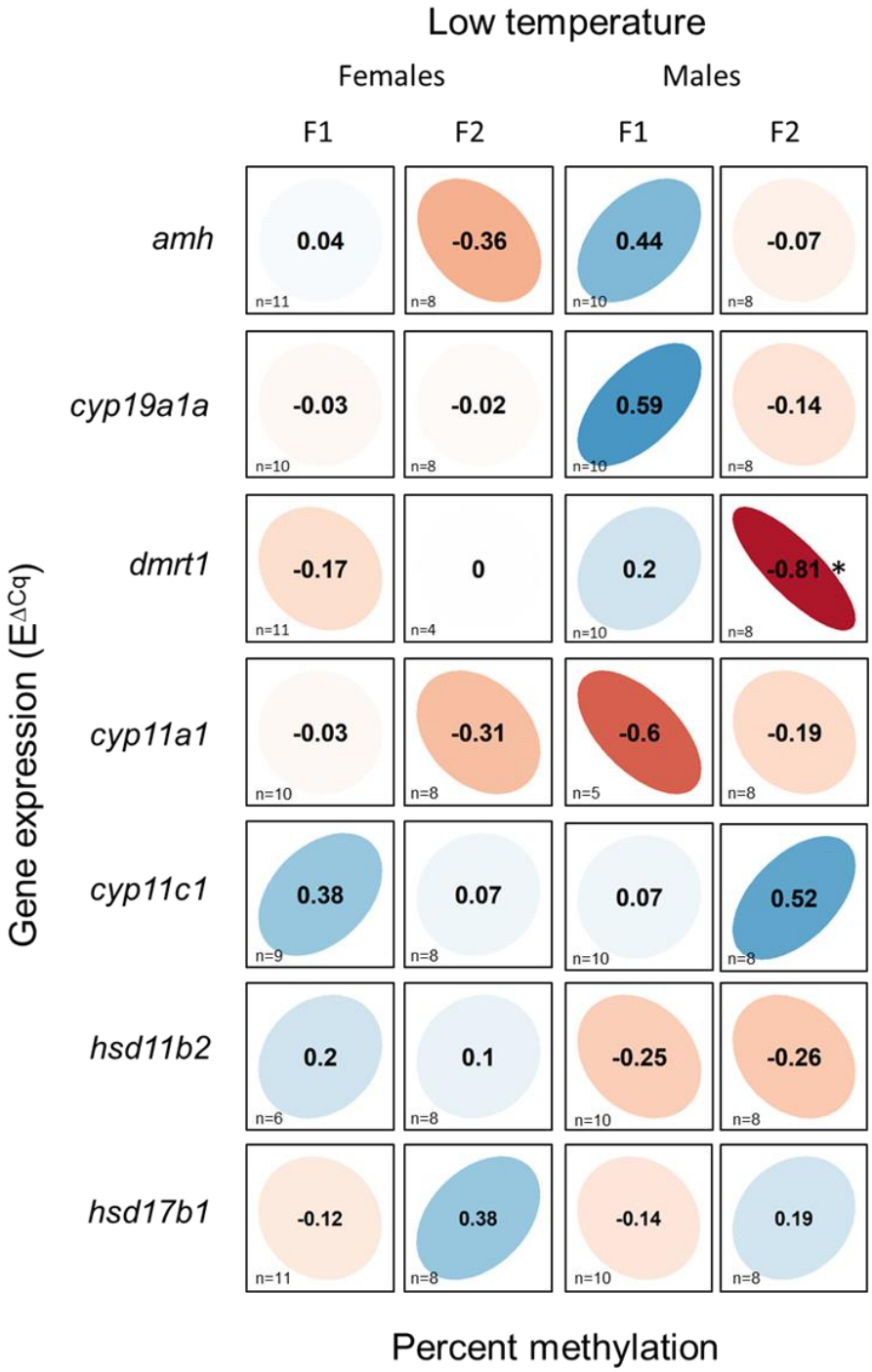

Figure S4. Correlations of gene expression and mean DNA methylation of the promoter regions of *amh*, *cyp19a1a*, *dmrt1*, *cyp11a1*, *cyp11c1*, *hsd11b2* and *hsd17b1* genes in the gonads of female and male offspring from family 1 (F1) and 2 (F2) at control temperature (LT). Correlations are shown by Spearman's rank correlation coefficient (ρ). The direction of the long axis of the ellipses and the color indicate the type of correlation: negative is shown in red and positive is in blue shade. The short axis of the ellipse and the intensity of the color are proportional to the correlation coefficients. Significant differences are considered when * = $P < 0.05$.

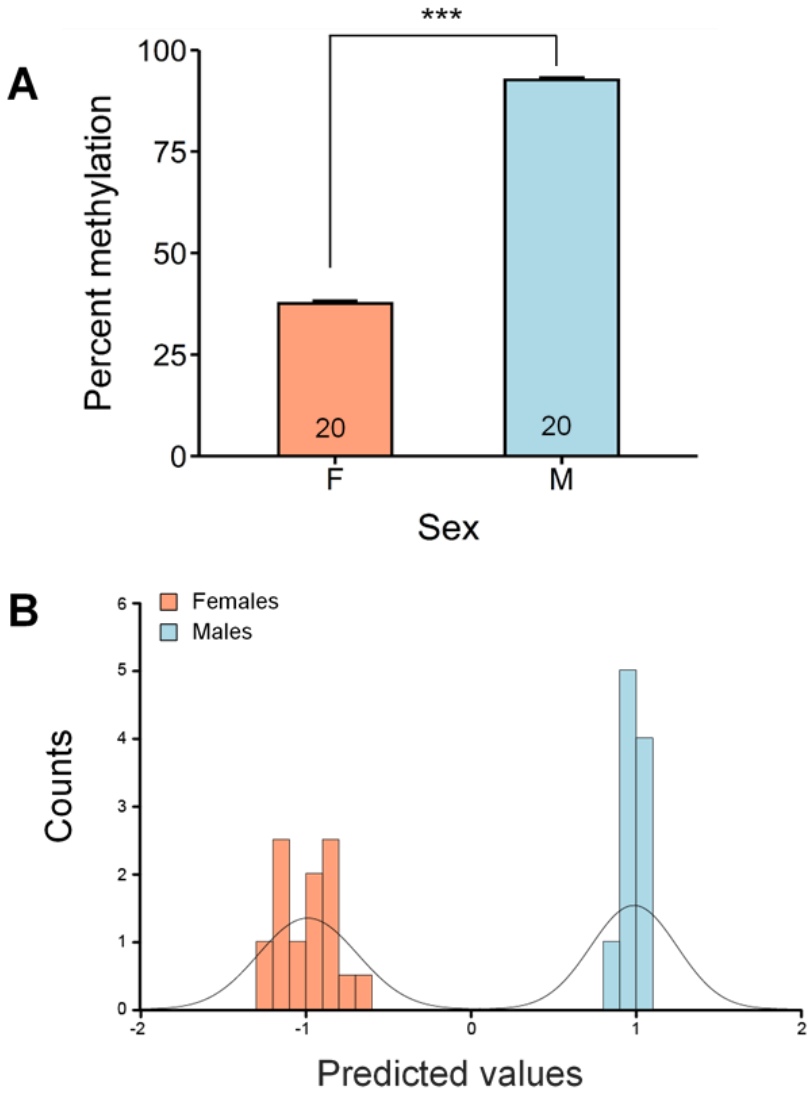

Figure S5. (**A**) DNA methylation levels of the promoter regions of *cyp19a1a* gene according to sex female (F) and male (M) in the offspring of family 3 (used for cross-validation) raised at 28°C. The numbers of fish analyzed in each group are shown inside the bars. A Kruskal-Wallis test was applied (*** $P < 0.001$). Data is shown as mean ± s.e.m. (**B**) Histogram of the 40 samples (20 males and 20 females) from family 3 classified using the training test from machine learning (Flexible Discriminant Analysis) using the methylation levels of the 15 CpGs located in the *cyp19a1a* amplicon.

Supplementary Tables

Table S1. Information of samples used in this study. Number of fish per family, number of fish per treatment (control temperature, LT; high temperature, HT), fish sex (males, M; females, F) and number of samples selected for DNA methylation analysis

Family	Treatment	Sex	Number of fish	Number of samples for analysis
1	LT	M	39	11
		F	29	11
	HT	M	77	12
		F	0	0
2	LT	M	17	12
		F	22	10
	HT	M	32	5
		F	18	13
Total			**234**	**74**

Table S2. Information of the genes selected for this study. Gene name, symbol, detailed sequences for the bisulfite primers and information related to the amplicon: positions in the genome, number of CpG sites, CpG island and annealing temperature

Gene name	anti-Mullerian hormone	androgen receptor	double-sex and mab-3 related transcription factor 1
Gene symbol	*amh*	*ar*	*dmrt1*
Reference Sequence	NM_001007779	NM_001083123	NM_205628
Chromosome	22	5	5
Strand	−	+	+
Forward primer (5' to 3')	TTTTATTTTATTAAAATTTAAGGTATGTGA	TGTAAGTATGATTGGGATTGAATAAAGT	AATATTTTTTATTGTGGTGTTTGTT
Reverse primer (5' to 3')	AACACTCCTCCCAAAATATAAAAAC	TAAAACTAAAAATATCCAAACTCTC	CAAAATAACCTCTCATAACCTTATC
Start coordinate amplicon	20736331	34961276	44344542
End coordinate amplicon	20736847	34961685	44345024
Amplicon size bp and relative position from TSS	517 (-217, +300)	410 (-274, +136)	483 (-249, +234)
Total number of CpG	18	34	24
Number of CpG before TSS	8	21	10
Number of CpG island	1	2	2
Temperature annealing ([Tº A])	51	53	53

Table S2 (Continuation**)**

Gene name	double-sex and mab-3 related transcription factor 3a	fanconi anemia, complementation group L	forkhead box L2a
Gene symbol	*dmrt3a*	*fancl*	*foxl2a*
Reference Sequence	NM_001005779	NM_212982	NM_001045252
Chromosome	5	13	15
Strand	+	+	+
Forward primer (5' to 3')	TTTTGTAATTTTTATGTTGTAAATAAGTTA	TGATTGATTTTAAATTTTTTATTTTT	TTTATAAATAGGTTTAATTTATAAAAA
Reverse primer (5' to 3')	CACACTTAAAAATCCTCTAAAAAAAC	AAACTATCTCTCATCAACAAACAACTC	ACTATTCTACCATCCCTTCTTATTCTTCT
Start coordinate amplicon	44400282	26573169	7060278
End coordinate amplicon	44400732	26573677	7060715
Amplicon size bp and relative position from TSS	451 (-366, +85)	509 (-480, +29)	438 (-159, +279)
Total number of CpG	18	18	26
Number of CpG before TSS	12	18	5
Number of CpG island	2	1	2
Temperature annealing ([Tº A])	53	50	50

Table S2 (Continuation**)**

Gene name	cytochrome P450, family 19, subfamily A, polypeptide 1a	hydroxysteroid (11-β) dehydrogenase 2	hydroxysteroid (17-β) dehydrogenase 1
Gene symbol	*cyp19a1a*	*hsd11β2*	*hsd17β1*
Reference Sequence	NM_131154	NM_212720	NM_205584
Chromosome	18	7	3
Strand	–	–	–
Forward primer (5' to 3')	TATTTTTTGTTTGTTTGTAGGTTTGAT	GTAAAGTTTTGGTTATTGGGTTTTG	ATTGTGATTAAAAATTTATAAGATGTATGA
Reverse primer (5' to 3')	CAACAATATTATAACTATAACTCCACAC	CAACCATACCACCACTAAAAACATA	CAATAATCATACATTAAACACAAAATAATA
Start coordinate amplicon	39655109	34854510	17897321
End coordinate amplicon	39655578	34854994	17897746
Amplicon size bp and relative position from TSS	470 (-326, +144)	485 (-362, +123)	426 (-394, +30)
Total number of CpG	15	9	6
Number of CpG before TSS	9	5	6
Number of CpG island	1	0	0
Temperature annealing ([Tº A])	53	59	53

Table S2 (Continuation**)**

Gene name	hydroxysteroid (17-β) dehydrogenase 3	hydroxy-Δ-5-steroid dehydrogenase, 3 β-and steroid delta-isomerase 2	nuclear receptor subfamily 3, group C, member 1
Gene symbol	*hsd17β3*	*hsd3β2*	*nr3c1*
Reference Sequence	NM_200364	NM_212797.1	NM_001020711
Chromosome	8	20	14
Strand	+	+	–
Forward primer (5' to 3')	TTTTGGTGGATTTATATAATAATAGG	GAATTAATTTTTTAAATGAAAATGA	TAGTTTTTGTATTTTTGTTTGTTTTTATTT
Reverse primer (5' to 3')	ACTCTATCAAACTTCTCTTAATTTCTACTA	CAAACATTAATATCTATTAAATACACACTA	TATTACAAAACCCTCTACTCCTTCC
Start coordinate amplicon	29856058	1252939	23501800
End coordinate amplicon	29856474	1253389	23502202
Amplicon size bp and relative position from TSS	417 (-370, +47)	451 (-209, +242)	403 (-107, +296)
Total number of CpG	4	10	10
Number of CpG before TSS	4	2	1
Number of CpG island	0	0	0
Temperature annealing ([T° A])	53	53	53

Table S2 (Continuation**)**

Gene name	cytochrome P450, family 11, subfamily A, polypeptide 1	cytochrome P450, family 11, subfamily C, polypeptide 1	DNA (cytosine-5)-methyltransferase 1
Gene symbol	*cyp11a1*	*cyp11c1*	*dnmt1*
Reference Sequence	NM_152953	NM_001080204	NM_131189
Chromosome	25	16	3
Strand	+	–	–
Forward primer (5' to 3')	GGGGTTAAATTTGTTATTGATATATTTTG	TTTTTTTTGATTTGTGTTTTTTTATAGTAA	AAATTTATGGGTAAATATGTATTTTTGAG
Reverse primer (5' to 3')	ATAAACATTCCTAAAACCTCCCATT	AACTCCTCCATATATTTATACATAC	AAATCTTAATAAACATTTCAAATTC
Start coordinate amplicon	22224239	54645580	54352441
End coordinate amplicon	22224672	54645995	54352912
Amplicon size bp and relative position from TSS	434 (-230, +204)	416 (-201, +215)	472 (-456, +16)
Total number of CpG	8	19	26
Number of CpG before TSS	0	2	26
Number of CpG island	0	1	1
Temperature annealing ([Tº A])	53	57	50

Table S2 (Continuation**)**

Gene name	euchromatic histone-lysine N-methyltransferase 2	myxovirus (influenza) resistance A	tumor necrosis factor α
Gene symbol	*ehmt2*	*mxa*	*tnfα*
Reference Sequence	NM_001113615	NM_182942	NM_212859
Chromosome	19	1	19
Strand	–	–	+
Forward primer (5' to 3')	AATATAATAAAAGTGTAGATATAATTAAAA	ATAAAGATAATATAGGTTAGGTGTTTTTTT	TTTGAAAGTTTTTGTTGTATATAAATTATT
Reverse primer (5' to 3')	CAATAATCATACATTAAACACAAAATAATA	TTTTTAATATATTATTCTCACCACTACCC	AATCCAACTTTCCTCCTAAACACT
Start coordinate amplicon	1356362	6886483	27434897
End coordinate amplicon	1356819	6886934	27435394
Amplicon size bp and relative position from TSS	458 (-202, +256)	452 (-232, +220)	498 (-409, 89)
Total number of CpG	18	20	7
Number of CpG before TSS	8	6	4
Number of CpG island	0	1	0
Temperature annealing ([Tº A])	53	58	53

Table S2 (Continuation**),** adapters sequences and sanger primers

Nextera Adapters sequences	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG+[forward primer]	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG+[reverse primer]
Primer for Sanger squencing	CGGCAGCGTCAGATGTGTAT	GTGGGCTCGGAGATGTGTAT

Table S3. Primer sequences used for the gene expression analysis

Gene	Gene name	Gene symbol	Reference sequence	Forward (5' to 3')	Reverse (5' to 3')	Reference
Target	anti-Mullerian hormone	*amh*	NM_001007779	ACAACCCGAAGGTCAACCCGC	GTGGCATGTTGGTCAGTTGGCTG	(Tzung et al., 2015)
	cytochrome P450, family 11, subfamily A, polypeptide 1	*cyp11a1*	NM_152953	GAGGCCTCAGGAATGTCCAC	GGTCCACGCGTCTACATTGA	(Hao et al., 2013)
	cytochrome P450, family 11, subfamily C, polypeptide 1	*cyp11c1*	NM_001080204	CCTCGGGCCCATATACAGAGA	CGTCCCGTTCTTGAGGAAGA	(Tzung et al., 2015)
	cytochrome P450, family 19, subfamily A, polypeptide 1a	*cyp19a1a*	NM_131154	GATATTTGCTCAGAGCCATGGA	GCTCTGGCCAGCTAAAACACT	(Tzung et al., 2015)
	doublesex and mab-3 related transcription factor 1	*dmrt1*	NM_205628	TGCCCAGGTGGCGTTACGG	CGGGTGATGGCGGTCCTGAG	(Tzung et al., 2015)
	hydroxysteroid (11-β) dehydrogenase 2	*hsd11b2*	NM_212720	GGGGGTCAAAGTTTCCACTA	TGGAAGAGCTCCTTGGTCTC	(Tokarz et al., 2013)
	hydroxysteroid (17-β) dehydrogenase 1	*hsd17b1*	NM_205584	GTCTGATGGGTCCTCTGGAA	TGCCGTGTCTCTTTTTCTTCA	(Ma et al., 2016c)
Reference gene	eukaryotic translation elongation factor 1 α 1 like 1	*eef1a1l1*	NM_131263	GGAGGCTGCCAACTTCAACGC	GGCGATGTGAGCAGTGTGGC	(Tang et al., 2007)
	ribosomal protein L13a	*rpl13a*	NM_212784	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	(Tang et al., 2007)

Table S4. Information of the sequencing data for each sample: quality of the raw data, number of reads before and after trimming, efficiency alignment and bisulfite conversion efficiency

Sample	Quality Scores and Base Calling Accuracy >=Q30	Total number of reads before trimming	Total number of reads after trimming	Alignment efficiency	Bisulfite conversion efficiency
128_G1_MHT	84.0%	663,270	660,193	87.4%	99.7%
128_G1_MLT	86.8%	579,689	576,949	89.1%	99.7%
128_G10_FLT	85.6%	870,587	865,560	35.4%	99.1%
128_G10_MHT	70.6%	374,875	373,317	63.2%	99.7%
128_G11_MLT	70.4%	85,423	84,977	53.7%	99.5%
128_G12_FLT	87.0%	510,934	508,119	42.9%	99.7%
128_G12_MHT	77.2%	502,960	500,941	75.3%	99.7%
128_G13_MHT	81.4%	414,473	412,616	84.6%	99.7%
128_G13_MLT	75.7%	478,134	476,238	68.1%	99.5%
128_G14_FLT	85.5%	784,384	780,630	45.6%	99.3%
128_G14_MHT	78.1%	654,094	651,414	84.0%	99.6%
128_G15_FLT	84.2%	612,595	609,742	59.6%	99.6%
128_G15_MHT	82.5%	410,448	408,414	95.1%	99.6%
128_G16_MLT	76.5%	522,846	520,469	73.8%	99.6%
128_G17_FLT	83.6%	744,496	741,491	54.7%	99.7%
128_G17_MHT	84.3%	491,794	489,523	88.5%	99.6%
128_G18_MLT	78.3%	661,699	658,812	74.2%	99.6%
128_G19_MHT	56.5%	438,897	436,493	46.1%	99.6%
128_G19_MLT	73.4%	745,698	742,215	66.3%	99.6%
128_G2_MHT	72.5%	473,882	472,157	82.4%	99.7%
128_G2_MLT	82.8%	491,815	489,408	86.3%	99.5%
128_G20_MLT	84.8%	730,424	726,889	52.4%	99.6%
128_G21_MLT	77.4%	561,900	560,018	61.6%	99.7%
128_G22_MLT	87.7%	745,466	741,832	59.0%	99.5%
128_G3_FLT	89.2%	311,504	310,089	53.0%	99.5%
128_G4_MHT	78.1%	288,904	287,760	78.5%	99.7%
128_G4_MLT	81.6%	278,121	276,585	78.2%	99.5%
128_G5_MHT	75.4%	221,927	220,856	77.9%	99.7%
128_G5_MLT	84.2%	242,394	241,227	84.5%	99.6%
128_G6_MHT	74.3%	631,565	629,024	72.5%	99.7%
128_G6_MLT	79.4%	377,090	375,559	69.7%	99.5%
128_G7_FLT	87.2%	333,887	332,213	57.7%	99.3%
128_G8_FLT	87.3%	346,344	344,728	68.3%	99.6%
128_G9_FLT	86.4%	776,875	772,537	51.7%	99.6%
204_G0_MLT	67.3%	619,509	615,936	66.3%	99.7%
204_G1_MHT	83.9%	302,069	300,544	83.4%	99.6%
204_G10_FHT	83.7%	517,175	513,880	80.3%	99.6%
204_G10_FLT	80.6%	239,459	238,005	74.6%	99.6%
204_G11_FLT	54.7%	393,647	391,249	41.1%	99.6%

#204_G11_MHT	#86.6%	#471,489	#469,075	#82.9%	#96.1%
204_G12_FHT	88.3%	236,425	235,230	82.1%	99.6%
204_G12_FLT	60.2%	712,937	708,940	53.3%	99.7%
204_G13_FLT	71.0%	818,943	815,503	60.1%	99.7%
204_G13_MHT	89.7%	232,734	231,501	83.8%	99.3%
#204_G14_FHT	#67.4%	#86	#86	#73.3%	#97.7%
204_G14_MLT	71.1%	545,839	541,353	75.0%	99.5%
204_G15_FHT	80.3%	533,056	527,861	86.0%	99.6%
204_G15_MLT	70.6%	589,836	586,707	73.1%	99.7%
204_G16_FHT	87.0%	373,490	371,253	84.4%	99.5%
204_G16_FLT	84.5%	508,395	505,826	75.5%	99.6%
204_G17_MHT	87.1%	240,551	238,992	84.0%	99.4%
204_G17_MLT	74.0%	367,667	365,510	72.0%	99.6%
204_G18_MHT	85.6%	513,416	510,763	82.9%	99.5%
204_G19_FLT	83.8%	312,899	311,175	83.9%	99.6%
204_G19_MHT	91.1%	379,215	377,245	89.4%	99.5%
204_G2_MHT	90.4%	685,302	681,900	92.3%	99.6%
204_G2_MLT	72.3%	306,695	304,636	71.1%	99.5%
204_G20_FHT	87.9%	410,936	408,532	84.9%	99.5%
204_G20_FLT	73.7%	602,202	599,546	68.9%	99.6%
204_G3_FHT	75.1%	549,351	547,091	69.0%	99.6%
204_G3_MLT	81.7%	333,823	332,216	83.7%	99.5%
204_G4_FHT	81.7%	725,085	721,156	75.9%	99.5%
204_G4_FLT	69.5%	68,151	67,667	70.1%	99.7%
204_G5_FHT	86.1%	428,220	426,024	87.4%	99.6%
204_G5_MLT	58.6%	510,120	503,954	53.0%	99.6%
204_G6_FHT	80.5%	344,182	342,546	76.1%	99.5%
204_G6_FLT	72.2%	318,457	316,750	76.2%	99.7%
204_G7_FHT	83.5%	383,269	381,043	81.4%	99.6%
#204_G7_MLT	#38.3%	#759,440	#755,330	#22.6%	#97.2%
204_G8_MHT	83.6%	719,377	715,464	78.5%	99.5%
204_G8_MLT	68.9%	166,694	165,697	63.2%	99.7%
204_G9_FHT	84.2%	277,488	276,009	78.0%	99.6%
204_G9_MLT	77.6%	258,539	256,769	73.9%	99.7%
Mean	79.3%	469,779	471,392	71.9%	99.6%
S.D.	8.08%	185,638	186,554	13.63%	11.38%
s.e.m	0.97%	22,188	22,297	1.63%	1.36%

This values no computed because did not pass the quality control

Chapter 4

Hot sex in wild zebrafish: can the natural genetic sex determination mechanism buffer environmental effects on population sex ratios?

Hot sex in wild zebrafish: can the natural genetic sex determination mechanism buffer environmental effects on population sex ratios?

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Abstract

Fish exhibit remarkable sexual plasticity and sex can be determined by genetic factors, environmental factors or a combination of both. The most studied environmental factor is temperature, which when abnormally elevated during early sensitive stages generally has a masculinizing effect. Thus, fish that under normal conditions would become females end up being males (neomales). Neomales are being discovered in wild populations of many species. Thus, understanding the genetic basis and environmental influences behind the appearance of neomales is of great interest for conservation biology in a global warning scenario. Here, we assessed the rate of sex reversal under normal and elevated temperature conditions in two wild strains of zebrafish, Nadia (NA) and EkkWill (EKW), where sex determination is controlled by a major locus in a ZZ/ZW system, (*sar4*); and in the AB laboratory strain, which lacks a Z chromosome leading to a polygenic or environmental sex determination mechanism. Results showed that, surprisingly, wild zebrafish of both the NA and EKW strains have the same susceptibility to the masculinizing effects of exposure to elevated temperature as the AB laboratory strain. The existence of genotype-by-environment interaction in sex ratio response in the natural strains suggests that factors other than *sar4* may also contribute to phenotypic sex in the

wild. Results also show the spontaneous presence of neomales at control temperature and genotypic-dependent differences in the susceptibility of neomales to elevated temperature-induced masculinization. Finally, we found that NA ZW neomales but not regular ZZ males increased sperm production when exposed to elevated temperature. Taken together, our results suggest a genetic basis underlying both the production of neomales and their response to temperature with potential functional consequences in reproductive capacity. This information is of relevance since it can help to better understand the possible consequences of global warning on natural fish populations.

Key words: sex determination, sex differentiation, sex reversal, neomales, global warning

1. Introduction

Zebrafish (*Danio rerio*) is a gonochoristic member of the Cyprinidae family and is native to South Asia (mainly India and Bangladesh). Its natural habitat includes rivers and freshwater streams subjected to monsoon seasons and survives from sea level into the mountains. Thus, zebrafish is adapted to survive in a broad range of environmental conditions: temperature oscillations (16.5°C–38.6°C), different pH values (5.5–9) and variable amounts of dissolved salts that result in different water conductivities (10 to >1000 µS) (McClure et al., 2006; Spence et al., 2006; Engeszer et al., 2007; Arunachalam et al., 2013). Further, due to its biological attributes —including small size, short generation cycle life, ease of maintenance in captivity, together with many available genomic resources (Howe et al., 2013)— zebrafish is now a well-established model organism (Khan and Alhewairini, 2018). Thus, zebrafish is widely used as a model in several research areas, including developmental biology (Lele and Krone, 1996), human diseases (Dooley and Zon, 2000), tissue regeneration (Gemberling et al., 2013), toxicology (Tanguay, 2018) and aquaculture (Ribas and Piferrer, 2014). Although many laboratories around the world rear zebrafish, its maintenance still presents some drawbacks (Aleström et al., 2019), a major one being that different strains often exhibit highly-skewed sex ratios (Lawrence et al., 2008). This problem complicates experiments requiring the mating of specific rare genotypes (Postlethwait and Braasch, 2020).

Sex determination in zebrafish has been a subject of interest for some time (Schreeb et al., 1993; Amores and Postlethwait, 1998; Traut and Winking, 2001; Wallace and Wallace, 2003). In wild populations, a "sex-associated region" (*sar4*) can be found in the telomeric part of chromosome 4 (Wilson et al., 2014). This region is compatible with a ZW/ZZ system, in which at least one W chromosome is necessary for female sex differentiation, although its presence does not always guarantee ovarian development (Tong et al., 2010; Wilson et al., 2014). In contrast, due to inbreeding during the process of domestication, laboratory strains have lost the genetic sex determination mechanism embedded in the *sar4* locus (Wilson et al., 2014). Consequently, once devoid of the main genetic sex-determinants, laboratory strains accrue a still unidentified number of interplaying autosomal loci responsible for polygenic sex determination (PSD) (Liew et al., 2012).

Gonadal sex differentiation in zebrafish laboratory strains (AB and TU) involves genetic, epigenetic, hormonal and environmental influences (Nagabhushana and Mishra, 2016). Several genes involved in zebrafish testis and ovarian differentiation have been described (Rodríguez-Marí et al., 2005; von Hofsten and Olsson, 2005; Wang et al., 2007a; Siegfried and Nüsslein-Volhard, 2008; Orban et al., 2009; Webster et al., 2017). Because of PSD, sex ratios in laboratory strains can easily be altered by environmental factors such as elevated population density (Ribas et al., 2017c; b), temperature (Uchida et al., 2004; Abozaid et al., 2011; 2012; Ribas et al., 2017a; Hosseini et al., 2019), and hypoxia (Shang et al., 2006) when fish are exposed during early stages of development. Inbreeding

could also contribute to skewed sex ratios in laboratory strains (Lawrence et al., 2008).

Sex can be regarded as a quantitative trait with continuous variation based on the combined effects of genetic, environmental and random influences that is resolved in a binary fashion directing the sexual phenotype to become either male or female (Beukeboom and Perrin, 2014; Perrin, 2016). Thus, combining genotypic and environmental variation and a lability trait, for example, how some enzyme might respond to temperature when a specific threshold is crossed, might affect the resulting phenotypic or gonadal sex (Baye et al., 2011). In fish, this phenomenon has been observed with elevated temperatures in gonochoristic species with genetic sex determination (GSD) harboring sex chromosomes as in, for example, medaka XY/XX (Matsuda et al., 1998) and the half-smooth tongue sole (*Cynoglossus semilaevis*) ZZ/ZW (Shao et al., 2014), but also in PSD species such as the European sea bass (*Dicentrarchus labrax*) (Saillant et al., 2002). The substantial increase in the number of males due to stressful conditions (Adolfi et al., 2019), regardless of the environmental stressor, implies that some fish that would otherwise develop as females finally end up as males (Goikoetxea et al., 2017).

Sex-reversed females that develop into males are commonly referred to as "*neomales*" or "*pseudomales*" (Nagahama, 2005; Baroiller and D'Cotta, 2016). Neomales have also been observed in reptiles (Quinn et al., 2007) and amphibians (Wallace et al., 1999). The mismatch between the genotypic and phenotypic sex can be distinguished in GSD species, either with a XY/XX or a ZW/ZZ system, if genetic markers capable of identifying genotypic sex are available (Shinomiya, 1999; Shinomiya et al., 2004; Martínez et al., 2009; Zhang et al., 2019). However, the detection of neomales with PSD or environmental sex determination (ESD) systems is challenging because markers of genotypic sex are difficult or essentially impossible to obtain, respectively.

A higher number of males in wild zebrafish populations (unspecified strain) exposed to elevated temperature has also been observed (Brown et al., 2015), suggesting that despite the possession of an intact sex determining region, these wild fish are also susceptible to masculinization by high temperature. A rigorous study on the response of different sexual genotypes to elevated temperature, however, has not been carried out in the zebrafish, not in natural strains containing the *sar4* nor in laboratory strains devoid of *sar4*. Recently, the presence of male-biased sex ratios and, in some cases, the identification of neomales has been reported in wild populations of different fish species including Nile tilapia (*Oreochromis niloticus*) (Bezault et al., 2007) and medaka (*Oryzias latipes*) (Shinomiya et al., 2010), both with a XX/XY sex determination system. This finding has been linked to exposure to abnormally high temperature (Sato et al., 2005; Azaza et al., 2008). The underlying mechanisms behind the spontaneous appearance of neomales, however, are far from clear. Thus, understanding the genetic basis and environmental influences behind the appearance of neomales is of great interest for conservation biology in a global warning scenario.

Here, we characterized the sex ratio response to elevated temperature in different families of two different wild strains: Nadia (NA) and EkkWill (EKW), which have the *sar4* sex locus, and compared to the common laboratory (AB) strain, which lacks *sar4*. Under one hypothesis, the genetic sex determination mechanism of *sar4* should buffer natural strains from the effect of high temperature, making natural strains much less sensitive to environmental sex change than the domesticated strain. An alternative hypothesis, however, would suggest that different sex ratios in different natural environments might be advantageous for ecological reasons. Furthermore, if there is a dominant female sex determinant, even if not totally penetrant (Wilson et al., 2014), it can be also hypothesized that WW fish should be more resistant to the effects of elevated temperature than ZW fish. In this study, we used previously validated (NA) or developed new (EKW) primers linked to *sar4*, which allowed the identification of the genotypic sex. Importantly, these primers not only enabled distinguishing neomales from genetic males for the first time, but also allowed a careful characterization of the reaction norms of the sex ratio response to temperature of different and novel sexual genotypes not reported previously. Finally, we also evaluated the effects of temperature on gonadal morphology to study differences at the cellular level between wild and laboratory strains, comparing mature gonads of males, neomales and females.

2. Materials and Methods

Animal care and husbandry

Zebrafish were housed in the Aquatic Animal Care Services Zebrafish Facility at the University of Oregon under standard conditions (Westerfield, 1995). The recirculating closed water system has been previously described in detail in Mason et al. (2016). Water conditions were: pH = 7.6, alkalinity = 30–40 mg/L, temperature = 28±0.5°C, conductivity = 750 μS/cm^2, ammonia = 0 mg/L, nitrites = 0 mg/L and nitrates < 30 mg/L. The photoperiod was set at 14 h light/10 h darkness. The feeding regime was according to the development stages of zebrafish: from 4–10 days post fertilization (dpf) larvae were fed with rotifers (*Brachionus plicatilis*, L-type Reed Mariculture, USA) four times per day *ad libitum*. From 10–21 dpf, larvae were fed with a mix of rotifers and brine shrimp nauplii (*Artemia franciscana*, Artemia International, Texas, USA), and from 21–60 dpf with a mix of brine shrimp nauplii with dry crumble feed (55.0% crude protein, 15% crude fat, 1.5% crude fiber, vitamins A, B12, C, D3 and E; Zeigler Adult Zebrafish Irradiated Diet, USA) twice per day. From 60–90 dpf, fish were fed only with dry crumble twice per day. The experimental procedures were approved by the University of Oregon Institutional Animal Care and Use Committee (IACUC protocol # 15–19).

Zebrafish strains and the family crosses

Two wild strains, Nadia (ZDB-GENO-030115-2, NA) and EkkWill (ZDB-GENO-990520-2, EKW), and the laboratory strain AB (ZDB-GENO-960809-7 were used in this study. Embryos were obtained by natural spawning (one male and one female mature fish). At least three different families originating from different pairs of parents were created for each strain. For each family, at 4 dpf batches of 20 larvae each were distributed into 1-liter weaning tanks (Thoren Aquatics) with a total of six tanks per family. At 10 dpf, larvae were transferred to 3.5-liter tanks (Tecniplast) and were reared until 18 dpf under the environmental conditions described above.

Temperature treatment and sampling procedures

At 18 dpf, fish were counted and the number of alive fish in each tank according to strain and family was taken as the initial number of fish used for the thermal treatments (**Table S1**). No fish replacement took place. The thermal treatment was carried out according to Ribas et al. (2017a). The six tanks of each family were randomly assigned to two groups with three tanks (technical replicates) per group. One group, consisting of three of the six 3.5-liter tanks, was always reared at 28°C (control temperature). In the other group, consisting of the remaining three tanks, starting at 18 dpf, temperature was gradually increased at 0.5°C every 6 h until it reached 36°C at 22 dpf and was maintained at this temperature until 30 dpf. Tanks making up the 36°C-group were placed into an isolated stand-alone rack (Tecniplast, USA) to ensure proper temperature maintenance. At 30 dpf, temperature was gradually decreased at 0.5°C every 6 h until it reached 28°C at 34 dpf. Then, the 36°C-tanks were placed next to the 28°C-tanks in the same recirculating system facility at 28°C. Because all the three NA families fish exposed to 36°C resulted in 100% males and thus no females were available for further analysis, the experiment was completely repeated using three new NA families, which were exposed to 34°C instead of 36°C under the same schedule of temperature changes.

Larval survival was recorded from 4–18 dpf (before the thermal treatment) and from 18–34 dpf (during the thermal treatment). Survival was calculated by dividing the number of alive fish at the end of each period by the number of alive fish at the beginning of that period and expressed as a percentage. Fish were euthanized by hypothermic shock at 90 dpf. A total of 1,217 fish were sampled (**Table S1**). At sampling, a finclip was cut and kept in 70% ethanol until further analysis. Phenotypic sex was visually determined by examining the gonads with the aid of a dissecting scope. For histological analysis, five males and five females were randomly selected from each tank and a cross-section of the body trunk was fixed in Bouin's solution (HT10132, Sigma-Aldrich) for 72 hours at room temperature, washed and stored in 70% ethanol until analysis.

Sex genotyping the NA and EKW fish

DNA was isolated using the DNeasy Blood & Tissue kit (69506, Qiagen, Hilden, Germany). Primers for sex genotyping the NA and EKW fish were designed sex-linked loci from the zebrafish sequence RAD-tags under accession no. SRP044635 (Wilson et al., 2014). Because the AB strain does not contain male *vs.* female versions of *sar4*, sex genotyping was not possible in fish from this strain.

The sequences of sex genotyping primers for NA fish appear in **Table S2**. In each sample, 2 µl of DNA was mixed with 0.25 µl of GoTaq Flexi DNA polymerase (M8298, Promega, Madison, Wisconsin, USA), 10 µl Turbo Master Mix 2X (9PIM750, Promega), 0.6 µl F/R primers (10 µM) and 10 µl water. The PCR was carried out in a thermocycler with the following conditions: one cycle of 94°C for 6 min, 45 cycles of 94°C for 25 s, 61°C for 25 s, 72°C for 30 s and a final step of 72°C for 10 min. After the PCR, a restriction endonuclease reaction was carried out by adding 1 µl of CviQI enzyme (R0639L, New England Biolabs, Massachusetts, USA), 4 µl 1X NEBuffer 3.1 (B7203S, New England Biolabs) and 16 µl water, followed with an incubation for 2 h at 25°C. The NA primers amplified a region of 119 nt length and this differed in one SNP: G in the Z chromosome and A in the W chromosome (**Figure S1A**). The CviQI enzyme recognizes the sequence (5'-GTAC-3') present in the Z chromosome and cut the fragment in two parts (35 and 84 nt in length), whereas the amplified fragment from the W chromosome remained intact (**Figure S1A**). Bands were visualized in a 2.5% agarose gel (**Figure S1B**). NA primers were available at the start of the experiment and thus they could be used not only to sex genotype the offspring but also the parents. In the NA strain, all the mattings were genetic males (ZZ) and females (ZW) previously genotyped.

To sex genotype EKW fish, two primer pairs (P_1 and P_2) were designed and applied to each sample (**Table S2**). These EKW primers became only available after the experiment had started and thus it was not possible to select the genotype of the parents as we did for the NA fish. For EKW samples, the reactions and the volumes for the PCR were the same as described above for NA samples. Thermocycler conditions were: one cycle of 94°C for 6 min, 45 cycles of 94°C for 25 s, 59°C for 25 s, 72°C for 30 s and a final step of 72°C for 10 min. Bands were visualized in a 3% agarose gel. The combination of primer pairs P_1 and P_2 together allowed us to identify two different SNPs present in the W chromosome (W_1 and W_2) (**Figure S2A**) and based on the specific pattern of the bands, we could distinguish the different genotypes (**Figure S2B**). To validate the EKW primers, first we checked how many possible different genotypes a male fish theoretically could have: ZZ (regular male) and ZW_1, ZW_2, W_1W_1, W_1W_2, and W_2W_2 (different types of neomales, because WW males had been found before) (Wilson et al., 2014). Likewise, females could have the following genotypes: ZW_1, ZW_2, W_1W_1, W_1W_2 and W_2W_2. A total of 30 possible combinations were obtained (**Table S3**). Expected genotypic frequencies were calculated for each cross and linked to each genotype with the bands resulting from the combination of primer pairs P_1 and P_2, thus obtaining a unique banding pattern associated

with offspring frequencies (**Figure S3**). We genotyped the offspring of each EKW family and we compared observed against expected frequencies of the 20 unique possible outcomes based on the genotype of the parents. In this way, the parental genotypes were thus inferred.

Histomorphometrical analysis

To examine the differences in the abundance of the different germ cell types between the gonads of fish exposed to low and high temperature, we selected AB and NA samples from fish exposed to either 28 or 36°C from different families. In total, 57 fish were histologically analyzed (**Table S4**). Samples were dehydrated through a graded series of ethanol and embedded in paraffin. To cover the whole gonad, between 25 and 30 evenly spaced cross sections (7 µm) per slide with a total of 7–8 slides per fish were obtained using a Leica Reichert-Jung 2040 microtome. After rehydration, sections were stained with hematoxylin and eosin (H&E) and mounted with DPX. We used the mid part of the gonad (slides 3 and 4) for analysis. In each sample, two photomicrographs of non-contiguous cross-sections were taken per slide using a Leica DFC310 FX microscope fitted with a Leica DFC425C camera and controlled by the Leica Application Suite software (v. 3.8.0). To analyze the images of the gonadal cross sections, the ImageJ software (v. 1.8.0.112) (Schneider et al., 2012) with Grid plugging (46 x 33 = 1518 intersections or points on the histological field) were used for counting the proportion occupied by different germ cell types. For each sample, four histological fields were counted, totalizing 6072 intersections per animal. The final value (mean ± s.e.m) was obtained from the four histological fields of each sample. In testes, we calculated the proportion of germ cells at different stages of development according to Leal et al. (2009a): type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (B), spermatocytes (Spc), spermatids (Spd), and spermatozoa (Spz). The relative number of spermatozoa in each sample was estimated as described by Fallah et al. (2019) using the histological images (40x objective) aligned with the ImageJ optimized parameters (magnification, 8-bit image type). In this analysis, background subtraction, threshold adjustment, and watershed separation of particles resulted in a black and white picture of the highlighted spermatozoa were used to count the relative number of spermatozoa in the control and treatment groups. In ovaries, we calculated the proportion of different stages of oocyte development according to Brown-Peterson et al. (2011): primary growth oocyte (PG), cortical alveolar oocyte (CA) and vitellogenic oocyte (Vtg). We also recorded the proportion of postovulatory follicles (Pofs), which indicates recent spawning, and the presence of degenerating oocytes (atretic oocytes - A).

Statistical analysis

Statistical analyses were carried out using R software (v. 3.0.2) (Team, 2013). Significant results were considered when $P < 0.05$. All graphs were created using the "*ggplot2*"

package (v. 3.1.0) (Wickham, 2016). Global sex ratios per each strain were analyzed by fitting a generalized linear mixed model (GLMM, binomial error distribution, logit link function) using the "*lme4*" package (v. 1.1–2.1) (Bates et al., 2014). In the GLMM the temperature was considered as fixed factor and the family and tank as random effects with the replicates included. For survival analysis, the Kruskal-Wallis test was applied followed with a Bonferroni test to compare survival rates between strains during the period 4–18. To determine the differences of sex ratio between temperature treatments in each family and also for the differences in genotypic frequencies in NA and EWK families the χ^2 test (Fisher's exact) was used (McHugh, 2013). For each strain and group, the Student's *t*-test was applied to compare the proportion of germ cell types and the relative number of spermatozoa between treatments for each sex separately.

3. Results

Survival

Survival from 4 to 18 dpf (mean ± s.e.m) was 69.7±7.0%, 93.9±3.2% and 88.6±2.0% in the AB, NA and EKW strains, respectively. We found significant differences in survival rates between the wild strains and the AB strain of zebrafish during 4 to 18 dpf, whereas during the heat treatments between 18 and 34 dpf, it was nearly 99% in all strains, showing no effect of high temperature on genotype-specific lethality (**Table S1**).

Sex ratio response to temperature in wild *vs*. laboratory strains

The sex ratio had a genetic and an environmental component in all tested strains because it varied across families at the control temperature; the number of males increased with elevated temperature in all but one of the 12 families used in this study (**Table 1**). The percent males ranged from 26.0 to 62.9% at 28°C in the three families of the AB strain, but increased to 76.2% and 91.9% in a significant manner only in families 2 and 3 (χ^2 = 21.0 and 11.4, respectively; $P < 0.001$ in both cases) at 36°C (**Figure 1A**). The overall sex ratio summing the three AB families increased males from 49.5% at 28°C to 85% at 36°C (χ^2 = 35.4; $P < 0.001$) (**Figure 1B**). We conclude that elevated temperature masculinizes AB strain fish, confirming prior studies (Uchida et al., 2004; Abozaid et al., 2012; Ribas et al., 2017a).

Even at 28°C, the NA strain sex ratio was male-skewed with relatively low variation (68.5–89.5% males (**Figure 2A**). Of the three families exposed to 34°C, only family 5 significantly (χ^2 = 7.72; $P < 0.01$) increased the proportion of males over controls. All three families exposed to 36°C, however, were fully masculinized (χ^2 = 14.7; 7.7 and 15.9; $P < 0.001$; 0.01 and 0.001, respectively) (**Figure 2A**). Thus, masculinization was temperature-dependent, reaching 100% at 36°C (**Figure S4**). We conclude that elevated temperatures masculinize NA strain fish, and surprisingly, to a greater extent than the

domesticated strain AB. The three EKW families showed different frequencies of males at 28°C, all of which were consistently and significantly masculinized at 36°C (family 1, 29.6% to 75.5%; χ^2 = 25.2, P < 0.001; family 2, 43.5% to 98.1%; χ^2=34.9, P < 0.001; family 3, 70.9% to 92.2%; χ^2=6.4, P < 0.05) (**Figure S5A**). Thus, the mean sex ratio was also significantly increased by temperature in EKW as in NA fish (χ^2 = 35.4; P < 0.001) (**Figure S5B**).

Table 1. Generalized linear mixed model (GLMM) with binomial errors and a logit link function to test effects of temperature, family and replicate on the sex ratio and in each strain

Strain	Fixed effects	Temperature (°C)	Estimate	Std. Error	*P*(>|z|)
AB	Temperature	28 (Intercept)	-0.04	0.43	0.92
		36	1.92	0.33	**7.18E-09**
	Random effects	**Variance**	**s.d.**		
	Family	0.38	0.62		
	Replicate	0.05	0.23		
Nadia	Temperature	28 (Intercept)	1.40	0.30	**2.17E-06**
		34	0.78	0.31	**0.01**
	Random effects	**Variance**	**s.d.**		
	Family	0.15	0.39		
	Replicate	4.462E-10	2.11E-05		
	Temperature	28 (Intercept)	1.08	0.22	**8.10E-07**
		36	28.43	915.89	0.975
	Random effects	**Variance**	**s.d.**		
	Family	0.05	0.22		
	Replicate	0.00	0.00		
EkkWill	Temperature	28 (Intercept)	-0.08	0.43	0.86
		36	2.31	0.32	**3.97E-13**
	Random effects	**Variance**	**s.d.**		
	Family	0.47	0.69		
	Replicate	1.57E-10	1.25E-05		

Genotypic frequencies and neomales in wild strains

In any case, in NA and EKW strains no intersex fish (no ovotestis) were found, so the neomales exhibited complete sex reversal. Because primers for genotyping NA fish were already available at the start of this study, they were used for broodstock selection. Thus, only verified genotypic ZZ males and ZW females were used. We calculated genotypic frequencies in NA offspring in the 28°C, 34°C and 36°C groups. As expected, offspring genotypes were ZZ and ZW at 1:1 ratio (162:174, χ^2= 0.0025, P=0.96) in the 28°C group. The same statistical frequency was observed in fish of the 34°C (82:96, χ^2=0.01, P= 0.91) and 36°C (71:82, χ^2=0.01, P= 0.91) groups (**Figure 2B**), indicating that observed phenotypic sex ratio distortions were not due to differential mortality of specific genotypes due to temperature. The percent of neomales, however, increased significantly

with temperature, from 28.6% in the 28°C controls to 42.7% and 53.6% in the 34°C and 36°C groups, respectively (χ^2 = 15.8; P < 0.001 in 28°C *vs.* 34°C and χ^2 = 51.1; P < 0.001 in 28°C *vs.* 36°C) (**Figure 2C**).

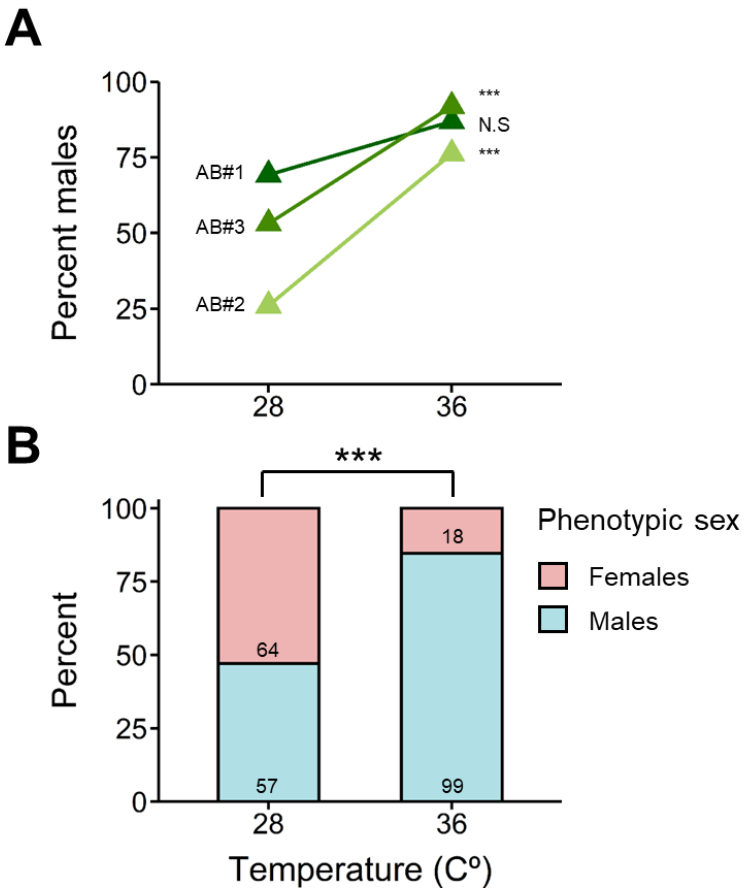

Figure 1. Sex ratio response to temperature in the AB strain when fish were exposed to high temperatures at 18–32 days post fertilization. **(A)** Data from three independent families (**B**) Mean sex ratio. Numbers indicate sample size. A χ^2 test is applied. (N.S. no significant; * = P < 0.05, **= P < 0.01 and *** = P < 0.001).

In the EKW strain, genotypes of the parents varied among the three families because we lacked sex-genotyping primers when the EKW crosses were set up. Thus, different genotypic frequencies were found in the offspring of each family (**Figure 3**), compounded with the situation that EKW has two different W chromosomes. Importantly, in all three families, the fish used as males turned out to be ZW neomales. In EKW family 1, the parental genotypes were ZW_1 male and W_2W_2 female and the genotypic frequencies in the offspring were ZW_2 and W_1W_2 in a ~1:1 ratio as expected for this type of cross (**Figure 3A**). In EKW family 2, the parental genotypes were ZW_1 male and ZW_1 female and the frequencies of the offspring were ZZ, ZW_1 and W_1W_1 in a ~1:2:1 Mendelian ratio (**Figure 3B**). In EKW family 3, the parental genotypes were ZW_1 and ZW_2 and the frequencies of the offspring were ZZ, ZW_1, ZW_2 and W_1W_2 in a ~1:1:1:1 Mendelian ratio (**Figure 3C**). None of the EKW families showed significant differences in genotypic frequencies between the 28°C and 36°C groups, again indicating the lack of differential mortality of any given genotype due to temperature.

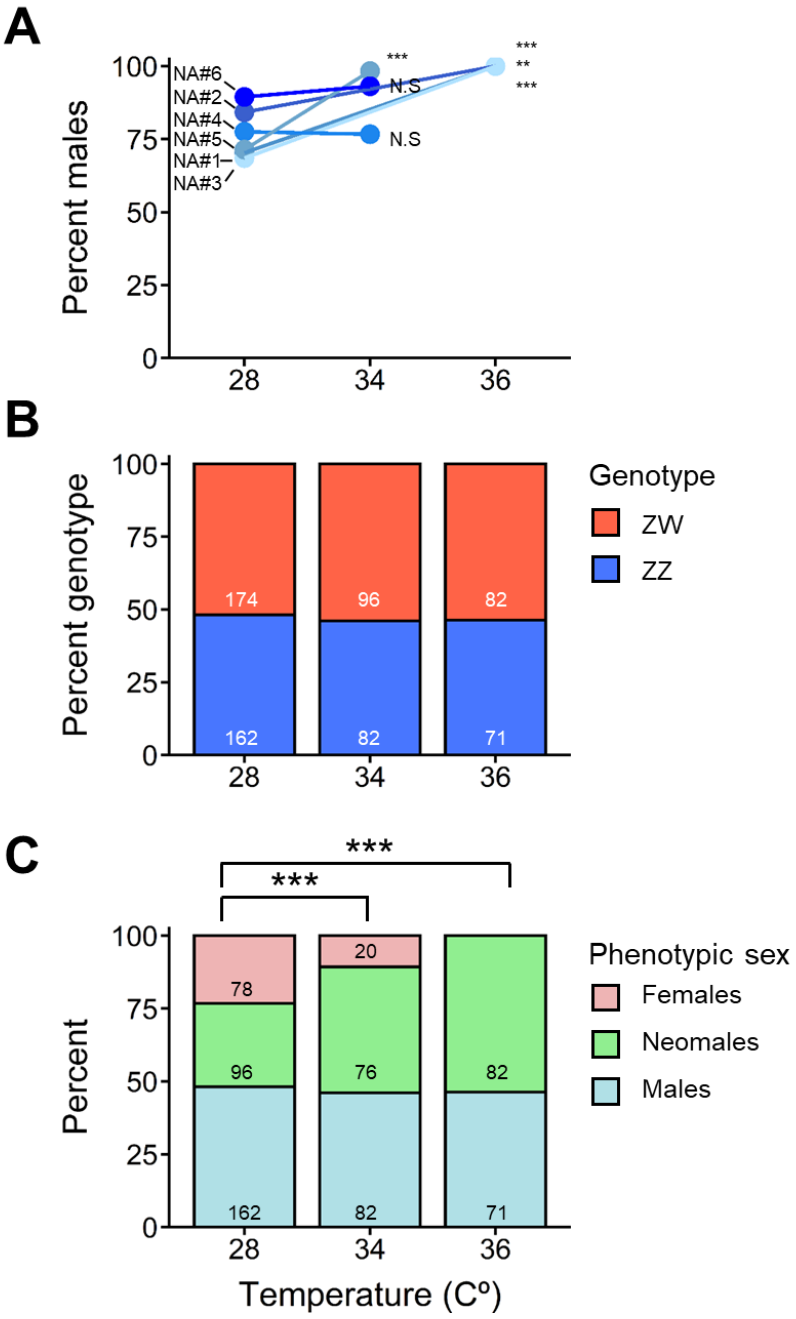

Figure 2. Sex ratio response to temperature in the Nadia (NA) strain when fish were exposed to high temperatures at 18–32 days post fertilization. (**A)** Data from six separate families (three for the 28–34°C comparison and three for the 28–36°C comparison). (**B**) Genotypes of the fish in each temperature treatment. (**C**) Mean sex ratio, distinguishing neomales (ZW) from regular males (ZZ). Numbers inside bars indicate sample size. A χ^2 test was applied (N.S. no significant; * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

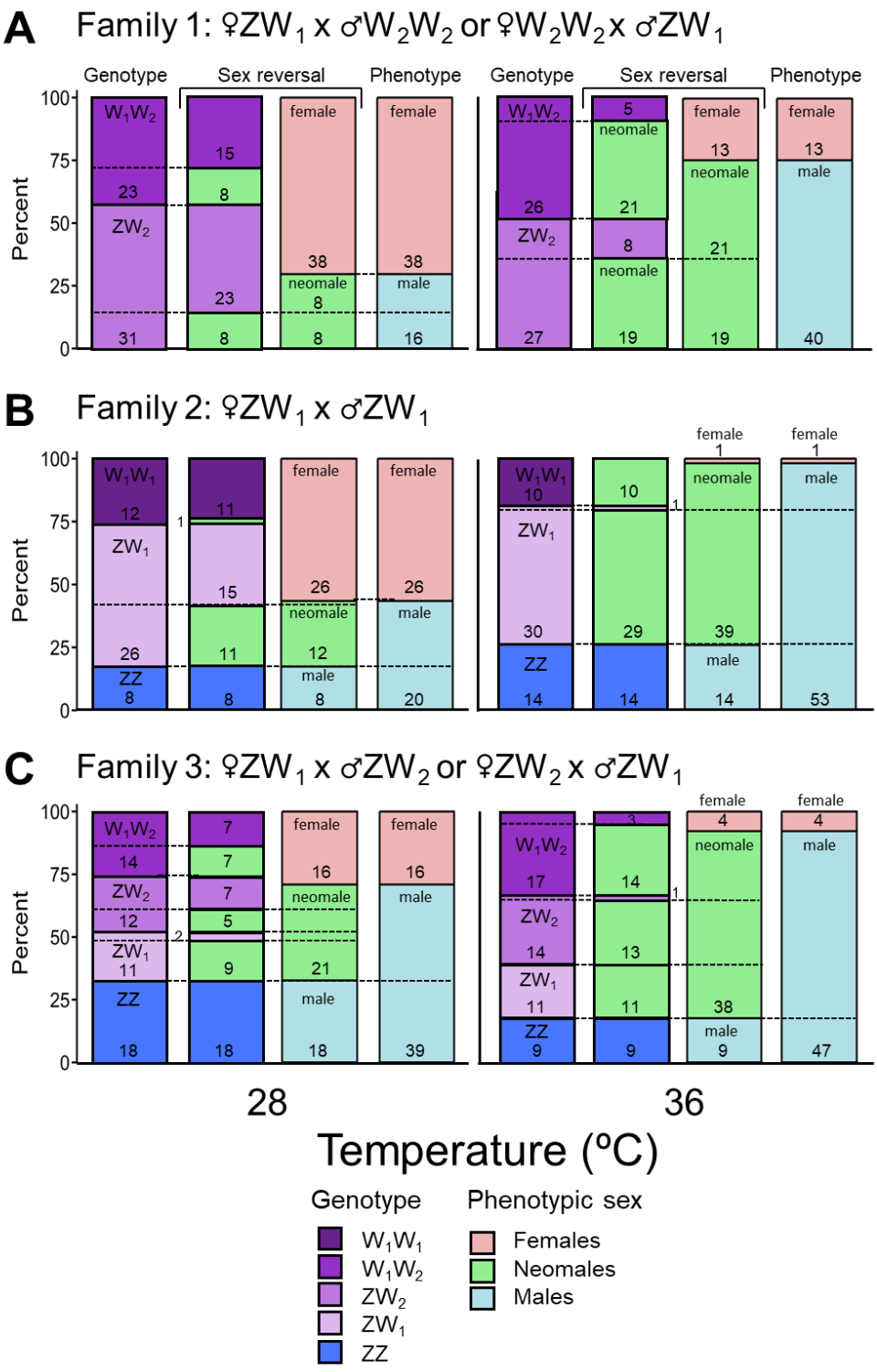

Figure 3. Genotypic sex and phenotypic sex ratio response to temperature distinguishing neomales (either ZW_X or W_XW_X, with X = 1 or 2) from regular males (ZZ) and the sex genotypes in the three (**A**, **B**, **C**) separate EkkWill (EKW) families. (**A**) Family 1. (**B**) Family 2. (**C**) Family 3. Numbers inside bars indicate sample size. For each family, the four panels on the left correspond to 28°C and the four on the right to 36°C.

Different EKW genotypes showed different rates of masculinization by elevated temperature

We quantified the number of neomales from the ZW_1, ZW_2, W_1W_1 and W_1W_2 genotypes for each family and observed that temperature increased the number of neomales in all these genotypes when compared to their respective controls (**Figure 3A-C**). In EKW family 1, ZW_2 and W_1W_2 genotypes had 25.8% and 34.7% neomales at 28°C and 70.3% and 80.7% neomales at 36°C. In EKW family 2, ZW_1 and W_1W_1 genotypes had 43.3% and 8.3% neomales at 28°C and 96.6% and 100% neomales at 36°C. Finally, in EKW family 3, the ZW_1, ZW_2 and W_1W_2 genotypes had 81.8%, 41.6% and 50.0% neomales at 28°C, and 100%, 92.8% and 82.4% neomales at 36°C (**Figure 4A**).

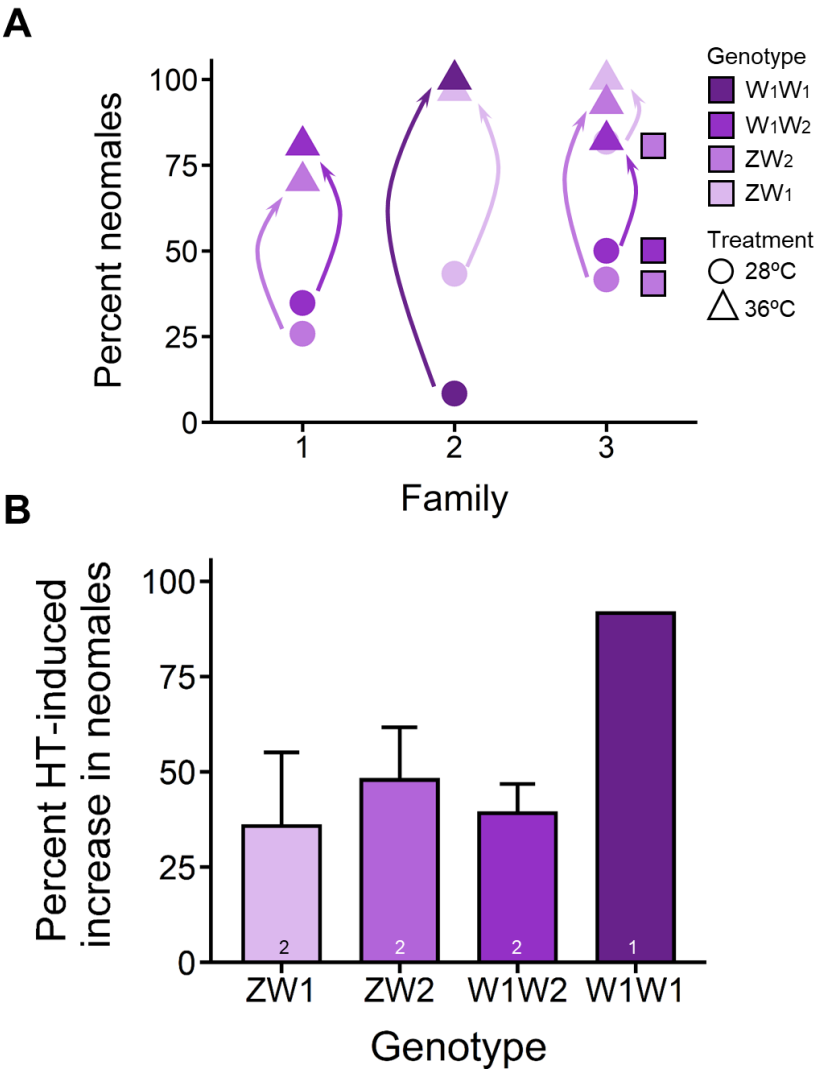

Figure 4. Response to temperature in the EkkWill (EKW) strain. (**A**) Percent of neomales as a function of the genotype and temperature in family 1, 2 and 3. (**B**) Percent increase in the number of neomales due to temperature irrespective of family. Data as mean ± SD. Numbers inside bars indicate the sample size.

We sought to calculate masculinization induced by elevated temperature above that occurring when fish were raised under control conditions, i.e., neomales found at elevated temperature in excess of the number in control treatments. To this end, in each family and for each genotype (ZW_1, ZW_2, W_1W_1 and W_1W_2), we took the percent of neomales at 36°C and subtracted the percent of neomales at 28°C (**Figure 4B**). Then, we averaged the increase of neomales due only to elevated temperature for each genotype that appeared among the different families. Results showed that for the ZW_1 genotype, the increase of neomales was 35.7±17.7% (present both in family 2 and 3). For the ZW_2 and W_1W_2 genotypes, the average increase in neomales was 47.9±3.3% and 39.2± 6.8%, respectively (present both in family 1 and 3). For the W_1W_1 genotype, the increase in neomales was 91.2% (present only in family 2) (**Figure 4B**).

The reaction norms of the different genotypes in NA and EKW strains were also plotted (**Figure 5**). Results showed that, regardless of the strain, the reaction norms of sex ratio to temperature were genotype-dependent.

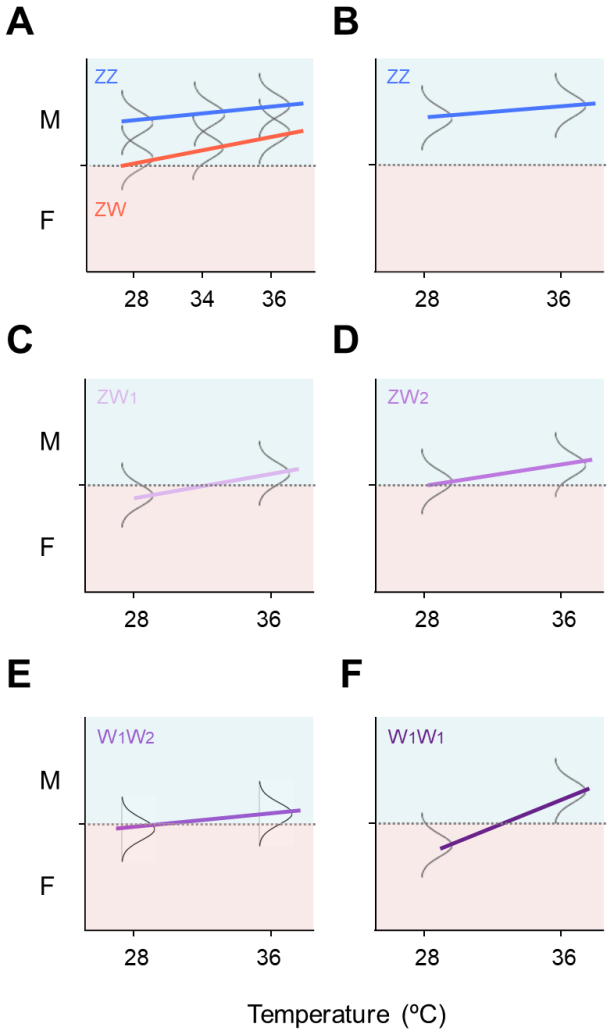

Figure 5. Reaction norms of the genotypes in response to temperature (**A**) ZZ and ZW genotypes in Nadia (NA) strain and the different (**B**) ZW_1, (**C**) ZW_2, (**D**) W_1W_2 and (**E**) W_1W_1 genotypes EkkWill (EKW)strain. Abbreviations: male (M) and female (F).

Histomorphometrical changes in the gonads due to elevated temperature

To determine if elevated temperature caused gonads to have abnormal development, we examined histological sections. Histological analysis revealed that testes in AB strain males and in ZZ males of the NA strain had similar proportions of different germ cell stages regardless of temperature (**Figure S6**). In testis of ZW neomales of the NA strain, however, the proportion of A_{diff}, B, Spc, and Spd germ cell types were significantly lower in the 36°C fish compared to control fish (*t*-student, $P < 0.05$; 0.01; 0.001; 0.001 and 0.001, respectively), whereas the proportion of Spz was significantly higher in heated fish (*t*-student, $P < 0.001$) (**Figure 6A**). The number of spermatozoa (mean±SD) in NA ZW neomales (1,178± 207) was lower than in NA ZZ males (1,997.7±428) and in the AB males (2,525±608) at 28°C, but significantly higher proportions (*t*-student, $P < 0.001$) in NA neomales at 36°C (**Figure 6B**).

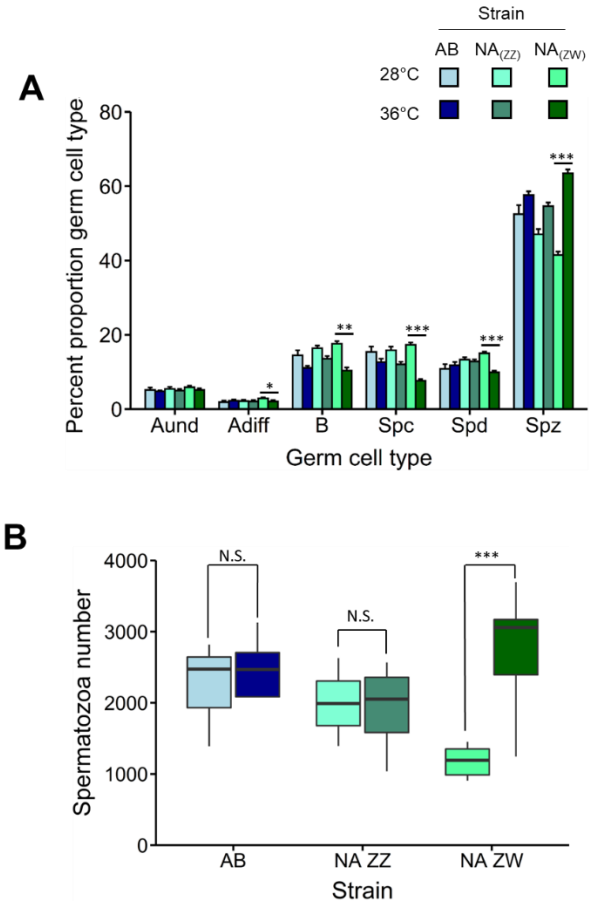

Figure 6. Effects of temperature (36°C) during sex differentiation (18–34 days post fertilization, dpf) on testis morphology of adult zebrafish in AB and Nadia (NA) strains sampled at 90 dpf. (**A**) Proportion of the different germ cell types in AB and Nadia strains. Abbreviations: type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (B), spermatocytes (Spc), spermatids (Spd) and spermatozoa (Spz). (**B**) Spermatozoa quantification per field generated by using ImageJ from AB males, NA males $_{(ZZ)}$ and neomales $_{(ZW)}$. Sample size in each group: AB (3 and 5), NA $_{(ZZ)}$ (7 and 7) NA $_{(ZW)}$ (6 and 8) for low (28°C) and high (36°C) temperature, respectively. Data as boxplots (line: median). Abbreviations: NS, not significant; *** = $P < 0.001$. The Student *t*-test was used to compare the number of each germ cell type between the two temperatures.

In females, histological analysis of the ovaries (**Figure S7**) showed that the proportion of Pofs (postovulatory follicles) cells was significantly (*t*-student, $P < 0.05$) lower in the gonads of AB females at 36°C than the AB females at 28°C whereas the other germ cell stages remained unaffected (**Figure 7**). At 28°C, the NA ZW females showed lower proportions of oocytes at PG and CA stages, and also reduction of Pofs in comparison to AB females at 36°C. On the other hand, the Vtg and A showed higher values in NA ZW females when compared to AB females between treatments although these differences were not significant (**Figure 7**).

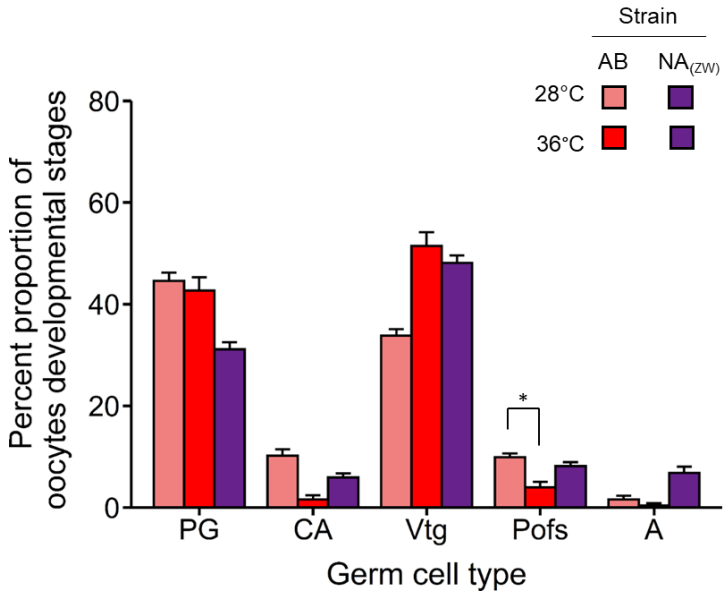

Figure 7. Effects of temperature (36°C) during sex differentiation (18–34 days post fertilization, dpf) on ovarian morphology of adult zebrafish in AB and Nadia (NA) strains sampled at 90 dpf. Abbreviations: primary growth (PG) oocytes, cortical alveolar (CA) oocytes, vitellogenic (Vtg) oocytes, postovulatory follicles (POFs) and atretic (A) oocyte. Data as mean ± SD. * = $P < 0.05$. The Student *t*-test was used to compare the number of each germ cell type between the two temperatures. Note: NA $_{(ZW)}$ females at high temperature (36°C) were not available due to 100% masculinization.

4. Discussion

Sex ratio response to temperature

Prior to this study, it was known that sex determination in the laboratory zebrafish AB strain occurs in the absence of sexual chromosomes (Wilson et al., 2014) and is governed by a polygenic sex determination system (Liew et al., 2012). In wild zebrafish, a single locus in the telomeric part of chromosome 4 was highly correlated to sex, and sex determination relies on a GSD (ZZ/ZW) system (Anderson et al., 2012; Wilson et al., 2014). It was also known that ZZ fish always develop as males and that ZW and WW fish usually become females, although a small proportion of natural fish with these two genotypes developed male phenotypes as neomales (Wilson et al., 2014).

In the present study, when we exposed NA, EKW and AB juveniles to elevated temperature during gonadal development, all strains showed significant masculinization. These results are in line with previous findings obtained with laboratory zebrafish reared at high temperature (Abozaid et al., 2012; Ribas et al., 2017a) and with those observed in nature for a wild zebrafish population (Brown et al., 2015). In this study, we also confirmed, as previously described in laboratory zebrafish (Ribas et al., 2017a; Hosseini et al., 2019), family-dependent sex ratios at the control temperature and family-dependent sex ratio response to elevated temperatures.

Spontaneous production of neomales

In fish, neomales can occur spontaneously (Nanda et al., 2003; Wilson et al., 2014) or can be environmentally induced by a variety of stressors, such as temperature, density or hypoxia (Santos et al., 2017). On the other hand, neomales can be also induced after treatment with androgens or aromatase inhibitors (Takatsu et al., 2013; Lee et al., 2017), with morpholinos targeting dead end protein 1 gene (*dnd*) essential for germ line survival (Siegfried and Nüsslein-Volhard, 2008) or by knock-out mutations in genes involved in gonadal development (Rodríguez-Marí et al., 2010; Lau et al., 2016; Ramanagoudr-Bhojappa et al., 2018). When using laboratory strains of zebrafish, or other PSD species such as the European sea bass, the problem arises that the frequency of neomales is calculated from the increase in the frequency of males at high *vs*. low temperature because no genetic sex markers are available (Palaiokostas et al., 2015; Ribas et al., 2017a; Anastasiadi et al., 2018b). In this study, in contrast, by using sex-specific primers for the NA and EKW strains, we were able to identify a direct relationship between the genotypic sex and the phenotypic sex of each fish and hence we could distinguish between genotypic males and neomales among the phenotypic males.

In the NA strain, Mendelian ratios of ZZ and ZW genotypes in the offspring of ZZ males to ZW females was expected at 28°C and this is what occurred. Thus, the number of ZZ males (mean = 48.1±0.5%) plus the number of ZW males (neomales) (mean = 28.5±3.5%) explained the highly skewed male sex ratios observed in all NA families (mean=77.0±2.5%). This means that nearly half of the individuals with the ZW genotype developed spontaneously into males at 28°C. Given that stress in many fish species appears to masculinize sex ratios, this finding suggests that under the standard laboratory culture conditions, NA strain natural zebrafish experience a substantial amount of stress. However, at present the source of that stress is not known and it could include inbreeding, nutrition, crowding, pH, salinity, the fact that a constant temperature of 28°C is too high or other unsuspected factors such as detergent film on the aquaria and also perhaps noise in the building.

In EKW, sex genotyping primers were not available at the start of the experiments, so crosses were carried out according only to phenotypic sex. Thus, the actual genotypic sex

was unknown. After we developed primers to sex genotype EKW fish, we found that EKW families also produced neomales at 28°C and, importantly, they had several different genotypes (ZW_1, ZW_2, W_1W_1, and W_1W_2). Differences in sex ratios among families can then be attributed to the parental genotypes of each individual cross that gave different genotypic frequencies in the offspring. This explanation clarifies differences in the percent of genetic males (ZZ) and neomales (ZW) among the three EKW families. Further, it is interesting to note that the reaction norm of the sex ratio response to temperature of AB family 2 (**Figure 1A**) is similar to that of EKW family 1 **(Figure 5A**) (progenitors: ♀ZW1 x ♂W2W2 or ♀W2W2 x ♂ZW1), both being female-biased at 28°C. Likewise, AB family 1 is similar to EKW family 3 (progenitors: ♀ZW1 x ♂ZW2 or ♀ZW2 x ♂ZW1), in this case both being male-biased. It is, then, tempting to suggest that despite the polygenic sex determination system of the AB strain, family 2 would have a sex genotype similar to EKW family 1, whereas AB family 1 would have a sex genotype in line with that of EKW family 3. One possibility, however, is that the AB strain, after the process of domestication over the years is actually composed of WW fish. Some WW become males and some females, indicating that the fact that the ratios are similar does not necessarily mean that the genetics behind it are similar. Then, similarity between the sex ratios of different families would be due to chance.

Care was taken to avoid the masculinizing effects of high density (Ribas et al., 2017b; c). However, other environmental factors could have inadvertently interfered with the final sex phenotypes. Thus, for instance, the amount of food available for some fish in the tanks might be limited due to the presence of dominant conspecifics, provoking stress and consequently skewed sex ratios (Lawrence et al., 2008; Paull et al., 2010; Dreosti et al., 2015). Likewise, high doses of metals such as copper in the water can also affect sex ratios (Craig et al., 2009) although this was unlikely in our case. The appearance of neomales at 28°C in both wild strains tested indicates that the presence of *sar4* does not prevent male development under a thermal regime theoretically considered to be neutral in terms of effects on sexual development. Thus, the presence of neomales can be due to the effects of still unidentified secondary loci, or to unknown environmental stressors, or both. Uncovering the actual underlying mechanism that generates neomales should provide useful insights on sexual development in zebrafish in particular and in fish in general. One possible explanation is inbreeding. Inbreeding can result in masculinization in both laboratory (Lawrence et al., 2008; Monson and Sadler, 2010; Nasiadka and Clark, 2012) and wild zebrafish (Brown et al., 2015). In our study, the NA and EKW broodstock fish had been in captivity for several generations by mating siblings or close relatives, thus likely resulting in inbreeding (Wilson et al., 2014).

Neomales at elevated temperatures

In zebrafish exposed to elevated temperature, the number of neomales increased significantly in both NA and EKW populations. The induction of neomales by temperature has also been observed in species such as medaka (XX/XY) (Sato et al.,

2005) and half-smooth tongue sole (ZW/ZZ) (Shao et al., 2014), indicating that even in species with a strong genetic sex determination mechanism, the phenotypic sex can be affected by environmental conditions. In NA families, we observed a temperature-dependent masculinization, and surprisingly, all ZW fish developed as males at 36°C.

In the EKW strain, we were able to identify for the first time that different SNPs in the W chromosome gave different combinations of genotypes. Each genotype showed different responses of masculinization due to temperature, although a small fraction of fish from these genotypes represented heat-resistant individuals that developed as females. The question arises whether some of the allelic combinations of these genotypes have an intrinsic resistance to masculinization by temperature. For instance, the W_1W_1 genotype, which occurred only in the offspring of EKW family 2, showed lower sex ratio reversal in regular conditions when compared to the other genotypes, but in contrast it was the most sensitive to elevated temperature, the significance of this not being clear at the moment. A similar situation was described in the half-smooth tongue sole, with a SNP (A/T) located on the Z chromosome. Thus, while the allele Z_TW was strongly associated with neomales, fish with the Z_AW genotype always developed as females (Jiang and Li, 2017). These results indicated that sex reversal was mediated mainly by the presence of T in the SNP because the A variant always results in females (Jiang and Li, 2017), although epigenetic mechanisms are thought to be involved in the appearance of neomales in half-smooth tongue sole (Chen et al., 2014; Shao et al., 2014). Based on our results with the EKW strain, the three family crosses have in common that none of the progenitors was a genetic male (ZZ), therefore implying that parental male fish in each cross, was a neomale. Thus, it would be interesting to know whether the W variant (W_1 or W_2) and the dosage of W (ZW or WW) inherited from the progenitors could explain the different masculinization ratios observed in the offspring and that sex phenotype is controlled epigenetically in these fish as is the case of ZW males in the half-smooth tongue sole (Shao et al., 2014). The fact that none of three males taken at random were ZZ males suggests that during its laboratory culture since taken from large outdoor ponds at the EkkWill Waterlife Resources fish farm in Gibsonton, Florida, the EKW strain may be losing its Z chromosome and becoming an all WW stock, which our results show produces both males and females.

Genotype-dependent reaction norms of sex ratio responses to temperature

One important finding of this study is that the sex ratio response to elevated temperature of the wild strains containing the natural *sar4* locus resembled the response of the laboratory AB strain, as evidenced by similar reaction norms. We observed a range of phenotypic variation when individuals with the same genotype (i.e., ZW and WW) either from NA or EKW strains were exposed to different temperatures. Aside from the ZZ genotype, which always produced males, the ZW genotype of the NA strain could produce both males and females in the offspring. In contrast, in the EKW strain we identified genotypes that varied in the dosage of the W chromosome (ZW *vs*. WW) and

in SNP content in the chromosome W (W_1 *vs.* W_2). Under the hypothesis that the W chromosome has a dominant incompletely penetrant female determining gene, we expected that individuals possessing two W chromosomes (WW) would strongly resist the masculinizing effects of elevated temperature when compared to fish with only one W chromosome (ZW). Our results clearly show, however, that fish with the W_1W_1 or W_1W_2 genotypes (the W_2W_2 genotype was never detected) had essentially the same susceptibility to temperature as fish with the ZW genotype. Taken together, our results suggest genotype-dependent differences in the rate of spontaneous sex reversal at the control temperature but genotype-independent response to elevated temperature.

Histomorphometrical analysis reveals alterations of germ cell proportions due to high temperature

Exposure to elevated temperature during sex differentiation also significantly altered the proportion of the different germ cell types of NA neomales (ZW) but not of NA males (ZZ) and males of the AB strain. Temperature reduced type B spermatogonia, spermatocytes and spermatids in the mature testis of heat-treated fish, but effects were only significant in the NA neomales. Likewise, the amount of spermatozoa was higher, but again only to a statistically significant degree in NA neomales. In fish, temperature is considered an important modulator in reproduction, including aspects related to testicular function and spermatogenesis (Borg, 1982; Quintana et al., 2004). The reduction of testicular germ cells through induction of apoptosis after exposure to elevated temperatures was observed in Nile tilapia (Alvarenga and França, 2009) and also in mice (Setchell, 2006). Apoptosis plays an important role during spermatogenesis (Roosen-Runge, 1977; Shaha, 2007) because it reduces the number of cells that produce sperm (Nóbrega et al., 2009). We found higher levels of sperm in NA neomales exposed to high temperature during gonadal development. This increase would be explained as an adaptation or compensatory mechanism to cope with a situation where temperatures would be higher once they reached maturity. This phenomenon was described in the Eastern mosquitofish (*Gambusia holbrooki*) males exposed to high temperature, as they produced about three times more sperm than those kept in much colder water (Adriaenssens et al., 2012). Nevertheless, amount of spermatozoa in neomales does not necessarily mean higher quality, as it was shown in Atlantic salmon (*Salmo salar*), where sperm quality of chromosomally XX neomales did not compare favorably with that of regular (XY) males (de Castro and Patil, 2019). In contrast, in Nile tilapia, temperature did not alter the frequency of spermatocytes and spermatozoa between males and temperature-induced neomales (Sun et al., 2016).

In the ovaries of AB zebrafish strain exposed to elevated temperature, we found a significant decrease of oocytes at CA stage, and also a reduction of Pofs, as previously described in the adult females of Japanese flounder (*Paralichthys olivaceus*) treated for 27 or 48 hours from a range of 9.2° C and 22.6°C (Kurita et al., 2011). Effects on oogenesis in temperature-treated females was also observed in Nile tilapia in oocytes at

the pre-vitellogenic and vitellogenic stages (Sun et al., 2016). In this study, the effects of temperature remained visible at the level of oogenesis but not at the level of gonadal maturation as described in laboratory strains of zebrafish using the same experimental design (Ribas et al., 2017a).

5. Conclusions

We hypothesized that the genetic sex determination mechanism of *sar4* should buffer natural strains of zebrafish from the effect of high temperature, making natural strains much less sensitive to environmental sex change than the domesticated strain. In this study, we show that, contrary to predictions, zebrafish of two wild strains, NA and EKW, which have the *sar4* locus in a ZW/ZZ system, have similar or even higher susceptibility to the masculinizing effects of exposure to elevated temperature during gonadal development as laboratory strains, which do not have the *sar4*. Likewise, wild strains also have genotype-by-environment interaction in sex ratio response, suggesting that in addition to *sar4*, other genetic factors may contribute to phenotypic sex. In this study, we developed primers to identify the genotypic sex of the EKW strain as was done before for the NA strain. We demonstrate the spontaneous presence of neomales at control temperatures and genotypic-dependent differences in the susceptibility of genotypes to elevated temperature-induced masculinization. However, the WW genotype does not confer increased resistance to the effects of masculinization than those fish with ZW genotypes, indicating that female dominant sex determinant homozygosity does not necessarily protect against masculinization. Finally, we found higher amount of spermatozoa in Nadia ZW neomales but not in regular ZZ when exposed to elevated temperature. Taken together, our results reveal the susceptibility of wild strains to temperature, a genetic basis underlying both the production of neomales and their response to temperature with potential functional consequences in reproductive capacity. Further research is needed to determine the underlying mechanisms responsible for the production of neomales and whether ZW or WW individuals that become neomales have genetic modifiers different from ZW or WW fish that become females. This information is essential to properly gauge the consequences of global warning on natural populations.

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Competing interests

The authors declare that there is no conflict of interests.

Author contributions

FP, JP, LR and AV designed the study. AV and CW conducted the experiments and sampling. AV did the subsequent analysis and graphs. AA and CW designed the primers for EKW and NA strains, respectively. MDSR and RHN did the Histomorphometrical analysis. AV, LR and FP wrote the manuscript. All authors read, corrected and approved the final manuscript.

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Supplementary Figures

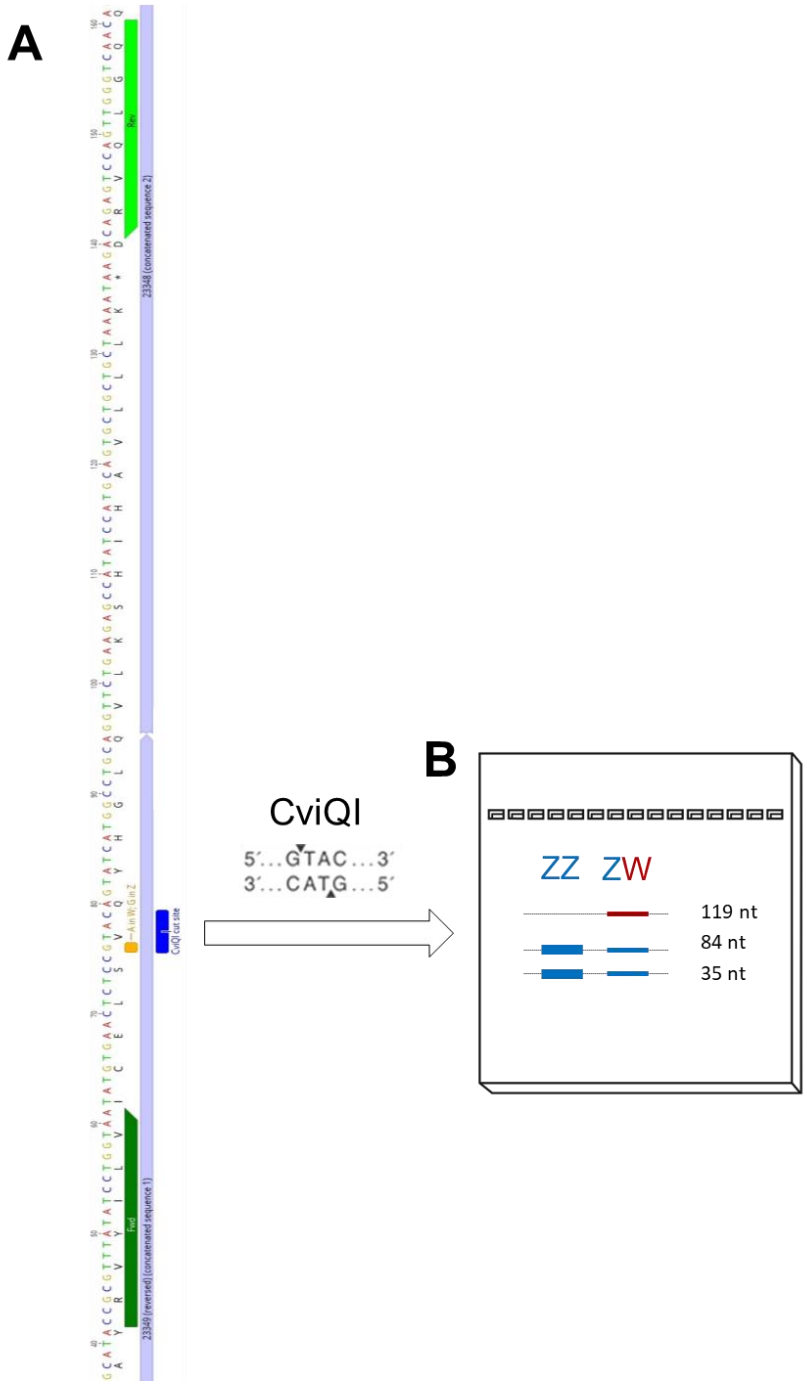

Figure S1. Identification of the sexual genotype in the Nadia (NA) strain**. (A)** Primer sequences: forward, dark green; reverse, light green. Notice the presence of a SNP (yellow band) in position 76: G in the Z chromosome and A in the W chromosome. (**B**) Illustration of the resulting bands associated with each sexual genotype after digestion with the CviQI restriction enzyme. Abbreviation: nucleotides, nt.

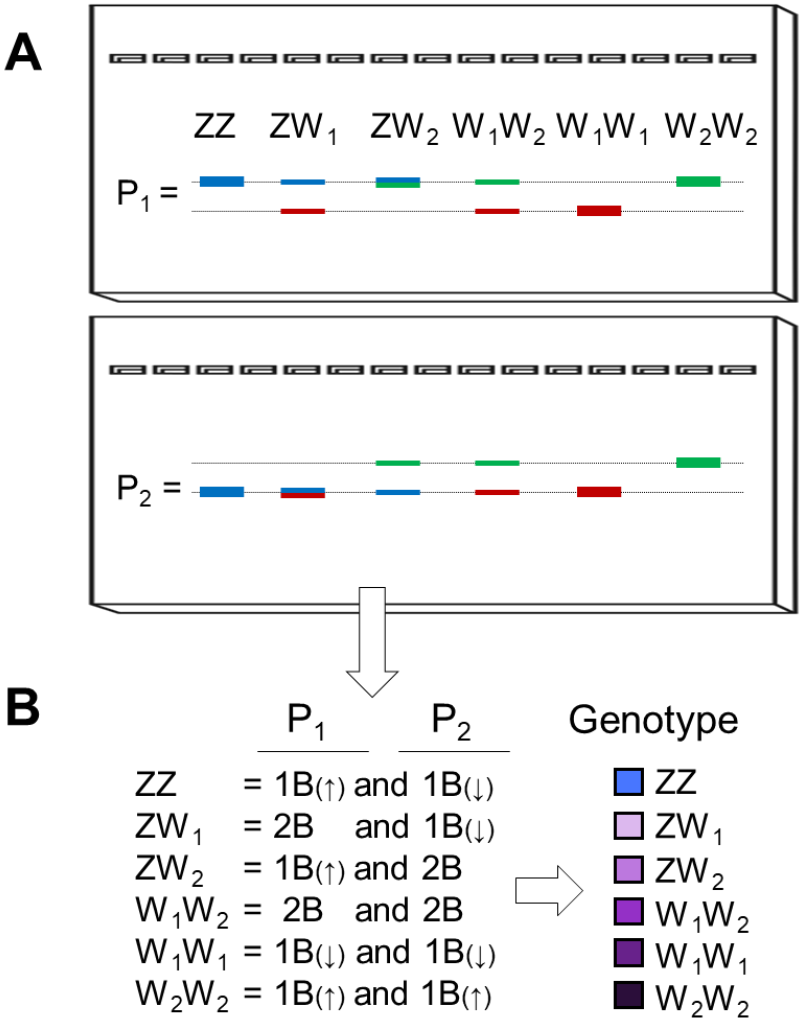

Figure S2. Identification of the sexual genotype in the EkkWill (EKW) strain**.** (**A**) Illustration of the bands of the different genotypes after PCR using the primer pairs P_1 and P_2. (**B**) The combined banding pattern allows the unambiguous identification of the sexual genotype. Arrows (↑) and (↓) indicate different positions of the bands (B) on the agarose gel.

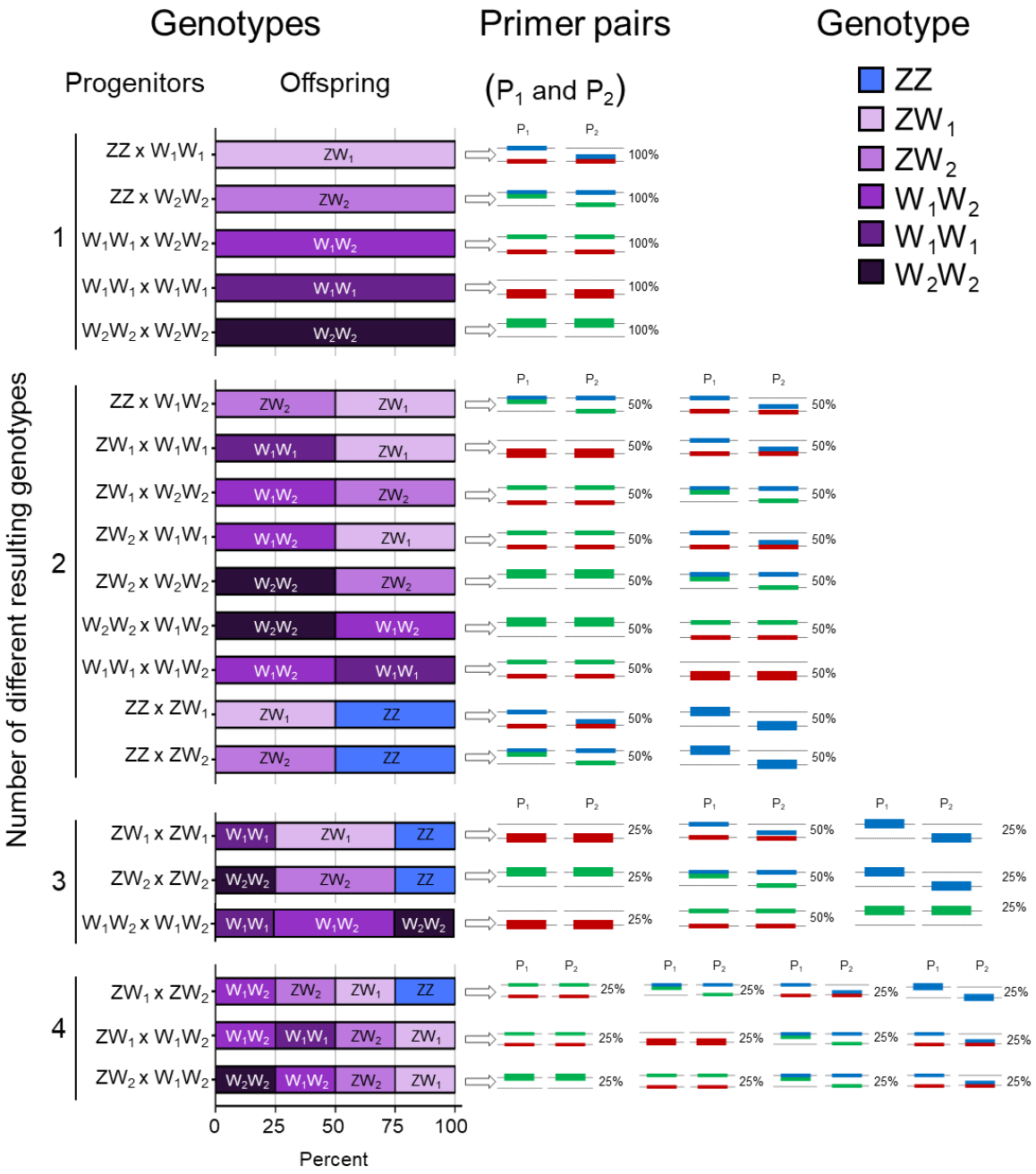

Figure S3. The 20 hypothetical crosses given the presence of two different SNPs in chromosome W in the EkkWill (EKW) strain. For each cross, the expected genotypic frequencies in the offspring were calculated together with the frequency of bands associated with each genotype.

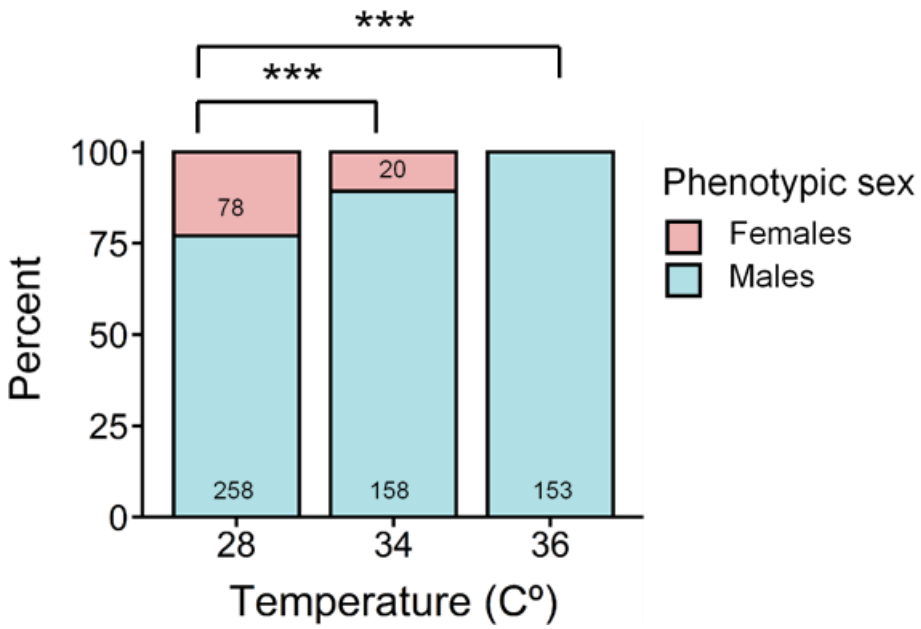

Figure S4. Mean sex ratio from six separate families of the Nadia (NA) strain (three for the 28–34°C comparison and three for the 28–36°C comparison). Numbers indicate sample sizes. A χ^2 test was applied. *** = $P < 0.001$).

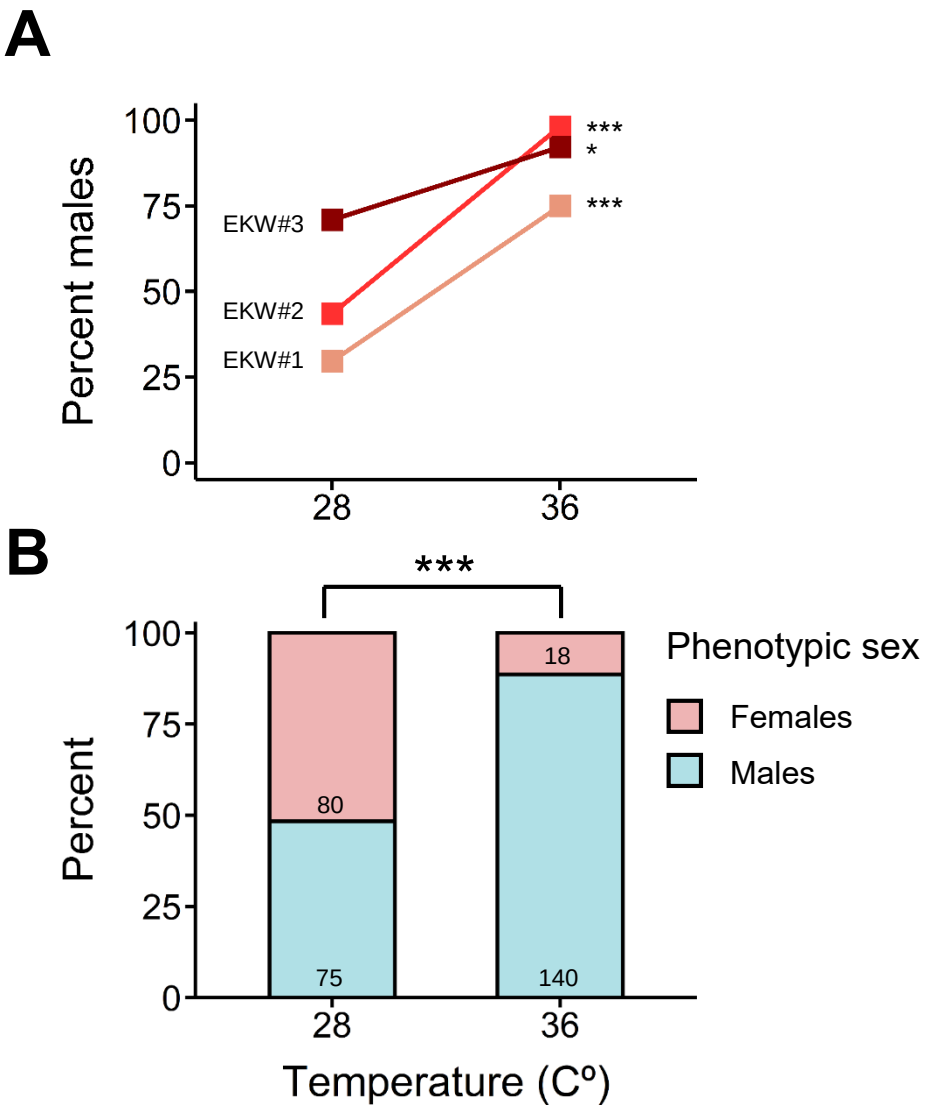

Figure S5. Sex ratio response to temperature in the EkkWill (EKW) strain when fish were exposed between 18–32 days post fertilization. **(A)** Data from three separate families **(B)** Mean sex ratio. Numbers indicate sample size. A χ^2 test was applied. (*** = $P < 0.001$).

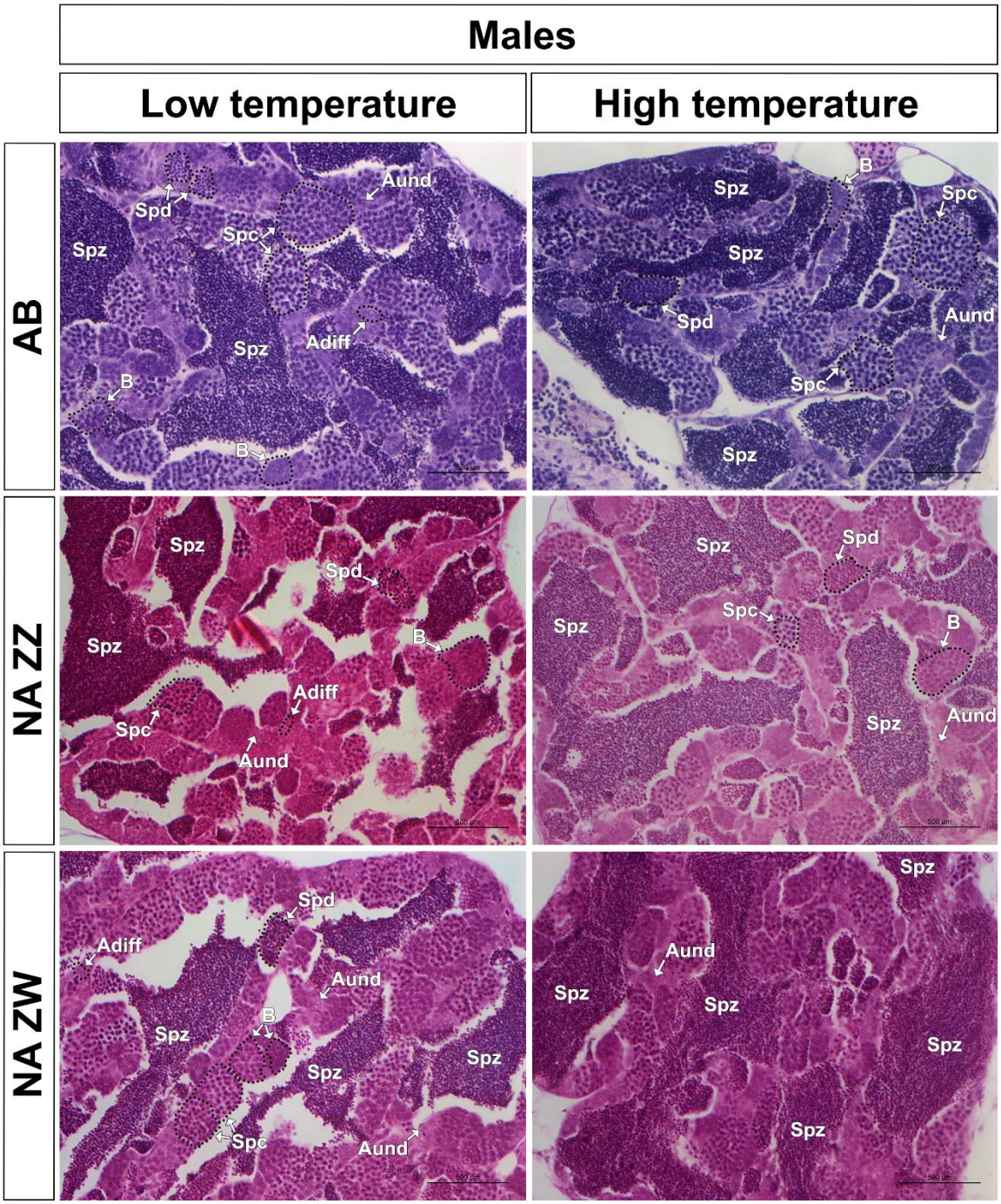

Figure S6. Morphological sections from adult zebrafish testis: AB males, NA males (NA ZZ) and NA neomales (NA ZW) exposed to different temperatures during sex differentiation. type A undifferentiated spermatogonia (Aund), differentiated spermatogonia (Adiff), type B spermatogonia (B), spermatocytes (Spc), spermatids (Spd) and spermatozoa (Spz). Staining: hematoxylin and eosin. Scale bar = 500 µm.

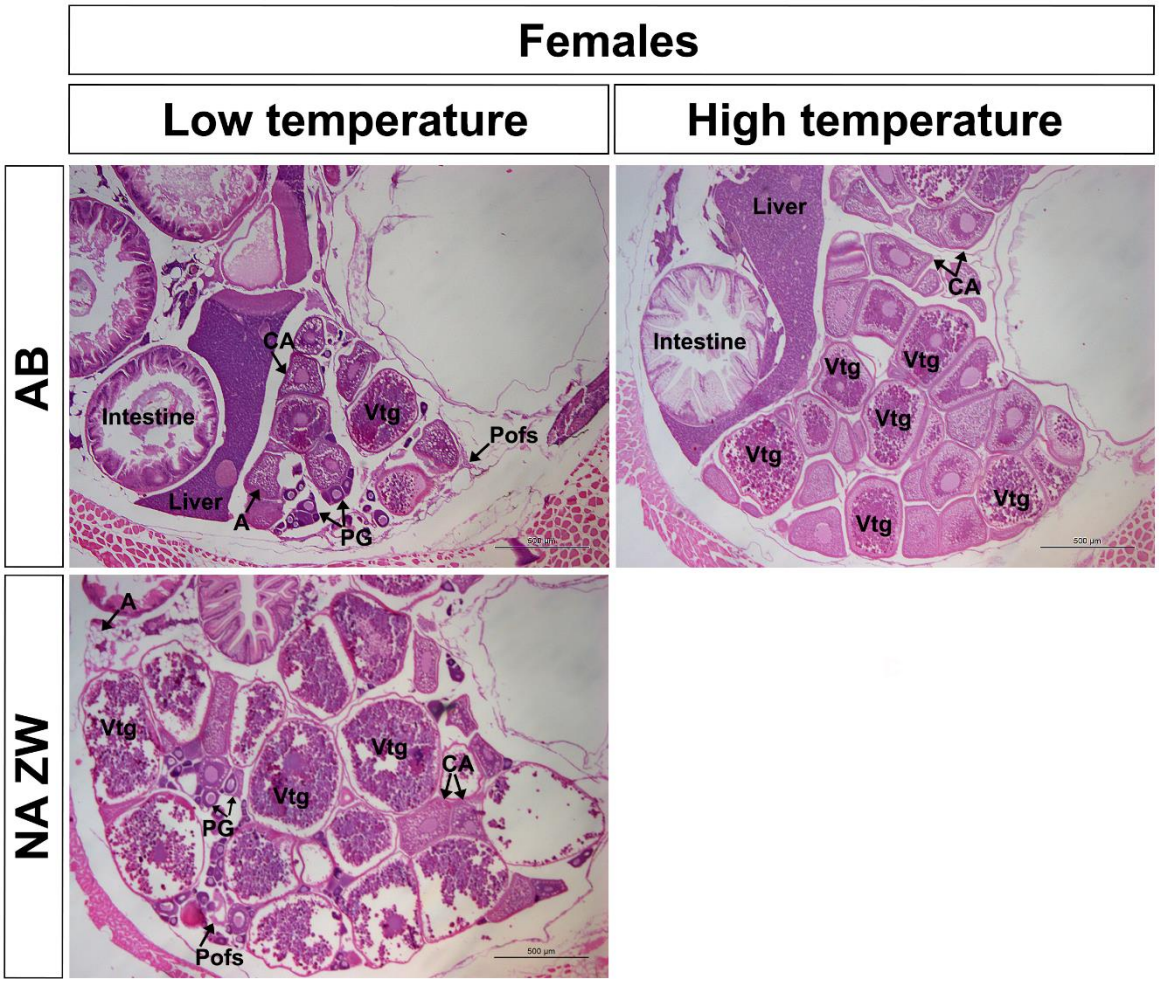

Figure S7. Morphological sections from adult zebrafish ovaries: AB and NA (NA ZW) females exposed to different temperatures during sex differentiation. Primary rowth oocytes (PG), cortical alveolar oocytes (CA), vitellogenic oocytes (Vtg), postovulatory follicles (Pofs) and atretic oocytes (A). Staining: hematoxylin and eosin. Scale bar = 500 µm. Note: high temperature resulted in 100% males in NA, so no NA ZW females were available for analysis.

Supplementary Tables

Table S1. Survival of zebrafish at 18, 34 and 90 days post fertilization (dpf), i.e., before, during and the end of the thermal treatment, respectively. The number of males at 90 dpf per strain and temperature is also indicated

Strain	Family	Tank	S18 dpf (%)	Family mean	Strain mean	Temp. (°C)	S34 dpf (%)	Family mean	S90 dpf (%)	Males (%)	Females (%)	Mean males (%)
AB	1	1	60	68.3 ± 4.0%	69.7 ± 7.0%	28	100	100 ± 0.0%	100 ± 0.0%	67	33	69.4 ± 3.9
		2	60				100			75	25	
		3	75				100			67	33	
		4	85			36	82	91.6 ± 5.1	97.6 ± 2.4%	77	23	87.0 ± 7.1
		5	65				92			92	8	
		6	65				100			92	8	
	2	1	95	82.5 ± 6%		28	95	96.6 ± 1.7%	100 ± 0.0%	22	78	24.8 ± 8.9
		2	100				95			37	63	
		3	65				100			15	85	
		4	65			36	92	89.6 ± 6.9%	100 ± 0.0%	67	33	76.5 ± 11.3
		5	85				100			71	29	
		6	85				76			92	8	
	3	1	60	58.2 ± 4.8%		28	100	100 ± 0.0%	100 ± 0.0%	42	58	53.9 ± 18.5
		2	50				100			80	20	
		3	50				100			40	60	
		4	80			36	94	97.9 ± 2.1%	100 ± 0.0%	87	13	92.2 ± 5.7
		5	50				100			90	10	
		6	60				100			100	0	
Nadia	1	1	80	87.5 ± 6.6%	93.9 ± 3.2%	28	100	96.7 ± 1.7%	100 ± 0.0%	69	31	70.3 ± 2.4
		2	100				95			68	32	
		3	100				95			74	26	
		4	85			36	94	98.0 ± 2.0%	100 ± 0.0%	100	0	100 ± 0
		5	100				100			100	0	
		6	60				100			100	0	
	2	1	100	96.7 ± 2.1%		28	100	98.3 ± 1.7%	100 ± 0.0%	75	25	84.6 ± 2.4
		2	100				95			84	16	
		3	90				100			94	6	
		4	100			36	100	98.3 ± 1.7%	100 ± 0.0%	100	0	100 ± 0

		5	100				95			100	0	
		6	90				100			100	0	
	3	1	90	87.5 ± 3.4%		28	100	98.1 ± 1.9%	100 ± 0.0%	89	11	68.5 ± 14.4
		2	90				94			59	41	
		3	95				100			58	42	
		4	75			36	100	96.5 ± 3.5%	100 ± 0.0%	100	0	100 ± 0
		5	95				89			100	0	
		6	80				100			100	0	
	4	1	95	98.3 ± 1.1%		28	100	100 ± 0.0%	100 ± 0.0%	74	26	77.6 ± 4.7
		2	95				100			84	16	
		3	100				100			75	25	
		4	100			34	100	100 ± 0.0%	100 ± 0.0%	85	15	76.7 ± 8.5
		5	100				100			65	35	
		6	100				100			80	20	
	5	1	100	96.7 ± 1.7%		28	100	98.1 ± 1.9%	100 ± 0.0%	60	40	72.0 ± 9.2
		2	95				100			74	26	
		3	90				94			82	18	
		4	100			34	100	100 ± 0.0%	100 ± 0.0%	95	5	98.3 ± 2.4
		5	95				100			100	0	
		6	100				100			100	0	
	6	1	100	96.7 ± 2.1%		28	95	98.3 ± 1.7%	100 ± 0.0%	100	0	89.3 ± 9.1
		2	100				100			90	10	
		3	90				100			78	22	
		4	100			34	100	100 ± 0.0%	100 ± 0.0%	100	0	93.1 ± 6.2
		5	90				100			94	6	
		6	100				100			85	15	
EkkWill	1	1	90	91.7 ± 1.1%	88.6 ± 2.0%	28	100	100 ± 0.0%	98.2 ± 1.8%	28	72	29.6 ± 6.9
		2	90				100			22	78	
		3	95				100			39	61	
		4	90			36	100	100 ± 0.0%	96.4 ± 1.8%	76	24	75.5 ± 2.4
		5	90				100			78	22	
		6	95				100			72	28	
	2	1	85	85.0 ± 3.7%		28	100	95.6 ± 4.4%	100 ± 0.0%	41	59	44.7 ± 12.6
		2	75				87			62	38	
		3	80				100			31	69	

	4	100		36	100	100 ± 0.0%	100 ± 0.0%	95	5	98.3 ± 2.4
	5	90			100			100	0	
	6	80			100			100	0	
3	1	90	89.2 ± 2.4	28	100	100 ± 0.0%	100 ± 0.0%	72	28	71.2 ± 10.4
	2	90			100			83	17	
	3	95			100			58	42	
	4	80		36	100	98.0 ± 2.0%	100 ± 0.0%	94	6	92.3 ± 2.0
	5	85			94			94	6	
	6	95			100			89	11	

Abbreviations: S18 dpf (%), Percent survival during 4–18 dpf; S34 dpf (%), Percent survival during 18–34 dpf; S90 dpf (%), Percent survival during 34–90 dpf; Temp. (°C), Temperature (°C).

Table S2. Primers used for sex genotyping zebrafish of the Nadia and EkkWill strains

Strain	Primer pair	Forward primer	Reverse primer	Tm (C^{o})
Nadia	P_1	CCGCGTTTATATCCTGGTAA	GTTGACCCAACTGGACTCTG	61
EkkWill	P_1	AGACCTGACTTTGTCAAGCTGT	TTTCTCGGATTTTTGCAGCCT	59
	P_2	ACTCTTGACAGCAGAATCGCA	AGTGTGAAGCGGTGACTGTAG	59

Table S3. The 30 different possible genotypic frequency combinations based on the genotypes of the parents in the EkkWill zebrafish strain. This takes into account the paternal or maternal origin of the Z chromosome. If such distinction is not made, then the number of different genotypic combinations is reduced to 20 (see Figure S3)

Phenotype		Female				
	Genoytpe	ZW_1	ZW_2	W_1W_1	W_1W_2	W_2W_2
Male	ZZ	ZZ, ZW1 — 50% ZZ, 50% ZW_1 — **1**	ZZ, ZW2 — 50% ZZ, 50% ZW_2 — **7**	ZW1, ZW1, ZW1, ZW1 — 100% ZW_1 — **13**	ZW1, ZW2, ZW1, ZW2 — 50% ZW_1, 50% ZW_2 — **19**	ZW2, ZW2, ZW2, ZW2 — 100% ZW_2 — **25**
	ZW_1	ZZ, ZW1, W1Z, W1W1 — 25% ZZ, 50% ZW_1, 25% W_1W_1 — **2**	ZZ, ZW2, W1Z, W1W2 — 25% ZZ, 25% ZW_2, 25% ZW_1, 25% W_1W_2 — **8**	ZW1, ZW1, W1W1, W1W1 — 50% ZW_1, 50% W_1W_1 — **14**	ZW1, ZW2, W1W1, W1W2 — 25% ZW_1, 25% ZW_2, 25% W_1W_1, 25% W_1W_2 — **20**	ZW2, ZW2, W1W2, W1W2 — 50% ZW_2, 50% W_1W_2 — **26**
	ZW_2	ZZ, ZW1, W2Z, W2W1 — 25% ZZ, 25% ZW_1, 25% ZW_2, 25% W_1W_2 — **3**	ZZ, ZW2, W2Z, W2W2 — 25% ZZ, 50% ZW_2, 25% W_2W_2 — **9**	ZW1, ZW1, W2W1, W2W1 — 50% ZW_1, 50% W_1W_2 — **15**	ZW1, ZW2, W2W1, W2W2 — 25% ZW_1, 25% ZW_2, 25% W_1W_2, 25% W_2W_2 — **21**	ZW2, ZW2, W2W2, W2W2 — 50% ZW_2, 50% W_2W_2 — **27**
	W_1W_1	W1Z, W1W1, W1Z, W1W1 — 50% ZW_1, 50% W_1W_1 — **4**	W1Z, W1W2, W1Z, W1W2 — 50% ZW_1, 50% W_1W_2 — **10**	W1W1, W1W1, W1W1, W1W1 — 100% W_1W_1 — **16**	W1W1, W1W2, W1W1, W1W2 — 50% W_1W_1, 50% W_1W_2 — **22**	W1W2, W1W2, W1W2, W1W2 — 100% W_1W_2 — **28**
	W_1W_2	W1Z, W1W1, W2Z, W2W1 — 25% ZW1, 25% W_1W_1, 25% ZW_2, 25% W_1W_2 — **5**	W1Z, W1W2, W2Z, W2W2 — 25% ZW_1, 25% W_1W_2, 25% ZW_2, 25% W_2W_2 — **11**	W1W1, W1W1, W2W1, W2W1 — 50% W_1W_1, 50% W_1W_2 — **17**	W1W1, W1W2, W2W1, W2W2 — 25% W_1W_1, 50% W_1W_2, 25% W_2W_2 — **23**	W1W2, W1W2, W2W2, W2W2 — 50% W_1W_2, 50% W_2W_2 — **29**
	W_2W_2	W2Z, W2W1, W2Z, W2W1 — 50% ZW_2, 50% W_1W_2 — **6**	W2Z, W2W2, W2Z, W2W2 — 50% ZW_2, 50% W_2W_2 — **12**	W2W1, W2W1, W2W1, W2W1 — 100% W_1W_2 — **18**	W2W1, W2W2, W2W1, W2W2 — 50% W_1W_2, 50% W_2W_2 — **24**	W2W2, W2W2, W2W2, W2W2 — 100% W_2W_2 — **30**

Table S4. Number of fish selected from experiment 1 (AB and Nadia strains, 28°C *vs*. 36°C) for histological analysis of the gonads

Strain	Temperature (Cº)	Sex	Family	Number of fish	Total number of fish per group
AB	28	male	1	1	
			3	2	3
		female	1	2	
			2	3	
			3	2	7
	36	male	1	1	
			2	2	
			3	2	5
		female	1	1	
			2	2	
			3	1	4
Nadia	28	male	1	3	
			2	2	
			3	2	7
		female	1	3	
			3	3	6
		neomale	1	2	
			2	2	
			3	2	6
	36	male	1	3	
			2	2	
			3	2	7
		neomale	1	2	
			2	3	
			3	4	8

Chapter 5

Family-dependent variation in the multigenerational effects on sex ratios in zebrafish exposed to elevated temperature: changes in the testicular epigenome of unexposed offspring

Family-dependent variation in the multigenerational effects on sex ratios in zebrafish exposed to elevated temperature: changes in the testicular epigenome of unexposed offspring

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Abstract

In many organisms, sex can be determined by a combination of genetic and environmental influences. Recently accumulated evidence shows that changes in environmental conditions can affect population sex ratios through epigenetic regulation of key genes involved in gonadal development. Further, altered sex ratios can persist in the offspring even when the environmental cue is no longer present (a multigenerational effect) and that epigenetic changes tend to be paternally transmitted. However, evidence of transgenerational effects (i.e., beyond the first non-exposed generation) in subsequent generations is scarce and a matter of debate. Here, we used a laboratory strain of zebrafish, where sex determination is dependent on genetic and environmental influences, to study possible multi-and transgenerational effects of exposure to abnormally elevated temperature during the critical period of sex differentiation. To this end, we used eight different families of which we selected five to capture sufficient biological variability in sex ratio at control (28°C) and sex ratio response to elevated (35°C) temperature in the parental (P) generation. Sex ratios were determined at the non-exposed F_1 and F_2 offspring generations from parents kept at 28°C (control) and 35°C (treated). All offspring were raised at 28°C. Global DNA methylation was also assessed in males of the P, F_1 and F_2 generations derived from exposed and non-exposed fathers. Results showed a consistent increase in the number of males in the P generation in all five families but only a persisting male-skewed sex ratio in the 35°C-derived, unexposed offspring of the

F1 generation in two families, denoting family-dependent multigenerational effects. However, no transgenerational effects were observed in any of the families. Likewise, DNA methylation was significantly decreased only in the testis of the 35°C-derived males in the F_1 generation but not of the F_2 generation and, surprisingly, neither in the 35°C-exposed males of the P generation. Taken together, our results show great interfamily variation, not only in sex ratio response to elevated temperature, but also on its multigenerational effects, denoting a strong influence of genetics. Alterations in the testicular epigenome in F_1 males calls for attention to possible, previously-unnoticed effects of temperature in the unexposed offspring of heat-exposed parents in a global warning scenario.

Key words: sex determination, epigenetics, DNA methylation, sex ratio, transgenerational effects, global warming

1. Introduction

Throughout vertebrate evolution, different sex-determining mechanisms have been co-opted (Bachtrog et al., 2014). In vertebrates, sex determination can be controlled by genetic factors (genetic sex determination, GSD), by environmental factors (environmental sex determination, ESD) or a combination of both types (Devlin and Nagahama, 2002; Sarre et al., 2004; Heule et al., 2014). Sex determination produces the sex ratio, defined as the number of males and females in a population (Hardy, 2002). The sex ratio is a key demographic parameter in ecology and evolution since it has direct influence on the reproductive capacity of populations and thus is crucial for species survival (Beissinger and McCullough, 2002). Many environmental cues (among them, temperature) can change the sex ratio set by sex determination. Particularly in lower vertebrates, even in GSD species, environmental influences may thus sometimes override the genetic factors, resulting in a phenotypic sex different from the genotypic sex (Devlin and Nagahama, 2002). Temperature is the most common environmental factor affecting population sex ratio, and exposure to heat in fish usually results in an increase in the number of males (Ospina-Alvarez and Piferrer, 2008; Baroiller et al., 2009; Ribas et al., 2017a). However, there are other factors such as population density, pH and hypoxia that can alter the sex ratio as well (Baroiller et al., 2009). Thus, prolonged changes in population sex ratio as a result of persistent environmental disturbances can have dramatic demographic consequences, threatening population viability (Le Galliard et al., 2005; Zhou et al., 2010; Piferrer, 2016).

Responses to environmental changes are mediated by epigenetic mechanisms, which have the potential to modulate gene expression without changes in the nucleotide sequence (Turner, 2009). Epigenetic changes thus act as organismal responses to cope with new environmental scenarios (Kronholm and Collins, 2016). DNA methylation is the most studied epigenetic mechanism involved in the acquisition of the sexual phenotype during the process of gonadal development, although histone modifications or regulation by microRNAs have been shown to play a role as well (Piferrer, 2019a). Evidence regarding the integration of environmental cues such as temperature through changes in DNA methylation and affecting sexual fate was described for the first time in the European sea bass (*Dicentrarchus labrax*). Elevated temperature during early development hypermethylated the promoter region of the *cyp19a1a* gene (gonadal aromatase) resulting in transcriptional silencing (Navarro-Martín et al., 2011). Aromatase is the sole enzyme involved in the synthesis of estrogen such as estradiol-17ß, and estrogens are essential for ovarian differentiation in all but eutherian vertebrates (Guiguen et al., 2010). Similar results involving *cyp19a1a* and other key genes for sex differentiation have been found in vertebrates susceptible to the effects of temperature, notably reptiles and fish (Piferrer, 2019a). Further, in the European sea bass elevated temperature applied during larval, but not juvenile, development had long-lasting effects on global DNA methylation and affected many key genes involved not only in sex differentiation but also in survival and organ formation (Anastasiadi et al., 2017). However, the extent at which these changes

can persist in the exposed individuals as well as their possible transmission to the offspring is currently a matter of debate (Wang et al., 2017).

Intergenerational epigenetic inheritance represents the transmission of epigenetic marks from one generation to the next (Anway et al., 2005; Jirtle and Skinner, 2007; Skinner, 2008; 2014; Pang et al., 2017). Germ cells can transmit not only genetic, but also epigenetic information (Magnúsdóttir and Surani, 2014). Studies in vertebrates, mainly in mammals, have shown that the genome undergoes two main epigenomic reprogramming events, each of which involves waves of DNA demethylation and *de novo* methylation (Cantone and Fisher, 2013; Seisenberger et al., 2013b). In spite of the extensive DNA demethylation in preimplantation embryonic cells, some of the genomic regions escape this reprogramming process and are transmitted to the newborn individual in methylated form (Borgel et al., 2010; Daxinger and Whitelaw, 2012). This means that DNA methylation changes, induced by environmental stressors in germ cells, may not only have consequences on the exposed individual but can potentially be heritable across generations and cause transgenerational adverse effects (Pacchierotti and Spanò, 2015). During an organism's lifespan, there are critical windows where environmental factors can affect germ cell lineages. The first window occurs in the primordial germ cells (PGCs) which, after fertilization, migrate into the genital ridges to develop future gonads, whereas the second occurs at adult stages during gametogenesis (Jammes et al., 2010; Hales et al., 2011; Seisenberger et al., 2013a).

The typology of the environmental exposures which directly induce epigenetic changes in the germinal line can range from temperature, nutrition and stress, to a large number of endocrine disruptors (Jirtle and Skinner, 2007). Consequently, these induced epigenetic marks in the PGCs can be theoretically inherited across generations either via parental, maternal or both (Heard and Martienssen, 2014). Thus, by virtue of inherited epigenetic patterns, offspring are able to manifest the same phenotype without having been exposed to the same environmental cue as their progenitors (Richards, 2006). In fish, due to ovipary and external fertilization, transgenerational effects can be already observed in the F_2 generation, in contrast to what occurs in mammals, in which transgenerational effects are not observed until F_3 generation if the exposed female happens to be pregnant (Skinner, 2008; Skinner et al., 2010; Nilsson and Skinner, 2015; Hanson and Skinner, 2016). Thus, fish are useful models for studying long-term, transgenerational effects of environmental stressors (Baker et al., 2014; Corrales et al., 2014; Bhandari et al., 2015). In fish, several cases of multigenerational and transgenerational effects due to endocrine disruptors or environmental factors affecting sex development and reproduction have been reported. For example, in medaka (*Oryzias latipes*), exposure to bisphenol A (BPA) and 17α-ethynylestradiol (EE_2) during embryonic development, induced reproductive impairment and compromised the fertilization rate in offspring of the two following generations (Bhandari et al., 2015). In *Oryzias melastigma*, exposure to hypoxia caused transgenerational impairment of several reproduction traits, delayed gonad development along with a diminished sperm count and motility in the F_1 and F_2 (Wang et al., 2016).

Epigenetic inheritance may contribute to adaptation to new environmental conditions. This is beneficial for the survival of the exposed population and future offspring as they are expected to cope with the changing environment (Burggren, 2016), such as acclimation of poikilothermic animals to current climate changes (Munday, 2014; Veilleux et al., 2015). Nevertheless, some of these inherited epigenetic changes could also constitute what is known as an epigenetic trap, i.e., any epigenetic change that arises in response to novel environmental cues that produce maladaptive phenotypes, with no increase in phenotypic variance (Consuegra and Rodríguez López, 2016; Piferrer, 2016). In a climate change scenario, there is an interest in understanding how epigenetic marks are transmitted to subsequent generations and their possible physiological consequences in the new environment (Consuegra and Rodríguez López, 2016; Piferrer, 2016). Recent findings from our laboratory have shown that temperature exposure during sex differentiation was able to alter methylation levels in the promoter regions in a set of reproduction-epigenetic and stress-related genes in adult zebrafish (*Danio rerio*) gonads (Chapter 3). In the present study, we used the zebrafish model to determine if the masculinization observed when a given generation (generation P) was exposed to elevated temperature during the critical sensitive period could be inherited in subsequent generations. To do so, we analyzed population sex ratios and DNA methylation levels not only in the P generation but also in their offspring up to the F_2 generation.

2. Materials and Methods

Fish and husbandry

Zebrafish (AB strain; ZFIN ID: ZDB-GENO-960809-7) from the European Zebrafish Resource Centre (EZRC, Germany) were housed at the experimental facilities of the Institute of Marine Sciences (ICM-CSIC, Barcelona). The housing conditions, the physicochemical water parameters and the feeding regimes have been described elsewhere Ribas et al. (2017b). Eggs were reared in Petri dishes at ~50 eggs per dish filled with embryo medium (pH 7.2 ± 0.5), supplemented with 0.1% Methylene Blue until 6 days post fertilization (dpf). The animal facility used for the present study is licensed by the Bioethical Committee of the Government of Catalonia for animal experimentation with reference code no. 9977.

Experimental design

The study involved three generations: parental or the exposed generation (P), and the subsequent first (F_1) and second (F_2) generations (both unexposed; **Figure 1A**). Eight independent families of the P generation, each consisting of the offspring from a cross between a single male and a single female (i.e., the P_{-1} generation), were used. At 18 dpf, larvae from each family were randomly divided into two groups with equal numbers. In one group, larvae were always raised at control temperature (28°C), while in the other

water temperature was gradually increased at 2ºC/day to 35°C from 18 to 34 dpf, the sensitive period (Ribas et al., 2017b). Thereafter, fish of both groups were raised under the same conditions until they became sexually mature (> 90 dpf). To create the F_1 and F_2 generations, siblings of an unrelated, untreated family (from here onwards external family), raised at 28°C, were mated with fish of the 28 and 35°C groups. Thus, an external male was mated with a female from the 28°C group and then the same male was mated with a different female from the 35°C group. The same principle was used with external females, which were mated first with a male from the 28°C group and then another male from the 35°C group. Thus, the effect different allelic combinations due to the PSD nature of the AB zebrafish and their contribution to variation in offspring sex ratio was hopefully reduced, as the two groups to be compared consisted of half-sibs.

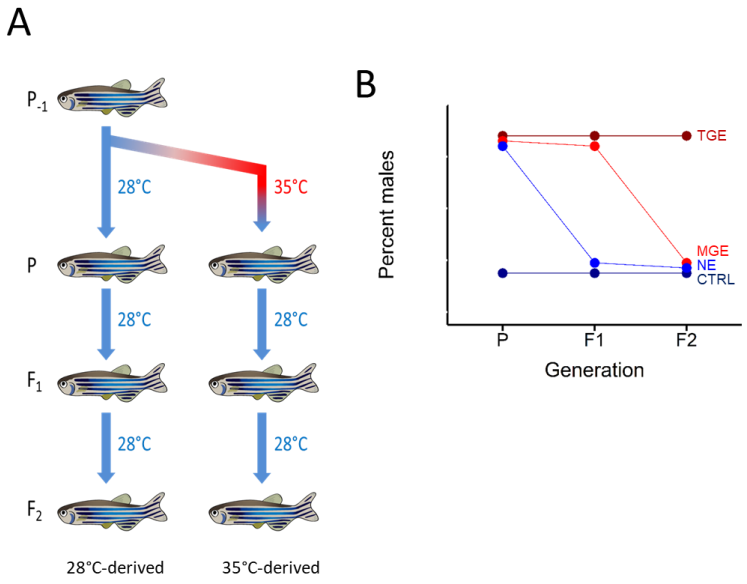

Figure 1. Study of possible transgenerational effects of exposure to elevated temperature during sex differentiation on resulting zebrafish adult sex ratios. (**A**) Experimental design applied in each one of the five families used. An untreated male and female (defined as P_{-1}) were crossed and larvae were divided in two groups. Coinciding with gonadal development of zebrafish (18–34 days post fertilization) one group was exposed to 28°C (control) and the other to 35°C (treated), and constituted the parental (P) generation. Populations of first generation (F_1) and second generation (F_2) were obtained from each temperature treatment using both males and females from the previous generation, which were outcrossed with unrelated, untreated females and males, respectively. Sex ratio were determined when fish were sexually mature (> 90 days post fertilization). (**B**) In families with sex ratio response to temperature as evidenced by an increase in the proportion of males, the following scenarios could be expected, as depicted by lines of different colors: control (CTRL), with no significant departures from the 1:1 sex ratio along the different generations; transgenerational effects (TGE), effects of elevated temperature persist at least until the F_2; multigenerational effects (MGE), with effects seen only at the F_1 but not at the F_2; and no effect (NE).

To obtain the 28°C- and 35°C-derived groups of the F_2 generation the same procedure was applied. All F_1 and F_2 were raised exclusively at 28°C and it was taken that the number of larvae in the tanks, i.e., the rearing density, prior and during the thermal stress and across generations was below the density threshold needed for masculinization, as observed in our previous study (Ribas et al., 2017b). Thus, observed deviations among sex ratios of the 28°C-derived groups was ascribed only to the PSD in zebrafish and deviations of sex ratios in the 35°C-derived groups *vs.* the 28°C ones was due to primarily temperature, not to rearing density.

Sampling

Fish were euthanized by shock hypothermia using ice and water (**Table S1**). Their phenotypic sex was visually checked with the aid of a dissecting scope and the number of males and females were counted to determine the sex ratio. Gonads were dissected out and sexed without discrepancy between the visual and gonad-based sexing, snap frozen with liquid nitrogen to finally stored at –80°C until further analysis.

DNA extraction

Samples were digested overnight at 56°C with buffer containing 1 µg of proteinase K (Sigma-Aldrich, St. Louis, Missouri) to eliminate proteins. Then, the standard phenol-chloroform-isoamyl alcohol protocol (PCI 25:24:1) with 0.5 µg ribonuclease A (PureLink RNase A, Life Technologies, Carlsbad, California) was performed to isolate DNA and eliminate RNAs. The quality and quantity of DNA was measured by a NanoDrop (ND-1000) spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Isolated DNA samples were stored at –20°C until further analysis.

Global DNA methylation quantification assay

For DNA methylation analysis, testes of fish from the 28°C and 35°C groups of the P generation in family #2 were selected. Both for the F_1 and F_2 generations, testes from offspring of a pair of males (28°C and 35°C) mated with the same external female were used. All fish were from family #2. Global DNA methylation was estimated using the 5m-C DNA ELISA Kit (Zymo Research, D5326, USA) according to the manufacturer's instructions. A total of 100 ng of genomic DNA was used for each biological sample with two technical replicates. To quantify the percentage of 5m-C methylated DNA (%5m-C), a standard curve was built and assayed in parallel with samples. The negative and positive controls (0% and 100% methylation, respectively, from *Escherichia coli* DNA) were premixed to generate a 7-datapoint standard curve (each in duplicate) with known %5m-C DNA methylation concentration. Samples were denatured at 98°C for 5 min in a thermal cycler and then transferred immediately to ice for 10 min. All denatured DNA samples were transferred to plate wells, which were then coated with 5m-C coating buffer by incubation at 37°C for 1 h. After washing three times with 200 µl of 5m-C ELISA buffer, wells were incubated at 37°C for 30 min in 200 µl of 5m-C ELISA buffer.

Antibody mix was prepared by diluting anti-5-methylcytosine (1:1000) and horseradish peroxidase Developer (HRP, secondary antibody) (1:1000) in 5m-C ELISA buffer. A total of 100 µl antibody mix was added to each well and incubated at 37°C for 1 h. After washing three times with 200 µl of 5m-C ELISA buffer, 100 µl of HRP Developer was added to each well and then incubated at room temperature for 60 min. Absorbance at 405 nm was measured using an Infinite M200 PRO microplate reader (Tecan, Männedorf, Switzerland). To determine %5m-C of the biological samples, a logarithmic second-order regression was generated with the relation of %5m-C and absorbance from the standard curve using the function $e^{\frac{(Absorbance - y_{intercept})}{Slope}}$. Percent 5m-C values were corrected with the zebrafish CpG density according to the manufacturer instructions. To obtain the most accurate values, the latest zebrafish genome was downloaded (GRCz11; Assembly accessions: GCA_000002035.4, version 98.1) from Ensemble (www.ensembl.org). We extracted the length of the genome (L) = 1,674,207,132 bp, and we calculated the total numbers of cytosine (C) and the number of CpG dinucleotides (CG) using the command: "grep -v '>' zebrafish_genome.fa | grep -o -i 'X' | wc –l", (where X is for 'C' and then for 'CG', respectively) in Linux operative system (Ubuntu v. 16.04.6 LTS). Once we obtained the total number of C = 306,412,859 and CG = 29,220,867 from the zebrafish genome, we calculated the fold difference of CpG density (total CG genome / L) between the genomes of (*E. coli / D. rerio*) = (0.07472 / 0.0175) = 4.2811. Finally, to obtain the global methylation values, the percent 5m-C/CpG density values were multiplied by the value obtained from the total number of C/ L zebrafish = 0.1830.

Statistical analysis

The sex ratio at 28°C and the sex ratio response to temperature in the P generation was analyzed separately in each one of the eight tested families with the χ^2 test with Yates' correction (Yates, 1934). A two-way ANOVA was used with family and temperature as independent factors and the sex ratio as the dependent variable to analyze the contribution of family and temperature in the sex ratio of the parental generation in the five selected families. A Linear Mixed Model (LMM) was used (West et al., 2014) with generation and temperature as independent factors and the sex ratio as the dependent variable to analyze the contribution of generation and temperature in the sex ratio of the parental, F_1 and F_2 generations in the five selected families. Because the number of fish available for sex ratio analysis varied between families and generations weighed averages were used for ANOVA and LMM. The Student's t-test was used to compare the global DNA methylation levels in the testes of males exposed at 28°C and 35°C in each generation. Values were logit transformed prior analyses. Two outliers, as assessed with a 95% confidence test, were removed (one from the F1 and another from the F2 generation data). All statistical analyses were carried out using R software (v. 3.6.1) (Team, 2013). Significant differences were considered when $P < 0.05$. All graphs were generated using the *ggplot2* package (v. 3.2.1) (Wickham, 2011).

3. Results

Sex ratio and sex ratio response to temperature in the P generation was family dependent
Sex ratio in the P generation of families 1 through 8 raised at 28°C varied greatly, ranging from 18 to 72% males in accordance to PSD in the zebrafish AB strain (Ribas et al., 2017b). When siblings from the same families were subjected to 35°C, the number of males increased in all families but only in five showed a significant increase (χ^2-test: families #2 and #7 $P < 0.05$; families #3 and #8 $P < 0.01$; and family #5 $P < 0.001$) (**Figure S1A**). Thus, when considering the eight families together, a temperature-dependent significant increase (t-student; $P < 0.01$) in the number of males was observed, as expected (**Figure S1B**).

There was a significant overall relationship (Adjusted R^2=0.7419; $P < 0.00371$) between the number of males at 28°C and at 35°C across the eight families, meaning that the higher number of males at 28°C meant a higher number of males at 35°C (**Figure S2A**). However, the actual increase in the number of males due to temperature varied between ~15% and ~45%, but was independent of the number of males at 28°C, as evidenced by the lack of correlation, and with an average increase between 25 and 30% (**Figure S2B**).

In order to capture sufficient biological variation in sex ratio at 28°C, sex ratio response to elevated temperature and, importantly, also considering the number of available fish for experimentation, we selected five families out of the initial eight: families #1 and #2 (female-skewed initial sex ratio) and families #4, #6 and #7 (male-skewed initial sex ratio; **Figure S1C**) to study possible transgenerational effects. Family #5, for example, despite being highly influenced by temperature could not be selected because there were no females at 35°C (**Table S1**) and thus no female-derived F_1 generation could be created. Considering the five selected families together, two-way ANOVA showed that the sex ratio in the P generation was significantly influenced by both genetics (i.e., the different families) and environment (i.e., temperature; **Table 1**). This reflected the variability typically associated with sex ratio in the AB laboratory strain of zebrafish, confirms previous observations (Ribas et al., 2017a)and as such it was suitable for the study of possible transgenerational effects (up to the F_2 generation) of elevated temperature in the P generation.

Table 1. The contribution of family, temperature and the interaction of both factors in the sex ratio of the parental generation in the five selected families

		d.f.	SS	F-value	*P*-value
Factors	Family	4	6463	38.275	**0.000608**
	Temperature	1	2122	50.276	**0.000864**
Interaction	Family x Temperature	4	229	1.354	0.367314
	Residuals	5	211		

A two-way ANOVA was used with family and temperature as independent factors and the sex ratio as the dependent variable. Statistically significant differences are shown in bold. (Abbreviations: d.f., degrees of freedom; SS, Sum of Squares).

Sex ratio in the F_1 and F_2 generations was also family-dependent without consistent effects

The sex ratios of the five selected families in the F_1 and F_2 generations in addition to the P generation varied considerably (**Figure 2)**. Except for family #1, which was highly female-biased (**Figure 2A**), and regardless of the number of males in the 35°C groups of the different families in the P generation, the sex ratio of the different families tended to equilibrium along the F_1 and F_2 generations (**Figure 2B**, **C**, **D** and **E**). This, without exception, was particularly evident in all 28°C-derived groups, since the sex ratio was close to 1:1 in the F_2 (blue lines in **Figure 2**). However, when analyzing the contribution of generation and temperature in the sex ratio of the P, F_1 and F_2 generations in these families, LMM results showed a significant effect of temperature ($P < 0.05$) but not of the generation (**Table 2**), indicating that, despite the increase in the number of males in the P generation (ANOVA, **Table 1**), male-skewed sex ratios were not globally maintained in the subsequent generations.

Table 2. The contribution of generation and temperature and interaction of both factors in the sex ratio of the parental, F_1 and F_2 generations in the five selected families

		d.f	SS	F-value	*P*-value
Factors	Generation	1	76	0.19	0.6645
	Temperature	2	2798	3.503	**0.0371**
Interaction	Generation x Temperature	2	2265	2.836	0.0674
	Residuals	54	21567		

A Linear Mixed Model (LMM) was used with generation and temperature as independent factors and the sex ratio as the dependent variable. Statistically significant differences are shown in bold. (Abbreviations: d.f., degrees of freedom; SS, Sums of Squares)

However, these overall results could be influenced by the great variability of the sex ratios in the 28°C groups of the P generation (18-72% males). These extreme values coincided with family #1 (18% males at 28°C in the P generation), which showed a difference from the other 35°C-derived groups at the subsequent generations since sex ratios kept departing away from 1:1 (**Figure 2A**). The other extreme value (72% males at 28°C in the P generation) corresponded to family #7, in which we had no data in the 35°C-derived groups in the F2 generation due to poor viability of the offspring (**Figure 2E**). Thus, in order to determine which one of the three possibilities (namely, no effect, multigenerational effect or transgenerational effect; **Figure1B**) was actually taking place, the weighted average of the sex ratios of families #2, #4 and #6 at each generation and for both the 28°C- and 35°C-derived lines were calculated in order to account for differences in sample size. The proportion of males was 57.7% in the 28°C derived groups and 72.8% at 35°C derived groups in the F_1 generation, suggesting multigenerational effects. Sex ratios of both groups were similar and close to 50% in the F2, so no transgenerational effects were present (**Figure 2F**). Significant differences in the sex ratio at the F_1 generation between the 28°C-and 35°C- derived groups was only found in family #2 (**Figure 2B**). Therefore, only this family was used for global DNA methylation analysis. We selected only the male offspring.

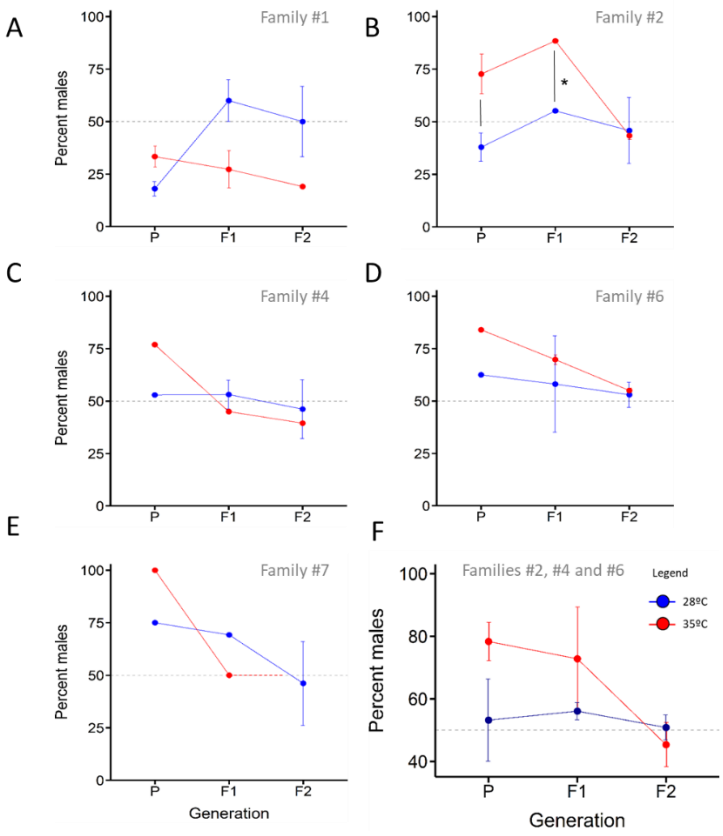

Figure 2. Sex ratios in the parental (P), first (F_1) and second (F_2) generations according to temperature experienced in the P generation in the five selected families: **A)** family #1; **B**) family #2; **C)** family #4; **D**) family #6; **E)** family #7; **F)** combined data from families #2, #4 and #6; **G**) Observed sex ratios along the P, F_1 and F_2 generations in the control and treated lines. Each datapoint is the weighted mean ± SEM of percent males of families #2, #4 and #6. Families are arranged according to increasing proportion of males at 28°C. Data as mean ± S.E.M.

Global DNA methylation

When testicular global DNA methylation levels across generations for the 28°C- and 35°C-derived groups in family #2 were tested, we found no significant differences in global methylation levels in the P generation (**Figure 3**.). In contrast, significant differences (*t*-test, $P < 0.001$) in methylation levels in the F_1 in testes of the 35°C-derived group when compared to testes of the 28°C-derived group were observed. Differences were lost in the F_2 generation.

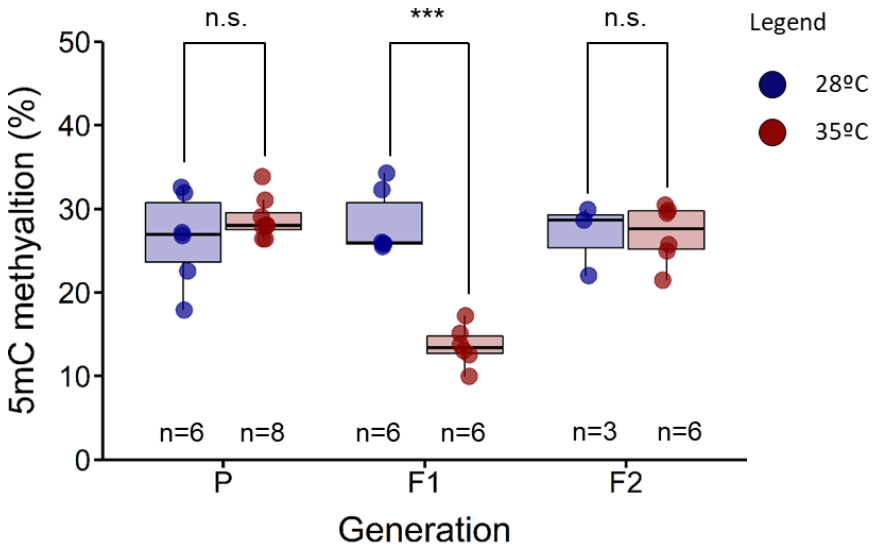

Figure 3. Global DNA methylation in testis of adult (> 90 days post fertilization) zebrafish (*Danio rerio*) in the parental (P), first (F_1) and second (F_2) generations of family #2. Half of the P generation was kept at control temperature (28°C), whereas the other half was exposed to elevated temperature (35°C). Controls and their offspring (28°C-derived) are shown on the left (light blue), whereas heat-exposed and their offspring (36°C-derived) are on the right (pink) at every generation. In the F_1 and F_2 generations all fish were grown at 28ºC. Means were compared with the Student's *t*-test. In the boxplot, each datapoint corresponds to an individual sample, and the thick line represents the median. Abbreviations: n = sample size; n.s. = not significant; *** = $P < 0.001$.

4. Discussion

Our results confirm the wide variation in sex ratio when zebrafish of the AB strain were reared at the control temperature of 28°C, as previously described (Liew et al., 2012; Ribas et al., 2017a). Our results also confirm that heat-induced masculinization in zebrafish is family-dependent (Ribas et al., 2017b). Thus, population sex ratio in the laboratory AB strain of zebrafish, which lost the sex-determining gene during the process of domestication (Wilson et al., 2014), reflects the polygenic nature of sex determination, where sex depends primarily on genetic factors, but can be overridden by environmental factors.

To the best of our knowledge, this is the first study in zebrafish examining possible multi- or transgenerational effects of elevated temperature on population sex ratio. Analysis of the sex ratios across the generations (F_1 and F_2) derived from the 35°C-exposed P generation showed that population sex ratio tended towards 1:1 (male:female), with the exception of family #1, as one would expect if frequency-dependent selection was taking place. Our results regarding the male-skewed sex ratios of the 35°C-derived groups in the F_1 are consistent with the presence of multigenerational effects when only the three tested families (#2, #4 and #6) that initially did not depart too far from 1:1 at 28°C in the P generation are considered, but not when all five families are taken into account (**Fig. 2**). However, we could not find any significant transgenerational effect on the sex ratio either globally or in any individual family. Our results with the male-skewed sex ratio in the F_1 of 35°C-derived groups of families #2, #4 and #6 are in agreement with results obtained in the half-smooth tongue sole (*Cynoglossus semilaevis*), having a ZZ/ZW system, where male-skewed sex ratios when compared to control populations were found in the F_1 generation derived from heat-exposed parents (Shao et al., 2014). These observations were taken as evidence for the presence of multigenerational inheritance of the masculinizing effect induced by high temperatures (Shao et al., 2014). Our results also resemble those obtained with the Atlantic silverside (*Menidia menida*), a GSD-ESD species, in the sense that progressively balanced sex ratios were obtained in the subsequent generation after the environmental effect (Conover et al., 1992). Masculinizing effects of high temperature were gradually reduced from one generation until Fisherian sex ratios were obtained after 8-10 generations (Conover et al., 1992), suggesting that balanced sex ratios evolve by frequency-dependent selection of the minority sex (Fisher, 1930), and thus a balanced sex ratio constitutes an evolutionary stable strategy (Smith and Maynard-Smith, 1978).

In fish, evidences of transgenerational effects caused by exposition elevated temperature mostly concern metabolic scope rates (Donelson et al., 2012), aerobic capacity and acclimation (Veilleux et al., 2015; Ryu et al., 2018), and growth (Salinas and Munch, 2012; Shama et al., 2014) but not in the resulting sex ratio. In fact, transgenerational effects on sex ratios and reproductive capacity have been mostly investigated after exposure to endocrine disrupting chemicals (EDCs), not temperature. This is the case of bisphenol-A (BPA) exposure of zebrafish that led to epigenetic changes affecting expression of genes involved in reproduction and caused a reduction of fertilization rates (Laing et al., 2016; Santangeli et al., 2019). Similar results were observed in the F_2 and F_3 generations in medaka and in fathead minnows (*Pimephales promelas*) exposed to EDCs (Schwindt et al., 2014; Bhandari et al., 2015), although possible mutagenic effects of BPA and not strictly transgenerational effects should not be discarded (Jalal et al., 2018). When fertilized zebrafish embryos were exposed to 5-azacytidine (5AC) until 6 dpf, a significant shift towards male bias in the F_1 generation was found when compared to the exposed F_0 population (Kamstra et al., 2017). However, female-biased sex ratios were obtained after treatment of young zebrafish with a 5-azadeoxycytidine, DNA-methyltransferase inhibitor (Ribas et al., 2017d).

Although we did not find transgenerational effects on sex ratios, we tested whether exposure to elevated temperature could lead to DNA methylation changes. Since previous studies showed that epigenetic changes are transmitted mostly through the sperm (Bogdanović et al., 2011; Jiang et al., 2013; Potok et al., 2013) – but see also Santangeli et al. (2019) – we examined the testes of males of the P, F_1 and F_2 generations for both the 28°C- and the 35°C-derived groups through the father. Interestingly, no global DNA methylation differences were observed in fish from the P generation. These results are similar to those found in threespine stickleback (*Gasterosteus aculeatus*), where exposure to elevated temperature during embryonic development did not change global methylation in adult testes from males of the same exposed generation (Metzguer and Shutle 2017). It should be noted that germ cells of the testes in the exposed P generation are diluted among somatic cells and this could explain why there were no differences in global DNA methylation in the testes. In contrast, the testes of the F_1 males from the 35°C-derived groups exhibited significantly lower DNA methylation levels when compared to 28°C-derived groups. Albeit these fish never experienced any thermal episode, it appears that they inherited the effects from the exposed father.

In mammals, epigenetic reprogramming of PGCs occurs in two sequential steps, the first during PGC expansion and migration, and the second upon entry of PGCs into the gonads (Sasaki and Matsui, 2008; Seisenberger et al., 2013a). During epigenetic reprogramming, there is an epigenetic state for totipotency in which most of the environmentally-induced epigenetic changes are erased (Santos et al., 2002; Surani et al., 2007; Seisenberger et al., 2012). Unlike mammals, and in contrast to what occurs in medaka (Wang and Bhandari, 2019), studies in zebrafish post-fertilization embryos suggested that their genome does not undergo genome-wide DNA demethylation and, consequently, a global erasure is absent (Bogdanović et al., 2011; Jiang et al., 2013; Potok et al., 2013). In addition, in zebrafish embryos, global DNA methylation is largely preserved during germline differentiation, where the paternal methylome is preserved (Ortega-Recalde et al., 2019; Skvortsova et al., 2019). Thus, along the zebrafish lifespan, global methylation levels in PGCs at early stages showed similar values as the somatic cells, suggesting that the putative epigenetic changes acquired are maintained (Ortega-Recalde et al., 2019). In our experiments, we exposed zebrafish larvae to high temperature during gonadal development (18 to 34 dpf), i.e., past the period when the highest number of PGCs are found (6–14 dpf) (Uchida et al., 2002; Orban et al., 2009; Tzung et al., 2015). Consequently, only a few PGCs were still present in the differentiation gonads and, in contrast, spermatogonia in the of males would have been mostly affected by temperature. Thus, these cells and their derived stages would be responsible to transmit the epigenetic modifications in the F_1generation. Thus global hypomethylation found in the testes of 35°C-derived males in the F_1 generation could be explained by taking into account that F_1 fish are in fact derived from the presumably affected germ cells of the P generation. Further studies, targeting different periods, e.g., germ cell differentiation, gonad formation, sexual differentiation and gametogenesis, should be carried out to determine sensitive windows in which external perturbations affect the germline with potential transmission to the following generations.

5. Conclusions

In this study carried out using the laboratory AB strain of zebrafish, we confirm a genetic and environmental component in sex ratios and great interfamily variation in response to elevated temperature. We provide evidence suggestive of multigenerational effects of elevated temperature in sex ratios. However, we could not demonstrate effects beyond the first non-exposed generation, i.e., in the F_2 generation. Interestingly, although no changes in global DNA methylation in the testis of exposed fish was observed, a clear hypomethylation in the testes of the offspring of exposed fathers was recorded. This calls attention to possible effects of high temperature in fishes that was previously unnoticed. These aspects deserve further study for a better understanding of the effect of global changes in natural populations.

Acknowledgments

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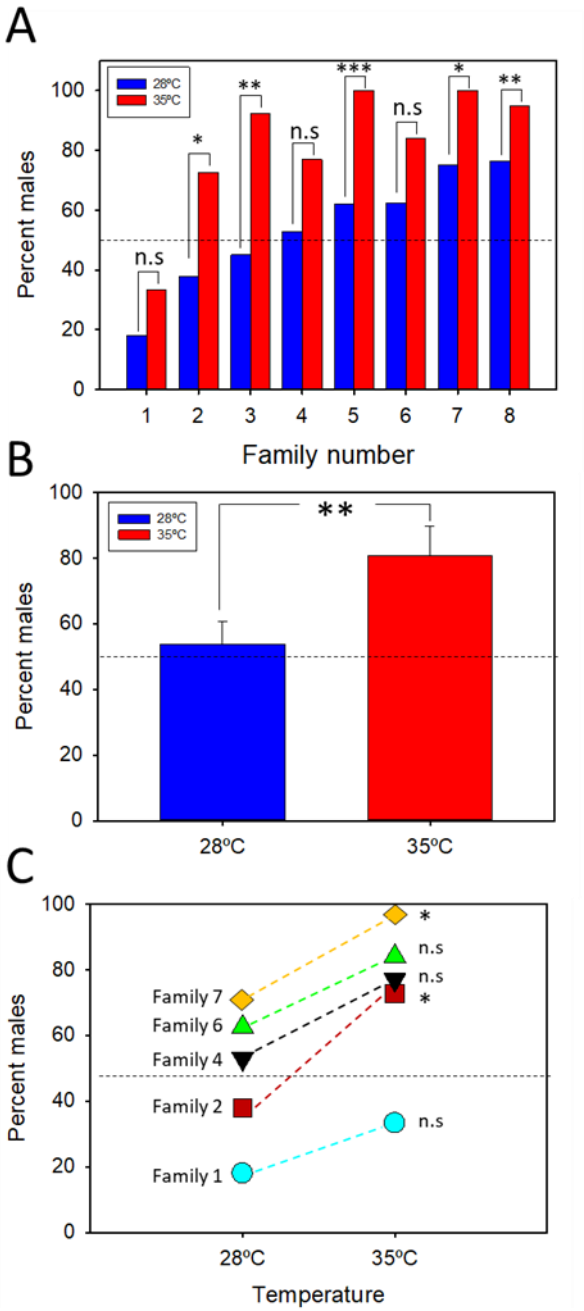
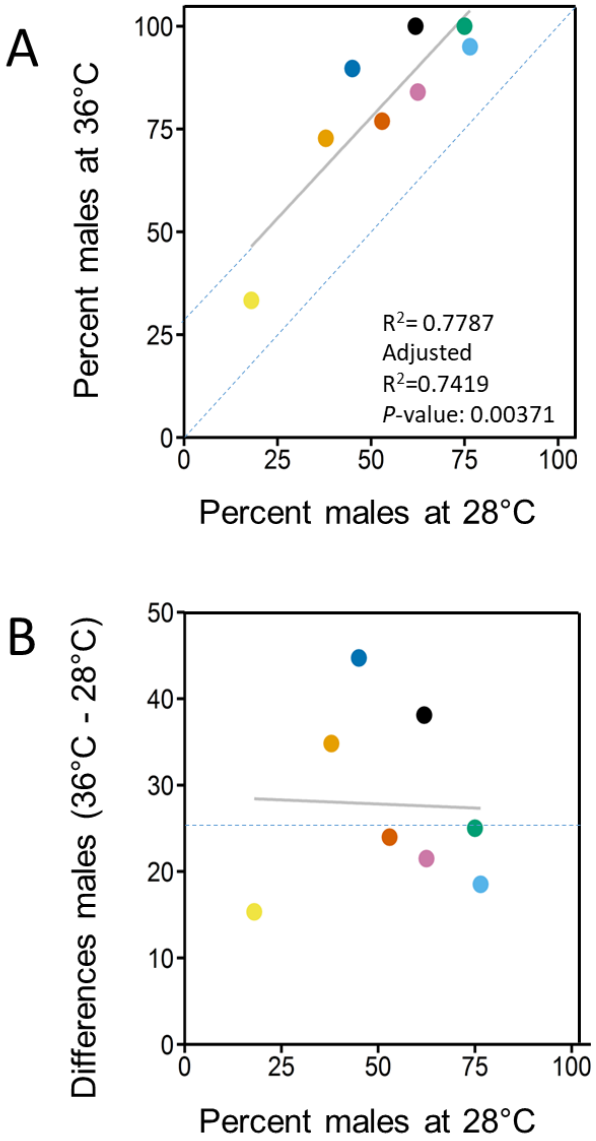
Supplementary Figures



Supplementary figure 1. Sex ratio response to temperature in zebrafish. **A**) Sex ratio (percent males) in the eight initially tested families (families #1–8) at control (28°C) and elevated (35°C) temperature. Larvae were exposed during the period of 18–34 days post fertilization. Families are arranged and numbered according to increasing amount of males at 28°C. **B**) Average sex ratio at normal and elevated temperature of the eight families. Data as mean ± S.E.M. **C**) Reaction norms of the five families (families 1, 2, 4, 6 and 7) selected to study possible transgenerational inheritance of effects of elevated temperature. The *t*-student test was used. Abbreviations: n.s. = no significant; * = $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$.



Supplementary figure 2. Sex ratio variation in zebrafish. **A)** Relationship between sex ratio at control (28°C) and elevated (35°C) temperature. **B**) Relationship between sex ratio at 28°C and the differential of change at 35°C. Each datapoint represents one family.

Supplementary Tables

Family	Generation	Temp. (°C) (P – F_1 – F_2)	Replicate	Cross came from	Percent males	Number of males	Number of females	Total fish per tank	Total fish per family
1	P	28	1		21.74	5	18	23	
			2		14.81	4	23	27	
		35	1		37.50	6	10	16	
			2		27.27	3	8	11	
	F_1	28-28	1	male	50.00	5	5	10	
			1	female	70.00	7	3	10	
		35-28	1	male	33.33	5	10	15	
			1	female	14.29	1	6	7	
	F_2	28-28-28	1	male	83.33	5	1	6	
			1	female	45.00	9	11	20	
			2	female	43.75	7	9	16	
		35-28-28	1	male	NA	NA	NA	NA	
			1	female	19.05	4	17	21	182
2	P	28	1		33.33	5	10	15	
			2		42.86	6	8	14	
		35	1		66.67	8	4	12	
			2		80.00	8	2	10	
	F_1	28-28	1	male	90.00	9	1	10	
			1	female	55.56	10	8	18	
			2	female	54.55	6	5	11	
		35-28	1	male	37.50	3	5	8	
			1	female	96.30	26	1	27	

			2	female	100.00	17	0	17	
	F_2	28-28-28	1	male	52.94	9	8	17	
			1	female	28.57	2	5	7	
		35-28-28	1	male	41.67	10	14	24	
			2	male	42.86	12	16	28	
			3	male	45.16	14	17	31	
			1	female	NA	NA	NA	NA	249
4	P	28	1		52.94	9	8	17	
		35	1		76.92	10	3	13	
	F_1	28-28	1	male	55.56	10	8	18	
			1	female	58.33	14	10	24	
			2	female	45.45	10	12	22	
		35-28	1	male	NA	NA	NA	NA	
			1	female	45.00	9	11	20	
	F_2	28-28-28	1	male	25.00	1	3	4	
			1	female	55.56	5	4	9	
		35-28-28	1	male	NA	NA	NA	NA	
			1	female	40.00	8	12	20	
			2	female	38.89	7	11	18	165
6	P	28	1		61.90	13	8	21	
			2		62.96	17	10	27	
		35	1		84.00	21	4	25	
	F1	28-28	1	male	40.74	11	16	27	
			2	male	37.04	10	17	27	
			1	female	75.00	18	6	24	
			2	female	81.48	22	5	27	

		35-28	1	male	68.42	13	6	19	
			2	male	70.59	12	5	17	
			1	female	67.86	19	9	28	
			2	female	72.73	16	6	22	
	F2	28-28-28	1	male	58.62	17	12	29	
			2	male	52.38	11	10	21	
			1	female	44.44	8	10	18	
			2	female	53.33	8	7	15	
		35-28-28	1	male	NA	NA	NA	NA	
			1	female	56.00	14	11	25	
			2	female	53.33	8	7	15	387
7	P	28	1		75.00	15	5	20	
		35	1		100.00	22	0	22	
	F1	28-28	1	male	100.00	3	0	3	
			1	female	69.23	9	4	13	
		35-28	1	male	50.00	7	7	14	
			1	female	NA	NA	NA	NA	
	F2	28-28-28	1	male	25.00	1	3	4	
			1	female	55.56	5	4	9	
		35-28-28	1	male	NA	NA	NA	NA	
			1	female	NA	NA	NA	NA	85

General discussion

The objective of this thesis was to study the effects of environmental factors on zebrafish during sex differentiation and the implication of DNA methylation in shaping the resulting sexual phenotype. To that end, different experiments were carried out. The results of these experiments have been discussed in detail in the corresponding preceding chapters. However, there might be more to be learn when experiments are contemplated together rather than separately. This is the goal of this general discussion.

1. Importance of family variation in zebrafish

Liew et al. (2012), when experimenting with laboratory zebrafish strains, proposed that genetic variation (G) was the main component explaining sex ratio variation among families but recognized also the influence of the environment (E). This situation was compatible with polygenic sex determination (PSD) in these laboratory strains. These results were confirmed with further experiments involving the study of the effects of elevated temperature during sex differentiation, evidencing G×E interactions and thus different rates of masculinization among families (Ribas et al., 2017a). This further confirmed PSD in laboratory strains of zebrafish (Liew et al., 2012; Ribas et al., 2017a).

1.1 Rearing density

In experiment 1 of *chapter 1*, four different pairs were bred to account for biological variation. Unfortunately, not all families could be tested in the four density treatments (Ribas et al., 2017b). For instance, the offspring from family #1 were tested only at the two lower density whereas family #3 was the only one that was tested at the four density treatments. In addition, although the sex ratio results of family #3 gave a high number of males (≥ 60%) in all density treatments tested, a significant increment of males was only observed in the two highest density treatments. Based on this scenario, it would be difficult to draw conclusions about the existence of interfamily variation in sex ratios in response to elevated density, as pointed out by Delomas and Dabrowski (2017).

From previous results on zebrafish with temperature in our lab (Ribas et al., 2017a), the importance of genetic variation in response to sex ratio was acknowledged. However, carrying out experiments to test whether density effects depend on family-variation in zebrafish come with biological limitations. The main one was the amount of eggs required to test different rearing densities starting with 25, 50, 100 and 200 fish per tank with at least two technical replicates for an individual family. That means that the minimum number of fertilized eggs required per family was 750. The fecundity of zebrafish females is typically around 200–300 eggs (Nasiadka and Clark, 2012; Ribas and Piferrer, 2014) and this creates an experimental problem, because it is very difficult to test the four rearing densities as proposed in a single batch of eggs. We could have pooled the eggs from different families but then we would have lost the individual genetic component.

For this reason, to better determine the effect of genetic variation in the masculinizing response to elevated rearing density a new experiment was carried out. In this case, in order to avoid the drawbacks of not having enough eggs, only the two extreme density treatments were considered: 11 and 40 fish/liter density, i.e., low (LD) and high density (HD), respectively, during the 18–45 days post fertilization (dpf) period. Larvae of 6 dpf from five independent families were equally represented in both treatments with two technical replicates per treatment. The results showed that all the families tended to increase the number of males with density although in different amounts, confirming G×E interactions (**Figure 1**). These new data, along with the data of *chapter 1*, clearly illustrates that a rearing density of 40 fish/liter results in masculinization in laboratory zebrafish strains (Ribas et al., 2017b; c).

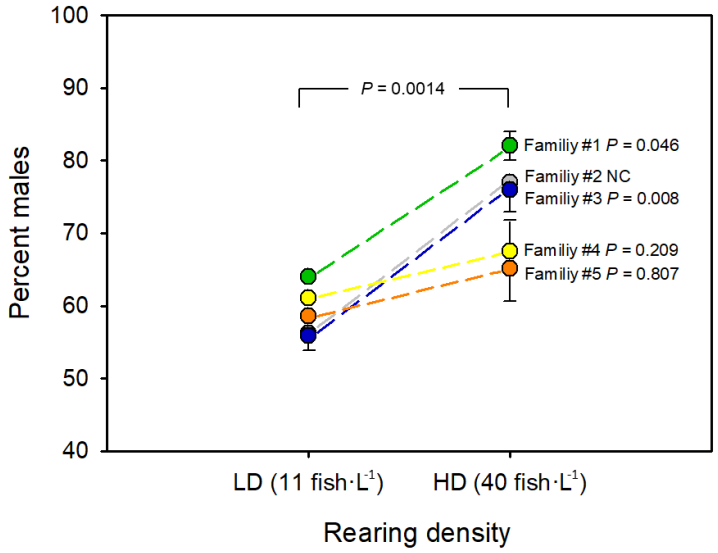


Figure 1. Genotype-dependent sex ratio response of five different zebrafish families (AB strain) as a function of rearing density: low (LD) and high density (HD) during the sex differentiation (18–45) days post-fertilization. Data as mean ± s.e.m. of two technical replicates for each family/density combination. The χ^2 test was applied between treatments for each family. Error bars that are similar in size or smaller than the datapoints are not shown. Abbreviations: NC, significance level not computed owing to insufficient sample size (Ribas et al., 2017c).

1.2 Sex ratio: density and temperature in different strains of zebrafish

Until recently, most of the studies on zebrafish sex ratio responses to environmental perturbations such as temperature (Ribas et al., 2017a), density (Ribas et al., 2017b; c) and hypoxia (Shang et al., 2006) were carried out using the AB strain. In this thesis, most of the results were also obtained using this strain (*chapter 1*, *2*, *3* and *5*), but in *chapter 4* two wild strains were also studied. Using different strains has been recognized as an aid to get a better picture of the interplay between genetics and environment (van den Bos et al., 2017). The goal of this section is to analyze how different strains of the same species respond to environmental pressure either density or temperature. For this reason, a

comparison between AB strain and the two wild zebrafish strains with the two environmental factors are discussed (*chapter 1*, *3*, *4* and *5*).

To perform this analysis, sex ratios were taken from all the 27 families used and sorted from the lowest to the highest percent of males, arranged by environmental factor (density and temperature), treatment (low and high) and strain (AB, Nadia and EkkWill). The chapters were the data come from and the sex determining mechanism is also indicated (**Figure 2**).

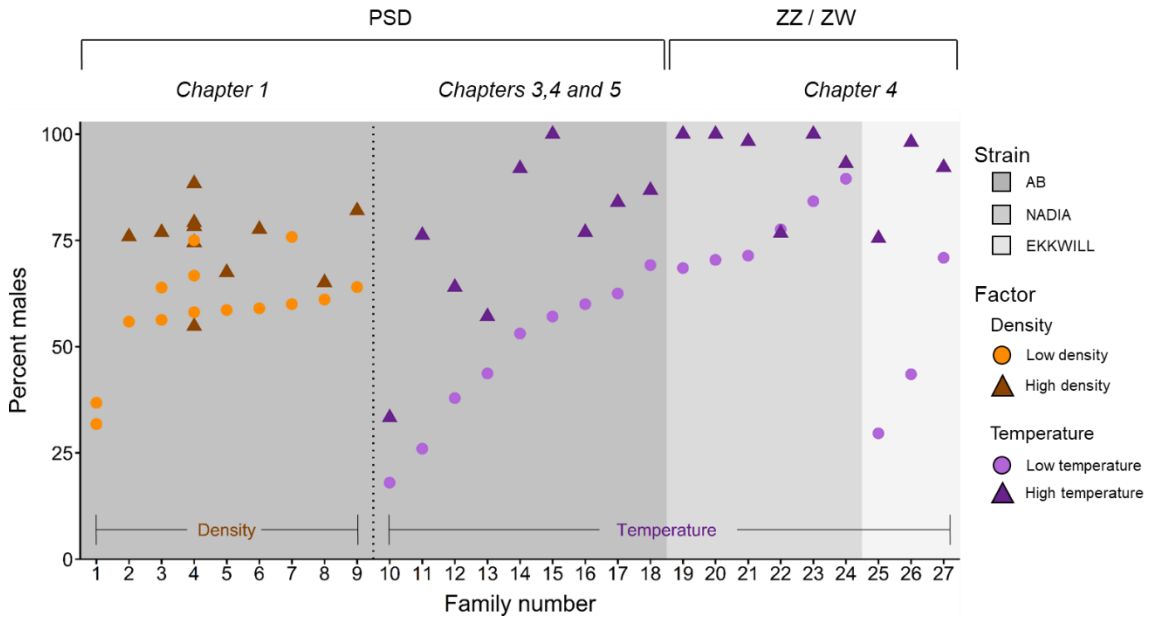


Figure 2. Genotype-dependent variation of the sex ratio response to either high density (orange) or elevated temperature (purple) from the 27 families used in this thesis. Polygenic sex determination (PSD).

This figure clearly shows, regardless of sex determination system and strain, a wide variation in sex ratios among families at control conditions in accordance to what had been previously described in Liew et al. (2012). Interestingly, in the Nadia strain, despite possessing *sar4*, a strong variation of the sex ratio among the six families was also observed (families # 19–24) despite having a ZZ /ZW system (Wilson et al., 2014), with a higher amount of males than the expected, also due to its ZZ/ZW system.

A second aspect worth mentioning is the difference in the masculinization response elicited between density and temperature. Thus, while none of the families subjected to the highest density produced a sex ratio with >90% males, in contrast, elevated temperature resulted in full or almost full masculinization in several families (families # 15, 19–21, 23 and 26). These results could be interpreted as that elevated temperature has a stronger masculinization effect than elevated density (but see below). This was also observed in the comparison of the ovarian transcriptomes exposed to density and temperature (*chapter 2*). A relationship between the degree of masculinization from the two environmental factors together with the average fold-change of the expression of certain genes in the ovarian transcriptome were observed (see **figures 5** and **6** in *chapter*

2). The main difference between the impact of these two factors could be explained by physiology since fish are poikilotherms and, consequently, one could expect that they are naturally vulnerable to abnormally elevated temperature. This, in turn, affects growth by changes in metabolic and physiological aspects (Cech and Moyle, 2000). For instance, in the European sea bass, elevated temperature induced masculinization (Saillant et al., 2002) whereas elevated density had no effect (Saillant et al., 2003b). In our case, both factors clearly masculinize populations but we cannot confirm whether zebrafish are inherently more sensitive to elevated temperature or elevated rearing density because further dose–response experiments should be carefully performed. Further, the periods in which the two factors were studied were not the same. In temperature was 18–32 dpf (Ribas et al., 2017a) and density was 6–90 dpf and 18–45 dpf (Ribas et al., 2017b; c).

In order to shed light on this issue, we propose the following experiment to determine if the effects of temperature have more incidence than the effects of density in masculinization. To this end, we proposed using a pool from different batches of larvae from different families with dose-response of different densities and temperatures treatments in a fixed period of the gonadal development should be used. From our experience, the most convenience period is 15 to 35 dpf, because it is sensitive period to temperature (Ribas et al., 2017a) and density (Moraleda-Prados et al., unpublished). In addition, larvae zebrafish at the age of 15 dpf have better survival rates than earlier stages of life. Performing these experiments by using exactly the same families, we would elucidate whether elevated density reaches the same percentage of masculinization than temperature and determine the LD_{50} for each factor.

2. Ovarian transcriptomic plasticity in zebrafish

Among all the organs in a fish, no other organ displays the plasticity of the gonad in response to environmental cues. Also, the gonad shows extreme regeneration/transformation capability as found in hermaphrodites, in which the function and structure of the gonad of one sex can degenerate and reform as a functional gonad of the opposite sex (DeFalco and Capel, 2009). Hermaphroditism is classified into two types: simultaneous and sequential (Avise, 2011). In sequential hermaphrodites, some individuals are able to produce first one type of gametes and then able to change to the opposite sex during their life cycle. This type includes protogynous (female to male), protandrous (male to female) and bidirectional sex changers (Devlin and Nagahama, 2002; Kobayashi et al., 2013; Todd et al., 2016; Liu et al., 2017a; Pla, 2019). Thus, in sequential hermaphrodites there is a genetic program in which the genes specific or differentially-expressed in one sex must be repressed to promote the expression of genes specific or differentially expressed in the opposite sex.

To understand the control of gene expression, it is important to link the relationship between genotype and phenotype. Ovaries and testes are structurally and functionally analogous organs originated from the same gonadal primordium. In this Ph.D. thesis, we

focused mainly on the expression of genes involved in sex determination and differentiation in the zebrafish gonads to understand how the environment affect the sex during their gonadal development (*chapter 2 and 3*).

Many studies have compared mature ovaries and testes at the transcriptomic level in gonochoristic species such as the half-smooth tongue sole (Wang et al., 2019b), Nile tilapia (Sun et al., 2018), olive flounder (Zhang et al., 2016), zebrafish (Ribas et al., 2017a), turbot (*Scophthalmus maximus*) (Hu et al., 2015; Ma et al., 2016a; Ribas et al., 2016); and also in hermaphrodite species such as the bluehead wrasse (*Thalassoma bifasciatum*) (Liu et al., 2015; Todd et al., 2019), seabream (*Diplodus puntazzo*) (Manousaki et al., 2014), pandora (*Pagellus erythrinus*) and the red porgy (*Pagrus pagrus*) (Tsakogiannis et al., 2018). In these studies, and regardless of the reproductive strategy of the species being examined, testes and ovaries had very specific and distinct gene expression patterns. Taking into account this information and based on our results (*chapter 2*), we would like to introduce the concept of "*Gonad transcriptomic difference*", defined as the difference between the identity of genes that are significantly up- or down-regulated in a specific gonadal type when compared to another sexual phenotype. This difference is maximum between control testis and control ovaries. A diagram of this concept is presented (**Figure 3**).

When we compared ovarian transcriptomes of laboratory zebrafish strain from high-*vs.* low-density treatments of experiment 1 (*chapter 2*), we observed that ovaries from females subjected to elevated density expressed a set of upregulated "male" genes when compared to the ovaries of control females. This was also described with females exposed to temperature (*chapter 2*), and previously referred to as "superfemales" (Ribas et al., 2017a). These heat-treated females, here better defined as "pseudofemales" ("superfemales", as used in ZZ/ZW systems would mean WW females), although having ovaries, were transcriptomically similar to regular males and neomales but different from regular females. Therefore, the gonads of these pseudofemales would be placed midway between the ovaries and testes (**Figure 3)**. In contrast, neomales, i.e., masculinized females, showed a similar number of DEG with respect to regular females than regular males and thus neomales and males were essentially transcriptomically identical, confirming previous observations (Ribas et al., 2017a). Then these testes of males and neomales would be represented approximately at the same distance from regular females (**Figure 3)**.

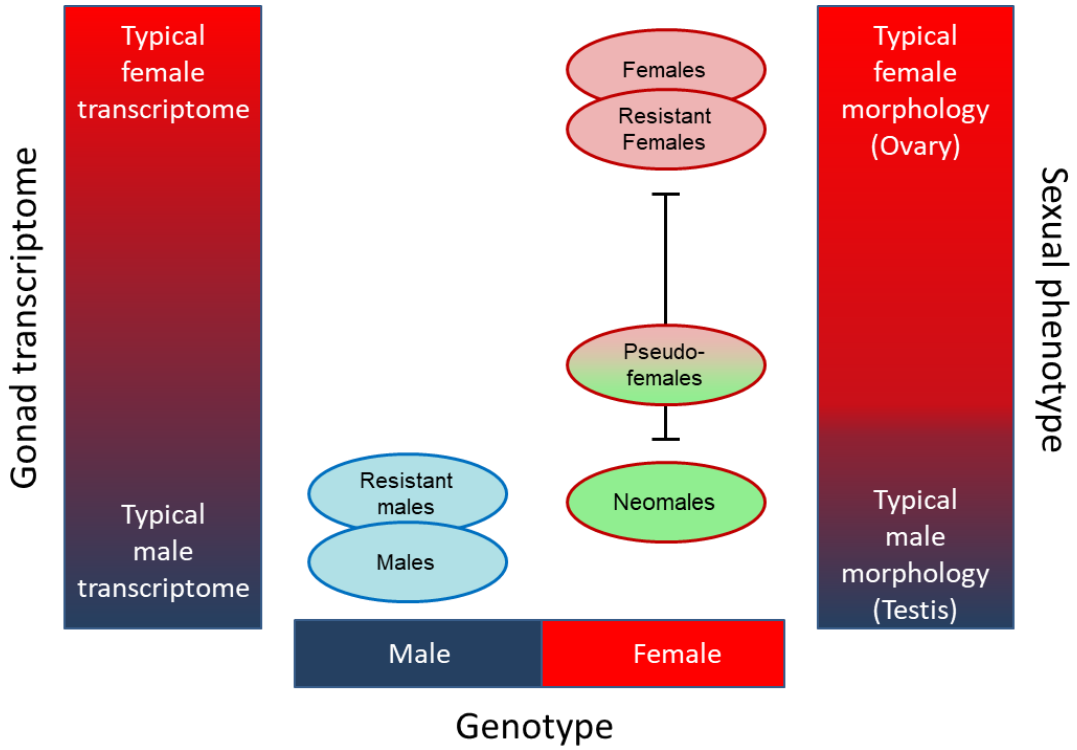


Figure 3. Schematic representation of transcriptomic and morphological differences between distinct types of zebrafish gonads of fish exposed to masculinizing conditions based on the underlying sexual genotype.

These examples were also described in other fish when they were exposed to thermal stress. In the European sea bass, upregulation of male genes was also found in ovaries from females previously exposed to heat during the initial stages of sex differentiation (Díaz and Piferrer, 2015). In Nile tilapia (XY/XX), neomales (XX) expressed a largely different gonadal transcriptome than their control females and the heat-resistant females, and these two showed almost identical transcriptomes (Sun et al., 2018). In a study with half-smooth tongue sole, ovaries of females exposed to elevated temperature (28°C) showed a substantial increase in the number of upregulated genes compared with the ovaries of control females (22°C) (Wang et al., 2019b).

Thus, transcriptomically speaking, ovaries can range from those of regular females to those of pseudofemales, i.e., with about two-thirds of a male transcriptome. In contrast, testes of neomales are transcriptomically essentially identical to those of genetic males.

Although further research is needed, this plasticity in the ovarian tissue could be understood as a mechanism of resistance to the masculinization effects of the environment in order to facilitate the viability of the population under adverse conditions occurring during development. Nevertheless, not considering hermaphrodites, the following question could be placed concerning gonochoristic species: do the different levels of male-like transcriptomes in ovaries of heat resistant females and pseudofemales correlate with the degree of exposure to the environmental factor? In another words, whether

exposure to increasing masculinizing conditions would result in, first, heat-resistant females and then pseudofemales with gonad transcriptomic progressively closer to the transcriptomes of males. Then, how much can an ovary be transcriptomically stretched until no longer has the morphology of an ovary? Is the change to testis (to a neomale) progressive or is there a threshold that when crossed the gonad no longer can maintain the morphology of an ovary? From our results in adults it seems that the second option is more likely (Figure 3) but studies analyzing juvenile and not adult gonads, on one hand, and fish exposed to feminizing treatment, on the other, should be conducted to confirm this viewpoint. However, it should be remembered that these observations have been made in ESD or PSD species, and that this range of male-like transcriptome in heat resistant females is feasible because many loci are involved in sex determination and influenced by the environment. However, in species with GSD, environmental factors can override the genetic sex component from the beginning of the sex determination cascade and then sex differentiation be switched to a regular male gene expression program (Wang et al., 2019b).

More studies are needed to further understand the ovarian plasticity in gonochoristic and hermaphroditic fish. In our case, although different strains can show different transcriptomic results (Holden and Brown, 2018), it would be interesting nevertheless to analyze the gonadal transcriptomes of genetic males (ZZ), neomales (ZW and WW) and females (ZW and WW) of Nadia and Ekkwill wild strains reared at control and high temperature of those individuals obtained in *chapter 4*.These efforts are currently underway.

3. Epigenetic biomarkers and potential applications

A biomarker is a trait the nature of which can be very varied and that can be precisely measured and validated as an indicator of normal biological processes, pathogenic responses or exposure to pollutants (Group et al., 2001).

In aquaculture, broodstock fish, larvae, juveniles and adults are typically raised in different types of tanks and under proper conditions for each stage (Liu et al., 2017b). Periods of early development are usually the most sensitive to the effects of the environment and where external perturbations can have long-lasting effects in many traits. Avoiding stress is of major importance in aquaculture, otherwise populations could see compromised their development, growth and health (Rehman et al., 2017). Aspects affected by conditions during early development include growth, metabolism, sex determination, fecundity and behavior (Jonsson and Jonsson, 2014). These changes on the phenotype can be controlled by epigenetics. Recently, the importance of epigenetics including, but not limited to, DNA methylation, has been recognized for aquaculture (Moghadam et al., 2015; Gavery and Roberts, 2017). As explained in the general introduction, epigenetic modifications are relatively easy to measure with the currently available technologies.

The use of zebrafish for improving production process of aquaculture has emerged as an important research field (Ulloa et al., 2011; Ribas and Piferrer, 2014), and the information obtained in this Ph.D. thesis could be useful for aquaculture. Temperature exposure during early development in zebrafish changed the methylation levels of genes involved in sexual development (*chapter 3*). We were able to identify some CpG changes in gonads tissues that allowed to detect, first, the sex of the fish and, second, those fish that were previously exposed to temperature perturbations. Thus, DNA methylation as epigenetic markers can be used to better detect phenotypic outcomes of early environmental exposures as stressors. It would be interesting to analyze the same set of genes (*chapter 3*) but from fish stressed by density (*chapter 1*) and explore whether methylation changes in specific loci in response to different stressors are conserved. Thus, finding universal methylation regions or set of CpG highly sensitive to stress situations, either due to by abiotic factors (e.g., temperature or density), chemical (e.g. pollutants) or infections (e.g, bacteria or virus) would become a powerful tool to aid in broodstock management, assess larvae quality or assess the susceptibility to diseases. In particular, and based on what has been shown in this Ph.D. thesis, epigenetic markers can offer information on whether fish have been exposed to substandard environmental conditions. This would be beneficial not only in controlled aquaculture settings, but also in the analysis of natural populations in conservation biology

4.Transgenerational effects using wild zebrafish strains

In the study of the effects of temperature across generations (*chapter 5*) one of the main limitations was that it was not possible to distinguish between regular males and neomales in the AB strain, which the lack of sex associated region (*sar4*) (Wilson et al., 2014). However, we took into account the genetic component in this experiment, i.e., siblings exposed to 28°C and 36°C were outcrossed with siblings of an unrelated family, not only to avoid inbreeding but also to reduce possible differences in sex tendency because of the PSD nature of sex in the AB strain. Then, the question is whether neomales and pseudo-females (Ribas et al., 2017a) can reproduce normally and, if so, whether they would give rise to altered sex ratios in the offspring.

In the half-smooth tongue sole, the F_1 offspring derived from heat-induced neomales (ZW) mated with a regular females (ZW) had male-biased sex ratios even when reared at control temperature. The methylome of these neomales (ZW) of F_1 population resembled the paternal DNA methylation and also the genetic males (ZZ) (Chen et al., 2014; Shao et al., 2014). In this species, F_1 offspring carrying WW chromosomes are not viable and cannot survive even produced from artificial induction of mitogynogenesis (Chen et al., 2012; Chen et al., 2014), in contrast to the situation in wild strains zebrafish (Wilson et al., 2014). Unlike the zebrafish AB strain, in the half-smooth tongue sole genotypic sex can be identified and then neomales in the population can be selected (Liao et al., 2014).

We discussed above the susceptibility of the wild strains to the masculinizing effects of elevated temperature during gonadal development (**Figure 2**). Based on our results (*chapter 4* and 5), we proposed a new experiment to solve the randomization of selecting genetic males and neomales within the same population masculinized by temperature effects when we did the transgenerational effects experiments with AB strain. To evaluate the transgenerational effects by temperature using wild strains of zebrafish we could intentionally remove the Z chromosome from the population since to obtain males and females with the W chromosome is sufficient. A situation like this was described in the Australian bearded dragon (*Pogona vitticeps*), where under laboratory conditions the W sex chromosome was eliminated from the lineage in the first generation, creating a lineage exclusively with ZZ temperature-sensitive individuals (Holleley et al., 2015). In *chapter 4*, random selection of broodstock could lead by chance to the cross of a ZW female with a neomale ZW, and produce WW individuals in the progeny, which can either develop into females or males (Wilson et al., 2014). In our case, this was observed in family 1 of the EkkWill strain (see **Figure 3A** in *chapter 4*). In a population in which the Z chromosomes is removed, we change from a GSD (ZZ/ZW) system to a system of sex-reversed WW females influenced by environment, as similar to what would have happened with the AB strain (Postlethwait and Braasch, 2020), but in our case we can genotype fish by PCR.

Then, following the same experimental design of *chapter 5*, using a fixed WW genotype (**Figure 4**), all the individuals would be homogametic (i.e., only W_1W_1 or W_2W_2 but not W_1W_2) and so the effects of temperature and epigenetic inheritance could be studied. Having the genetic background fixed and no ZZ males being present, we could assure that all males would be neomales. Therefore, temperature-induced masculinization, when observed, would be entirely dependent on epigenetic modifications, not by changes in the genotype. This would be an elegant way to study the actual contribution of epigenetic inheritance in zebrafish across generations.

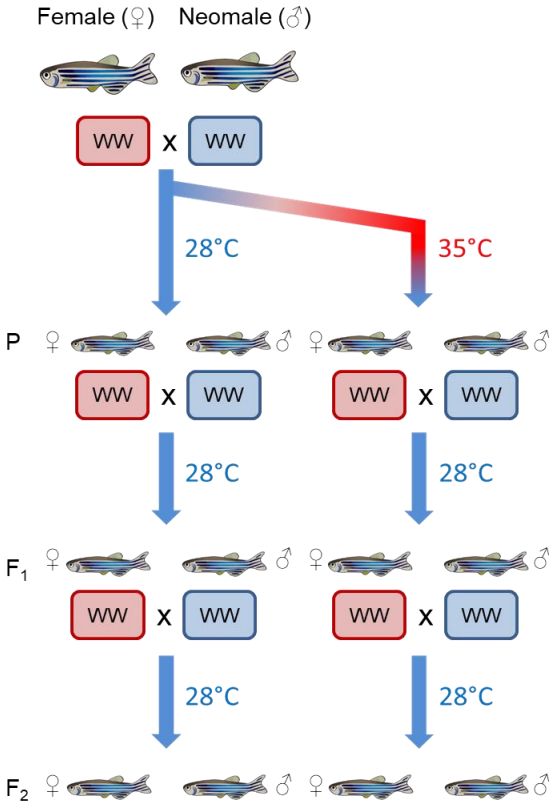


Figure 4. Proposal of a transgenerational experiment, parental (P), first (F_1) and second (F_2) generation to determine the effects of elevated temperature during sex differentiation on resulting wild zebrafish strains. Fixed WW genotype is in the population.

5. Future directions

The results presented in this doctoral thesis may serve as a guide for future research. After analyzing the results of each chapter, new questions arrive and a few future experiments came to my mind. These would complement the results of some of the questions from the previous chapters.

5.1 Cortisol and masculinization

Steroidogenic enzymes and sex steroids play a crucial role during sex differentiation (Bogart, 1987). Specifically, *cyp19a1a* is essential for ovarian differentiation in fish (Piferrer et al., 1994; Guiguen et al., 1999). In contrast, *cyp11c1* and *hsd11b2* are enzymes involved in 11-ketotestosterone synthesis (Kusakabe et al., 2002; Kusakabe et al., 2003) and also involved in testicular differentiation in zebrafish (Wang and Orban, 2007) and the stress response (Alderman and Vijayan, 2012).

When administrating exogenous cortisol during gonadal development all populations were fully masculinized (*chapter 1*). Cortisol seems to be the link between stress and

masculinization in fish. Stressful conditions can affect many steroidogenic enzymes and several authors have emphasized that the role of glucocorticoids in influencing gonadal fate (Hattori et al., 2009; Hayashi et al., 2010; Yamaguchi et al., 2010). In our experiments, we used metyrapone, which selectively inhibits cortisol synthesis acting through *cyp11c1* (*chapter 2*). In order to elucidate the role of cortisol in masculinization additional experiments using inhibitors acting in other enzymes directly involved with cortisol hormone (i.e., cortisol metabolism by *hsd11b2* and interaction with glucocorticoid receptor by *nr3c1*) should be carried out. Inhibitors like the 18β-glycyrrhetinic acid for *hsd11b2* activity (Alderman and Vijayan, 2012) and the mifepristone (RU486) for the *nr3c1* glucocorticoid receptor (Wilson et al., 2013) have been tested in zebrafish but in tissues other than gonads. .

Another option to understand the actual action of cortisol on sex differentiation in zebrafish would be the generation of transgenic lines by knockout of *hsd112* and *nr3c1* with gene editing techniques such as CRISPR-Cas9 (Hruscha et al., 2013; Ran et al., 2013).Yet, these genes could be essential for development and the procedure result in the no viability of the embryos.

5.2 Time course epigenetic levels of sex-related genes

We described a series of DNA methylation changes associated with the effects of temperature during early development (*chapter 3*). Temperature was applied during larval development, from 18–32 dpf, but not in the embryos, juveniles or adults. Additional time-course experiments at various stages of development using moderately elevated temperature could be performed to further narrow down, if possible, the most sensitive period. In addition, this would help to identify the affected genomic positions and allow to deduce the processes affected underlying masculinization by temperature in zebrafish at the methylation level.

The tools developed in this thesis (primers for genotyping, multiplex DNA methylation analysis) and the knowledge gained will hopefully contribute to further investigate sex determination and differentiation in zebrafish. They can also facilitate similar work in other species, including biologically important complex traits other than sex.

Conclusions

Conclusions

1. Sex differentiation in domesticated zebrafish is affected by elevated stocking density, leading to lower survival, lower growth and male-skewed sex ratios. Masculinization is likely to be mediated by an increase in cortisol through the stress response. These results should be of practical use for the husbandry of zebrafish in research facilities across the world. However, the mechanism underlying density-induced masculinization needs further study.

2. Elevated density during sex differentiation until adulthood sets permanent changes in male- and female-related sex genes even in non-masculinized females. By comparing the gonadal transcriptomes of heat- and density- resistant females, a set of conserved genes involved in developmental processes and anatomical structure morphogenesis were identified. These altered gene-expression profiles constitute novel biomarkers, indicative of previous exposure to environmental stress, that are not linked to conspicuous alterations in sex ratios or gonadal morphology. Thus, they could aid in identifying hidden effects of environmental perturbations.

3. DNA methylation of genes coding for steroidogenic enzymes, growth and transcription factors and sex steroid receptors in the adult zebrafish gonad is dependent on the genotype and influenced by the environment. Elevated temperature affected genes related to sex and the steroidogenic pathway more in males than in females.

4. Specific CpG loci located in *cyp19a1a*, *amh* and *foxl2a* were identified and allowed the accurate identification of the sex of the individual using a DNA sample and to discern whether fish had been exposed to abnormally high temperatures when young, i.e., a long time after the environmental stressor disappeared, even in the absence of morphological alterations of the gonads. These set of CpGs represent epigenetic biomarkers that correctly recapitulate past thermal history and pave the way for similar findings in other species.

5. The wild strains of zebrafish, Nadia and EkkWill, despite possessing an intact major sex determining region (*sar4*), had, surprisingly, the same sensitivity to the masculinizing effects of elevated temperature during gonadal development than the common laboratory AB strain. Thus, the presence of *sar4* does not confer higher resistance to temperature. The existence of genotype-by-environment interactions in sex ratio response in the natural strains suggests that factors other than *sar4* may also contribute to phenotypic sex.

6. Elevated rates of spontaneous sex reversal at control temperature and genotypic-dependent differences in the susceptibility to elevated temperature-induced masculinization plus changes in the amount of sperm were detected in the wild zebrafish strains. Taken together, these results suggest a genetic basis underlying both the production of neomales and their response to temperature, with potential functional consequences in their reproductive capacity.

7. The effects of elevated temperature on sex ratio and DNA methylation in the gonads were inherited, at least in males, in the first non-exposed generation but only in a family-dependent manner, while effects were never detected in the second generation. These results show not only wide interfamily variation in sex ratio response to elevated temperature but also on its possible multigenerational effects, denoting a strong influence of genetics in that response.

8. Taken together, these results contribute to our understanding of the role of DNA methylation in shaping the gonadal phenotype. These results can also aid towards obtaining a better picture of how environmental changes may affect natural populations in a global warning scenario, and in the development of epigenetic biomarkers to aid in monitoring aquatic production.

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Annex

Publications

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RESEARCH ARTICLE

Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response

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ABSTRACT

The zebrafish (*Danio rerio*) has become a well-established experimental model in many research fields but the loss of the primary sex-determining region during the process of domestication renders laboratory strains of zebrafish susceptible to the effects of environmental factors on sex ratios. Further, an essential husbandry aspect – the optimal rearing density to avoid stress-induced masculinization – is not known. We carried out two experiments: the first focusing on the effects of density on survival, growth and sex ratio by rearing zebrafish at different initial densities (9, 19, 37 and 74 fish per litre) for 3 months (6–90 days post-fertilization, dpf), and the second focusing on the effects of cortisol during the sex differentiation period (15–45 dpf) for zebrafish reared at low density. The results showed an increase in the number of males in groups subjected to the two highest initial rearing densities; we also observed a reduction of survival and growth in a density-dependent manner. Furthermore, zebrafish treated with cortisol during the sex differentiation period showed a complete masculinization of the population; treatment with the cortisol synthesis inhibitor metyrapone negated the effects of exogenous cortisol. Our results indicate that the process of sex differentiation in domesticated zebrafish can be perturbed by elevated stocking density and that this effect is likely to be mediated by an increase in cortisol through the stress response. However, the underlying mechanism needs further study.

KEY WORDS: Sex differentiation, Stress, Sex ratio, Cortisol, *Danio rerio*

INTRODUCTION

The zebrafish (*Danio rerio*) is a small tropical freshwater fish of the family Cyprinidae (Mayden et al., 2007) and a well-established animal model for many research fields (Streisinger et al., 1981; Chakrabarti et al., 1983; Whitfield et al., 1996; McGonnell and Fowkes, 2006; Ribas and Piferrer, 2014). Studies looking at genetic polymorphisms and using a variety of screening methods on domesticated zebrafish strains have identified putative sex-linked loci in different chromosomes: 3 and 4 (Anderson et al., 2012), 5 (Bradley et al., 2011) and 16 (Howe et al., 2013). Moreover, using different families and several crosses, family-dependent sex ratios were obtained, which led to the proposal that domesticated zebrafish have a polygenetic sex-determining system in which genetic factors and environment determine the sex (Liew et al., 2012; Ribas et al., 2017). Recently, in wild zebrafish populations it has been found that a locus at the telomeric region of chromosome 4 is strongly linked with sex and compatible with a WZ/ZZ sex determination system (Wilson et al., 2014). Interestingly, this region is not found in most laboratory strains and it has been argued that domestication has caused the loss of the sex-linked region as a result of continuous breeding and mutations (Wilson et al., 2014). Thus, because they lack the master sex-determining gene, laboratory strains of zebrafish probably have several minor sex-linked loci that determine sex, although under strong environmental influence. This would explain why laboratory zebrafish behave as though they have a polygenic sex-determining system (Liew et al., 2012). Environmental influences during early development are able to influence sex ratios in species with polygenic sex determination (Penman and Piferrer, 2008). Surprisingly, and opposite to what happens with rodents, where universal husbandry protocols are available, the zebrafish community lacks universal rearing guidelines. Although many authors have focused on the different aspects of rearing conditions (Westerfield, 1995; Casebolt et al., 1998; Trevarrow, 2004; Lawrence, 2007; Pavlidis et al., 2013; Giacomini et al., 2015), a main variable such as rearing density is still not clearly established. Thus, it is urgent to clarify the influence of rearing density on zebrafish sex differentiation.

Regardless of whether they are a laboratory model such as zebrafish or farmed species, rearing fish at a sufficiently elevated density has obvious advantages regarding space and resource utilization optimization. The influence of stocking density on fish sex ratios has only been documented in a few fish species, e.g. paradise fish, *Macropodus opercularus* (Francis, 1984), some coral reef fish species (Kuwamura et al., 2014; Lutnesky, 1994), European eel, *Anguilla anguilla* (Huertas and Cerdà, 2006; Krueger and Oliveira, 1999; Roncarati et al., 1997), European sea bass, *Dicentrarchus labrax* (Saillant et al., 2003) and zebrafish (Hazlerigg et al., 2012). However, as for any confined animal, a rearing density beyond a certain threshold has evident detrimental consequences in fish, including lower survival, decreased growth, higher incidence of deformities, increased susceptibility to diseases, and altered reproduction (e.g. Iguchi et al., 2003; North et al., 2006). The last of these is manifested in lower fecundity or higher larval mortality (Coman et al., 2007) due to an increase in plasma cortisol levels when fish are reared at elevated densities (Schreck, 1981; Barton, 2002). Cortisol is a glucocorticoid hormone regulating gluconeogenesis and other metabolic processes where glucose is needed as an energy substrate, and plays a role also in osmoregulation, growth and reproduction (Barton et al., 1987; Mommsen et al., 1999). Cortisol is considered to be a primary stress indicator with detrimental effects in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Thus, elevated cortisol reduced survival in Atlantic cod, *Gadus morhua*, eggs (Kleppe et al., 2013) and inhibited puberty in common carp, *Cyprinus carpio* (Consten et al., 2002).

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In recent years, it has become evident that elevated plasma or whole-body cortisol levels during early development can also affect the process of sex differentiation, resulting in an increase in the number of males in different species, including medaka, *Oryzias latipes* (Hayashi et al., 2010), pejerrey, *Odontesthes bonariensis* (Hattori et al., 2009), southern flounder, *Paralichthys lethostigma* (Mankiewicz et al., 2013), and Japanese flounder, *P. olivaceus* (Yamaguchi et al., 2010). However, each one of these studies proposed a distinct, albeit not necessarily mutually exclusive, mechanism on the underlying mechanism responsible for masculinization by cortisol. Furthermore, cortisol synthesis can be inhibited by metyrapone, a chemical compound that blocks the conversion of 11-deoxycortisol to cortisol (Lisansky et al., 1989). Metyrapone has been used in some fish species (e.g. zebrafish or rainbow trout, *Oncorhynchus mykiss*), as a strategy to elucidate cortisol effects (Leach and Taylor, 1980; Miranda et al., 1998; Zanuzzo and Urbinati, 2015). For example, metyrapone treatment through the diet was able to inhibit masculinization induced by high temperature in Japanese flounder (Yamaguchi et al., 2010).

Studies on the effects of rearing density in zebrafish are scarce and sometimes controversial. In a study involving eight major zebrafish facilities across the world, it was found that holding densities as high as 12 fish per litre after 4 months of age did not result in negative effects on clutch size, spawning success or egg viability (Castranova et al., 2011). When mating, changes in egg production, hatching rate or larval length were not observed until a density of 60 fish per litre (Goolish et al., 1998). Cortisol levels increased up to fourfold in adult zebrafish confined at high densities of 40 fish per litre (Ramsay et al., 2006). Differences in the number of reared zebrafish required to observe alterations in behaviour are present in the literature: 0.025 fish per litre in 38 l tanks (Larson et al., 2006), 0.25 fish per litre in 60 l tanks (Spence and Smith, 2005) and 1.4 fish per litre in 21 l tanks (Moretz et al., 2007). In contrast, other authors did not find differences at 1.2 fish per litre using 45 l tanks (Gronquist and Berges, 2013). Despite these studies, the influence of density during sexual development, when fish are more susceptible to the effects of external perturbations, is far from clear in zebrafish. One study found that elevated density caused a decrease in growth and survival rates without a clear link with sex ratios (Hazlerigg et al., 2012). Another study found that high density increased the number of males, although there was a high inter-family variation in the response, suggesting that other factors, both genetic and environmental, could also be affecting sex ratios (Liew et al., 2012).

In this study, we took advantage of the fact that domesticated zebrafish are sensitive to environmental perturbations to address the general question of how environmental factors can influence the process of sex differentiation in fish and, in particular, how stocking density affects the sex ratio. In addition, and in order to study the possible role of cortisol in the masculinization of zebrafish subjected to stress confinement as a result of high density, synthetic cortisol and metyrapone were administered during the sex differentiation period.

MATERIALS AND METHODS

Animal rearing conditions

Domesticated zebrafish (AB strain) were housed in a commercial rack (Aquaneering, San Diego, CA, USA) fitted with a recirculating water system (supplied with a water pump of 6000 l h^{-1}) and placed in an *ad hoc* chamber facility in our institute subjected to a constant photoperiod (12 h light:12 h dark), air temperature of 26±1°C and humidity of 50±3%. Water quality parameters were monitored daily and included: temperature 28±0.2°C, pH 7.2±0.5, conductivity 750–900 µS and dissolved oxygen 6.5–7.0 mg l^{-1}. Other water quality parameters were checked periodically (2–3 times a month) by the Water Analysis Service of our institute and maintained in the appropriate ranges (Ribas and Piferrer, 2014): ammonium 0.03± 0.00 mg l^{-1}, nitrite 0.25±0.14 mg l^{-1}, nitrate 66.42±8.04 mg l^{-1}, silicate 15.22±2.53 mg l^{-1} and phosphate 32.34±7.43 mg l^{-1}.

Breeding was always performed by natural spawning after single-pair crossings. The total number of eggs and fertilized embryos was counted to ensure that fecundity was according to the reference values for this species (Ribas and Piferrer, 2014) and post-hatch viability in accordance with the OECD's guidelines for the Fish Sexual Development Test (OECD, 2011; Fig S1). Eggs were reared in Petri dishes (ThermoFisher Scientific, Waltham, MA, USA) at ~50 eggs per dish filled with embryo medium (pH 7.2±0.5), supplemented with 0.1% Methylene Blue (Sigma-Aldrich, Madrid, Spain) at 26±1°C until 6 dpf. Then, hatched larvae were transferred to tanks at 6 dpf and housed in the commercial rack described above.

Fish were fed *ad libitum* 3 times a day with a commercial food according to their developmental stages: 6–15 days post-fertilization (dpf) larvae were fed with Micron (Sera, Heinsberg, Germany), which contains natural plankton (50% spirulina and 16% krill); 15–40, 40–60 and 60–90 dpf fish were fed with pellets of increasing size: ST1, ST2 and ST3, respectively (AquaSchwarz, Göttingen, Germany), containing 54–59% crude protein, 15–16% lipids, 12% crude ash, vitamins A, D3, E, C (C at 100–2000 mg kg^{-1}) and omega-3 (HUFA at 28–30 mg g^{-1}). The commercial feed in all stages was supplemented with live *Artemia* nauplii (AF48, INVE Aquaculture, Dendermonde, Belgium). The debris at the bottom and walls of the tanks was cleaned 3 times a week. Fish were kept in accordance with 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).

Experiment 1: effects of stocking density

Fish were reared in tanks (Aquaneering, model ZT280) of a nominal volume of 2.8 l (the actual capacity was 2.7 l). Different densities were achieved by placing 6-dpf larvae in the following numbers in the 2.7 l actual available volume: 25, 50, 100 and 200 larvae. This gave initial densities of 9.25, 18.51, 37.03 and 74.07 fish per litre. For clarity purposes, the rounded values of 9, 19, 37 and 74 fish per litre will be used from now on. The experiment was repeated 4 times with four different families, and each density treatment was replicated between 3 and 7 times, depending on the fecundity of each family. In total, 1625 fish were used. Survival for each density treatment was recorded at 15, 20, 30, 50, 70 and 90 dpf. At 50 dpf, juvenile fish from the 9 and 74 fish per litre groups were euthanized by immersion into ice for cortisol analysis (see below for further details). At 90 dpf, fish were euthanized on iced water followed by decapitation and total body mass (M_b, precision ±0.05 g), standard length (SL, precision ±0.01 cm) and sex ratios were recorded. The Fulton's condition factor (k) was calculated following the formula: $k=(M_b\times100)/SL^3$ (Fulton, 1902).

Experiment 2: cortisol treatment

The spawn of three different pairs were pooled in each experimental tank (with a total of 36 fish per 2.8 l tank), and received one of the following randomly assigned treatments: control (C), cortisol (F; hydrocortisone; ref. H0888, Sigma-Aldrich), metyrapone (M; 2-methyl-1,2-di-3-pyridyl-1-propanone; ref. 856525, Sigma-Aldrich), cortisol plus metyrapone (F+M), and the synthetic

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androgen 17α-methyltestosterone (MT; ref. M7252, Sigma-Aldrich) as a positive control for masculinization. Treated feed was prepared with the following concentrations (µg g^{-1} feed). F group: 50; F+M group: 50+500; M group: 500; MT group: 50. All compounds were diluted with 3 ml of 96% ethanol and sprayed directly on feed. Feed in the control group was also sprayed with 96% ethanol. The treated feed was then air dried in a ventilated hood for 3 h to remove ethanol traces and then stored at −20°C until consumption. For each group, at least two replicates were used, with a total number of 444 fish in the whole experiment. Fish survival calculations and sampling procedures were performed as described above for experiment 1.

Coinciding with the sex differentiation period (15–45 dpf), tanks were removed from the recirculating water system housing rack and placed in groups of three inside a large tub filled with water, maintained at a constant water temperature of 27.63±0.11°C with the use of electric waterproof heaters. Tanks were individually oxygenated with an independent air flow source but without altering environmental conditions and natural swimming. Renewal of three-quarters of the tank water was performed 3 times a week, together with removal of the debris at the bottom of the tanks. During this period, fish were fed 3 times a day with the treated feed. Afterwards (at 45 dpf), tanks were moved back to the housing rack.

Whole-body cortisol measurement

Whole-body cortisol levels were measured in juvenile 50 dpf zebrafish samples using a commercial enzyme immunoassay kit (ref. 402710, Neogen, Lansing, MI, USA), following the manufacturer's instructions with slight modifications. The specificity of the test was evaluated by comparing the samples with a standard curve. The linear regression of the standard curve was R^2=0.979. The mean intra-assay coefficient of variation for all tests was always <10% with an assay sensitivity of 0.03 ng ml^{-1}. Frozen fish were homogenized individually in 100 µl cold phosphate-buffered saline (PBS, pH 7.4) using a glass pestle, then suspended in 2 ml of pre-cooled diethyl ether for 15 min at 4°C. Homogenates were centrifuged at 2500 ***g*** for 2 min and frozen at −80°C for 30 min to recover the organic phase. This step was repeated 3 times. The diethyl ether from each sample was evaporated using a dry heater at 30°C in a ventilated hood. Samples were immediately resuspended in extraction buffer supplied by the manufacturer and diluted 1:5. Tubes containing samples and standards were measured at 650 nm in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland). All samples were measured in duplicate. Cortisol levels of a total of 23 juvenile fish were measured: eight fish in the low-density group (from the 9 fish per litre group) and 15 fish in the high-density group (from the 74 fish per litre group). The mean M_b of the fish at each density was used to calculate whole-body cortisol per gram of fish.

Statistical data analysis

Data normality and the homoscedasticity of variances were checked with Kolmogorov–Smirnov's and Levene's tests, respectively. When data did not follow normality, a Box Cox transformation was applied. One-way analysis of variance (ANOVA) was used to detect possible differences among groups in survival, M_b, SL and k calculations. *Post hoc* multiple comparisons were carried out with Tukey's test. For male SL and male k data, the Kruskal–Wallis test was used. Student's t-test was used to detect differences in cortisol levels. The Chi-squared test with Yate's correction was used for sex ratio analysis (Fowler et al., 2008). All data analyses were performed with Stats Graphics software (version 17). Data are expressed as means±s.e.m. In all tests, differences were accepted as significant when P<0.05.

RESULTS

Experiment 1

Effect of rearing density on survival

Survival was inversely related to rearing density (Fig. 1). Fish survival at 90 dpf was 76%, 64%, 45% and 35% relative to the initial number of fish in each case for the 9, 19, 37 and 74 fish per litre groups, respectively. The two highest tested densities significantly decreased (P<0.05) survival when compared with the two lowest densities at all sampling points (Fig. 1A). The highest mortality was observed between 6 and 15 dpf but survival was density independent after this point (Fig. 1B).

Effect of rearing density on growth

Growth was also inversely related to rearing density, with sex-related differences (Fig. 2). M_b was significantly decreased (P<0.05 and P<0.01) in males for groups reared at a density of 19 fish per litre or higher, but in females such an effect was observed only with densities of 37 fish per litre or higher (P<0.05) (Fig. 2A). SL was

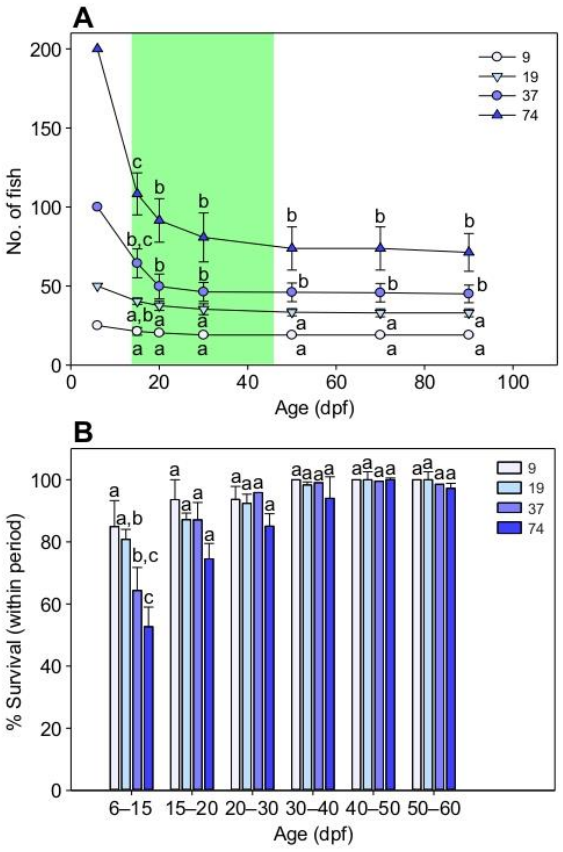

Fig. 1. Zebrafish survival as a function of stocking density during the first 3 months of age. Fish were held at densities of 9, 19, 37 and 74 fish per litre. (A) Absolute survival from 6 to 90 days post-fertilization (dpf). The sex differentiation period (15–45 dpf) is shaded in green. (B) Survival relative to different age periods. Data are presented as means±s.e.m. of 3–6 biological replicates per group. Significant differences (P<0.05) among groups at a given sampling age period were tested by one-way ANOVA and are indicated by different letters.

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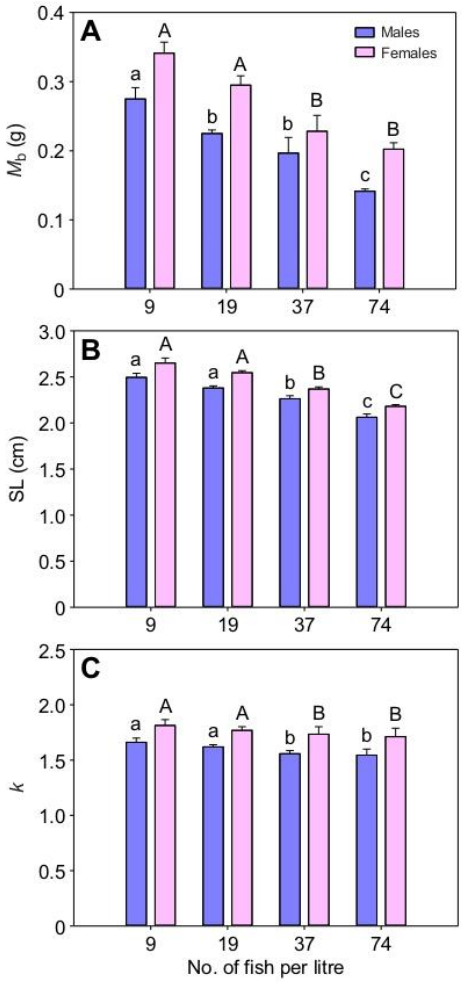

Fig. 2. Zebrafish growth as a function of different stocking density during the first 3 months of age and sex. (A) Body mass (M_b), (B) standard length (SL) and (C) condition factor (k). Data are presented as means±s.e.m. of 3–6 biological replicates per group. The number of fish at 90 dpf was 57, 231, 181 and 280 for the 9, 19, 37 and 74 fish per litre groups, respectively. Within the same sex, different letters indicate significant differences (a–b or A–B, P<0.05; and a–c or A–C, P<0.01) between groups analysed by ANOVA, except for SL and k male data, which were analysed by a Kruskal–Wallis test.

significantly (P<0.05) decreased in both sexes at densities of 37 fish per litre and higher (Fig. 2B). A similar trend of density effects was observed for k (Fig. 2C).

Effect of rearing density on sex ratio

There was a density-dependent effect on zebrafish sex ratio as the number of males observed at 90 dpf increased with rearing density (Fig. 3A). At a density of 9 and 19 fish per litre, the number of males was 54.4±11.06% and 61.4±6.04%, respectively, a proportion not different from the expected Fisherian sex ratio. Significant differences with respect to the 9 fish per litre group were recorded with densities of 37 fish per litre (71.6±6.5%; P<0.05) and 74 fish per litre (80.1±3.4%; P<0.01).

In order to determine density effects when sex differentiation takes place (15–45 dpf), the number of fish alive in the tank during this process was calculated by averaging observed values at 15, 20, 30 and

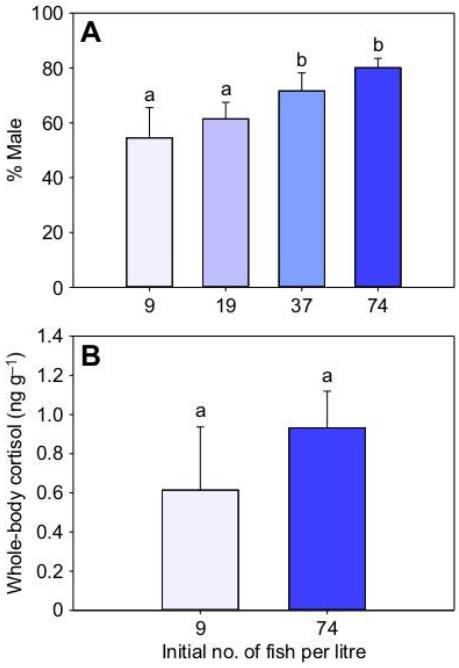

Fig. 3. Sex ratio and whole-body cortisol levels in zebrafish reared at different densities. (A) Zebrafish sex ratio as a function of stocking density during the first 3 months of age. Data are presented as means±s.e.m. of 3–6 biological replicates per group. The final number of fish per group is as in Fig. 2. Significant differences among groups (indicated by different letters) were analysed by a Chi-squared test with Yate's correction (P<0.05 in 37 fish per litre group and P<0.01 in 74 fish per litre group) with respect to the group with the lowest rearing density. (B) Whole-body cortisol levels in juvenile zebrafish at 50 dpf as a function of stocking density. Data are presented as means±s.e.m. of 8 (9 fish per litre) or 15 biological replicates (74 fish per litre). There was no significant difference between groups (Student's t-test, P=0.49).

50 dpf for each replicate treatment (Table 1). The mean±s.e.m. number of fish per litre was 7.4±0.4, 13.5±0.3, 18.3±0.7 and 31.9±1.0 in the 9, 19, 37 and 74 fish per litre groups, respectively.

Effect of rearing density on whole-body cortisol levels

Whole-body cortisol levels in fish subjected to high density confinement over 45 days (from 6 dpf to 50 dpf) increased at the end of this period by ~50% in the 74 versus the 9 fish per litre groups (Fig. 3B). However, these differences were not significant (P=0.49).

Experiment 2

Effect of cortisol on survival

At the end of the experiment (90 dpf), survival of the control group in experiment 2 was lower than that of the control group in experiment 1. Treatment with M or MT did not affect survival. However, treatment with F, particularly when it was administered alone, significantly (P<0.05) increased survival (Fig. 4).

Effect of cortisol on growth

Male M_b in the F, F+M and MT groups was significantly reduced (P<0.05) when compared with the control group but not with the M group (Fig. 5A). Treated females did not show any statistical differences in M_b or SL (the MT group contained only two females; Fig. 5A,B). Males treated with synthetic cortisol showed a significant decrease in SL (P<0.05; Fig. 5B). No significant differences were found in k in either males or females when compared with control fish (Fig. 5C).

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Table 1. Stocking density effects on zebrafish survival during the period of sex differentiation and on adult sex ratio

		Initial		Sex differentiation period			Final		
Pair	No. of replicates	No. of fish	No. of fish per litre	No. of fish per tank	No. of fish per litre	No. of fish per litre per group	No. of fish per tank	% Males	*P*-value
1	3	25	9	19.0±0.0	7.0	7.4±0.4	19	36.8	
2			9	20.5±0.4	7.6		20	60	
3			9	20.5±0.7	7.6		18	66.7	ns
1	7	50	19	27.3±0.5	10.1	13.5±0.3	22	31.8	
2			19	36.8±0.6	13.6		33	75.8	
3			19	37.8±0.0	14.0		36	63.9	
3			19	46.7±1.2	17.3		40	75	
3			19	31.3±0.8	11.6		30	66.7	
3			19	34.3±0.9	12.7		31	58.1	
4			19	42.0±0.7	15.6		39	59	ns
3	4	100	37	31.0±0.0	11.5	18.3±0.7	31	54.8	
3			37	49.3±0.0	18.2		48	79.2	
3			37	60.0±0.7	22.2		53	79.2	
4			37	57.8±1.3	21.4		49	77.6	<0.05
3	4	200	74	121.5±1.6	45.0	31.9±1.0	98	74.5	
3			74	74.0±1.7	26.3		69	88.4	
3			74	78.5±1.0	29.1		60	78.3	
3			74	71.0±1.7	24.9		53	79.2	<0.01

Initial number of fish per litre has been rounded to the nearest whole number. The sex differentiation period was from 15 to 45 days post-fertilization (dpf); data are means±s.e.m. Final values were measured at 90 dpf. *P*-value indicates departure from Fisherian sex ratio; ns, not significant.

Effect of cortisol on sex ratio

Treatment with cortisol resulted in a complete masculinization of the population ($P< 0.001$; Fig. 6). The same effect occurred with the positive control treatment MT, where 92.3±6.7% of the fish were masculinized ($P<0.001$). No differences in sex ratios were found in the M and M+F groups (61.9±0.6% and 48.6±2.7% of males, respectively) with respect to the control group (44.1±3.2%), meaning that metyrapone counteracted cortisol effects, preventing masculinization.

DISCUSSION

In this study, we tested four stocking densities for their effect on survival, growth and sex ratio of domesticated zebrafish. Consistent with the results of Hazlerigg et al., (2012), stocking zebrafish in high densities was detrimental to their survival, as also observed in other fish species including the Nile tilapia, *Oreochromis niloticus* (Huang and Chiu, 1997), pufferfish, *Takifugu rubripes* (Kotani et al., 2009), vundu catfish, *Heterobranchus longifilis* (Coulibaly et al., 2007), and pigfish, *Orthopristis chrysoptera* (DiMaggio et al., 2014).

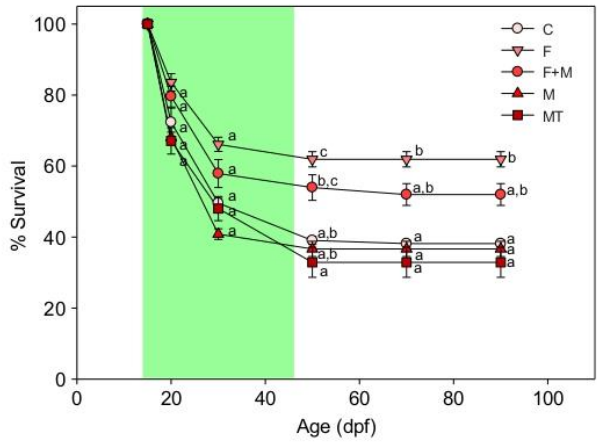

Fig. 4. Zebrafish survival during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. The final number of fish was 34, 75, 21, 37 and 26 fish per group, respectively. The sex differentiation period (15–45 dpf) is shaded in green. Data are presented as means±s.e.m. of 2–3 biological replicates per group. Significant differences ($P<0.05$) among groups at a given sampling age were examined by one-way ANOVA and are indicated by different letters.

The stocking densities used in this study were set up at the time of transfer of the larvae to the rearing tanks but no attempt was made to replace dead fish with new ones to maintain the initial number of fish during the experiment. Thus, effects on growth and sex ratios determined at 90 dpf have to be seen as the result of the cumulative effects of varying stocking density as some fish died while others grew. Initial rearing densities of 9 and 19 fish per litre did not have any effect on sex ratios at 90 dpf. In contrast, initial densities of 37 and 74 fish per litre significantly increased the proportion of males. It should be noted that rearing fish at 9 and 19 fish per litre did not affect survival during 6–15 dpf, or SL and condition factor *k*, presumably indicating no influence of conditions other than rearing density, although a density of 19 fish per litre decreased M_b. Furthermore, survival of the group reared at 9 fish per litre was around 76%. This value is standard for zebrafish and suggests that rearing conditions other than density were not deleterious. Thus, it is probably safe to state that, based on our results, a stocking density in the range of 13–20 fish per litre in a ∼3 l commercial tank starting at 6 dpf would not cause masculinization. However, with improvements in feeding or diet formulation (e.g. supplemented by rotifers), survival in the initial stages could be higher than the survival recorded in this study. Thus, based on our data, in order to avoid density-induced masculinization, the stocking density should be taken into account, especially during the sex differentiation period. Ongoing experiments in our lab involving additional families show that elevated rearing density during this period results in a clear sex bias towards males, confirming the present results. Following the above-mentioned guidelines, in recent experiments in our lab, we did not observe masculinization. We do not know for sure whether, taking into account other factors (e.g. social interactions, behaviour, husbandry strategies, etc.) applied in

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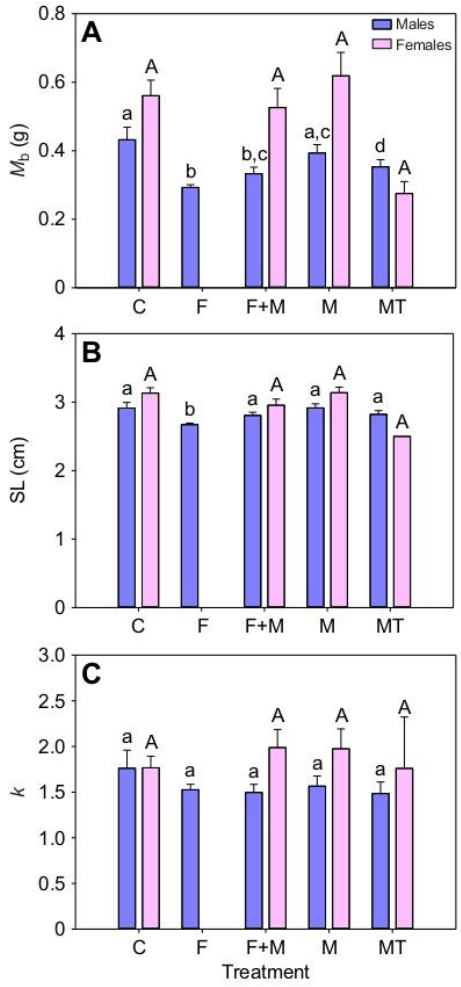

Fig. 5. Zebrafish growth during the first 3 months of age as a function of cortisol treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. (A) M_b, (B) SL and (C) *k*. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Within the same sex, different letters indicate significant differences (P<0.05) between groups analysed by ANOVA, except for SL and *k* male data, which were analysed by a Kruskal–Wallis test.

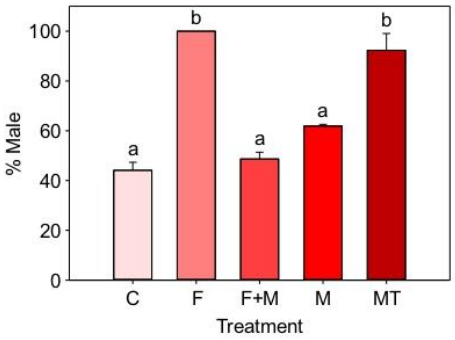

Fig. 6. Zebrafish sex ratio at 3 months of age as a function of treatment. C, control; F, cortisol; F+M, cortisol+metyrapone; M, metyrapone; and MT, 17α-methyltestosterone. Data are presented as means±s.e.m. of 2–3 biological replicates per group. The final number of fish per group is as in Fig. 4. Significant differences among groups were analysed by the Chi-squared test with Yate's correction. Different letters indicate a significant difference (P<0.001) with respect to the control group.

other laboratories, this density range would also work well with tanks of much larger volume, e.g. 45 l tanks, as used by Gronquist and Berges (2013) for behavioural studies. In our experiments with domesticated zebrafish, other than density, environmental conditions (e.g. water quality, feeding regime, etc.) were the same in all tanks. Thus, although the sex ratio of domesticated zebrafish may be influenced by several factors, in this study we focused on rearing density, and aimed to minimize other possible environmental influences.

An interesting aspect is whether the results obtained in this study would apply to other zebrafish strains. We used domesticated zebrafish from the AB strain, but other laboratories have carried out density experiments using other zebrafish strains. Liew et al. (2012), using a Tübingen (TU) strain, found an increase in males of ∼20% with rearing densities of 66.6 fish per litre when compared with 33.3 and 16.6 fish per litre. In contrast, Hazlerigg et al. (2012), using the WIK (Wild Indian Karyotype) strain, found that densities from 2 to 40 fish per litre had no effect on sex ratios when combined with different feeding regimes (constant or limited). Here, it is interesting to note that while the WIK strain has a WZ/ZZ sex determination system with a putative sex-determining gene at the tip of chromosome 4, the TU and AB strains have lost this gene, presumably during the many manipulations in the process of domestication (Wilson et al., 2014). Because of this loss, the TU and AB strain sexual development is more sensitive to environmental cues. This concurs with the fact that domesticated zebrafish show a wide range of inter-family variation in sex ratios and that a polygenic system of sex determination has been proposed for domesticated zebrafish (Liew et al., 2012; Ribas et al., 2017; see Table 1). Taken together, it can be concluded that the effects of density found in this study with the AB strain would also apply to the TU strain, while experiments with the WIK strain are needed in order to determine whether the presence of the WZ/ZZ system confers greater resistance to the influence of environmental cues on sex ratios.

We also found that growth is inversely related to stocking density in zebrafish, as also found by Hazlerigg et al., (2012) and conforming to what has been observed in other species (Barton et al., 1987; Björnsson, 1994; Holm et al., 1990). The fish reared at the two lowest densities in this study had a mean M_b of ∼0.25±0.01 and ∼0.32±0.01 g and a mean fork length of ∼2.4±0.04 and ∼2.6±0.05 cm at 90 dpf for males and females, respectively, and corresponding with the range for the typical M_b and length of an adult zebrafish at this age (reviewed in Ribas and Piferrer, 2014). However, the highest stocking density tested produced a decrease in M_b, with mean values of ∼0.14±0.003 and ∼0.20±0.003 g for M_b and ∼2.1±0.03 and ∼2.2±0.01 cm for SL in males and females, respectively. Similar results were found in other experiments stocking zebrafish at a density of 60 fish per litre with a constant feeding regime, in which a decrease to ∼2.0 cm in length was also observed (Hazlerigg et al., 2012). In addition, in experiment 2, males treated with different compounds showed significant differences in growth rate, whereas in females those differences were not present. Specifically, M_b was reduced in fish from groups treated with cortisol, in agreement with what has previously been described for other fish species, e.g. goldfish (Bernier et al., 2004), largemouth bass, *Micropterus salmoides* (O'Connor et al., 2011),

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and sturgeon, *Huso huso* (Poursaeid et al., 2012), where cortisol treatment induced a decrease in food intake and hence a decrease in growth rate in a dose-dependent manner.

It has been observed that fast growth during the period of gonad formation promotes female development in zebrafish, suggesting that the growth rate during this period could be regarded as an environmental factor capable of affecting sex differentiation (Lawrence et al., 2008). In our experiments, fish were fed *ad libitum*, so although food intake was not strictly controlled, food was not a limiting factor for any of the groups. This reinforces the view that stocking density rather than possible differences in growth is responsible for the observed changes in sex ratio in the groups exposed to the highest stocking density.

High stocking density may be experienced as a stressful situation by the fish and if so it is then possible that the observed masculinization is somehow related to this stress response. As cortisol is the most common indicator of the stress response, to address this we measured whole-body cortisol levels in the fish reared at the lowest versus the highest stocking density tested in this study. Although whole-body cortisol levels were ∼50% higher in the latter group, differences were not significant because of the large inter-individual variability within each group. However, the most important aspect to consider is that the cortisol levels measured in our fish (<1 ng g^{-1} M_b) cannot be considered representative of a stressful situation, as reported values in zebrafish subjected to different types of stress (e.g. crowding, handling, visual predator) are much higher, in the range 4–12 ng g^{-1} (Ramsay et al., 2006; Barcellos et al., 2007; Pavlidis et al., 2013). The lack of a cortisol-related stress response has been documented in other species such as the gilthead sea bream, *Sparus aurata* (Barton et al., 2005), and the wedge sole, *Dicologoglossa cuneate* (Herrera et al., 2015), suggesting that acclimation to a chronic stressor allowed the attenuation of cortisol release, which is more related to the acute rather than the chronic stress response. This, of course, does not preclude that elevated stocking density caused elevated cortisol levels during the sensitive period of sex differentiation. Recently, it has been shown that feeding zebrafish with cortisol over a period of 5 days did not change whole-body cortisol levels but increased ovarian cortisol levels twofold (Faught et al., 2016).

Thus, in order to further explore the possible link between the stress response and masculinization, we conducted experiment 2, focusing on the possible role of cortisol in sex differentiation. Treatment with cortisol resulted in 100% masculinization, demonstrating that cortisol was able to strongly influence the process of sex differentiation in zebrafish. Similar effects were observed in other fish species, i.e. medaka (Hayashi et al., 2010), Japanese flounder (Yamaguchi et al., 2010) and pejerrey (Hattori et al., 2009) – in the latter, fish showed elevated cortisol levels after being exposed to high temperature. In the Southern flounder, the stress caused by the background colour of the tank increased cortisol levels and skewed sex ratios towards males (Mankiewicz et al., 2013).

Cortisol may also influence survival. In experiment 2, survival of the control group was lower than in experiment 1 and this is probably due to the fact that in experiment 2 all groups, including the control group, were removed from the main rack to administer the different treatments. However, the two groups treated with cortisol showed higher survival rates when compared with the rest of the groups. This runs counter to what is described in the literature in the sense that elevated cortisol plasma levels are detrimental to fish survival, and we have no satisfactory explanation for this observation. However, it has been shown that cortisol also possesses some benefits because in fish it can activate the innate immune system to better cope with adverse situations (MacKenzie et al., 2006). Thus, the increase in survival after cortisol treatment, which is clear based on our results, may be linked to these supposed benefits of cortisol. However, further work needs to be done to provide more evidence.

Treatment with metyrapone alone, a cortisol synthesis inhibitor (Leach and Taylor, 1980), had no effect on zebrafish sex ratios but completely suppressed cortisol-induced masculinization. This is interesting because in Japanese flounder treated with equal doses of the two compounds, cortisol was able to override the inhibitory effects of metyrapone on cortisol synthesis and, consequently, complete masculinization was observed (Yamaguchi et al., 2010). The metyrapone dose used in our study was 10 times higher than the cortisol dose, following the principle of a 1:10 ratio of stimulator to inhibitor in pharmacology. Thus, it may be that the effect of metyrapone as a blocker of endogenous cortisol synthesis was stronger than the effect of exogenous cortisol.

Our study demonstrates a link between high stocking density and masculinization, probably mediated by cortisol through the stress response. However, the underlying molecular mechanism is not known and thus the question of how cortisol interacts with gonadal differentiation remains unclear. To date, different – although not necessarily mutually exclusive – molecular mechanisms by which cortisol could masculinize fish gonads have been proposed. In medaka, it has been suggested that cortisol suppresses germ cell proliferation, which is associated with female development in teleosts, through inhibition of expression of the follicle-stimulating hormone receptor (*fshr*) gene (Hayashi et al., 2010). In the pejerrey, cortisol interacts with the glucocorticoid response element in the promoter of the 11β-hydroxysteroid dehydrogenase type 2 (*hsd11β2*) gene, a key enzyme that is shared between the glucocorticoid and androgen pathways, increasing its expression. This led Fernandino et al., (2012, 2013) to suggest that while increased *hsd11β2* expression contributes to the degradation of cortisol to cortisone, it also helps to convert 11β-hydroxyandrogens into 11-ketotestosreone, the typical teleost androgen, with more masculinizing potency than testosterone (Piferrer et al., 1993). In contrast, in the Japanese flounder, it was suggested that cortisol suppressed gonadal aromatase (*cyp19a1a*) expression by blocking the cAMP response element located in the *cyp19a1a* promoter (Yamaguchi et al., 2010). Thus, a clear and unifying view of the molecular mechanisms responsible for cortisol-induced masculinization is not available in fish and further research is needed.

In conclusion, we have shown that the rearing of domesticated zebrafish – which lack the master sex-determining gene present in their wild counterparts, and hence behave as though they possess a polyfactorial system of sex determination – is very susceptible to environmental perturbations in terms of sex ratio. Thus, stocking domesticated zebrafish larvae at high densities has an impact on the subsequent adult sex ratio by increasing the number of males, but also by decreasing growth and fish survival in an inversely dependent manner. We suggest that masculinization by high density may be related to the stress response and that cortisol may have a prominent role, but the underlying mechanism needs further elucidation. Although inter-family variation in the final sex ratio is a factor that cannot be underestimated in domesticated zebrafish, we suggest that when transferring larvae to the tanks, typically at 6 dpf, the initial stocking density should not be higher than 13–20 fish per litre. In any case, and with the information available now, during the process of sex differentiation (15–45 dpf), it is advisable that the lower figure of this range is not surpassed. This will avoid

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detrimental effects on survival and growth, and will prevent male-biased sex ratios in the subsequent adult population. In addition to providing new information on environmental effects on fish sex ratios, our study thus offers useful information on how to rear zebrafish, filling a gap in an essential husbandry aspect in this important experimental model.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
F.P. and L.R. designed the study. L.R., A.V. and N.D. conducted the experiments and the subsequent analysis. L.R. drafted the initial manuscript. L.R., A.V., N.D. and F.P. wrote the manuscript. All authors read and approved the final manuscript.

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Response to "The importance of controlling genetic variation – remarks on 'Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response'"

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We appreciate the comment by Delomas and Dabrowski to the Ribas et al. (2017a) paper and want to mention that we were very much aware of the possible effect of genetic differences in the sex ratio response to increasing rearing density from the beginning. This is why, in each one of the four replications of experiment 1, we avoided using pooled eggs and sperm from different progenitors, and instead used eggs and sperm derived from a single dam and sire, respectively. Thus, each time we used a different pair (pairs 1–4), hence creating four different families (biological replicates). Although some sources (e.g. FishBase) claim higher values, the absolute fecundity of zebrafish females is typically around 300 eggs (Ribas and Piferrer, 2014). This creates an experimental problem when eggs from individual dams are used, as in the Ribas et al. (2017a) paper, because with that number of available eggs it is very difficult to test four rearing densities starting with 25, 50, 100 and 200 fish per tank, as even with only two technical replicates per density the amount of eggs needed climbs to 750. Thus, as Delomas and Dabrowski rightly point out, pairs were not tested at all densities, as we explicitly show in table 1 of Ribas et al. (2017a). In that study, pairs 2, 3 and 4 showed an increase in the number of males, while pair 1 showed the opposite effect but, again, the masculinizing effect of elevated density was seen when the sex ratios obtained at each density were compared with the sex ratios of the lowest density, as also stated. Genotype-by-environment (G×E) interactions in the sex ratio response to external factors in zebrafish have been recently described by Ribas et al. (2017b), in accordance with the existence of the sex ratio variation among, but not within, families of domesticated zebrafish, as previously proposed by Liew et al. (2012). This was reflected by different susceptibilities of elevated temperature among different families (see fig. 1C of Ribas et al., 2017b).

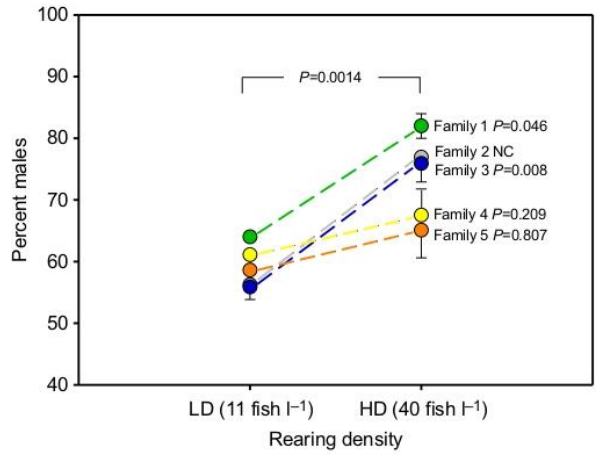

Fig. 1. Genotype-dependent sex ratio response of five different zebrafish (AB strain) families as a function of rearing density [low density (LD) =11 fish l^{-1}; high density (HD)=40 fish l^{-1}] during the sex differentiation period [18–45 days post-fertilization (dpf)]. The number of fish available for sexing at 90 dpf per family was as follows: *n*=95, 29, 201, 161 and 74 for families 1–5, respectively. Family numbers are arbitrary. Data are means±s.e.m. of two technical replicates for each family/density combination. For clarity, error bars that are similar in size to or smaller than the data points are not shown. NC, significance level not computed owing to insufficient sample size.

Furthermore, in Ribas et al. (2017a) we clearly stated that ongoing experiments in our laboratory confirm the masculinizing effects of elevated densities. We advance the sex ratio results of these experiments below, which are part of a larger study on other aspects of the effects of rearing density. We reared five additional pairs precisely to better determine the effect of genetic variation in the masculinizing response to elevated rearing density. In order to avoid the shortcomings of not having enough eggs, in this case we used only two densities: 11 and 40 fish l^{-1} in 2.7-liter tanks for the low density and high density groups, respectively, applied during the 18–45 days post-fertilization (dpf) period. Each density treatment was replicated twice for each family pair. This is possible with the typical ~300 egg batch. As previously observed in the temperature experiments (Ribas et al., 2017b), we found a G×E interaction in the response to elevated densities as evidenced by non-parallel, family-specific reaction norms (Fig. 1). Resulting sex ratios were analyzed at 90 dpf with the chi-squared test. Of the five families tested, family 2 was excluded from the statistical analysis because of insufficient fish numbers at the time of sampling. Of the remaining four families, families 1 and 3 showed statistical differences ($P<0.05$ and $P<0.01$, respectively), whereas for families 4 and 5, although there was also an increase in the number of males, differences were not statistically significant (Fig. 1). If the data for all five families are combined, differences are significant ($P=0.0014$). These data, along with the data presented in Ribas et al. (2017a), clearly illustrate that at a rearing density of 40 fish l^{-1} or higher, masculinization occurs in zebrafish. Although most families tend to increase the number of males in response to elevated density, some show statistical significance and some do not. Thus, as stated in the concluding remarks of our initial paper, there is an inter-family variation, meaning that there is, as in many other aspects, a genetic component in the sex ratio response to rearing density.

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RESEARCH ARTICLE

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Ovarian transcriptomic signatures of zebrafish females resistant to different environmental perturbations

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Abstract

Sex is remarkably plastic in fish and can be easily influenced by environmental cues, in which temperature has been the most studied abiotic factor. However, it has been shown that elevated population densities can increase the number of males in several species but 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 versus low population densities contained 4,634 differentially expressed genes. Among them, a set of promale genes (*amh*, *sypc3*, *spata6*, and *sox3*) were upregulated in the high-population density group. 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 *sycp3*) but also genes involved in other functions (e.g., *faima*, *ccm21*, and *ankrd6b*) and a downregulation of *cyp19a1a* together with other genes (e.g., *lgals9l1* and *ubxn2a*). We identified the common Gene Ontology terms involved in the reproduction and sexual development that were consistently affected in both environmental factors. These results show that regardless of the environmental perturbation there are common genes and cellular functions involved in the resistance to masculinization. These altered gene-expression profiles can be used as markers indicative of previous exposure to environmental stress independent of conspicuous alterations in sex ratios or gonadal morphology.

KEYWORDS
density, environmental stress, global change, gonad, masculinization, molecular markers, temperature

1 | INTRODUCTION

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

Sex determination in fish can be regulated by genetic factors (genetic sex determination, GSD), by influences of the environment (environmental sex determination, ESD), or a combination of both (Devlin & Nagahama, 2002; Guiguen, Fostier, & Herpin, 2019; Penman & Piferrer, 2008). In GSD species, sex can be determined by the action of a single or master gene, by the action of a master gene plus secondary loci or by the combined action of several autosomal genes, the latter called polygenic sex determination (PSD). In ESD, sex can be influenced by different abiotic and biotic factors, of which temperature and population density are the most common ones (Penman & Piferrer, 2008; Shen & Wang, 2019; Valenzuela, 2008). Nowadays, GSD and ESD are not considered necessarily

mutually exclusive and, hence, are regarded as the two ends of a continuum (Heule, Salzburger, & Böhne, 2014; Sarre, Georges, & Quinn, 2004; Yamamoto, Zhang, Sarida, Hattori, & Strüssmann, 2014).

For poikilothermic animals like fish, temperature is perhaps the most important abiotic factor. Abnormally elevated temperatures can masculinize many species if exposure takes place during the process of sex differentiation or earlier (Ospina-Alvarez & Piferrer, 2008; Valenzuela & Lance, 2004). The masculinizing effect of elevated temperature takes place regardless of the underlying sex-determining system and thus have been observed in species with male heterogamety (XX/XY) such as the rainbow trout (*Oncorhynchus mykiss*; Valdivia et al., 2014) and the Nile tilapia (*Oreochromis niloticus*; Baras, Jacobs, & Mélard, 2001); and species with PSD such as the European sea bass (*Dicentrarchus labrax*; Díaz & Piferrer, 2015; Saillant et al., 2002; Vandeputte & Piferrer, 2019).

Regarding population density, it was found that elevated density in the Paradise fish (*Macropodus opercularis*; Francis, 1984) and in the European eel (*Anguilla anguilla*; Huertas & Cerdà, 2006; Roncarati, Melotti, Mordenti, & Gennari, 1997) was able to skew the sex ratio toward males. In contrast, in the European sea bass, no effects were observed when fish were confined during the early stages of development (Saillant et al., 2003). In zebrafish (*Danio rerio*), elevated density resulted in male-biased sex ratios (Ribas, Valdivieso, Díaz, & Piferrer, 2017a) with interfamily variation (Liew et al., 2012; Ribas, Valdivieso, Díaz, & Piferrer, 2017b) due to genotype–environment interactions.

Zebrafish is a small cyprinid widely used as an animal model in many research areas. Wild zebrafish strains exhibit a GSD system of the ZW/ZZ type, with a putative sex master gene located in the telomeric region of chromosome 4 (Wilson et al., 2014). In contrast, in the two most used laboratory strains (AB and TÜ) this system was not detected probably due to the loss of the sex-determining region during the domestication process (Wilson et al., 2014). Due to this, domesticated zebrafish behave as a PSD system with elevated sensitivity to environmental perturbations (Liew et al., 2012; Ribas, Liew, et al., 2017) which explains why zebrafish facilities across the world usually encounter skewed sex ratios. Thus, laboratory strains of zebrafish constitute indeed a good model where to study the effects of environmental perturbations on the developing gonads and the resulting sex ratios.

Sex differentiation in zebrafish takes place from 10 until about 45 days postfertilization (dpf). In all individuals, gonads first develop as an ovary-like immature tissue (Takashi, 1977). Later, ovarian differentiation continues in about half the individuals, whereas in the remaining fish, apoptosis takes place and the ovarian tissue regresses and is progressively substituted by testicular tissue (Orban, Sreenivasan, & Olsson, 2009; Uchida, Yamashita, Kitano, & Iguchi, 2002; von Hofsten & Olsson, 2005). The stress resulting from exposure to elevated temperature during sex differentiation causes masculinization (Abozaid, Wessels, & Hörstgen-Schwark, 2012; Brown et al., 2015; Ribas, Liew, et al., 2017; Uchida, Yamashita, Kitano, & Iguchi, 2004) and thus there are some sex-reversed females (referred to as "neomales"; Pandian & Sheela, 1995). In addition to regular males and females, two additional transcriptomic profiles were identified in heat-treated zebrafish: the neomales and a novel type of females. These were phenotypic females but with a male-like transcriptomic profile, where an upregulation of male-related genes (e.g., *amh* and *dmrt1*) and a downregulation of female-related genes (e.g., *cyp19a1a*, *foxl2a*, and *vtg2*) was observed (Ribas, Liew, et al., 2017).

Further, regardless of the type of stressor (temperature or density), a common physiological response is increased plasma cortisol levels (Ramsay et al., 2006, 2009; Yeh, Glöck, & Ryu, 2013). This is a protective mechanism in response to stress that helps to regulate basal homeostasis (Wendelaar Bonga, 1997). It is well established that chronic stress has adverse effects on growth and reproduction (Schreck, 2010) but recently it has also been demonstrated that elevated cortisol levels due to stress are associated with masculinization (Fernandino, Hattori, Acosta, Strüssmann, & Somoza, 2013; Fernandino, Hattori, Kishii, Strüssmann, & Somoza, 2012; Hattori et al., 2009). Further, oral administration of cortisol during early development resulted in complete masculinization in zebrafish (Ribas et al., 2017a).

However, 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 the resistant females mentioned above, 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 (Small, Carney, Mo, Vannucci, & Jones, 2009; Sreenivasan et al., 2008). Few studies reported the influences of elevated temperature on the gonadal transcriptomes, one in zebrafish (Ribas, Liew, et al., 2017) 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 an elevated temperature from previous experiments reported in our lab (Ribas, Liew, et al., 2017). 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.

2 | MATERIALS AND METHODS

2.1 | Gonad samples and gonadal maturation data

Gonad maturation data and RNA samples of zebrafish exposed to different rearing densities were obtained from fish described in "*Experiment 1: effects of stocking density*" in Ribas et al. (2017a). 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/liter. 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, Liew, et al., 2017).

2.2 | RNA extraction, cDNA synthesis, and primers

RNA was extracted from eight fish per sex for each density (total $n = 16$ for each density) for quantitative polymerase chain reaction (qPCR) analysis (Table S1) using 400 μl of TRIzol reagent (Invitrogen, CA). 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, Carlsbad, CA), the complementary DNA (cDNA) was synthesized from 200 ng of RNA using SuperScript III reverse transcriptase (Invitrogen). Specific primers for target genes were designed using Primer Express® software (v. 3.0; Applied Biosystems, Foster, CA) and purchased from Invitrogen. Primers were designed within the interexon regions and their efficiencies were checked from 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 check the expected size of the PCR product. The qPCR reactions were carried out in triplicate and performed on a QuantStudio™ 12K Flex System (Applied Biosystems, Foster, CA) using 2 μl of SYBR Green PCR master mix (Applied Biosystems, Foster, CA), 0.5 μl for each forward and reverse primers and 2 μl cDNA (1/10 dilution) in a total reaction volume of 10 μl/well. The qPCR cycle parameters consisted of 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 and gene, the average threshold cycle (C_q) was calculated and normalized against a reference gene, the eukaryotic translation elongation factor 1α 1, like 1 (*eef1α1l1*), previously validated in zebrafish (McCurley & Callard, 2008). Relative gene expression data were obtained using the $2^{-\Delta\Delta C_q}$ method (Livak & Schmittgen, 2001). The gene symbol, gene name, accession number, and forward and reverse sequences for each primer pair used in this study are summarized in Table S2.

2.3 | Microarray analysis of ovaries obtained from fish exposed to HD

For microarray analysis, the control group (9 fish/liter) and the group exposed to the highest density (74 fish/liter) were used (from now on referred to as the LD and HD groups, respectively). From each group, six of the eight gonad samples used for the qPCR analysis were also used (Table S3) for microarray analysis and hybridized individually in a 4×44K Agilent platform (G2519F–026437) at the Barcelona Biomedical Research Park (PRBB). Briefly, the cRNA generated from the messenger RNA of each ovarian sample was prepared for overnight hybridization with the corresponding buffers during 17 hr at 65°C and washed on the following day. Hybridized slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies, Santa Clara, CA). The results were deposited in the Gene Expression Omnibus database with the accession number GSE116700.

2.4 | Common genes and pathways in ovaries of females exposed to either HD or high temperature

Gene expression differences of HD versus LD females were compared with those of high temperature (HT) versus low temperature (LT) females, specifically, the "FHT2" females described in Ribas, Liew, et al. (2017). The FHT2 were females with an 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: (a) both experiments were performed at the same fish facility, (b) the laboratory strain used was AB, (c) 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 ± 1°C, (d) exposure to the environmental perturbations included the sex differentiation period and (e) all fish were sampled at the same age, 90 dpf.

2.5 | Statistical analysis of the data

Differences in the proportions of gonad types were analyzed by the χ^2 test. For the qPCR $\Delta\Delta C_q$ data, normality was checked with the Kolmogorov–Smirnov test and logarithmic transformations were applied when necessary. The homoscedasticity of variances was checked with Levene's test. Means were compared by one-way analysis of variance 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 software (v. 17; Statgraphics Technologies, Inc. The Plains, VA).

Microarray raw data were processed by Agilent software to avoid saturation and generate feature extraction. The output data were statistically analyzed by R software (v. 3.4.2; Team, 2013) application and the quantile normalization method was implemented (Smyth & Speed, 2003) using the Limma package (v. 3.5). To determine differentially expressed genes (DEGs), an adjusted $P \leq 0.05$ and fold change (FC) ≥1.2 was used as a 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, Sherman, & Lempicki, 2008a, 2008b). 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, Bošnjak, Škunca, & Šmuc, 2011). The ggplots (v. 2.2.1) and gplots (v 3.0.1) packages were used to modify the REVIGO graphs and to generate heatmap figures, respectively.

3 | RESULTS

3.1 | HD-affected gonadal maturation in females and males

Elevated rearing density not only masculinized zebrafish in a density-dependent manner (Ribas et al., 2017aa) 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 (Figure S1).

3.2 | HD-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 (Figure 1a), whereas the expression of forkhead box L2a (*foxl2a*) was unaffected (Figure 1c). In testes, the expression levels of double sex and mab-3 related transcription factor 1 (*dmrt1*; Figure 1b) and the anti-Müllerian hormone (*amh*; Figure 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 significant down-regulation in both ovaries (Figure 1e) and testes (Figure 1f). These results indicated that elevated rearing density affected both sexes.

3.3 | Transcriptomic overview and microarray validation

Next, a species-specific and validated microarray was used to compare the transcriptomes of females of the HD group, that is, the females that did not become masculinized. These females were compared with the control females. Results showed a total of 4,634 DEGs (adjusted $P \leq 0.05$, FC > 1.2) between the ovaries of fish reared at HD versus LD (Data set S1). Microarray data was fully validated by qPCR in a subset of selected genes covering a range of ±4 FC values ($R^2 = 0.83$ and $P = 0.0001$; Figure S2). For a complete list of gene names and abbreviations see Data set S1.

3.4 | GO terms associated in ovaries of females exposed to HD

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

3.5 | HD promoted the upregulation of promale genes in the ovaries

All the DEGs were compared against two independent lists of genes related to sex and stress responses. One list included a total of 48 genes with proven sex-related functions in zebrafish, as described in Ribas, Liew, et al. (2017), whereas 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

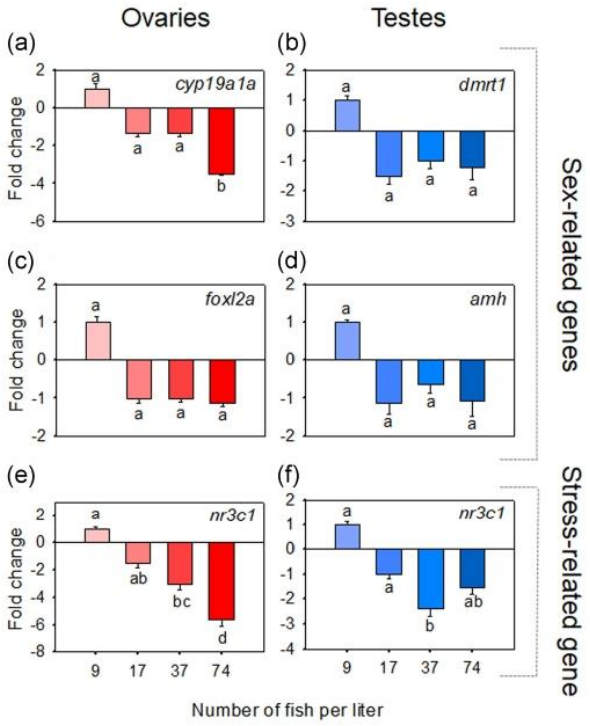

FIGURE 1 Expression of sex-related genes: *cyp19a1a* and *foxl2a* in ovaries (a,c) and *dmrt1* and *amh* in testes (b,d) and of the stress-related gene *nr3c1* in ovaries and testes (e,f), respectively. Average values ($n = 7–8$ individuals per treatment) are shown with a standard error of the mean. Fold change values are shown relative to the 9 fish/liter treatment (control). Analysis of variance followed by post hoc Tukey test. Statistically significant differences ($P < 0.05$) among treatments are indicated by letters

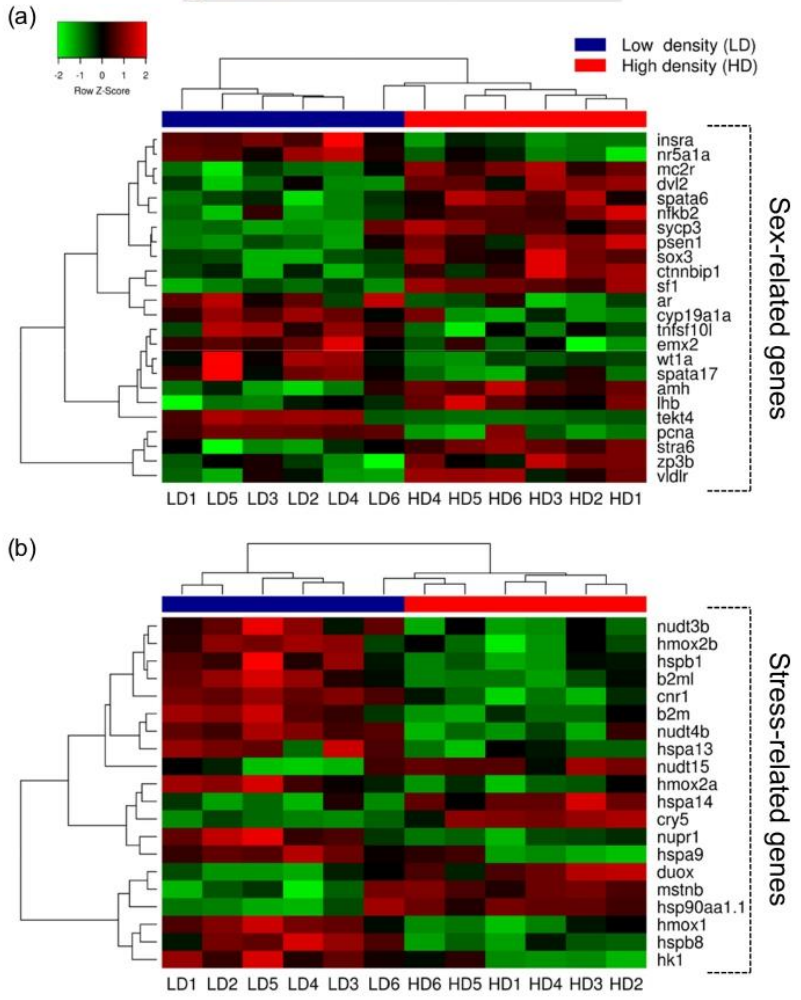

FIGURE 2 Gene expression represented by heatmaps of (a) sex-related and (b) stress-related genes in ovaries of females exposed to high density (HD, bright red) and low density (LD, blue). Key color (Row Z-score) represents the level of gene expression for each gene (red: high expression and green: low expression)

reproduction and stress, respectively, were identified (Figure 2), and allowed the clustering of all but one sample according to rearing density. Regarding sex-related genes (Figure 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 (Figure 2b), 15 of the 20 genes shown in the heatmap were downregulated in the HD group (e.g., *nudt3b*, *hmox2b*, *b2nl*, and *cnr1*) and five (i.e., *nud*, *hsp14*, *duox*, *mstnb*, and *hsp90aa.1.1*) were upregulated.

3.6 | Common genes in ovaries of females subjected to HD or HT

Elevated rearing density or temperature during the sensitive period of sexual development result in masculinization of some females (Figure S4), which is easy to quantify by sex ratio analysis. However, though all nonmasculinized females have apparently normal ovaries, at least morphologically, 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 the fish have been exposed to elevated density or temperature. Thus, we compared the reported ovarian transcriptomic results of the HD versus the LD groups with those obtained comparing the HT versus LT groups previously described in our laboratory (Ribas, Liew, et al., 2017). We found 1,269 DEGs (Figure S5) commonly regulated and were represented according to their FC (Figure 3) and their expression tendency (Figure 3b). We classified them in four sets (Data set 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 (*ptpdc1*), 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.

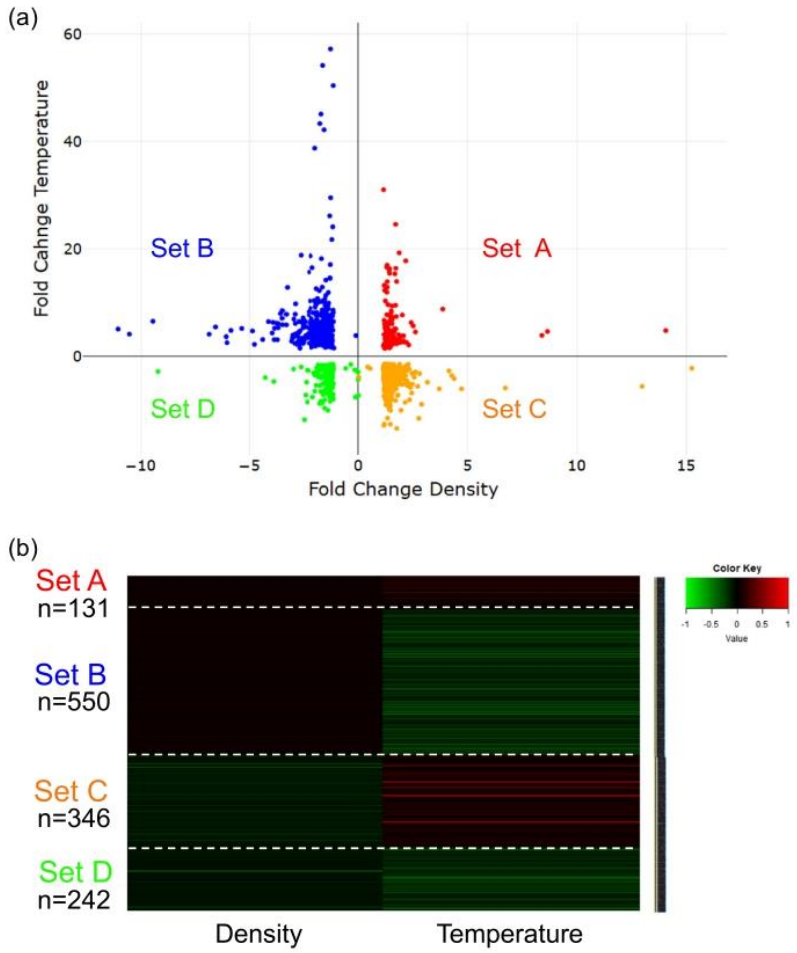

FIGURE 3 (a) Scatterplot of the fold change (FC) values of the common 1,269 differentially expressed genes (DEGs) found between density (*X* axis) and temperature (*Y* axis). (b) Heatmap of the FC values of the 1,269 DEGs found between density and temperature in Sets A–D. The *n* indicates the number of genes found for each set. Key color (Row Z-score) represents the level of FC for each gene (red: high expression and green: low expression)

3.7 | Common GO terms in ovaries of females subjected to HD or HT

We analyzed separately the genes of the Sets A and D by DAVID to inquire into the BP-GO terms. There were a total of nine GO terms in Set A (Figure 4a) including cellular component organization (GO: 0016043) and negative regulation of response to stimulus (GO: GO:0048583); and eight GO terms in Set D (Figure 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; Figure 5a–c), whereas in Set D were macromolecule localization (GO: 0033036), primary metabolic process (GO: 0044238), and cellular metabolic process (GO:0044237; Figure 5d–f). Next, for exploratory analysis, we looked at the top three genes (with the highest FC in the density experiment) 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. Interestingly, FC values of genes altered by temperature were higher than those altered by density (Figure 6a).

3.8 | KEGG pathways in ovaries of females subjected to HD or HT

There were no common KEGG pathways related to the DEGs conforming the A or D Sets, probably due to the relatively low number of genes in each set. However, the expression of genes involved in reproduction-related KEGG pathways in zebrafish (Ribas, Liew, et al., 2017), namely, the Wnt signaling (dre04310), homologous recombination (HR; dre03440), transforming growth factor-β (TGF-β; dre04350), oocyte meiosis (OM; dre04114), p53 signaling (dre04115), and steroid hormone biosynthesis (SHP; dre00140) pathways had the same direction of change regardless of perturbation (Figure 6b). In the Wnt signaling pathway, there were two downregulated genes (axin 2 conductin-axil, *axin2*, and protein kinase cAMP-dependent catalytic α genome duplicate b, *prkacab*) and one

TABLE 1 Top 10 upregulated and downregulated genes in the Sets A and D of genes commonly expressed in ovaries of females exposed to elevated density or temperature

Set	Gene name	Gene symbol	Ref. seq	Fold change	
				Density	Temperature
A	Fas apoptotic inhibitory molecule a	*faima*	NM_001002583	14.07	4.78
	Tyrosine phosphatase containing 1	*ptpdc1*	ENSDART00000081832	8.64	4.59
	Cerebral cavernous malformation 2-like	*ccm2l*	XM_692004	8.39	3.88
	Adenylate kinase 7b	*ak7b*	NM_001109698	3.87	8.76
	Eukaryotic translation elongation factor 1 α1, like 2	*eef1a1l2*	NM_001039985	2.62	4.51
	IQ motif containing with AAA domain 1	*iqca1*	NM_001098731	2.52	5.64
	WD repeat domain 66	*wdr66*	XM_692631	2.42	6.25
	zgc:92052	*zgc:92052*	BC075870	2.31	2.10
	Ankyrin repeat domain 6b	*ankrd6b*	NM_194423	2.21	2.56
	zgc:101737	zgc:101737	BC085428	2.17	17.75
D	Galectin 9	*lgals9l1*	NM_200072	−9.21	−2.85
	Deoxyribonuclease II lysosomal	*dnase2*	NM_001114738	−4.30	−3.98
	zgc:173693	*zgc:173693*	NM_001130657	−3.90	−4.70
	si:dkey-78l4.12	*si:dkey-78l4.12*	NM_001083067	−3.02	−2.40
	zgc:162913	*zgc:162913*	NM_001099250	−2.67	−1.99
	Cytochrome P450 family 19 subfamily A polypeptide 1a	*cyp19a1a*	NM_131154	−2.52	−11.81
	Pannexin	*panx1a*	NM_200916	−2.46	−7.22
	zgc:173480	*zgc:173480*	ENSDART00000106432	−2.43	−4.85
	Calcyon neuron-specific vesicular protein	*caly*	ENSDART00000052272	−2.39	−2.60
	UBX domain protein 2A	*ubxn2a*	ENSDART00000128612	−2.39	−8.52

upregulated gene (transforming protein RhoA, *rhoa*). *Rhoa* also belongs to the TGF-β pathway together with *amh*, which was also upregulated. In the HR signaling pathway, *sycp3* was upregulated. The phosphatase and tensin homolog B (*ptenb*), golgin rab6-interacting (*gorab*), the F-box protein 5 (*fbxo5*), and *cyp19a1a* genes belonging to the p53 signaling, OM, and SHP pathways, respectively, were downregulated.

The KEGG pathways from the 4,634 DEGs found in the density experiment were compared to the KEGG pathways reported in the ovaries of those resistant females treated with elevated temperature (Ribas, Liew, et al., 2017) and no common pathways were found, so we listed in Table 2, the significant upregulated and downregulated KEGG pathways found in the density experiment and the four most significant upregulated and downregulated KEGG pathways found in the temperature experiment. In particular, in the ovaries of fish subjected to density, we found an upregulation of the gonadotropin-releasing hormone receptor (GnRHr) pathway (dre04080) and downregulation of five KEGG pathways, for example, the integrin signaling pathway (dre04510). For the temperature factor, the most upregulated and downregulated pathways were the cardiac muscle contraction (dre04260) and the protein processing in the endoplasmic reticulum (dre04141).

4 | 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 (Baroiller & D'Cotta, 2016; Ospina-Alvarez & Piferrer, 2008; Piferrer, 2018). The masculinization of genotypic females generates phenotypic males (Hliwa, Bah, Kuźmiński, Dobosz, & Ciereszko, 2014) that are termed neomales (Pandian & Sheela, 1995). These neomales are capable of producing viable sperm (Piferrer, 2001). The remaining females, that is nonmasculinized 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, had a male-like transcriptome (Ribas, Liew, et al., 2017). In the present study, we showed that these females with apparently normal morphology but with a male-like transcriptome also appeared in populations partially masculinized by elevated rearing density and consequently we were interested in finding out what were 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., 2017a). This maturation delay was also observed in the gonads of zebrafish subjected to HT (Ribas, Liew, et al., 2017), 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, whereas the expression of *foxl2a*, a transcriptional regulator of *cyp19a1a* (D. S. 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, Wang, Li, Zhou, & Gui, 2017) and it is possible that the other variant could be expressed instead. The downregulation of

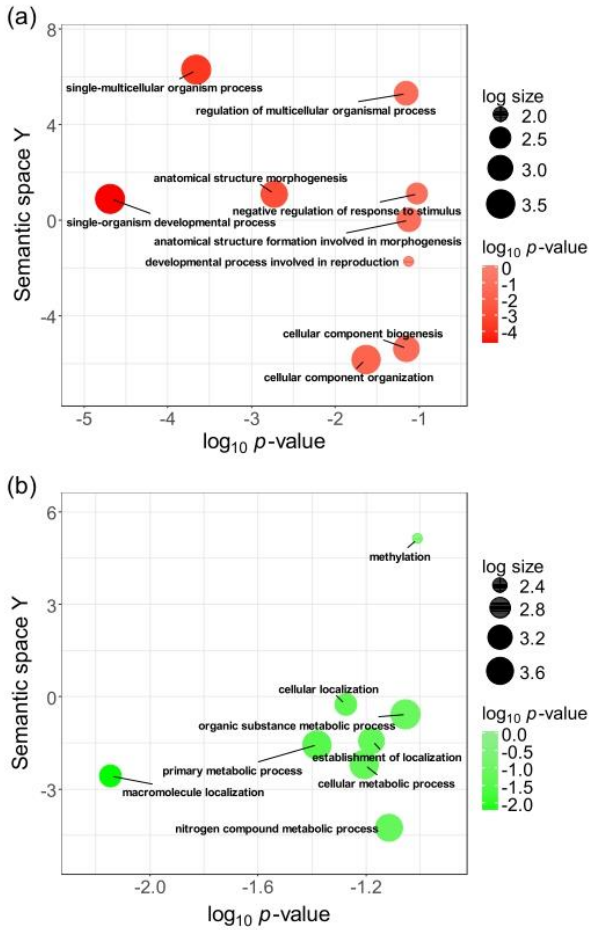

FIGURE 4 GO terms associated to the common genes affected by elevated density or temperature. (a) GO terms associated to Set A (red color), (b) GO terms associated to Set D (green color). For each GO term graph, the log size indicates the number of genes represented and the color saturation indicates the $\log_{10}$ Fisher's *P* value along the *X* axis. GO terms are distributed in multidimensional semantic similarities in the *Y* axis

cyp19a1a in ovaries was described in zebrafish exposed to elevated temperature (Ribas, Liew, et al., 2017) and to hypoxia conditions (Shang, Yu, & Wu, 2006), as well as in other fish species such as the olive flounder (Kitano, Takamune, Kobayashi, Nagahama, & Abe, 1999) and in the European sea bass (Díaz & Piferrer, 2015; Navarro-Martín et al., 2011) when subjected to HT. Thus, environmental stress reduced the expression of *cyp19a1a* in the gonads and this is in agreement with the observed masculinization since *cyp19a1a* is necessary for ovarian development, as demonstrated in the loss-of-function experiments (de Castro Assis, de Nóbrega, Gómez-González, Bogerd, & Schulz, 2018; Lau, Zhang, Qin, & Ge, 2016). In the 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 the estrogen production (Rodríguez-Marí et al., 2005), did not show differences in gene expression due to HD confinement, suggesting no conspicuous effects of density at least in these two important genes for male sexual differentiation. This, of course, does not exclude the possible effects on genes related to spermatogenesis, which were not the focus of this study.

It is known that the 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, Ings, & Vijayan, 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 the 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). As cortisol exerts different effects on various organ systems, it is likely that the functional targets of GR were different in each tissue (Le et al., 2005). The cortisol plasma levels increased after 7 and 14 days of chronic confinement in sea bream (*Sparus aurata*; Barton, Ribas, Acerete, & Tort, 2005) and rainbow trout (Kiilerich et al., 2018), respectively, but they were recovered in longer confinement experiments (~ 200 days) in wedge sole (*Dicologoglossa cuneate*; Herrera, Ruiz-Jarabo, Vargas-Chacoff, de la Roca, & Mancera, 2015). In a similar manner, no difference in cortisol levels was found in juvenile zebrafish subjected to HD during 45 days (Ribas et al., 2017a) but, when sexually mature, GR was downregulated in the gonads at the highest densities, especially in females. This is, to our knowledge, the first time that downregulation of GR in the gonads due to chronic stress is reported, as it was not observed in the reproductive tissues when zebrafish were exposed to elevated temperature (Ribas, Liew, et al., 2017). The downregulation found of GR in the gonads of fish exposed to elevated density was probably to attenuate the physiological long-term effects due to the stress.

Prolonged rearing at high population density affected the expression of more than 4,600 genes in the ovaries of nonmasculinized 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 the 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 promale pathway. These male genes were *amh*, *sycp3*, a gene that is a spermatocyte marker for meiotic cells (Ozaki, Saito, Shinya, Kawasaki, & Sakai, 2011; Saito, Siegfried, Nüsslein-Volhard, & Sakai, 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 &

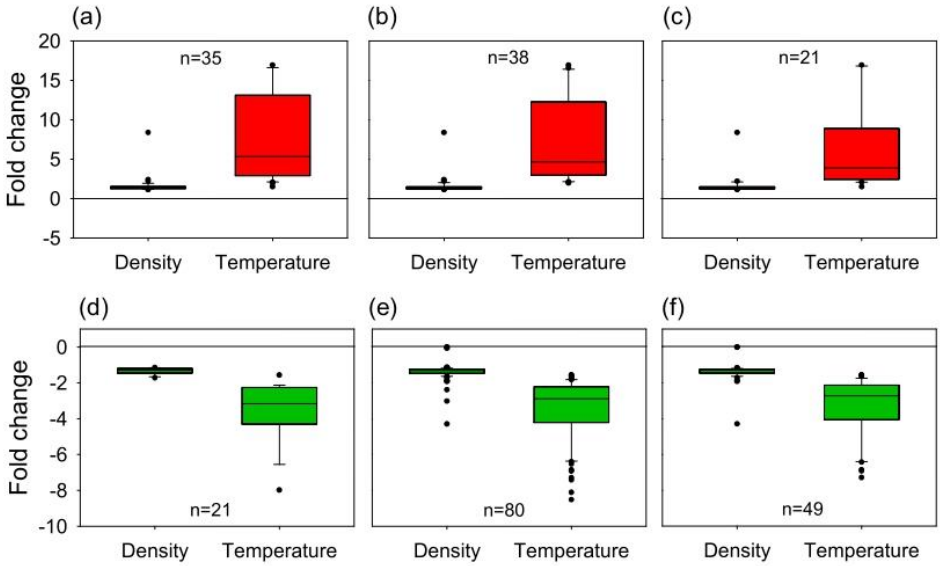

FIGURE 5 Fold change representation of common upregulated genes of (a) single-organism developmental process, (b) single-multicellular organism process, (c) anatomical structure morphogenesis and common downregulated genes in (d) macromolecule localization, (e) primary metabolic process, (f) cellular metabolic process of gene ontology terms affected by elevated density and temperature. "*n*" indicates the number of genes in each case

Englert, 2002), and the androgen receptor. The results were in concordance with those previously described in zebrafish females exposed to elevated temperature (Ribas, Liew, et al., 2017). Regarding genes related to the stress response, 14 of 20 were downregulated, including heme oxygenase enzymes (*hmox1*, *hmox2a*, and *hmx2b*), 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); four were downregulated (*hsp13*, *hsp14*, *hspa9*, and *hspb8*) and two upregulated (*hsp14* and *hsp90aa1.1*). The *hsp90aa1.1* is a crucial gene for cortisol activity as it acts as 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 versus chronic stress (Pottinger, 2008).

The comparison of the ovarian transcriptomes of females exposed to either elevated population density or temperature allowed insights into what were the conserved signatures irrespective of the type of perturbation. It is difficult to say whether zebrafish gonads are more sensitive to elevated temperature or elevated rearing density because a proper estimation would require carefully designed dose–response experiments and comparing, for example, the transcriptomes of females when, say, 50% of the initial females become masculinized either after temperature or density, which is not the case in this study. However, at least it is interesting to notice a certain relationship between the degree of masculinization and the average fold change of genes commonly affected by both factors (temperature > density).

Of the 1,269 DEGs shared in both type of perturbations, 373 DEGs were affected in the same direction regardless of the environmental perturbation. Among these common genes, *faima* was strongly upregulated (FC = 14.07 and 4.78 in density and temperature experiments, respectively). *Faima* is an inhibitor of the apoptotic response and it is expressed in mammalian ovaries during follicular atresia in the granulosa cells (Matsuda-Minehata, Inoue, Goto, & Manabe, 2006) with similar expression pattern than *foxl2* in the ovaries (Nikic & Vaiman, 2004). During sex differentiation, juvenile zebrafish ovaries activate apoptotic pathways and, although further research is needed, *faima* could play a role in the resistance to masculinization and, therefore, could be a potential maker for those females that although subjected to environmental perturbations they still manage to develop ovaries. *Ptpdc1*, upregulated in fish ovaries by elevated density and temperature exposure (FC = 8.64 and 4.59, respectively), is a pleiotropic gene that regulates a variety of cellular processes including cell growth and differentiation (Bonetti et al., 2014). Other examples of common upregulated genes were *ccm2l*, involved in malformation in heart and body axis in zebrafish (Cullere, Plovie, Bennett, MacRae, & Mayadas, 2015), and *ankrd6b*, which phosphorylates and inhibits the β-catenin signal during zebrafish development (Moeller et al., 2006). Among those downregulated

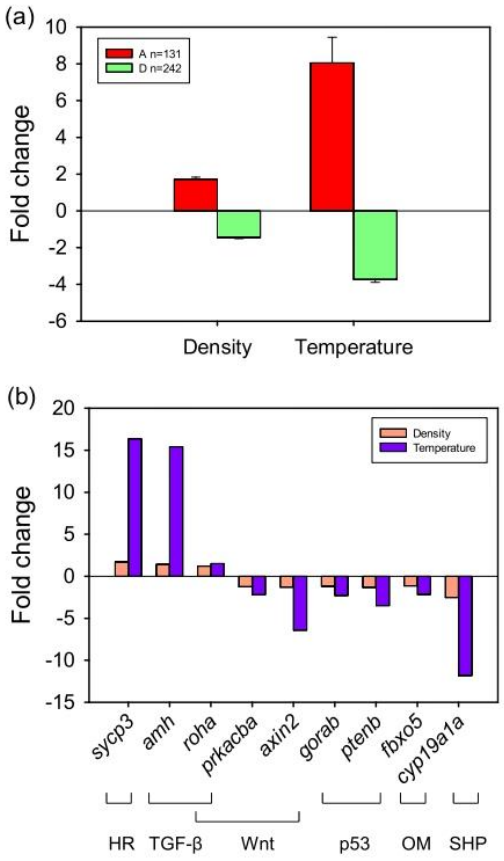

FIGURE 6 (a) Mean fold change for the genes in sets A and D for density and temperature. (b) Fold change of genes involved in homologous recombination (HR; dre03440), transforming growth factor-β (TGF-β; dre04350), Wnt signaling (dre04310), p53 signaling (dre04115), oocyte meiosis (OM; dre04114) and steroid hormone biosynthesis (SHP; dre00140) pathways for density and temperature factors. Note that *rhoa* gene belongs to both the Wnt and TGF-β pathways

genes, we found *lgals9l1* (FC density = −2.85 and temperature = −9.21), 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 larvae 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 nonmasculinized females. It is known that during gonadal development in zebrafish, testis development requires an upregulation of *amh* together with a downregulation of *cyp19a1a* in the immature ovary (X. 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 promale genes and pathways was also observed in ovaries of zebrafish treated with a demethylation agent (decitabine) during sex differentiation, which skewed the sex ratios toward females (Ribas, Vanezis, Imués, & Piferrer, 2017), may indicate that activation of some promale genes is the 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*, *lgals9l1*, 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 yielded 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 (Anastasiadi, Díaz, & Piferrer, 2017; Navarro-Martín et al., 2011). This GO term included the RNA (guanine-7-) methyltransferase (*mmt*), histamine *N*-methyltransferase (*hnmt*) and *suv420h2* genes, which are three methyltransferases that regulate the epigenome of developing organs and of the germline (Anway, Rekow, & Skinner, 2008) the dysfunction of which 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, Starmer, Shibata, Yee, & Magnuson, 2017), and the SET domain containing 3 (*setd3*) gene, which acts as a transcriptional activator of histone H3K36 (Kim, Kim, Kim, & Seo, 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 GnRHr pathway was activated. GnRH stimulates the secretion of luteinizing hormone and follicle-stimulating hormone, both acting synergistically in the reproductive system (Millar, 2005), and in the seabream it was observed that GnRH plays an important role in the regulation of gonadal differentiation, preventing the regression toward the testis from the ovary (Soverchia et al., 2007). In contrast, inflammation mediated by chemokine and cytokine, integrin signaling, and angiogenesis pathways were inhibited. These pathways are involved in the protective response of cells to pathogens, infection, or tissue damage, as observed in zebrafish larvae exposed to pathogens (Díaz-Pascual, Ortíz-Severín, Varas, Allende, & Chávez, 2017). Interestingly, these pathways are also activated during ovarian commitment in zebrafish sex differentiation, which resembles an inflammatory response process in which the activation of the nuclear factor-κβ and Wnt signaling pathways are required (Liew & Orbán, 2013).

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, Liew, et al., 2017)

Factor	Regulation	KEGG pathway description	KEGG ID	No. of genes affected	*P* value
Density	Upregulated	Gonadotropin releasing hormone receptor	dre04080	36	0.0490
	Downregulated	Integrin signaling	dre04510	33	3.10E−04
		Ras signaling	map04014	20	3.90E−03
		Inflammation mediated by cytokine–cytokine receptor interaction	dre04060	41	7.80E−03
		Angiogenesis	dre04370	29	0.0138
Temperature	Upregulated	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
		Cell adhesion molecules	dre04514	86	1.44E−03
	Downregulated	Protein processing in endoplasmic reticulum	dre04141	140	7.25E−10
		ErbB signaling pathway	dre04012	87	1.99E−09
		Ribosome biogenesis in eukaryotes	dre03008	65	1.11E−08
		Phosphatidylinositol signaling system	dre04070	63	1.52E−08

Note. ECM: extracellular matrix; KEGG: Kyoto Encyclopedia of Genes and Genomes.

5 | CONCLUSIONS

Previously, we had shown that exposure of zebrafish to constant, elevated population density since early life masculinized some females. In the present study, we show that it also delayed gonadal maturation in both sexes and, further, that resistant females had a gonadal transcriptome similar in many ways to the transcriptome of females resistant to the masculinizing effects of elevated temperature. These transcriptomic changes included the upregulation of some known promale genes such as *amh*, *sycp3*, and *spata6*, but also of other novel genes not previously related to reproduction, such as *faima*, *ccm2l*, and *ankrd6b*. The commonly downregulated genes included the *lgals9l1* and *ubxn2a* genes, in addition to *cyp19a1a*. These persistent gene expression changes can be used as markers indicative of previous exposure to environmental perturbations of both biotic and abiotic origin present in fish with otherwise apparently normal ovaries. If conserved in other species, these markers could be used in the assessment of the hidden effects of environmental stress. However, it remains to be determined the actual consequences in terms of reproduction in the affected females.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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