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Oakes, J.A., Li, N., Wistow, B.R.C. et al. (5 more authors) (2019) Ferredoxin 1b deficiency leads to testis disorganization, impaired spermatogenesis and feminization in zebrafish. Endocrinology, 160 (10). pp. 2401-2416. ISSN 0013-7227

https://doi.org/10.1210/en.2019-00068

Ferredoxin 1b deficiency leads to testis disorganization, impaired spermatogenesis and feminization in zebrafish

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Short title for page headings: Fdx1b deficiency and testis function in zebrafish.

Key words: Ferredoxin, fdx1b, zebrafish, testes, sperm, androgen.

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Grants and fellowships: This work was supported by the International Fund Congenital Adrenal Hyperplasia 2017 research grant (to N.K. and V.T.C) and the Deutsche Forschungsgemeinschaft (KR3363/3-1).

Disclosure Summary: Nothing to disclose.

Abstract

The roles of steroids in zebrafish sex differentiation, gonadal development and function of the adult gonad are poorly understood. Herein, we have employed a ferredoxin 1b (*fdx1b*) mutant zebrafish to explore such processes. Fdx1b is an essential electron-providing cofactor to mitochondrial steroidogenic enzymes, which are crucial for glucocorticoid and androgen production in vertebrates. *Fdx1b*$^{-/-}$ zebrafish mutants develop into viable adults, in which concentrations of androgens and the glucocorticoid, cortisol, are significantly reduced. Adult *fdx1b*$^{-/-}$ mutant zebrafish display predominantly female secondary sex characteristics but may possess either ovaries or testes, confirming that androgen signaling is dispensable for testicular differentiation in this species, as previously demonstrated in androgen receptor mutant zebrafish. Adult male *fdx1b*$^{-/-}$ mutant zebrafish do not exhibit characteristic breeding behaviors, and sperm production is reduced, resulting in infertility in standard breeding scenarios. However, eggs collected from wild-type females can be fertilized by the sperm of *fdx1b*$^{-/-}$ mutant males by IVF. The testes of *fdx1b*$^{-/-}$ mutant males are disorganized and lack defined seminiferous tubule structure. Expression of several pro-male and spermatogenic genes is decreased in the testes of *fdx1b*$^{-/-}$ mutant males, including pro-male transcription factor SRY-box 9a (*sox9a*) and spermatogenic genes insulin-like growth factor 3 (*igf3*) and insulin-like 3 (*insl3*). This study establishes an androgen- and cortisol-deficient *fdx1b* zebrafish mutant as a model for understanding the impacts of steroid deficiency on sex development and reproductive function. This model will be particularly useful for further investigation of the roles of steroids in spermatogenesis, gonadal development and regulation of reproductive behavior, thus enabling further elucidation of the physiological consequences of endocrine disruption in vertebrates.

Introduction

The development of the gonads and secondary sexual characteristics in zebrafish are highly plastic processes that are sensitive to a range of environmental and physiological signals. These sensitivities make zebrafish an experimentally tractable subject in which to elucidate how abnormalities of steroid metabolism and signaling contribute to the endocrine disruption of reproductive physiology and other sex-specific characteristics[1].

Mitochondrial cytochrome P450 enzymes are crucial for steroidogenesis, and their activity requires electron transfer from cofactors including Ferredoxin 1 (FDX1) (**Figure 1**)[2]. The pathways to mineralocorticoid and glucocorticoid biosynthesis are well conserved between humans and zebrafish (**Figure 1**), however some distinctions exist. Zebrafish possess two *CYP11A1* homologs: *cyp11a1* and *cyp11a2.* Cyp11a2 is thought to be the principal side chain cleavage enzyme in larval and adult steroidogenesis, whereas Cyp11a1 is thought to have important roles in very early development[3]. Cyp11c1 is the zebrafish homolog for CYP11B1, which encodes 11β-hydroxylase in humans. A homolog for the human gene CYP11B2, encoding aldosterone synthase, has not been identified in zebrafish; nor has aldosterone been detected, suggesting that zebrafish are unable to produce this steroid hormone[4].

The pathway to estrogen production is also highly alike in zebrafish and humans, with estradiol acting as the principal estrogen[4]. In contrast, the pathway to androgen production is somewhat different in zebrafish compared to humans (**Figure 1**). The principal active androgen in zebrafish is 11-ketotestosterone (11KT) and this may be produced via multiple pathways. Unlike the situation in humans, dihydrotestosterone is not thought to be a major androgen in teleost fish, although production may be possible[5]. The major pathway for androgen production in zebrafish begins with conversion of androstenedione to 11β-hydroxyandrostenedione by Cyp11c1, which is subsequently converted to 11-ketoandrostenedione by Hsd11b2[6]. Finally, 11-ketoandrostenedione is converted to 11KT by Hsd17b3[6,7].

In an alternative pathway androstenedione may be converted to testosterone by Hsd17b3[7], which is in turn converted to 11β-hydroxytestosterone[6] by Cyp11c1. 11β-hydroxytestosterone is finally converted to 11KT by Hsd11b2[4]. The contribution of this pathway to 11KT production is thought to be minimal as testosterone and 11β-hydroxytestosterone appear to be produced at extremely low levels[6]. The two pathways to 11KT production in zebrafish may be linked by conversion of 11β-hydroxyandrostenedione to 11β-hydroxytestosterone by Hsd17b3. This reaction has been demonstrated in cells expressing *hsd17b3*[7], but is unlikely to occur *in vivo*[6,8].

In humans, FDX1 is an important co-factor to the steroidogenic cytochrome P450 side-chain cleavage enzyme (CYP11A1), aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1)[2]. FDX1, and the associated ferredoxin reductase, act as sequential electron donors, facilitating transfer of electrons from NADPH to cytochrome P450 enzymes[2]. This process is crucial for processing of target substrates. The zebrafish ortholog of FDX1, Fdx1b, appears to be the electron-providing cofactor to the steroidogenic cytochrome P450 side-chain cleavage enzymes (Cyp11a1, Cyp11a2) and cytochrome P450 11β-hydroxylase (Cyp11c1), however, these functions have not been definitively proven. Cyp11a1 and Cyp11a2, homologs of CYP11A1, catalyze the conversion of cholesterol to pregnenolone, while Cyp11c1, the zebrafish homolog of CYP11B1, catalyses the 11β-hydroxylation of 11-deoxycortisol to produce cortisol[9]. Cyp11c1 may also facilitate conversion of the mineralocorticoid 11-deoxycorticosterone to corticosterone in teleost fish[10]. Unlike in mammals, 11β-hydroxylase is postulated to play a crucial role in androgen synthesis in zebrafish, namely the 11β-hydroxylation of androgen precursors (**Figure 1**). Zebrafish possess two ferredoxin 1 paralogs: *fdx1* and *fdx1b*. We have previously shown that *fdx1b*$^{-/-}$ mutant larvae are profoundly glucocorticoid deficient whereas *fdx1* morphants displayed a severe early developmental phenotype[9]. Fdx1b deficient larvae exhibit decreased expression of glucocorticoid responsive genes phosphoenolpyruvate carboxykinase 1 (*pck1*) and FK506 binding protein 5 (*fkbp5*), a glucocorticoid receptor chaperone protein. Expression of *pck1* and *fkbp5* does not appreciably increase after a stressor is applied, indicating an absent or heavily attenuated cortisol response to stress. The role of

Fdx1b in adult zebrafish has not been investigated, and the impact of Fdx1b deficiency on sex steroid hormone synthesis remains unknown.

Androgen signaling is pivotal in regulation of the development of male secondary sex characteristics, gonads, and reproductive behavior. Mutation of the androgen receptor (AR) results in adults with predominantly female morphological characteristics; however, some fish possess testes, indicating that the AR is dispensable for testis differentiation. Homozygous AR mutant males possess testes with disorganized seminiferous tubules, and are infertile[11,12]. Mutation of the AR has also been linked to impaired courtship behavior[13] and defective spermatogenesis[14]. Exposure of zebrafish to the androgen trenbolone during development causes robust masculinization[15,16], whilst anti-androgen treatment causes a shift towards female development and delayed testes maturation[17].

Herein, we have used *fdx1b*$^{-/-}$ mutant zebrafish to investigate the roles of Fdx1b in androgen biosynthesis, sex differentiation and development, gonadal function and reproductive behavior in zebrafish. Fdx1b-deficient male zebrafish exhibit feminization of secondary sex characteristics, decreased androgen production, disorganized testicular structure, decreased spermatogenesis, abnormal reproductive behavior and infertility. Our results suggest that Fdx1b is the key electron-providing co-factor to Cyp11c1 in glucocorticoid and androgen biosynthesis in zebrafish.

Methods

Zebrafish husbandry and ethics

The mutant zebrafish utilized in this study were previously created by our group using a TALEN approach to achieve targeted genetic disruption of *fdx1b.* This *fdx1b* mutant allele (allele number UOB205) contains a 12bp deletion in exon 4, resulting in a 4 amino acid in-frame deletion (c.295_306del; p.Cys99_Ile102del) in a functionally conserved motif[9]. Zebrafish were maintained in a recirculating system (ZebTECTM, Tecniplast®, Kettering, UK, and Sheffield, UK) at 28.5°C in a 10:14 dark/light photoperiod. Embryos were obtained by natural spawning and incubated at 28.5°C in E3 medium (5mmol/L NaCl, 0.17mmol/L KCl, 0.33mmol/L $CaCl_2$, 0.33mmol/L MgSO4). All fish were humanely euthanized by administration of the anesthetic tricaine mesylate (Pharmaq, Fordingbridge, Hampshire, UK) followed by destruction of the brain by piercing of the skull with a needle. All procedures involving zebrafish were performed in compliance with local and UK animal welfare laws, guidelines and policies.

Morphological Analysis

After humane euthanization, adult zebrafish were measured and their weight was recorded. Fish were photographed intact under a dissecting microscope and subsequently dissected to expose the gonads, which were also photographed. The gonads and liver were collected for RNA extraction.

Steroid measurements by liquid chromatography tandem mass spectrometry

Adult zebrafish were humanely euthanized and transferred to a silanized test tube and snap frozen on dry ice. Samples were further frozen in liquid nitrogen before fine grinding using a Mikro-Dismembrator S (Sartorius, Göttingen, Germany) and freeze drying. Approximately 50mg of the

dried samples were transferred to a 2ml microcentrifuge tube and resuspended in 900µl ultrapure deionized MilliQ water (Millipore, Burlington, Massachusetts, United States) and 100µl MilliQ water containing deuterated internal standard (15ng D4-cortisol, D8-17α-hydroxyprogesterone, D7-androstenedione and 1.5ng D2-testosterone). A small metal bead was added to each tube and the samples were subsequently homogenized using an Omni bead rupter 24 (Omni International, Inc., Kennesaw, Georgia, United States) for 1.5 minutes at 3.3m/s. The samples were then centrifuged at 8000 x g for 5 minutes. The resulting supernatant was collected in a glass test tube and the steroids were extracted twice using methyl tertiary-butyl ether (MTBE) (1:3). The pellet was resuspended in 1ml MilliQ water, homogenized again and extracted as described. All MTBE fractions for each sample were pooled and dried under a stream of nitrogen at 45°C. The dried residue was resuspended in 1ml methanol and eluted through a Phree column (Phenomenex, Torrance, California, United States) to remove remaining phospholipids. The column was washed with an additional 1ml methanol and the resulting 2ml eluent was dried and resuspended in 150µl 50% MeOH prior to analysis. Steroids were separated and quantified using an Acquity UPLC System (Waters, Milford, Connecticut, United States) coupled to a Xevo TQ-S tandem mass spectrometer (Waters) as previously described[18].

Breeding and *in vitro* fertilization (IVF)

Breeding was conducted to assess the fertility of Fdx1b-deficient zebrafish. Two common methods were employed: marbling [19] and pair mating. The pair mating technique was used to outcross *fdx1b*$^{-/-}$ fish and wild-type siblings to unrelated wild-type fish. Wild-type fish could be sexed by conventional sex differences whereas *fdx1b*$^{-/-}$ fish were sexed based on presence or absence of a rounded abdomen, which was indicative of an ovary. On the afternoon prior to a breeding trial an *fdx1b*$^{-/-}$ fish was transferred to a pair mating tank, along with an unrelated wild-type fish of the opposite sex. The following morning, shortly after illumination of the aquarium, a divider separating male and female

fish was removed and they were allowed to mate. Released eggs fell through slots in the bottom of an inner container and were collected in a larger outer container.

For IVF experiments, sperm was collected by dissection of humanely euthanized adult males in order to remove the testes, which were then homogenized in a 50x mass:volume dilution with 600mOsm/kg Hank's balanced salt solution prepared as previously described[20]. Eggs were collected from anesthetized wild-type females by gentle palpation of the abdomen. Eggs were transferred to a 35mm Petri dish and either 10µl of sperm solution collected by stroking of the abdomen or 50µl of sperm solution obtained by testes dissection was added, followed by 400µl of aquarium water to activate the sperm. After 2 minutes a further 2ml of aquarium water were added[19]. Fertilization of eggs was confirmed by visualization under a dissecting microscope at low magnification and raised at 28.5°C to 5 days post fertilization (dpf) before culling.

Analysis of breeding behavior

Fdx1b$^{-/-}$ male zebrafish or wild-type male siblings were paired with a wild-type female fish on the evening before breeding, as described above. The following morning, breeding was video recorded from above the tank using the Zebralab software (Viewpoint, Lyon, France). Following this, breeding behaviors in the first five minutes after removal of the divider were analyzed. Intimate contacts, where fish touched or crossed one another, and total time fish spent exhibiting chasing behavior was recorded. Any instance where one fish was closely following the other was classified as chasing behavior.

Haematoxylin and eosin (H&E) staining

Adult fish were humanely euthanized and then fixed in 4% paraformaldehyde (PFA) for 4 days at 4°C, followed by brief washing in PBS. Fish were decalcified in 0.25M EDTA pH8 for 4 days followed by transfer to 70% ethanol and storage at 4°C. The head and caudal and anal fins were removed from the zebrafish and the samples were transferred to a tissue processor (Leica TP2010) for dehydration and paraffin infiltration. Following this, the samples were embedded in paraffin wax and 5µm sections were cut through the gonad. For haematoxylin and eosin (H&E) staining, samples were dewaxed and rehydrated by transfer through a series of ethanol baths at decreasing concentrations. Samples were stained with Gill's haematoxylin for 1 minute before washing and dehydration by transfer through increasing ethanol concentration series. Samples were stained with 1% eosin in 95% ethanol for 30 seconds and subsequently washed in absolute ethanol. Samples were transferred to xylene and mounted using DPX mountant.

Sperm counting

Sperm was collected by dissection of humanely euthanized adult males to remove the testes followed by homogenization of testis tissue in a 100x mass:volume dilution with 600 mOsm/kg Hank's balanced salt solution prepared as previously described[20]. Alternatively, sperm was collected from anesthetized male fish by gentle stroking of the abdomen with blunt ended forceps (Millipore, Burlington, Massachusetts, United States) and aspiration of expelled semen into a capillary tube. Semen was immediately transferred to a 20µl aliquot of 600mOsm/kg Hank's balanced salt solution. 10µl of this sperm solution were transferred to each counting chamber of a dual-chamber Improved Neubauer haemocytometer (Hawksley, Sussex, UK) and the number of sperm in each sample was counted in duplicate, according to the protocol specified in the WHO Laboratory manual for the

examination and processing of human semen[21]. Gonadosomatic index (GSI) was calculated using the formula GSI = [gonad weight / total tissue weight] × 100.

qPCR

Total RNA was extracted from liver or gonad tissue using Trizol (Ambion, Texas, United States). 1µg of RNA was used for a first strand cDNA synthesis reaction using the Superscript III kit (Thermo Fisher Scientific, California, United States) and 20mer oligo(dT) primers (IDT, Coralville, Iowa, United States). qPCR primers were taken from previously published studies or designed using primer3[22] and are listed in **Table 1**. Each primer pair was determined to have an amplification efficiency of between 90-110% and an R^2 value of >0.98. 10µl reactions consisting of 5µl of PowerUP SYBR Green Master Mix (Applied Biosystems, California, United States), 1µl forward and reverse primers (1000nM), 1µl cDNA and 3µl H_2O were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, California, United States). qPCR data was analyzed using the Pfaffl method [23] with elongation factor 1 alpha (*ef1a*) as the reference gene. Fold changes in gene expression are displayed relative to expression in wild-type male tissue.

Statistical methods

All statistical analyses were conducted using Graphpad Prism (GraphPad Software, San Diego, California, United States). Data normality was assessed using either a Shairo-Wilk normality test or D'Agostino-Pearson normality test. When data was not normally distributed, outliers were identified using the ROUT method. Normally distributed data was analyzed using unpaired *t*-tests. Data that was not normally distributed was analyzed using Mann-Whitney tests. Statistical significances are reported using asterisks as follows: * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Results

Morphological characterization and feminization of secondary sex characteristics in *fdx1b*$^{-/-}$ zebrafish

Morphological secondary sex characteristics in zebrafish include pigmentation, abdominal shape and the prominence of the genital papilla. Androgens are involved in the development of male secondary sex characteristics, including pigmentation, in a wide range of fish species[24]. Upon raising the progeny of an *fdx1b* heterozygous mutant in-cross to adulthood, it was observed that the homozygous mutant population displayed only female pigmentation patterns. Dissection of these mutant fish revealed that adult *fdx1b*$^{-/-}$ zebrafish possessed either testes or ovaries, despite their external appearance as female. No bias towards ovary or testis development was observed in homozygous *fdx1b* mutant zebrafish compared to wild-types, and inheritance of the *fdx1b* mutant allele followed Mendelian ratios. At the earliest stage examined, which was 73dpf, directly after genotyping by fin-clipping, wild-type males and females could be readily distinguished by sex specific differences in pigmentation of the dorsal fin. Wild-type males exhibited a pale or transparent dorsal fin, whereas females exhibited a green-yellow pigmented fin (**Figure 2**). All *fdx1b*$^{-/-}$ fish displayed female type pigmentation of the dorsal fin, irrespective of gonadal sex. In older wild-type fish, differences in the coloration of the anal fin also became apparent. Wild-type male zebrafish have dark blue- and golden-striped anal fins. Blue pigmentation is similar in male and female wild-type zebrafish whereas the orange pigmentation is reduced or absent in wild-type females. (**Figure 2**). We observed an absence of dark golden pigmentation in the anal fins of *fdx1b*$^{-/-}$ fish and this was independent of their gonadal sex. Wild-type male zebrafish often display dark golden and purple body coloration, and this was also reduced in *fdx1b*$^{-/-}$ males. Wild-type females had large and prominent genital papillae in comparison to males, and also had a rounded abdominal shape due to presence of an ovary. Genital papilla prominence and abdominal shape were unaffected by mutation of *fdx1b* and could be used to robustly predict gonadal sex. *Fdx1b*$^{-/-}$ males were larger than wild-type

male siblings, and both length and weight were significantly increased. *Fdx1b*$^{-/-}$ females were found to be significantly heavier than wild-type female siblings, but no difference in length was recorded (**Supplementary figure 1**)[25].

Decreased concentrations of 11-ketotestosterone and cortisol in *fdx1b*$^{-/-}$ zebrafish

Fdx1b is a cofactor for the steroidogenic enzymes Cyp11a1, Cyp11a2 and Cyp11c1. Cyp11a1 and Cyp11a2 catalyze the first stage in steroid biosynthesis: conversion of cholesterol to pregnenolone[3]. Cyp11c1 is required for the final step of cortisol biosynthesis, as well as 11β-hydroxylation of androstenedione in the androgen biosynthetic pathway[4]. Cyp11c1 may also be required for 11β-hydroxylation of testosterone in the alternative pathway to 11KT production. (**Figure 1**).

In order to determine the impact of Fdx1b deficiency on steroid hormone production, LC-MS/MS was used to quantify whole body steroid hormone concentrations (**Figure 3**). Significantly decreased concentrations of 11KT and cortisol were measured in *fdx1b*$^{-/-}$ males in comparison to wild-type sibling males. To determine the precise impact of Fdx1b deficiency on steroidogenesis, we also measured the concentrations of glucocorticoid and androgen precursors **(Figure 3)**. As Fdx1b is involved in the first stage of steroidogenesis a blockage of the whole pathway might be expected; however, an apparent decrease in 17α-hydroxyprogesterone was not significant (p=0.069). This may be because concentrations of 17α-hydroxyprogesterone were close to the limit of detection; alternatively, Fdx1 may be compensating for loss of Fdx1b function in this reaction, as both are expressed in the testes[9]. Androstenedione concentrations were significantly increased in *fdx1b*$^{-/-}$ mutants, indicating an increase of sex steroid precursors, probably due decreased 11β-hydroxylation of this steroid and shunting of glucocorticoid precursors into the sex steroid pathway. In contrast, no significant change in testosterone concentration was observed. 11β-hydroxylated 11KT precursors were undetectable in *fdx1b*$^{-/-}$ mutant male zebrafish, providing strong evidence for the essential role

of Fdx1b as an electron-providing co-factor for the steroidogenic reactions facilitated by Cyp11c1 (**Figure 3**). Very similar results with comparable fold changes were recorded in a replicate experiment, confirming disruption of steroidogenesis in Fdx1b deficient zebrafish (**Supplementary figure 2**)[25].

In addition, systemic deficiency of glucocorticoids and androgens was confirmed by measuring the expression of established steroid responsive genes in liver tissue. *Fkbp5* and *pck1* are known to be robust glucocorticoid-responsive genes in zebrafish[9,26], with *FKBP5* expression in humans and mice also influenced by androgen receptor transactivation[27]. *Fkbp5* expression is also inducible by exogenous androgens in zebrafish larvae[28]. *Cyp2k22* was used as a well-established androgen-responsive gene in zebrafish[28,29]. The expression of all three genes was decreased in *fdx1b*$^{-/-}$ males compared to wild-type siblings (**Figure 4**).

In order to further characterize the impact of loss of Fdx1b function and decreased androgen and cortisol concentrations on gonadal steroidogenesis, we measured the expression of key steroidogenic enzymes in this pathway, as well as that of the transport protein *steroidogenic acute regulatory protein* (*star)*. *Cyp11a2* and *cyp11c1,* which are crucial for cholesterol side chain cleavage and conversion of androgen precursors respectively, were expressed at significantly higher levels in the testes of *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings (**Figure 5**). The expression levels of *star* and *hsd17b3* also suggested increased expression in *fdx1b*$^{-/-}$ testes*,* however these changes did not achieve statistical significance (**Figure 5**). No changes in the expression of *cyp17a1,* which is crucial for gonadal sex steroid production (**Figure 1**), or the estrogenic enzyme *cyp19a1a* were detected (**Figure 5**).

Fdx1b$^{-/-}$ males and females are infertile using conventional breeding techniques

Following the finding that Fdx1b mutant males exhibit mainly female secondary sex characteristics, we investigated whether loss of Fdx1b function affects fertility in response to the conventional marbling technique[19]. Tanks of *fdx1b*$^{-/-}$ zebrafish or wild-type siblings containing a mixture of males and females were marbled weekly for four weeks. Fertilized embryos were obtained from groups of wild-type siblings in all four trials. However, groups of *fdx1b*$^{-/-}$ fish failed to produce fertilized embryos in any trial and no unfertilized embryos were observed either. To investigate whether this apparent infertility was due to a defect in male or female reproductive physiology, or both, *fdx1b*$^{-/-}$ or wild-type siblings were outcrossed with unrelated wild-type fish. Outcrossing was repeated for four weeks to ensure that the fish were habituated to the technique. In four weekly trials, *fdx1b* wild-type sibling males (n=5) and females (n=4) successfully produced fertilized embryos in 70% and 75% of trials respectively. *Fdx1b*$^{-/-}$ males (n=5) and females (n=5) were unsuccessful in all trials (**Table 2**).

To investigate whether sperm from *fdx1b*$^{-/-}$ zebrafish could fertilize eggs from wild-type females *in vitro,* sperm was collected by dissecting out and homogenizing the testes from humanely euthanized *fdx1b*$^{-/-}$ mutant and wild-type males. Sperm collected from 2 out of 6 wild-type sibling male zebrafish and 2 out of 5 *fdx1b*$^{-/-}$ male zebrafish successfully fertilized eggs collected from wild-type females, indicating that at least some *fdx1b*$^{-/-}$ male zebrafish can produce functional sperm.

Fdx1b mutant male zebrafish exhibit reduced breeding behaviors

Steroid hormones are known to exert powerful impacts on the vertebrate brain and a wide range of behaviors. To investigate whether reproductive behaviors were affected by loss of Fdx1b function, we paired *fdx1b*$^{-/-}$ mutant males or wild-type sibling males with wild-type females and quantified two characteristic behaviors previously reported in zebrafish: intimate contacts between fish[30] and

chasing[31]. Both of these behaviors were significantly reduced in *fdx1b*$^{-/-}$ zebrafish (**Supplementary figure 3**)[25].

Testes of *fdx1b*$^{-/-}$ zebrafish are disorganized and have decreased sperm production

Our findings have shown that *fdx1b*$^{-/-}$ males are unable to reproduce by conventional breeding methods, however their sperm can fertilize eggs from wild-type females in IVF experiments. These findings led us to investigate the histological structure of *fdx1b*$^{-/-}$ mutant testes. Histological examination of the testes by H&E staining revealed that the testes of wild-type zebrafish were well organized and had defined seminiferous tubules bounded by a basement membrane. Within the centre of each tubule cross-section were mature spermatozoa, surrounded by groups of developing spermatogonia, spermatocytes and spermatids located more peripherally. By contrast, the testes of *fdx1b*$^{-/-}$ mutant zebrafish were considerably disorganized and had much less distinct seminiferous tubules. Despite this disorganization, developing and mature sperm were present (**Figure 6**).

Histological examination also revealed a reduction of mature sperm present in the testes of *fdx1b*$^{-/-}$ mutants compared to wild-type siblings (**Figure 6**). To quantify this finding, we employed sperm counting techniques to determine the concentration of sperm in whole testes relative to testes weight. Sperm concentrations were decreased in *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings when sperm was collected from dissected testes (**Figure 6**). Following activation of sperm by addition of water, no obvious difference in sperm motility was apparent between sperm from *fdx1b*$^{-/-}$ mutant and wild-type sibling zebrafish. Sperm were observed under a 40x microscope objective. Moreover, there was no difference in gonadosomatic index (GSI) between *fdx1b*$^{-/-}$ mutant males and wild-type siblings (GSI = [gonad weight / total tissue weight] × 100) (**Figure 6**).

The disorganized appearance of *fdx1b*$^{-/-}$ mutant testes led us to hypothesize that release of sperm from the testes may be compromised in the absence of Fdx1b function. To determine whether

fdx1b$^{-/-}$ mutant males were able to release sperm, wild-type (n=8) and *fdx1b*$^{-/-}$ mutant (n=5) zebrafish were subjected to manual gamete expression and expressed fluid was collected from the urogenital papilla. All wild-type samples contained microscopically visible sperm, however this was only true for 3 out of 5 *fdx1b*$^{-/-}$ mutant samples In a repeat experiment, sperm were observed in all nine wild-type samples and 3 out of 10 *fdx1b*$^{-/-}$ samples, indicating a decreased ability to release sperm in *fdx1b*$^{-/-}$ zebrafish. It is not clear if *fdx1b*$^{-/-}$ mutant zebrafish that produced no sperm failed to produce any semen, or produced semen devoid of sperm.

Qualitatively, samples obtained from *fdx1b*$^{-/-}$ mutants also appeared to be more transparent compared to wild-type samples, which were milky and opaque. Mutant samples also appeared to occupy less volume in collection capillary tubes, although this could not be accurately measured due to the small volume of each sample. Sperm concentration in semen collected from *fdx1b*$^{-/-}$ mutant males by abdominal massage was also significantly decreased compared to wild-type siblings (**Figure 6**).

Downregulation of key genes involved in testis development and spermatogenesis

To investigate the molecular mechanisms underlying the phenotype described above, we used qPCR to measure the expression of genes involved in sex differentiation and spermatogenesis. Sox9a is a pro-testis transcription factor expressed in Sertoli cells of the testes, which has a crucial role in testis differentiation that is conserved throughout most vertebrate species[32,33].We observed a significant downregulation of *sox9a* expression in the testes of adult *fdx1b*$^{-/-}$ males compared to wild-type siblings (**Figure 7**). Sox9a is a pro-male transcription factor expressed in Sertoli cells[32].

No significant difference in the expression of doublesex and mab-3 related transcription factor-1 (*dmrt1*) or anti-Müllerian hormone (*amh*) was found between *fdx1b*$^{-/-}$ males and wild-type siblings (**Figure 7**). *Dmrt1* is a crucial transcription factor essential for male development[34]. Amh is a pro-

testis hormone during zebrafish development[32] and negatively regulates spermatogenesis in the adult testis[35,36].

Igf3 and *insl3* encode spermatogenic signaling molecules expressed by Sertoli and Leydig cells respectively. Igf3 is required for proliferation and differentiation of type A undifferentiated (A_{und}) and type A differentiating (A_{diff}) spermatogonia[35,37], whereas Insl3 is required only for proliferation and differentiation of type A_{und} spermatogonia[38]. Expression of *igf3* was downregulated 10-fold, whereas expression of *insl3* was downregulated 25-fold in *fdx1b*$^{-/-}$ mutant testes compared to wild-type (**Figure 7**). As both factors are involved in positively regulating the proliferation and differentiation of type A spermatogonia in the early stages of spermatogenesis[37,38], our results are consistent with the decreased sperm quantity observed by histology and sperm counting.

We also observed a significant 4-fold downregulation of inhibin subunit alpha (*inha*) in *fdx1b*$^{-/-}$ mutant testes compared to wild-type (**Figure 7**). Inhibins exert negative feedback on the hypothalamus-pituitary-gonadal axis and suppress FSH secretion[39], and this mechanism has been suggested to occur in fish[40]. In mice, *inha* is important for Sertoli cell proliferation and function and may play a role in spermatogenesis[41].

Expression of spermatogenesis marker genes in *fdx1b*$^{-/-}$ mutant and wild-type sibling testes

We observed decreased sperm concentration in *fdx1b*$^{-/-}$ mutant zebrafish testes compared to wild-type siblings. To investigate how Fdx1b deficiency impacts spermatogenesis, the expression of markers of germ cells at several stages of spermatogenesis were measured (**Figure 8**). Significant upregulation of nanos homolog 2 (*nanos2*) (type A_{und} spermatogonia)[42,43] and piwi-like RNA-mediated gene silencing 1 (*piwil1*) (all type A spermatogonia)[44] was observed in *fdx1b*$^{-/-}$ testes, suggesting a possible impairment of spermatogenesis during differentiation of type A spermatogonia into type B spermatogonia, causing an accumulation of type A spermatogonia (**Figure 9**). No change

in expression of deleted in azoospermia-like (*dazl*), expressed mainly in type B spermatogonia[44], the spermatocyte marker synaptonemal complex protein 3 (*sycp3*)[45] or the spermatid marker outer dense fiber of sperm tails 3B (*odf3b*)[37,46] was observed (**Figure 8**).

Discussion

We have used *fdx1b*$^{-/-}$ mutant zebrafish to investigate the roles of steroids in the development and function of the male gonad and secondary sexual characteristics. By measuring concentrations of 11KT and cortisol, we have shown that this steroidogenic co-factor is important for their production through its activity as a co-factor to the enzyme Cyp11c1. In cortisol biosynthesis, Cyp11c1 catalyses 11β-hydroxylation of 11-deoxycortisol to produce cortisol. The requirement for Fdx1b in this reaction has been previously demonstrated in zebrafish larvae[9] and is confirmed here in adult zebrafish. In addition to its role in glucocorticoid biosynthesis, Cyp11c1 plays a vital role in the androgen biosynthetic pathway in zebrafish: 11β-hydroxylation of androstenedione and testosterone to produce 11β-hydroxyandrostenedione and 11β-hydroxytestosterone respectively[4]. Decreased concentrations of 11KT, as well as undetectable concentrations of 11β-hydroxylated precursors in our *fdx1b*$^{-/-}$ mutant zebrafish, define the requirement for Fdx1b as an essential steroidogenic co-factor to Cyp11c1 in the androgen biosynthetic pathway in adult zebrafish for the first time. In humans, FDX1 is also a cofactor to CYP11A1, crucial for conversion of cholesterol to pregnenolone[2]. Fdx1b is thought to be the corresponding cofactor in zebrafish, however, concentrations of 17α-hydroxyprogesterone were not significantly different between *fdx1b*$^{-/-}$ males and wild-type siblings (Figure 3). This may indicate that Fdx1b is less important as a co-factor to Cyp11a1/2 than Cyp11c1, or that Fdx1 is compensating for loss Fdx1b function, as both are expressed in the testes[9]. Analysis of the expression of steroidogenic enzymes in the testes of Fdx1b mutant zebrafish was suggestive of upregulation of several genes encoding proteins crucial for the production of androgens. This is in agreement with studies on AR mutant zebrafish, which also exhibit increased expression of several steroidogenic enzymes in the testes[11,14].

Both AR[11,12] and Fdx1b deficient male zebrafish exhibit feminized secondary sex characteristics, including coloration and abdominal shape, however genital papilla prominence is not affected. These

results indicate that androgen signaling plays a role in pigment cell fate specification, differentiation, or organization in adult zebrafish, but does not control the development of the genital papilla.

In contrast to male AR deficient zebrafish, which have a female-like rounded abdomen[12], male Fdx1b deficient fish had a streamlined shape closer to the appearance of wild-type males. Abdominal shape may be affected by the absence or presence of ovaries, which are much larger than testes, or presence of adipose tissue. Although no effect on abdominal shape was observed in our study, significant changes in biometric parameters were observed as a result of loss of Fdx1b function. *Fdx1b*$^{-/-}$ males had increased length and weight compared to wild-type siblings **(Supplementary figure 1)**[25], however, no difference between wild-type males and females was detected, indicating that this phenotype may be independent of sex. Length is not affected by AR mutation, whereas the effect on weight is unclear, with increased weight reported in one AR mutant[14] and no change reported in another AR mutant line[11]. Regarding the contrasting findings relating to abdominal shape and biometrics in AR and Fdx1b mutants, a number of explanations are possible. In addition to reduced or disrupted androgen signaling, *fdx1b*$^{-/-}$ mutant zebrafish also have reduced concentrations of cortisol, which may contribute to this phenotype. Interestingly, *in vitro* analysis has shown that testosterone transactivates the zebrafish AR to a similar degree as 11KT[6,47,48]; however, testosterone was apparently unable to replace 11KT as the principal androgen in our Fdx1b deficient zebrafish. Phenotypic differences between Fdx1b and AR deficient zebrafish may be explained by tissue specific sensitivity to different androgens. AR signaling is abolished in AR mutant zebrafish, whereas the AR may still be transactivated by testosterone to some degree in Fdx1b deficient zebrafish[6].

As with AR mutant zebrafish[11,12], we found that mutant males were infertile under normal breeding conditions and were either unable to release sperm or unable to induce egg laying by females. By contrast, another AR mutant line[13] was able to induce egg laying. However, whether or not these eggs were fertilized is unclear, and it has been speculated that this AR mutant represents a hypomorph due to translation of an alternate transcript[12], rather than a complete null allele. *Fdx1b*

mutant zebrafish have disorganized testes with poorly defined seminiferous tubules and fewer sperm than their wild-type siblings. Zebrafish and mouse AR mutants also exhibit disorganized seminiferous tubules[12,14,49] and seminiferous tubule dysgenesis is seen in some cases of complete androgen insensitivity syndrome[50]. Attempts to manually collect sperm from Fdx1b mutant males and wild-type siblings revealed that mutant fish could release semen but that the concentration of sperm in their ejaculates was decreased. This could be explained by dysgenesis or blockage of the tubules of the male reproductive tract or testes, resulting in reduced sperm release. Although we have shown that it is possible for functional sperm to be released through the urogenital orifice of Fdx1b deficient zebrafish by abdominal massage, it is not possible to know whether this happens under natural conditions. In addition to potential anatomical aberrations contributing to reduced sperm release, impaired breeding behavior may also contribute to this phenotype by the absence of behaviors that stimulate release of gametes of either male or female origin. Both possibilities have been suggested in AR mutant zebrafish[12,13], and androgen deficient *cyp17a1* mutant zebrafish also exhibit impaired breeding behavior[30].

We measured significant down-regulation of the pro-male transcription factor *sox9a* in *fdx1b*$^{-/-}$ mutant testes. In zebrafish, *sox9a* is crucial for juvenile ovary to testis transformation[32,33], implicating disruption of this process in the phenotype we have observed. An ortholog of this gene in another teleost fish (*Oryzias latipes*), *sox9a2*, is maintained at the initial stage of testicular tubule development in males, but is down-regulated in the developing female gonad, leading to speculation that Sox9 is important for testicular tubule development[51]. Taken together, these findings provide evidence for the role of androgens in testicular morphogenesis and add credence to the theory that Sox9 regulates formation of testicular tubule structure.

We have observed significant downregulation of *igf3* and *insl3* expression, genes which have been shown to be upregulated in Atlantic Salmon following treatment with 11KT[52]. Both *igf3* and *insl3* are downregulated in AR mutant zebrafish testes[11] as well as in fish exhibiting estrogen-induced

androgen insufficiency[53]. Decreased availability of spermatogenic factors igf3 and insl3 may result in accumulation of type A spermatogonia (**Figure 9**), and this was demonstrated by increased expression of *nanos2* and *piwil1* in *fdx1b*$^{-/-}$ mutant testes. *Nanos2* is a marker of type A_{und} spermatogonia, whereas *piwil1* is expressed in all type A spermatogonia. This suggests that expression of *igf3* and *insl3* was sufficient for proliferation of type A spermatogonia but was insufficient to promote their normal differentiation into type B spermatogonia. Surprisingly, expression of genes known to be markers of later stages of spermatogenesis were unaffected by Fdx1b deficiency. However, sperm counting demonstrated a clear decrease in sperm concentration in *fdx1b*$^{-/-}$ mutant zebrafish, confirming a significant impairment of spermatogenesis due to Fdx1b deficiency.

The expression of *inha* was significantly decreased in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Inha is involved in the regulation of FSH expression in mammals and fish and is implicated in Sertoli cell proliferation and function in mice[39-41] and zebrafish[35]. Decreased expression of *inha* suggests compromised Sertoli cell function, which may indirectly reduce spermatogenesis, and also implicates regulation of the HPG axis and gonadotropins in the phenotype we have described. Overall, our findings indicate that a reduction in the differentiation of type A spermatogonia due to decreased expression of spermatogenic factors *igf3* and *insl3,* as well as compromised Sertoli cell function, contribute to decreased spermatogenesis in Fdx1b deficient zebrafish.

Several genes expressed in Sertoli cells are downregulated in Fdx1b mutant zebrafish, including *sox9a, inha* and *igf3.* Sertoli cells are crucial for testis differentiation and morphogenesis in mouse[54], and Sox9 signaling is required for juvenile ovary to testis transformation in zebrafish[32]. The testicular phenotypes we have described may well result from Sertoli cell dysfunction as a result of androgen deficiency. The nature of this dysfunction remains an exciting topic for further study.

In zebrafish, only the roles of Fdx1b in glucocorticoid and androgen synthesis have been investigated. Many of our results are in line with previous studies, which have shown that impaired

androgen signaling results in a similar phenotype to that which we have described[12]. In addition to this, androgen insufficiency and androgen receptor mutations cause similar changes in gene expression, including downregulation of *igf3* and *insl3*[11,53]. This suggests that the phenotype we have described is likely to be due to androgen deficiency, however, other effects of Fdx1b mutation cannot be ruled out.

Increased stress and exposure to exogenous cortisol during the period of sex differentiation has been shown to have a masculinizing effect[55] and it is feasible that cortisol deficiency may have the opposite effect. In addition to this, cortisol is thought to play a role in several reproductive processes in a variety of teleosts[56]. However, no feminization of secondary sex characteristics or breeding difficulties were reported in a glucocorticoid receptor mutant zebrafish, indicating that absent signaling by cortisol is unlikely to play a major role in the phenotype we have observed[57,58].

In summary, our work establishes an androgen and cortisol deficient Fdx1b zebrafish mutant as a model for the impacts of steroid hormone deficiency on sex development and testicular function. Androgen deficiency in *fdx1b*$^{-/-}$ mutant zebrafish is likely to be causative for the observed phenotype comprising infertility, testicular dysfunction and structural disorganization, as well as impaired breeding behavior. This model will be particularly useful for further investigation of the roles of steroids in pigment patterning, spermatogenesis and gonadal development and maintenance, and represents a novel tool for investigation of endocrine disruption in vertebrates.

Acknowledgements

The authors would like to thank the University of Sheffield aquarium team for day-to-day maintenance of fish stocks, Freek van Eeden and Maggie Glover for technical advice and Helen Eachus, Jack Paveley and Dheemanth Subramanya for useful discussions contributing to this study.

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689

690 **Figure legends**

691 **Figure 1. Steroid biosynthesis pathways in zebrafish.** Biosynthetic pathways for production of
692 steroid hormones including mineralocorticoids, glucocorticoids and sex steroids. Reactions known or
693 postulated to depend on Fdx1b are shown in red boxes. The principal pathway for 11KT biosynthesis
694 is indicated with solid arrows, whereas minor alternative pathways are indicated with dashed
695 arrows.

696

697 **Figure 2. Feminization of secondary sex characteristics in *fdx1b*$^{-/-}$ zebrafish.** Wild-type male adult
698 zebrafish exhibit a pale dorsal fin and a blue and golden striped anal fin. Wild-type female adult
699 zebrafish exhibit a yellow-green pigmented dorsal fin and lack the strong stripes of gold pigment
700 seen in the male anal fin. Mutant male adult zebrafish exhibited female type coloration of the dorsal
701 and anal fins throughout adulthood. No obvious difference in macroscopic gonadal morphology was
702 observed.

703

704 **Figure 3. Profoundly altered glucocorticoid and androgen biosynthesis steroid profiles in male**
705 ***fdx1b*$^{-/-}$ zebrafish.** The concentration of steroids was measured in samples prepared from whole fish
706 bodies. The concentration of cortisol (**A**) was profoundly reduced in *fdx1b*$^{-/-}$ mutant male zebrafish
707 (n=3, p=0.0044). The concentration of 17α-hydroxyprogesterone (**B**) was not significantly decreased
708 (n=3, p=0.069). The concentration of the sex steroid precursor androstenedione (**C**) was significantly
709 increased in *fdx1b*$^{-/-}$ mutant male zebrafish (n=3, p=0.033); however, testosterone (**E**) concentrations
710 were unchanged (n=3, p=0.30). The presence of 11β-hydroxyandrostenedione (**D**) (n=3, p=0.0026)
711 and 11β-hydroxytestosterone (**F**) (n=3, p=0.025) was undetectable and 11KT (**G**) was significantly
712 decreased (n=3, p=0.022) in *fdx1b*$^{-/-}$ mutants. All results were analyzed using unpaired *t*-tests. **
713 indicates a p-value of <0.01, * indicates a p-value of <0.05.

Figure 4. Decreased expression of glucocorticoid and androgen responsive genes in *fdx1b*$^{-/-}$ mutant male zebrafish. The expression of glucocorticoid responsive genes *fkbp5* (**A**) (n=8, p=0.015) and *pck1* (**B**) (wild-type n=8, mutant n=5, p=0.0064) and of androgen responsive gene *cyp2k22* (**C**) (wild-type n=6, mutant n=5, p=0.013) was significantly decreased in *fdx1b*$^{-/-}$ mutant male zebrafish. All results were analyzed using unpaired *t*-tests. ** indicates a p-value of <0.01, * indicates a p-value of <0.05.

Figure 5. Expression profile of steroidogenic enzymes and *star* in the testes of *fdx1b*$^{-/-}$ zebrafish compared to wild-type siblings. The expression of *cyp11a2* (**B**) (n=7, p=0.04) and *cyp11c1* (**D**) (wild-type n=7, mutant n=4, p=0.0249) was significantly increased in the testes of *fdx1b*$^{-/-}$ zebrafish compared to wild-type siblings. There was no statistically significant difference in the expression of *star* (**A**) (n=7, p=0.2061)*, cyp17a1* (**C**) (wild-type n=6, mutant n=7, p=0.9941)*, hsd17b3* (**E**) (wild-type n=6, mutant n=7, p=0.1414) or *cyp19a1a* (**F**) (wild-type n=5, mutant n=5, p=0.98). All results were analyzed using unpaired *t*-tests. * indicates a p-value of <0.05.

Figure 6. Disrupted development of testes and decreased sperm concentration in *fdx1b*$^{-/-}$ zebrafish. A-D: *Fdx1b*$^{-/-}$ zebrafish had disorganized testes and poorly defined seminiferous tubules compared to wild-type males. Developing and mature sperm were observed in both *fdx1b*$^{-/-}$ and wild-type testes. SZ – mature spermatozoa, ST – spermatids, SG – spermatogonia. **E:** Testes dissected from *fdx1b*$^{-/-}$ fish had a decreased sperm concentration compared to wild-type siblings (unpaired *t*-test, wild-type n=11, mutant n=15, p=0.0097). **F:** There was no difference in GSI (unpaired *t*-test, wild-type n=11, mutant n=16, p=0.12). **G:** Semen samples collected by abdominal massage also had a reduced sperm concentration in *fdx1b*$^{-/-}$ fish compared to wild-type siblings (unpaired *t*-test, wild-type n=8, mutant n=4, p=0.0012). ** indicates a p-value of <0.01.

Figure 7. Downregulation of pro-testis, spermatogenic and hypothalamic-pituitary-gonadal (HPG) axis regulating genes in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Expression of the conserved pro-testis gene *SRY-box 9a (sox9a)* (wild-type n=10, mutant n=7, p=0.029) (**A**), the spermatogenic factors *insulin-like growth factor 3 (igf3)* (wild-type n=10, mutant n=8, p=0.030) (**D**) and *insulin-like 3 (insl3)* (wild-type n=8, mutant n=6, p=0.00060) (**E**) and HPG axis regulator *inhibin subunit alpha (inha)* (wild-type n=10, mutant n=7, p=0.023) (**F**) was downregulated in *fdx1b*$^{-/-}$ zebrafish. Expression of pro-male transcription factor *doublesex and mab-3 related transcription factor 1 (dmrt1)* (wild-type n=10, mutant n=8, p=0.25) (**B**) and pro-male *anti-Müllerian hormone (amh)* (wild-type n=10, mutant n=10, p=0.45) (**C**), were not affected by mutation of *fdx1b*. All results analyzed using unpaired *t*-tests. * indicates a p-value of <0.05, *** indicates a p-value of <0.001.

Figure 8. Expression of spermatogenesis marker genes in *fdx1b*$^{-/-}$ mutant and wild-type zebrafish testes. *Nanos2, piwil1, dazl, sycp3* and *odf3b* can be used as markers of spermatogenic stages. Expression of *nanos2* (**A**) (wild-type n=4, mutant n=5, p=0.024) and *piwil1* (**B**) (Mann-Whitney test, wild-type n=4, mutant n=7, p=0.012) was significantly increased in mutant testes (type A_{und} spermatogonia and all type A spermatogonia respectively). No change in expression of *dazl* (**C**) (wild-type n=5, mutant n=8, p=0.21) (type B spermatogonia), *sycp3* (**D**) (Mann-Whitney test, wild-type n=5, mutant n=6, p=0.18) (spermatocytes) or *odf3b* (**E**) (wild-type n=9, mutant n=6, p=0.64) (spermatids) was recorded. All results were analyzed using unpaired *t*-tests unless otherwise stated. * indicates a p-value of <0.05.

Figure 9. Germ cell markers and the roles of Igf3 and Insl3 in zebrafish spermatogenesis. Spermatogonial stem cells undergo several rounds of mitotic division and differentiation, eventually resulting in production of primary spermatocytes. During mitosis, spermatogonia retain some

762 capacity for self-renewal. Primary spermatocytes enter several cycles of meiosis and differentiation,
763 eventually maturing as spermatozoa. Igf3 and insl3 are important for differentiation and
764 proliferation of type A spermatogonia. *Nanos2, piwil1, dazl, sycp3* and *odf3b* can be used as markers
765 of different stages of spermatogenesis

766

Tables

Table 1. qPCR primer sequences.

Gene	Forward	Reverse	R^2	Efficiency (%)	Citation
amh	AGGTGGATAGCAGCAGTACG	AGATACGTTCGGGATGGGAG	0.9964	96	This paper.
cyp11a2	TGGAGGAACAGCCTGAAAAG	TTCACACTTTCATAGAATCCAACC	0.9980	93	This paper.
cyp11c1	AAGACGCTCCAGTGCTGTG	CCTCTGACCCTGTGATCTGC	0.9989	96	This paper.
cyp17a1	AGTTGCAAAGGACAGCTTGG	GCTGCACGTTATCACTGTAGG	0.9991	108	This paper.
cyp19a1a	ACAAACTCTCACCTGGACGA	AGTCTGCCAGGTGTCAAAGT	0.9999	103	This paper.
cyp2k22	CGCTGTCAAACCTACGAGAC	GGGGCAGTTTTGTTTCAAATGG	0.9979	108	This paper.
dazl	ACTGGGACCTGCAATCATGA	AATACAGGTGATGGTGGGGC	0.9998	98	This paper.
dmrt1	GGCCACAAACGCTTCTGTAA	ATGCCCATCTCCTCCTCTTG	0.9984	104	This paper.
ef1a	GTGGCTGGAGACAGCAAGA	AGAGATCTGACCAGGGTGGTT	0.9964	99	This paper.
fkbp5	TTCCACACTCGTGTTCGAGA	ACGATCCCACCATCTTCTGT	0.9989	104	[9]
hsd17b3	CCAAATACCCTGCAAGCTCC	TCTGCTGCATTCCTGGTAGT	0.9956	102	This paper.
igf3	GTAGACCAGTGTTGTGTGCG	ATTCCTCATCTCGCTGCAGA	0.9982	95	This paper.
inha	CAGAGCTGTGCACCATGTAG	CCAGGTCCAGCATCAGAAGA	0.9972	97	This paper.
insl3	TCGCATCGTGTGGGAGTTT	TGCACAACGAGGTCTCTATCCA	0.9994	110	[43]
nanos2	AAACGGAGAGACTGCGCAGAT	CGTCCGTCCCTTGCCTTT	0.9996	92	[43]
odf3b	GATGCCTGGAGACATGACCAA	CAAAGGAGAAGCTGGGAGCTT	0.9965	92	[38]
pck1	TGACGTCCTGGAAGAACCA	GCGTACAGAAGCGGGAGTT	0.9980	99	[9]
piwil1	ATACCGCTGCTGGAAAAAGG	GCAAGACACACTTGGAGAACC	0.9988	90	[43]
sox9a	CGGAGCTCAAAACTGTG	CGGGGTGATCTTTCTTGTGC	0.9981	106	This paper.
star	TTGAACAAGCTCTCCGGACC	TCACTGTATGTCTCCTCGGC	0.9979	110	This paper.
sycp3	AGAAGCTGACCCAAGATCATTCC	AGCTTCAGTTGCTGGCGAAA	0.9981	95	[38]

Table 2. Outcrossing of *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings.

Genotype and sex	Number of fish	Total number of trials*	Trials in which fertilized eggs were produced
***fdx1b* +/+ male**	5	20	70% (14/20)
***fdx1b* +/+ female**	4	16	75% (12/16)
***fdx1b* -/- male**	5	20	0% (0/20)
***fdx1b* -/- female**	5	20	0% (0/20)

*Trials were conducted weekly for four weeks. Fish were housed in their home tank and male and female fish were selected at random each week.

Figure 1

Click here to access/download;Figure;Figure 1.tif

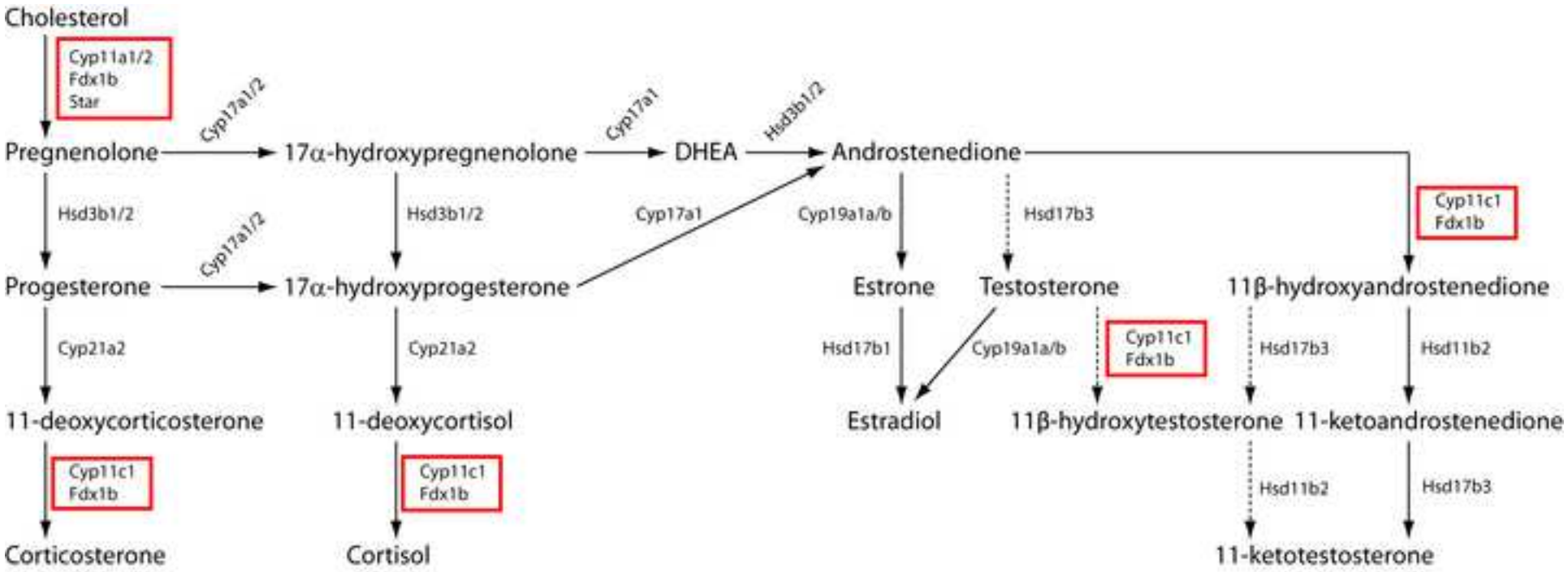

Figure 2

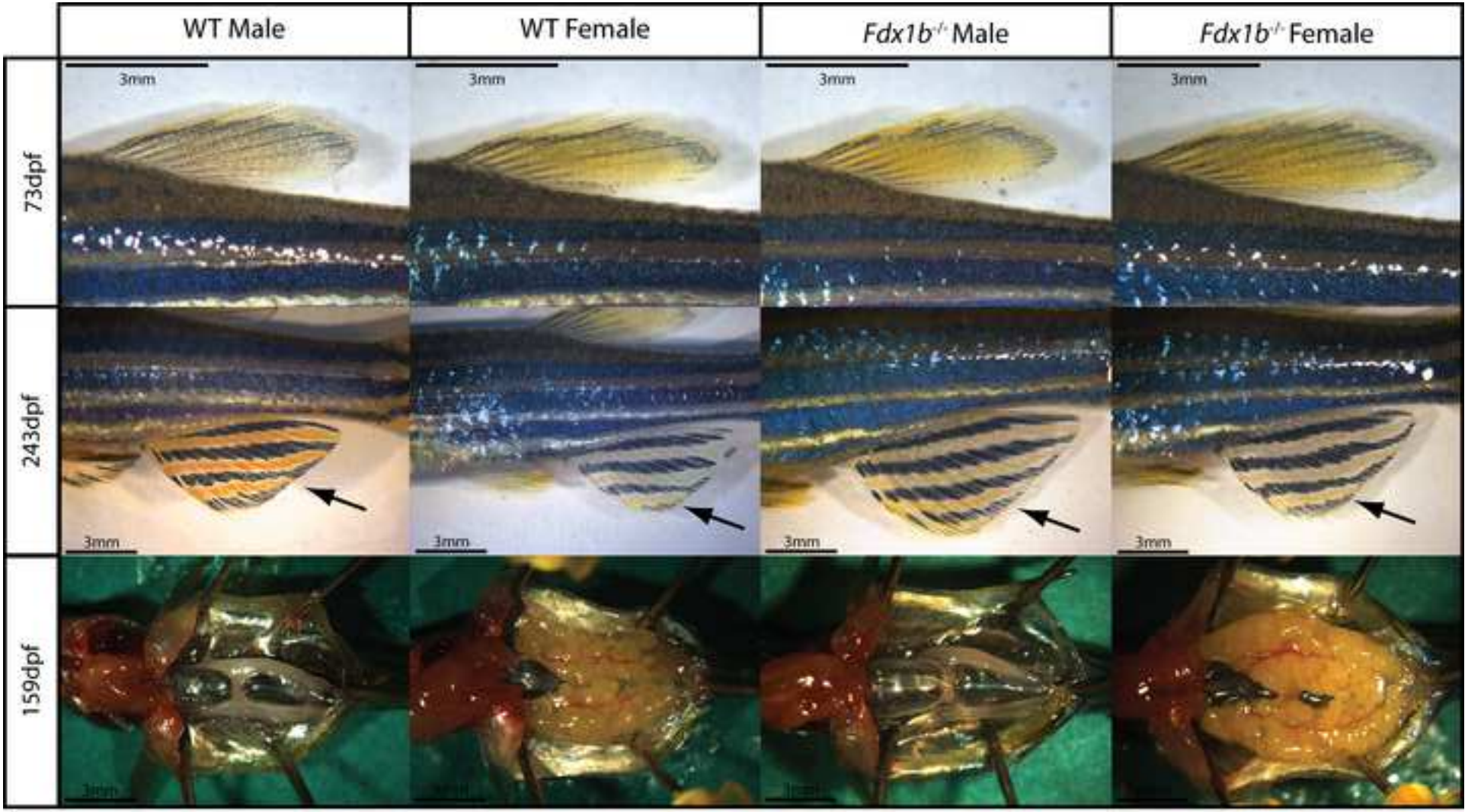

Figure 3

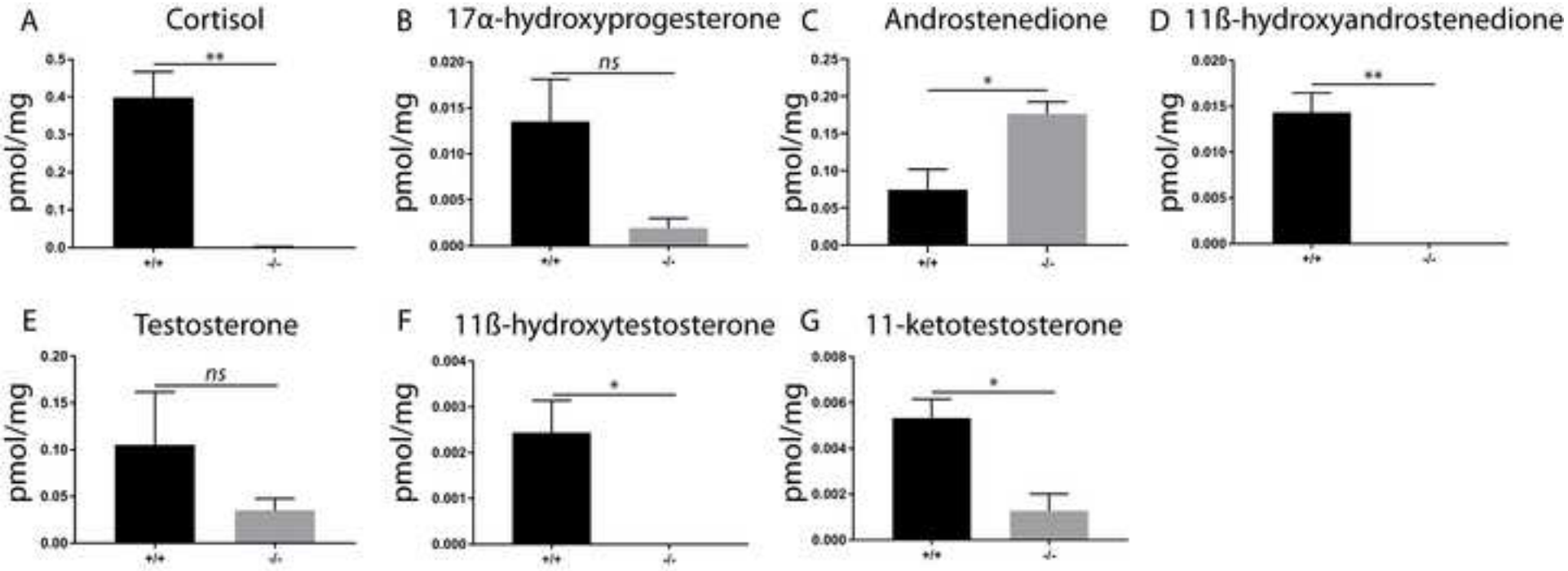

Figure 4

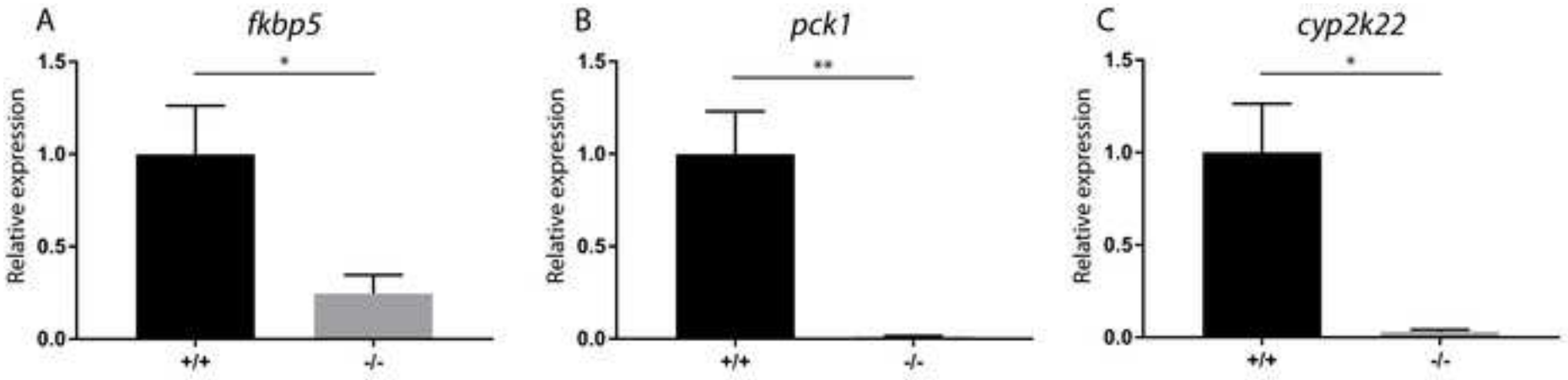

Figure 5

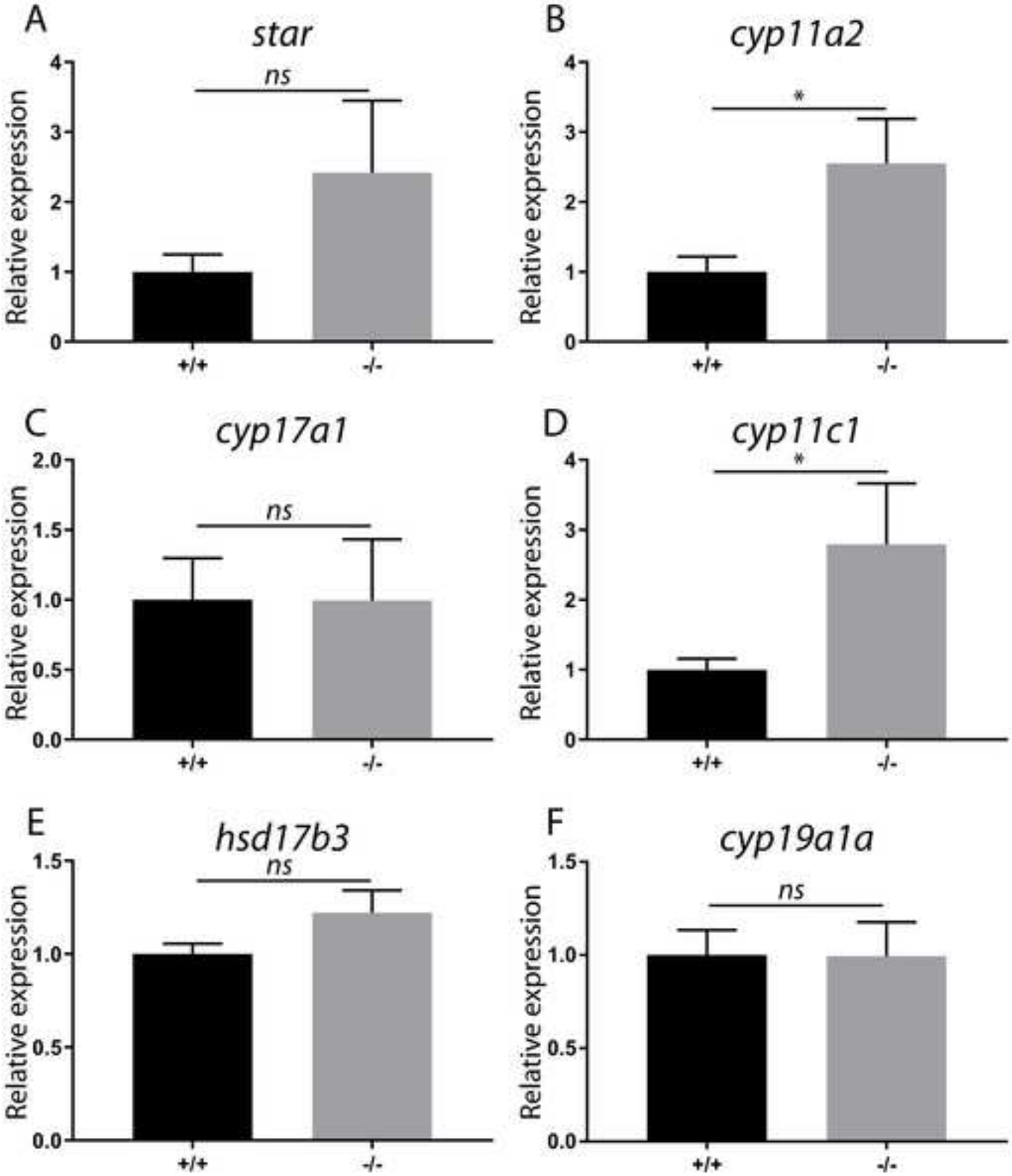

Figure 6

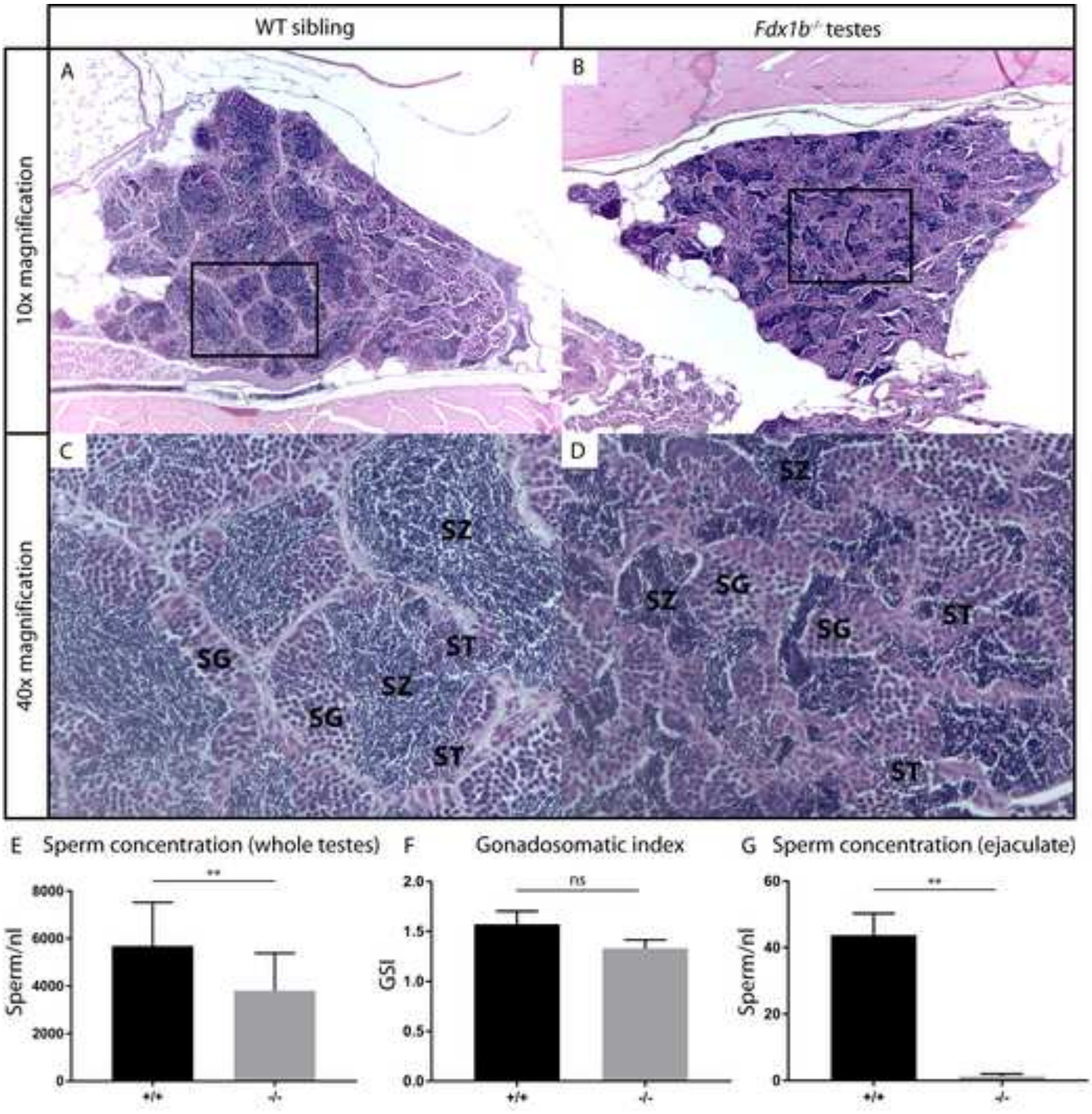

Figure 7

Click here to access/download;Figure;Figure 7.tif

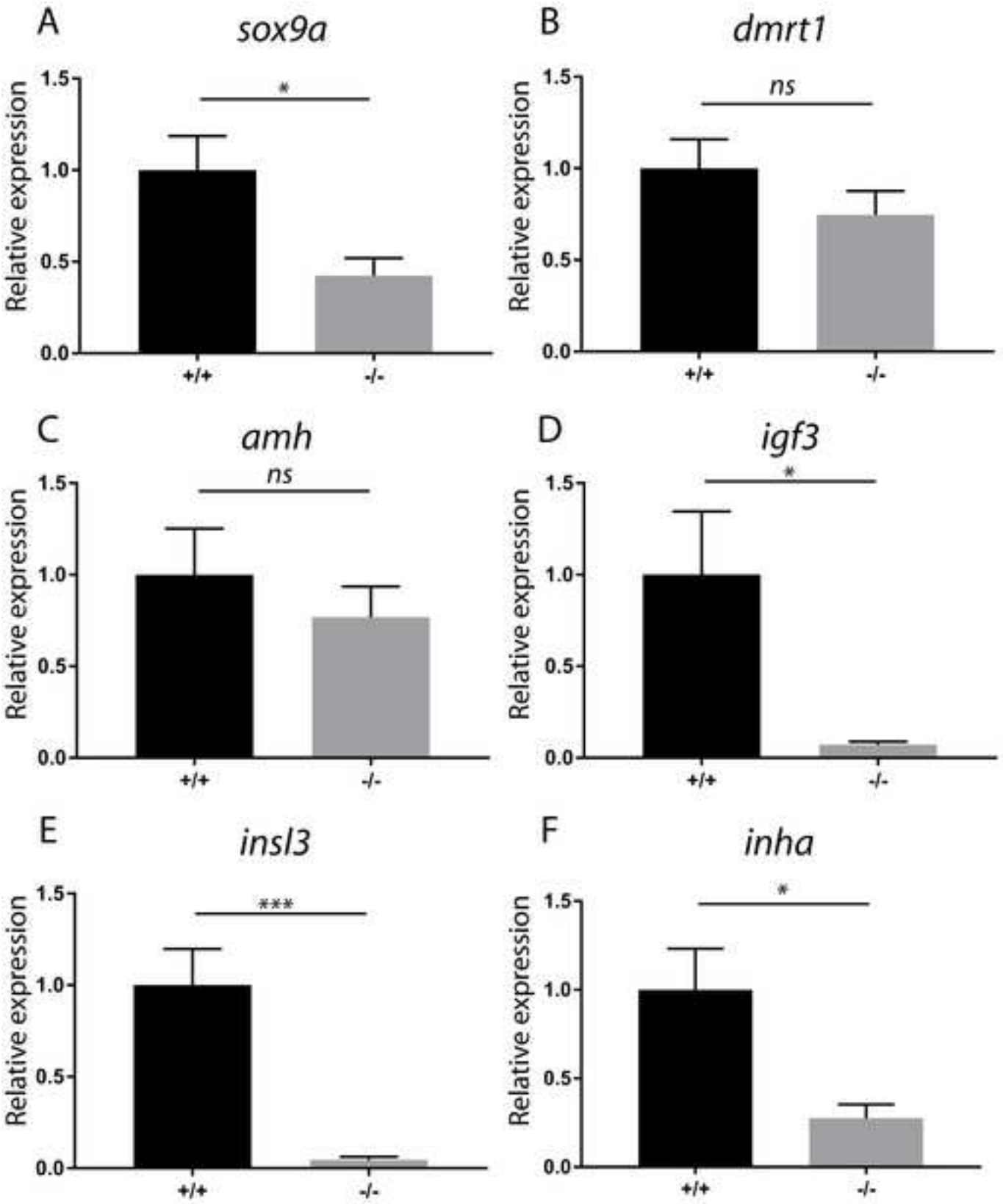

Figure 8

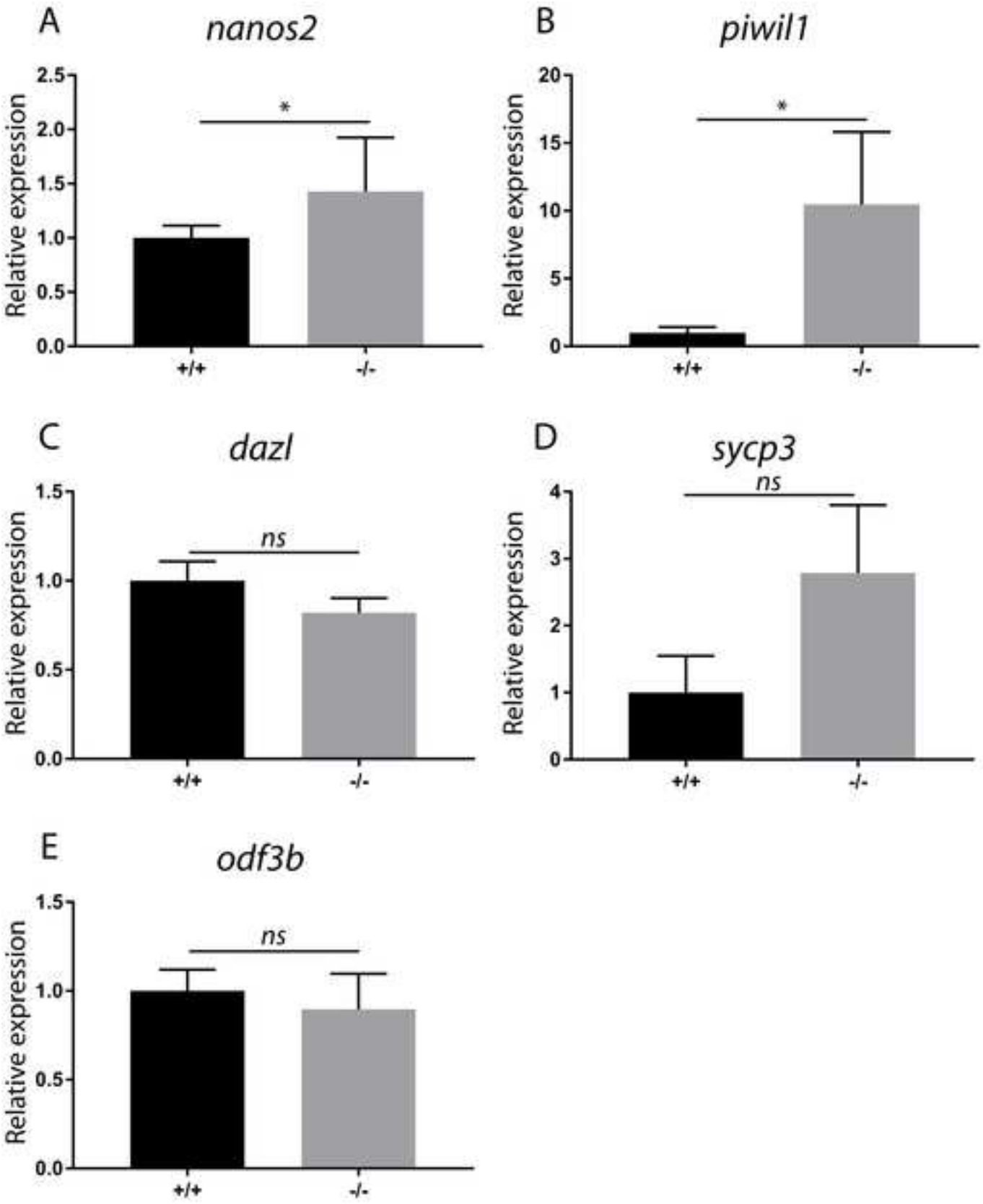

Figure 9