Investigating the roles of steroid signalling in gonadal development, maintenance and function through use of androgen and cortisol deficient mutant zebrafish lines

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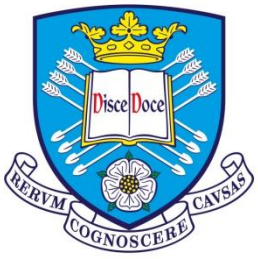

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Abbreviations

11KT	11-ketotestosterone
AR	Androgen receptor
bp	Base pairs
CPA	Cyproterone acetate
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAVID	Database for annotation, visualization and integrated discovery
DEG	Differentiatlly expressed gene
dpf	Days post fertilisation
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
GO	Gene ontology
GPER	G-protein coupled estrogen receptor
GSI	Gonadosomatic index
H&E	Haematoxylin and eosin
HPG	Hypothalamus-pituitary-gonadal
HPI	Hypothalamus-pituitary-interrenal
IVF	*In vitro* fertilisation
KEGG	Kyoto encyclopaedia of genes and genomes
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MeOH	Methanol
MIS	Maturation inducing steroid
MTBE	Methyl tertiary-butyl ether
nt	Nucleotide
PAM	Protospacer adjacent motif
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
SC	Spermatocytes
SG A_{diff}	Differentiating type A spermatogonia
SG A_{und}	Undifferentiated type A spermatogonia

SG type B	Type B spermatogonia
SSC	Spermatogonial stem cell
ST	Spermatids
SZ	Spermatozoa
TAE	Tris-Acetate-EDTA
TALEN	Transcription activator-like effector nucleases
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VBA	Visual background adaptation
wpf	Weeks post fertilisation

Summary

The roles of androgen signalling in the differentiation, maintenance and function of the zebrafish testis are poorly understood. This project aimed to explore this topic by characterising the phenotypes of androgen and cortisol deficient *fdx1b* and *cyp11c1* mutant zebrafish. Homozygous mutant adult zebrafish were found to exhibit female type pigmentation patterns, however these fish could possess either differentiated ovaries or testes. This demonstrates that androgens are dispensable for testis differentiation in zebrafish, as has also been found in androgen receptor mutant zebrafish. Androgen deficient male zebrafish were infertile in standard breeding scenarios, but their sperm could fertilise eggs collected from wild-type female zebrafish in IVF experiments. Various phenotypes in these fish are likely to contribute to their infertility, including perturbed breeding behaviours, disorganised testis organisation, reduced spermatogenesis and impaired sperm release. Targeted gene expression analysis by qPCR revealed downregulation of *igf3* and *insl3* in the testes of mutant fish, these genes encode important factors for spermatogonial differentiation. Histological examination of the testes and analysis of germ cell marker expression by qPCR revealed an accumulation of early germ cells. Taken together these findings indicate that spermatogonial differentiation is impaired, and that this process is highly dependent on androgens in zebrafish. Further investigations into the molecular mechanisms underlying the assorted phenotypes of these fish were undertaken by characterisation of the impacts of *fdx1b* mutation on the testis transcriptome. Several miRNAs with roles in repressing spermatogonial differentiation in mammals were upregulated in the testes of Fdx1b deficient zebrafish, indicating a potentially conserved role for these miRNAs in zebrafish and mammalian spermatogenesis. In addition to this, dysregulation of several structural elements of the testes was identified, including the basement membrane and Sertoli cell barrier. This is the first investigation into the mechanisms underlying disorganisation of testicular structure in androgen deficient or resistant zebrafish.

Chapter 1: Introduction - Steroid hormone biosynthesis and the roles of sex steroids in gonadal differentiation and function in zebrafish

The zebrafish is an established model organism for the study of hormones and endocrine disease (Lohr and Hammerschmidt, 2011). Steroid hormones are involved in a myriad of physiological and metabolic processes, ranging from the stress response, inflammation, salt homeostasis and gametogenesis. Steroids also play important roles in development, perhaps most notably in sex differentiation.

In contrast to humans and most mammals, laboratory strains of zebrafish lack sex chromosomes and instead exhibit polygenic sex determination (Liew et al., 2012). Zebrafish sex may also be influenced by environmental factors including stress and temperature (Ribas et al., 2017a; Ribas et al., 2017b). Pharmacological and genetic manipulation of sex steroid signalling in zebrafish has elucidated that sex determination is highly pliable in response to these hormones (Crowder et al., 2017; Lau et al., 2016; Morthorst et al., 2010). However, the specific roles of sex steroids, particularly androgens, in sex differentiation are poorly understood in this species. This chapter will describe the current literature pertaining to sex steroid biosynthesis and the roles of steroids in sex differentiation in zebrafish. In addition, zebrafish testis structure and spermatogenesis will be introduced.

1.1 - Steroidogenesis in zebrafish and other teleosts

The gonads and interrenal tissue, equivalent to the adrenal glands in mammals, are the principal sites of steroid hormone biosynthesis in zebrafish; the brain also has some capacity for steroid production (Diotel et al., 2011; Tokarz et al., 2015; Weger et al., 2018). Steroid biosynthesis (**Figure 1.1**) begins with side chain cleavage of cholesterol by Cyp11a1 or Cyp11a2 to produce pregnenolone (Parajes et al., 2013). Humans possess only one side chain cleavage enzyme homolog: CYP11A1. The activity of CYP11A1, and some other steroidogenic cytochrome p450 enzymes, is highly dependent

on electron transfer via ferredoxin reductase (FDXR) and ferredoxin (FDX1): FDXR receives electrons from NADPH, these are subsequently passed to FDX1 and finally donated to CYP11A1 (Miller and Auchus, 2011). This system is also present in zebrafish (Griffin et al., 2016). Pregnenolone may be converted to progesterone by Hsd3b1 (Lin et al., 2015) and this is followed by 17α-hydroxylation of either of these precursors by Cyp17a1 or Cyp17a2 (Pallan et al., 2015). Pregnenolone, progesterone and their 17α-hydroxylated forms are precursors for mineralocorticoid, glucocorticoid and sex steroid biosynthesis (Tokarz et al., 2015) (**Figure 1.1**).

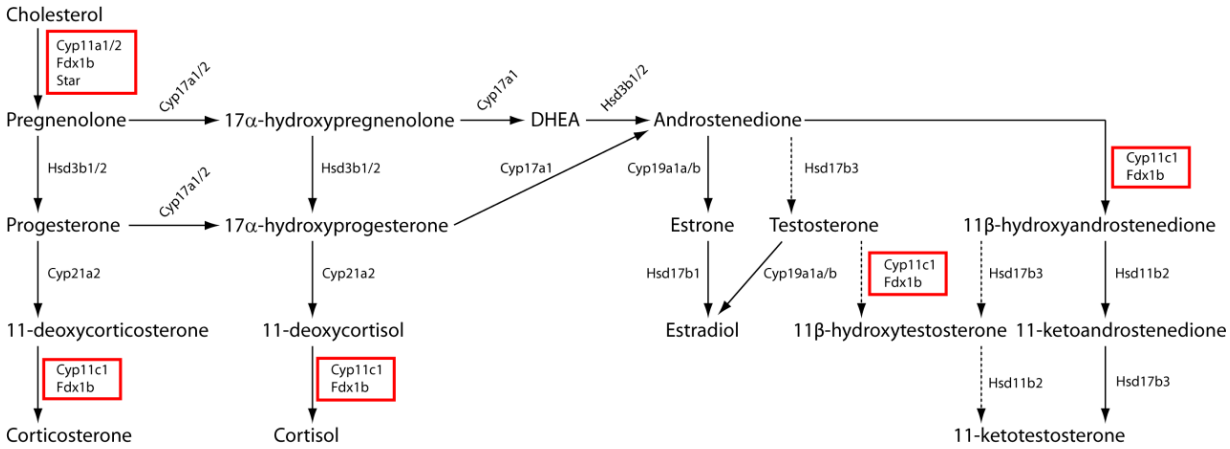

Figure 1.1. Postulated pathway for steroid hormone biosynthesis in zebrafish. Biosynthetic pathways for production of steroid hormones including mineralocorticoids, glucocorticoids and sex steroids in zebrafish. Reactions hypothesised to be affected by mutation of genes in this study are boxed in red. The minor pathway to androgen production in male zebrafish is indicated by dashed arrows.

In zebrafish and humans the principal glucocorticoid is cortisol, whereas in mice and rats it is corticosterone (Buckingham, 2006; Tokarz et al., 2015). The pathway to glucocorticoid biosynthesis is well conserved between humans and zebrafish (**Figure 1.1**). 17α-hydroxyprogesterone is converted to 11-deoxycortisol by Cyp21a2 (Eachus et al., 2017), and this is further converted to cortisol by Cyp11c1 (Jiang et al., 1998), the zebrafish homolog of CYP11B1. Like Cyp11a1/2, the activity of Cyp11c1 in this reaction is highly dependent on the activity of the mitochondrial electron donor Fdx1b (Griffin et al., 2016).

In the mineralocorticoid biosynthetic pathway, pregnenolone is thought to be converted to 11-deoxycorticosterone by Cyp21a2 followed by further conversion to corticosterone by Cyp11c1 (Jiang et al., 1998) (**Figure 1.1**). The identity of the principal mineralocorticoid in zebrafish is uncertain, however, 11-deoxycorticosterone is a likely candidate (Tokarz et al., 2015). 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 (Tokarz et al., 2015).

To enter the sex steroid biosynthetic pathway, 17α-hydroxypregnenolone or 17α-hydroxyprogesterone undergo further conversion by Cyp17a1, producing DHEA and androstenedione respectively (Pallan et al., 2015). These reactions cannot be catalysed by Cyp17a2 as it lacks the necessary 17α,20-lyase activity (Pallan et al., 2015). DHEA may be further converted to androstenedione by action of Hsd3b1 or Hsd3b2 (Lin et al., 2015) (**Figure 1.1**).

The gonad is the primary site of sex steroid biosynthesis, though production in the brain is also possible (Diotel et al., 2011). The principal oestrogen in zebrafish is estradiol, and this may be produced from androstenedione by two pathways (**Figure 1.1**). In the first pathway, androstenedione is converted to estrone by ovary specific Cyp19a1a (Yin et al., 2017) or brain specific Cyp19a1b (Diotel et al., 2011). Estrone is further converted to estradiol by Hsd17b1 (Mindnich et al., 2004). In the second pathway, androstenedione is converted to testosterone by

Hsd17b3 (Mindnich et al., 2005) before conversion to estradiol by Cyp19a1a or Cyp19a1b (Aggarwal et al., 2014).

The pathway to androgen production is somewhat different in zebrafish and humans (**Figure 1.1**). The principal active androgens in humans are testosterone and 5α-dihydrotestosterone, whereas the principal zebrafish androgen is 11-ketotestosterone (11KT) (Borg, 1994; Tokarz et al., 2015). This difference in androgen production is due to the fact that zebrafish favour production of 11-oxygenated androgens from androstenedione, rather than conversion of androstenedione to testosterone (de Waal et al., 2008). In humans testosterone is produced in the testes and this is converted to the more potent dihydrotestosterone at the target tissues (Miller and Auchus, 2011). Unlike the situation in humans, 5α-dihydrotestosterone is not thought to be a major androgen in teleost fish, although production may be possible (Margiotta-Casaluci et al., 2013).

The major biosynthetic pathway for 11-ketotestosterone production begins with the conversion of androstenedione to 11β-hydroxyandrostenedione (de Waal et al., 2008) by Cyp11c1 (Fernandes et al., 2007; Fernandes et al., 2014). 11β-hydroxyandrostenedione is further converted to 11-ketoandrostenedione, presumably by Hsd11b2, which is finally converted to 11KT by Hsd17b3 (Mindnich et al., 2005). As in the glucocorticoid biosynthetic pathway, reactions in the androgen biosynthetic pathway catalysed by Cyp11c1 are also thought to require activity of the electron providing co-factor Fdx1b.

In an alternative pathway, androstenedione may be converted to testosterone by Hsd17b3 (Mindnich et al., 2005), which is in turn converted to 11β-hydroxytestosterone (de Waal et al., 2008) by Cyp11c1. 11β-hydroxytestosterone is finally converted to 11KT by Hsd11b2 (Tokarz et al., 2015). The contribution of this pathway to 11KT production is thought to be minimal as testosterone and 11β-hydroxytestosterone appear to be produced at only extremely low levels (de Waal et al., 2008).

A reaction linking these two pathways of androgen production, the conversion of 11β-hydroxyandrostenedione to 11β-hydroxytestosterone by Hsd17b3, has been demonstrated *in vitro* (Mindnich et al., 2005), but is thought highly unlikely to occur *in vivo* (de Waal et al., 2008; Swart and Storbeck, 2015).

1.2 - Zebrafish sex differentiation is susceptible to disruption of sex steroid signalling

Sex determination and gonadal differentiation in zebrafish are poorly understood processes. Natural strains of zebrafish have a sex-linked region on chromosome 4, but this has been lost in laboratory strains which exhibit polygenic sex determination (Wilson et al., 2014) (Liew et al., 2012). This is in stark contrast to sex determination in humans, which is governed by the presence or absence of a Y chromosome. Human embryos first develop an indifferent gonad which is identical irrespective of karyotype. Appropriate temporal expression of the Y chromosomal testis determining factor *SRY* upstream of *SOX9* is crucial for testicular development. In the absence of *SRY* the indifferent gonad follows the female developmental pathway (Lucas-Herald and Bashamboo, 2014).

Zebrafish gonadal differentiation has been linked to a myriad of genes and cellular processes including tp53 mediated apoptosis, NFκB signalling, Wnt/β-catenin signalling, prostaglandins signalling and the production and action of sex steroids and other hormones (Pradhan et al., 2012; Rodriguez-Mari et al., 2010; Sreenivasan et al., 2014).

Environmental factors such as temperature and stress also play a role in sex determination and gonadal differentiation in zebrafish (Liew and Orban, 2014; Ribas et al., 2017a). For example, increased water temperature between 18-32dpf resulted in an increased proportion of males (Ribas et al., 2017a). Effects of temperature on sex determination are not restricted to fish species, many turtle and crocodilian species exhibit environmental sex determination. For example incubation of European pond turtle (*Emys obicularis*) eggs at higher temperatures leads to all male development.

In the alligator snapping turtle (*Macroclemys temminckii*) cooler or warmer incubation temperatures result in female development, whereas male development is more frequent at intermediate temperatures (Gilbert, 2000).

The importance of sex steroids in sex and gonadal differentiation in zebrafish has been widely demonstrated in both genetic and pharmacological studies. The steroidogenic enzyme ovarian aromatase (Cyp19a1a) is crucial for production of female sex steroids in zebrafish, and inhibition or mutation of this enzyme results in female to male sex reversal (Fenske and Segner, 2004; Lau et al., 2016; Takatsu et al., 2013; Yin et al., 2017). Treatment of developing zebrafish with an estradiol analogue (17α-ethinylestradiol / 17β-estradiol) has achieved conflicting results, with some studies reporting varying degrees of feminisation (Andersen et al., 2003; Brion et al., 2004; Orn et al., 2016), whilst others report limited effects (Luzio et al., 2015). Despite the impact of disrupted sex steroid signalling on gonadal differentiation ovotestis are rarely reported in adult fish, with nearly all fish possessing either differentiated testes or ovaries. Fish treated with oestrogens at different stages of development were found to possess ovotestes at 60dpf, this may be due to incomplete feminisation of the gonad or a delay in gonadal transformation leading to the presence of transforming gonads at a later stage than they are usually seen (Andersen et al., 2003).

Zebrafish possess three nuclear oestrogen receptors (esr1, esr2a and esr2b) as well as a G protein-coupled oestrogen receptor (GPER). Mutation of the GPER in zebrafish does not affect sex ratios or ovarian function, indicating that this receptor is of little reproductive importance, however, compensatory mechanisms may be masking functionally redundant roles of this protein (Crowder et al., 2018). Mutation of any of the three nuclear oestrogen receptors causes a shift in sex ratios towards male development. These receptors display functional redundancy as double or triple mutants exhibit further increases in the ratio of males:females (Lu et al., 2017).

Male sex steroids also play an important role in sex development. Exposure of zebrafish to the androgens 17β-trenbolone or 17α-methyltestosterone during development causes robust

masculinisation, producing a fertile entirely male population (Larsen and Baatrup, 2010; Lee et al., 2017; Morthorst et al., 2010). In contrast, exposure to the anti-androgen vinclozolin during zebrafish sex differentiation causes a shift in sex ratios towards females, as well as delayed testes maturation (Lor et al., 2015). Androgen resistance (Crowder et al., 2017; Tang et al., 2018; Yu et al., 2018) results in adult zebrafish with predominantly female morphological characteristics, however both testes and ovaries are observed. Several AR deficient zebrafish lines exhibit female bias in the homozygous mutant population (Crowder et al., 2017; Yu et al., 2018), indicating that AR function may influence sex in some individuals. Androgen resistant male zebrafish are infertile, exhibit disorganised testicular structure and impaired breeding behaviours (Yong et al., 2017).

Androgen resistant zebrafish are still able to develop testes despite absent androgen signalling (Crowder et al., 2017). However, oestrogen resistance or deficiency leads to heavily skewed sex ratios favouring male development, or complete masculinisation (Lau et al., 2016; Lu et al., 2017). This demonstrates that oestrogens are crucial for ovary differentiation, but that androgens are dispensable for testes differentiation. This notion is also backed up by studies of *cyp17a1* and *cyp11a2* mutant zebrafish which are both androgen and oestrogen deficient. *Cyp17a1* and *cyp11a2* mutant zebrafish develop as an entirely male population albeit with absent mating behaviour and feminised secondary sex characteristics (Li et al., 2019b; Zhai et al., 2018).

1.3 - Androgens are dispensable for testis differentiation in zebrafish

As described above, androgen resistance does not result in entirely female zebrafish populations (Crowder et al., 2017; Tang et al., 2018; Yu et al., 2018). This begs the question: what is the role of androgens in the process of gonadal development in zebrafish? Whilst androgens appear to be dispensable for testis differentiation in zebrafish, androgen resistant zebrafish lines are infertile and share similar testicular phenotypes at the adult stage, comprising disorganised testicular structure

with poorly defined seminiferous tubules (Crowder et al., 2017; Tang et al., 2018). Clearly, this demonstrates a role for androgens in the correct development, organisation or maintenance of the testes somewhere between the stage of gonadal differentiation and adulthood.

Development of the zebrafish gonad begins around 10dpf when primordial germ cells begin to differentiate into gonocytes (Tong et al., 2010), this is followed by proliferation of female germ cells. Up until 21dpf all zebrafish develop a juvenile ovary. In presumptive female zebrafish this structure is comprised mainly of meiotic germ cells which go on to complete meiosis and maturation (Sun et al., 2013). In presumptive male zebrafish this structure is characterised by fewer oocyte-like cells, asynchronous cell growth and increased somatic cell proliferation (Sun et al., 2013). This is followed by regression of the juvenile ovary through apoptosis, and development of testes (Uchida et al., 2002). Although all presumptive male zebrafish will at some point have developed a juvenile ovary, the extent to which this structure develops is highly variable (Wang et al., 2007).

The process of juvenile ovary-to-testis transformation involves regression of the juvenile ovary by apoptosis of oocytes, and development of spermatogonia (Uchida et al., 2002). The transition from juvenile ovary to testis is accompanied by transformation of the gonadal gene expression profile, characterised by increased expression of pro-male genes such as *sox9a* and *amh* and downregulation of pro-female genes such as *cyp19a1a* (Sun et al., 2013; Wang and Orban, 2007).

At the initiation of juvenile ovary-to-testis transformation, *sox9a* expressing stromal cells and extracellular matrix at the periphery of the gonad infiltrate the gonadal tissue and engulf oocytes and associated somatic cells, resulting in a pre-apoptotic state. This may be followed by complete apoptosis or transdifferentiation of follicle cells to form the testis cord (Sun et al., 2013).

Sox9a and *cyp19a1a* are both highly expressed in gonadal precursor tissue at around 17dpf, prior to the onset of gonadal sex differentiation. In contrast, *amh* is expressed at low levels at this stage (Rodriguez-Mari et al., 2005). The expression of *sox9a* and *cyp19a1a* becomes sexually dimorphic at

the initiation of gonadal differentiation around 21dpf; presumptive females exhibit high *cyp19a1a* expression and low *sox9a* expression, whilst the opposite pattern is observed in presumptive males (Tong et al., 2010). Expression of *amh* increases during juvenile ovary-testis transformation and this is accompanied by downregulation of *cyp19a1a* (Rodriguez-Mari et al., 2005; Sun et al., 2013; Tong et al., 2010; Wang and Orban, 2007). This expression pattern is consistent with Sox9a acting as a positive regulator of Amh, which in turn acts as a negative regulator of Cyp19a1a, as is the case in mammals (Rodriguez-Mari et al., 2005). The role of Amh in zebrafish testis differentiation is further supported by the finding that *amh* knock-out results in female biased sex ratios (Lin et al., 2017).

The initiation of gonadal differentiation is clearly susceptible to androgens, as treatment of zebrafish with exogenous androgens prior to and throughout this period results in robust masculinisation (Larsen and Baatrup, 2010; Lee et al., 2017). Indeed, *amh* is inducible by androgens, whereas *cyp19a1a* is repressed (Lee et al., 2017). Given that the initiation of gonadal differentiation appears to be susceptible to androgens, it may seem surprising that there is no apparent effect of impaired androgen signalling on this process. However, treatment of fish with exogenous androgens is unlikely to replicate the intricacies of normal hormonal regulation of this process.

Cyp11c1 is crucial for the production of androgens in zebrafish, and the expression of this steroidogenic enzyme only appears after the gonad has committed to testis differentiation (Wang and Orban, 2007). This indicates that androgens may not be essential for the initiation of this process, as production of these steroids may only increase after the initial stage of testis differentiation. This theory is further supported by the fact that gonadal expression of the androgen receptor only becomes sexually dimorphic at 5 weeks post fertilisation (wpf), well after the initiation of testis differentiation (Hossain et al., 2008).

In the above described studies (Larsen and Baatrup, 2010; Lee et al., 2017), androgen treatment began before the proposed onset of *cyp11c1* expression (Wang and Orban, 2007); suggesting that the initiation of gonadal differentiation may be susceptible to androgens, but that androgens do not

influence this process under natural conditions. Despite the plausibility of this theory it cannot be ruled out that exogenous androgen treatment causes gonadal masculinisation at a later stage of gonadal differentiation and does not affect the initiation of gonadal differentiation.

1.4 - Androgens may promote correct development and organisation of the testes by maintaining expression of pro-male genes

After the initial stage of testis differentiation, the expression of pro-male genes is maintained in the testes through to the adult stage. The crucial role of androgens in orchestrating organisation of the adult gonad may centre on maintaining expression of pro-male genes after the initial stage of gonadal differentiation. The abnormal testis structure observed in androgen resistant zebrafish is likely to result from dysregulation of androgen dependent genes, after the initial stage of testis differentiation. The necessity of androgens for correct regulation of pro-male genes is evidenced by dysregulation of these genes in androgen resistant zebrafish lines at the adult stage, for example *dmrt1* is downregulated in $ar^{-/-}$ mutant zebrafish (Crowder et al., 2017). The impact of impaired androgen signalling on *amh* expression is uncertain, as conflicting results are reported (Crowder et al., 2017; Tang et al., 2018; Yu et al., 2018).

In addition to its important role in juvenile ovary-to-testis transformation (Sun et al., 2013), *sox9a* plays important roles in further development and maintenance of the testes. A role for a *sox9* homolog, *sox9a2,* in testis tubule development has been suggested in medaka (*Oryzias latipes*), another teleost fish species (Nakamoto et al., 2005). The importance of Sox9 homologs in testis tubule maintenance is also supported by findings in mouse models. When *Sox9* expression was inactivated after sex differentiation, abnormal testis tubules were observed. When combined with mutation of the related transcription factor *Sox8*, tubular structures were completely absent in the testes (Barrionuevo et al., 2009).

Dmrt1 is a highly conserved pro-male transcription factor expressed in the germ cells and Sertoli cells of the testes in zebrafish (Webster et al., 2017). Expression of *dmrt1* becomes sexually dimorphic during gonadal differentiation with high expression detected in presumptive testes and low expression detected in presumptive ovaries at 28dpf and 5wpf (Schulz et al., 2007; Webster et al., 2017). Expression of *dmrt1* at 40dpf can be induced by androgen treatment during the period of gonadal differentiation (Lee et al., 2017). Mutation of *dmrt1* causes a sex bias favouring female development; some fish develop as males but these are infertile, with hypoplastic testes, lacking organised tubule structure and devoid of spermatozoa (Webster et al., 2017).

Overall, the maintenance of exogenous androgen treatment during the period of gonadal development results in upregulation of pro-male genes (*amh, dmrt1, gsdf*) subsequent to the initiation of gonadal differentiation (Lee et al., 2017). Whilst in-depth investigation into the roles of these pro-male genes in testis tubule development is currently lacking, it is clear that androgens play an important role in correct testis morphogenesis beyond the initial stages of gonadal differentiation.

1.5 - Testicular structure and spermatogenesis in zebrafish

Zebrafish possess paired testes which are connected to the urogenital orifice by the spermatic duct (Menke et al., 2011). Like in all vertebrates, in zebrafish the testis is split in to two compartments: interstitial and tubular. The interstitial space is home to the androgen producing Leydig cells and blood vessels, whereas the tubular compartment is the site of spermatogenesis. The seminiferous tubules are bounded by a basement membrane and peritubular myoid cells, and contain Sertoli cells and developing germ cells (Schulz et al., 2010) (**Figure 1.2**).

In contrast to humans, zebrafish exhibit cystic spermatogenesis. This is characterised by synchronously developing clonally identical germ cells enveloped by Sertoli cells – this is a

spermatogenic cyst, the functional unit of spermatogenesis in fish. Spermatogenic cysts are located at the periphery of seminiferous tubules whereas mature spermatozoa are found in the central lumen (**Figure 1.2**). Clonally identical germ cells will remain enveloped by Sertoli cells throughout spermatogenesis until mature sperm are released into the tubule lumen (Schulz et al., 2010). Spermatogenesis begins with an undifferentiated spermatogonium enveloped by Sertoli cells. Initially cysts are comprised of just one or two Sertoli cells but this number increases throughout spermatogenesis, particularly up until the spermatocyte stage (Leal et al., 2009). Type A spermatogonia undergo several mitotic divisions to eventually produce type B spermatogonia (Leal et al., 2009), these cells eventually differentiate into spermatocytes and enter the meiotic phase (Schulz et al., 2010), from which they emerge as spermatids. Following the meiotic phase of spermatogenesis, spermatids undergo significant differentiation to produce mature spermatozoa (Schulz et al., 2010), which are finally released from cysts into the tubule lumen (**Figure 1.2**).

Androgens are clearly important in zebrafish spermatogenesis as androgen receptor mutant zebrafish have a reduced number of germ cells (Tang et al., 2018). In addition to this, fish exhibiting oestrogen-induced androgen insufficiency exhibit impaired spermatogonial differentiation (de Waal et al., 2009).

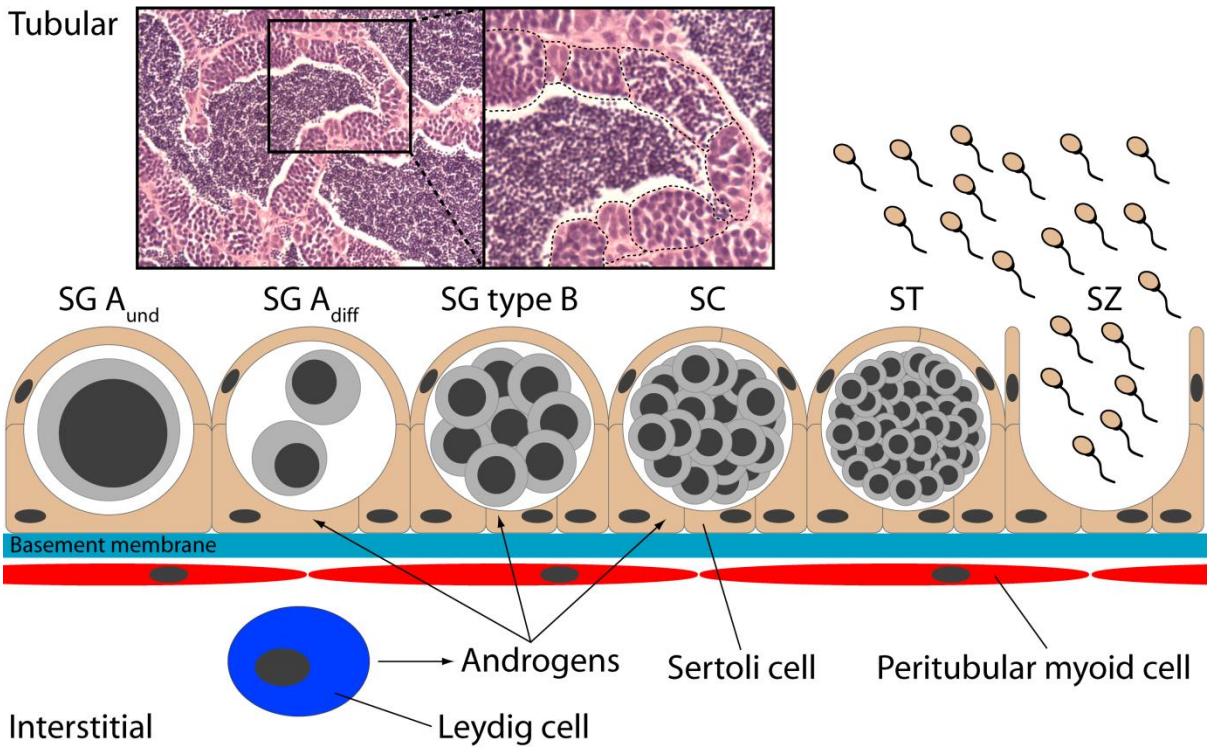

Figure 1.2. Spermatogenesis and organisation of the testes in zebrafish. The testes are organised into a basement membrane bounded tubular zone and an interstitial zone. The tubular zone comprises Sertoli cells and germ cells. Zebrafish spermatogenesis proceeds in a cystic fashion, where clonally identical germ cells are isolated in a cyst formed by Sertoli cells. These cysts are located towards the periphery of the seminiferous tubules. Eventually, mature spermatozoa are released into the seminiferous tubule lumen. Inset: H&E stained zebrafish testis section exemplifying the tubular structure of zebrafish testes; a close-up of the boxed zone is also displayed, with spermatogenic cysts demarcated by dashed outlines. Leydig cells are the steroid producing cells of the testes and are located in the interstitial zone; these cells produce androgens, which signal to a variety of cell types, including Sertoli cells. SG A_{und} = undifferentiated type A spermatogonia, SG A_{diff} = differentiating type A spermatogonia, SG type B = type B spermatogonia, SC = spermatocyte, ST = spermatid, SZ = spermatozoa.

1.6 – Aims and objectives

Sex steroids clearly play an important role in gonadal differentiation and function in zebrafish; however, the specific roles of these steroid hormones, particularly androgens, are poorly defined. This work aimed to enhance our understanding of steroid hormones in such processes, benefitting the development of the zebrafish as a model for endocrine disruption. This work may also aid our understanding of the impact of environmental endocrine disrupting chemicals on fish sex development and reproduction.

Previous studies have utilised androgen receptor (AR) mutant zebrafish to study androgen signalling in this species (Crowder et al., 2017). However, we have chosen to use androgen deficient zebrafish rather than androgen resistant zebrafish. In canonical androgen signalling, androgens bind the androgen receptor, which then translocates to the nucleus and influences gene expression. In addition to this canonical signalling mechanism, androgens may also exert biological effects in a non-AR mediated manner. For example the phenotype of *pkd2*$^{-/-}$ mutant zebrafish embryos could be modulated by androgen treatment in a manner independent of AR signalling (Metzner et al., 2020).

Initially, we investigated the impact of mutation of the steroidogenic co-factor Fdx1b on steroidogenesis. This was followed by characterisation of reproductive phenotypes in these fish, including feminisation of secondary sex characteristics, infertility, impaired breeding behaviour, decreased spermatogenesis and testis disorganisation. Targeted gene expression analysis was used to further characterise these phenotypes and to shed light on underlying molecular mechanisms.

In parallel to this work, CRISPR-Cas9 genomic editing was used to produce zebrafish carrying mutations in a steroidogenic enzyme to which Fdx1b is co-factor – Cyp11c1. Characterisation of this new zebrafish line was performed to confirm the roles of androgens and cortisol in reproductive phenotypes in zebrafish.

Finally, transcriptomic analysis of the testes of *fdx1b*$^{-/-}$ mutant zebrafish was performed. This allowed in-depth investigation of androgen regulated processes in the zebrafish testis, as well as investigation of molecular mechanisms underlying poorly understood phenotypes of perturbed androgen signalling, such as structural disorganisation of the testes.

Chapter 2: General materials and methods

2.1 - Zebrafish husbandry and ethics

Adult zebrafish were maintained in a recirculating system (ZebTECTM, Tecniplast, Kettering, UK, and Sheffield, UK) at 28.5°C on 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 anaesthetic tricaine mesylate (Pharmaq, Fordingbridge, Hampshire, UK) followed by decapitation or destruction of the brain by piercing of the skull with a needle. The ages of fish used in each experiment are indicated in **Table 2.1**. All procedures involving zebrafish were performed in compliance with local and UK animal welfare laws, guidelines and policies.

2.2 - Genotyping of the *fdx1b* mutant allele

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. As this mutation in *fdx1b* is an in-frame deletion, RNA stability is unlikely to be affected. However, the mutation affects highly conserved amino acid residues, specifically affecting 1 of 4 cysteine residues essential for Fe/S binding and Fe/S cluster stability (Griffin et al., 2016). Adult *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings were produced by incrossing heterozygotes carrying the *fdx1b* mutant allele. Progeny of *fdx1b* heterozygote incrosses were anaesthetised in 4.2% tricaine mesylate and a small biopsy of the caudal fin was transferred to 100µl of 50mM NaOH. Genomic DNA was isolated from the fin biopsy by heating to 98°C for 10 minutes followed by cooling and addition of 10µl 1M Tris pH8. Genotyping was achieved by PCR amplification (forward 5'-GTGTCTATATTAGGAGCATGCG, reverse 5'-

TTCCGTAAGCCAAATCCAGC) of the targeted genomic region and separation of the PCR products on a 3% TAE agarose gel. Single bands representing wild-type (133bp) or mutant (121bp) fish were observed, as well as triplet PCR products representing wild-type, mutant and heteroduplex PCR products produced from heterozygous fish (**Figure 2.1**).

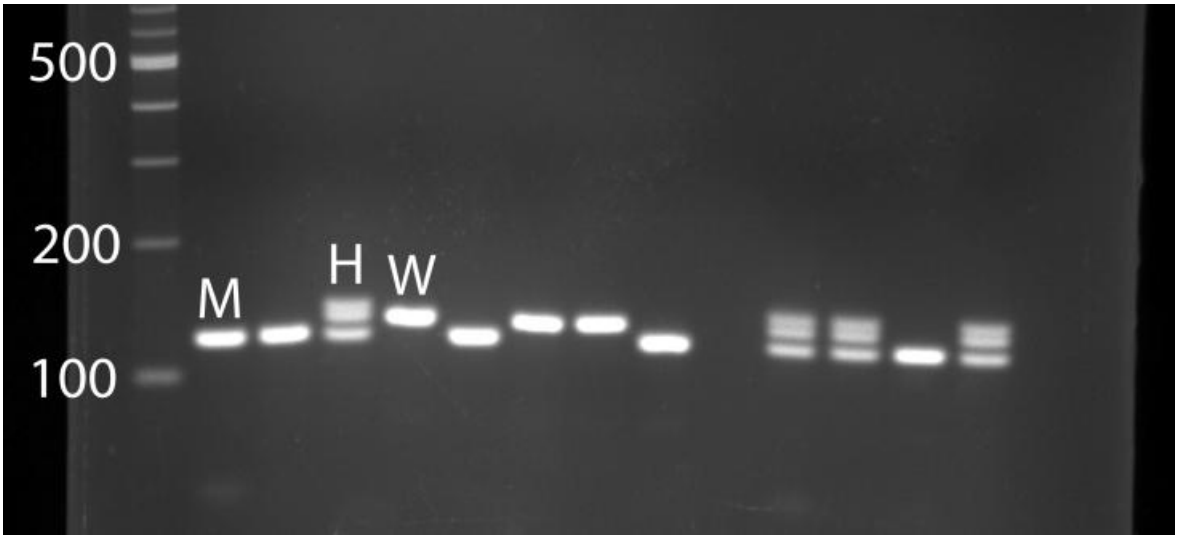

Figure 2.1. Genotyping of *fdx1b* heterozygous incrosses. PCR amplification of the genomic region containing *fdx1b* mutations, followed by separation of PCR products on a TAE agarose gel, allowed identification of wild-type (W), heterozygous (H) and *fdx1b*$^{-/-}$ mutant (M) zebrafish.

Table 2.1. Ages of fish used in each experiment.

Method	*fdx1b*	*cyp11c1*
Morphology	73dpf, 125dpf, 243dpf	11bp 146dpf 47bp 154dpf
Biometric	Many stages.	11bp 146dpf 47bp 154dpf
Steroids	205dpf	~153dpf
Fertility – marbling/ pair mating	~4-7 months post fertilisation	Males 11bp – 121-148dpf Females 11bp – 121-148dpf Males 47bp – 114-142dpf Females 47bp – 96-112dpf Males - (breeding behaviour) 96-110dpf
Fertility – IVF	<1 year	11bp – 127dpf 47bp – 145dpf
Sperm release	~4-9 months	~3-6 months
Breeding behaviour	~4-9 months	96-110dpf
Open field test	231-244dpf	99-112dpf
Histology	Juvenile – 21, 25, 31, 38dpf Adult ~160dpf	11bp 146dpf 47bp ~150dpf
Sperm counting	<1 year	127-145dpf
qPCR	Testes, ovary, liver - 159dpf Brain – M: 146dfp, F: 11 months	Testes, liver, brain - 136-138dpf
GSI	Females - 125dpf, Males - 243dpf	Males - 127-145dpf
RNA sequencing	131dpf	

dpf = days post fertilisation, bp = base pairs

2.3 - Targeted genetic disruption of *cyp11c1* by CRISPR-Cas9

To achieve genetic disruption of *cyp11c1* we have utilised the SygRNA two part system (Sigma, St. Louis, Missouri, United States). A crRNA targeting a 20 nucleotide sequence directly 3' to a protospacer adjacent motif (PAM) was designed to target exon 2 of *cyp11c1* (ENSDART00000185978.1). A 4µl mixture containing 0.1µM custom crRNA, 0.1µM tracrRNA (Sigma, St. Louis, Missouri, United States), 1µl phenol red and 1µl Cas9 (#M0646, NEB, Ipswich, Massachusetts, United States) was prepared and ~1nl was injected into embryos at the 1-cell stage. The targeted Cas9 cut site was overlapped by a BslI restriction site allowing for simple screening for mutant alleles by restriction digest (**Figure 2.2**). The targeted genomic region was amplified by PCR and digested with BslI (NEB, Ipswich, Massachusetts, United States) for 3-5 hours at 55°C. Injected embryos (F0) were raised to ~10wpf and outcrossed to unrelated wild-type fish. The progeny of these fish (F1) were screened for disruption of *cyp11c1,* and out of frame mutations were identified by DNA sequencing on a 3730 DNA Analyser (Applied Biosystems, Foster City, California, United States). F1 fish were outcrossed to unrelated wild-type fish to produce an F2 generation carrying stable mutations in exon 2 of *cyp11c1* in heterozygotes.

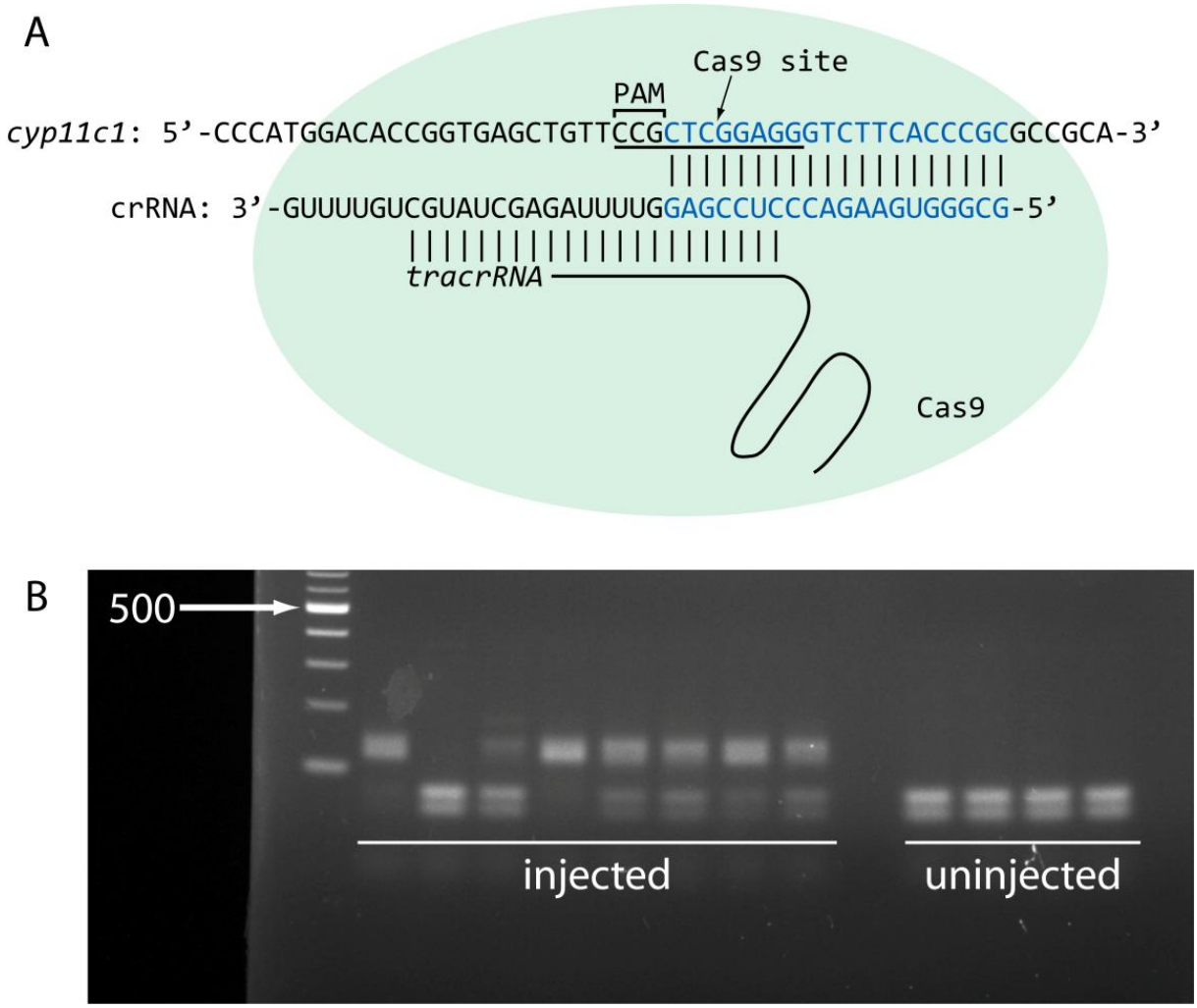

Figure 2.2. *Cyp11c1* CRISPR design and screen assay. A: A two part CRISPR system was used to generate mutations in *cyp11c1*. **B:** To screen for introduction of mutations in to *cyp11c1* the targeted genomic region was amplified by PCR and the resulting product was digested with BslI restriction enzyme (restriction site underlined), mutated alleles were identified by incomplete digestion.

2.4 - Morphological Analysis

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

2.5 - 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 deionised 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 samples were resuspended in 150µl 50% MeOH prior to analysis.

Prior to resuspension in 50% MeOH and after drying under nitrogen, an additional processing step was used for samples from *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings. Dried residue was resuspended in 1ml MeOH and eluted through a Phree column (Phenomenex, Torrance, California, United States) to remove remaining phospholipids. The column was washed with an additional 1ml

MeOH and the resulting 2ml eluent was dried and resuspended in 150µl 50% MeOH (as for *cyp11c1* samples) 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 (O'Reilly et al., 2017).

2.6 - Breeding, *in vitro* fertilisation (IVF), sperm counting and sperm release

Breeding was conducted to assess the fertility of Fdx1b and Cyp11c1 deficient zebrafish. Two common methods were employed: marbling (*fdx1b*$^{-/-}$ mutants and wild-type siblings only) (Westerfield, 2000) and pair mating. The pair mating technique was used to outcross mutant fish and wild-type siblings to unrelated wild-type fish. Wild-type fish could be sexed by conventional sex differences whereas mutant fish were sexed based on presence of a rounded abdomen or genital papilla, which are indicative of an ovary. On the afternoon prior to a breeding trial an mutant or wild-type sibling 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 lightly homogenized in a 50x mass:volume Hank's balanced salt solution. All Fdx1b IVF and sperm counting experiments utilised 600mOsm/kg Hank's balanced salt solution (Jing et al., 2009) whereas Cyp11c1 experiments utilised solution supplied by Sigma-Aldrich (H6648) (St. Louis, Missouri, United States). Eggs were collected from anaesthetised 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 (Westerfield, 2000). Fertilisation of eggs was confirmed by visualization under a dissecting microscope at low magnification and fertilised eggs were raised at 28.5°C to 5 days post fertilisation (dpf) before culling.

For sperm counting, samples were collected by dissection of humanely euthanized adult males to remove the testes followed by gentle homogenisation of testis tissue in a 100x (*fdx1b*$^{-/-}$ mutants and wild-type siblings) or 50x (*cyp11c1*$^{-/-}$ mutants and wild-type siblings) mass:volume dilution with Hank's balanced salt solution. 10µl of sperm solutions 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 (World Health Organization, 2010). Gonadosomatic index (GSI) was calculated using the formula GSI = [gonad weight / total tissue weight] × 100.

To assess sperm release, mutant and wild-type sibling male zebrafish were anaesthetised and semen was collected by stroking of the abdomen with blunt ended forceps (Millipore, Burlington, Massachusetts, United States) followed by aspiration of expelled fluid in a microcapillary tube and transfer to 25µl of ice cold Hank's balanced salt solution. Presence of mature sperm was confirmed by visualisation under a 20x objective.

2.7 - Behavioural analysis

For analysis of breeding behaviours, an *fdx1b*$^{-/-}$ or *cyp11c1*$^{-/-}$ mutant male zebrafish or wild-type sibling male was 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 behaviours in the first five minutes after

removal of the divider were analysed. Intimate contacts, where fish touched or crossed one another, and total time fish spent exhibiting chasing behaviour was recorded. Any instance when one fish was closely following the other was classified as chasing behaviour.

In addition to breeding behaviour, the response of *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant male zebrafish to an open field test was analysed. Fish were transferred individually to an opaque test tank, and following this the fish's movements were tracked for a duration of 5 minutes, using the Zebralab software (Viewpoint, Lyon, France). Fish were deemed to exhibit fast swimming behaviour at speeds of greater then 10cm/s. Fish were subjected to this test on at least three occasions with not less than three days between trials.

2.8 - Haematoxylin and eosin (H&E) and picrosirius red 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. Adult fish were then 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. Juvenile zebrafish aged 25-38dpf were fixed in 4% PFA for 2 days, and decalcified in 0.25M EDTA pH8 for a further 2 days before processing. Following this, 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 in tap water and dehydration by transfer through an 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.

For picrosirius red staining, slides were dewaxed in xylene and rehydrated by transfer through a series of ethanol baths at decreasing concentrations, as for H&E staining. Following washing in tap water for 10 minutes, slides were stained in picrosirius red for 1 hour. Slides were rinsed in three changes of acidified water (0.5% glacial acetic acid) and dehydrated in three changes of 100% ethanol, followed by mounting with DPX mountant.

2.9 - Gene expression analysis by quantitative PCR (qPCR)

For determining the expression of glucocorticoid responsive genes in *cyp11c1*$^{-/-}$ larvae, the progeny of an incross of *cyp11c1*$^{+/-}$ adults were sorted by visual background adaption (VBA). At 4 or 5dpf larvae were housed in dark conditions for 1 hour, followed by a 20 minute light exposure. Subsequently larvae were sorted into two groups: lightly pigmented (VBA+, mainly wild-type or heterozygotes) and darkly pigmented (VBA-, mainly homozygous mutants). VBA+ or VBA- larvae were pooled into groups of 20 and snap frozen on dry ice.

In order to quantify gene expression in adult zebrafish, fish were humanely euthanized, dissected, and the liver, testes and brain collected by snap freezing on dry ice. Total RNA was extracted from liver, testes and brain using Trizol (Ambion, Texas, United States). 1µg of liver or gonad RNA, or 3µg of brain 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 (Untergasser et al., 2012) and are listed in **Table 2.2**. Each primer pair was determined to have an amplification efficiency of between 90-110% and an R^2 value of >0.98.

For measurement of gene expression in *fdx1b*$^{-/-}$ mutant and wild-type sibling zebrafish 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 were analysed using the Pfaffl method (Pfaffl, 2001) 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.

For measurement of gene expression in *cyp11c1*$^{-/-}$ mutant and wild-type sibling zebrafish GoTaq qPCR master mix (Promega, Madison, Wisconsin, United States) was utilized in reactions containing 1µl cDNA synthesis product and specific primers at 1000nM. Reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, California, United States). *Cyp11c1* qPCR data were analysed using the Livak method (Livak and Schmittgen, 2001) 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.

Zebrafish brain tissue was collected by dissection of the head. Initially, the dorsal part of the skull and the mandible were removed. This was followed by removal of most non-brain tissue from around the brain. A small amount of bone including the sphenoid, which forms a protective fossa in which the pituitary resides, was left attached to the ventral aspect of the brain. The pituitary gland was routinely lost during dissection when this small amount of bony tissue was not retained. For analysis of gene expression in adult zebrafish brains, Taqman assays were utilised. Taqman probes specific to zebrafish *ef1a, pomca, fshb* and *lhb* (Thermo Fisher Scientific, California, United States) were used to quantify expression of these genes. Taqman qPCR data were analysed using the Livak method (Livak and Schmittgen, 2001).

Table 2.2. qPCR primer sequences.

Gene	Forward	Reverse	R^2	Efficiency (%)	Citation
amh	AGGTGGATAGCAGCAGTACG	AGATACGTTCGGGATGGGAG	0.9964	96	This thesis.
apoeb	CACGCAAACTGAAGAAACGC	TGGCTCATGTATGGCTGGAA	0.9989	105	This thesis.
ar	AGATGGGCGAATGGATGGAT	AGAACACTTTGCAGCTTCCG	0.9991	105	This thesis.
cyp11a2	TGGAGGAACAGCCTGAAAAG	TTCACACTTTCATAGAATCCAACC	0.9980	93	This thesis.
cyp11c1	AAGACGCTCCAGTGCTGTG	CCTCTGACCCTGTGATCTGC	0.9989	96	This thesis.
cyp17a1	AGTTGCAAAGGACAGCTTGG	GCTGCACGTTATCACTGTAGG	0.9991	108	This thesis.
cyp19a1a	ACAAACTCTCACCTGGACGA	AGTCTGCCAGGTGTCAAAGT	0.9999	103	This thesis.
cyp2k22	CGCTGTCAAACCTACGAGAC	GGGGCAGTTTTGTTTCAAATGG	0.9979	108	This thesis.
dazl	ACTGGGACCTGCAATCATGA	AATACAGGTGATGGTGGGGC	0.9998	98	This thesis.
dmrt1	GGCCACAAACGCTTCTGTAA	ATGCCCATCTCCTCCTCTTG	0.9984	104	This thesis.
ef1a	GTGGCTGGAGACAGCAAGA	AGAGATCTGACCAGGGTGGTT	0.9964	99	This thesis.
fkbp5	TTCCACACTCGTGTTCGAGA	ACGATCCCACCATCTTCTGT	0.9989	104	(Griffin et al., 2016)
foxl2a	CCCAGCATGGTGAACTCTTAC	CGTGATCCCAATATGAGCAGT	0.9945	91	(Crowder et al., 2017)
foxl2b	GCTCACTCTATCCGGCATCT	CTGTTCATGAAGCCCGACTG	0.9993	98	This thesis.
hsd17b3	CCAAATACCCTGCAAGCTCC	TCTGCTGCATTCCTGGTAGT	0.9956	102	This thesis.
igf3	GTAGACCAGTGTTGTGTGCG	ATTCCTCATCTCGCTGCAGA	0.9982	95	This thesis.
inha	CAGAGCTGTGCACCATGTAG	CCAGGTCCAGCATCAGAAGA	0.9972	97	This thesis.
insl3	TCGCATCGTGTGGGAGTTT	TGCACAACGAGGTCTCTATCCA	0.9994	110	(Safian et al., 2016)
nanos2	AAACGGAGAGACTGCGCAGAT	CGTCCGTCCCTTGCCTTT	0.9996	92	(Safian et al., 2016)
odf3b	GATGCCTGGAGACATGACCAA	CAAAGGAGAAGCTGGGAGCTT	0.9965	92	(Assis et al., 2016)
pck1	TGACGTCCTGGAAGAACCA	GCGTACAGAAGCGGGAGTT	0.9980	99	(Griffin et al., 2016)
piwil1	ATACCGCTGCTGGAAAAAGG	GCAAGACACACTTGGAGAACC	0.9988	90	(Safian et al., 2016)
sox9a	CGGAGCTCAAAACTGTG	CGGGGTGATCTTTCTTGTGC	0.9981	106	This thesis.
sox9b	AGACGCAGATCTCCACCAAT	CCGCTTCAGATCCGCTTTAC	0.9952	109	This thesis.
star	TTGAACAAGCTCTCCGGACC	TCACTGTATGTCTCCTCGGC	0.9979	110	This thesis.
sycp3	AGAAGCTGACCCAAGATCATTCC	AGCTTCAGTTGCTGGCGAAA	0.9981	95	(Assis et al., 2016)
znrf3	GCTAAGTTCAAGGGTCAGCG	TTCTTGTGGAATCGGTGTGC	0.9975	100	This thesis.

2.10 - Statistical methods

All statistical analyses were conducted using Graphpad Prism (GraphPad Software, San Diego, California, United States). Data normality was assessed using normality tests available in Graphpad Prism, including Shapiro-Wilk, D'Agostino-Pearson and Kolmogorov-Smirnov normality tests. When data were not normally distributed, outliers were identified using the ROUT method. Normally distributed data were analysed using unpaired *t*-tests or ANOVA. Data that was not normally distributed was analysed using Mann-Whitney or Kruskal-Wallis tests. Open field test data were analysed using multiple *t*-tests with the Holm-Sidak method to correct for multiple comparisons. Bar graph error bars represent the standard error of the mean. Statistical significances are reported using asterisks as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ **** $p<0.0001$.

Chapter 3 - Ferredoxin 1b deficiency leads to testis disorganisation, impaired spermatogenesis and feminisation in zebrafish

Summary

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 signalling 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 behaviours, and sperm production is reduced, resulting in infertility in standard breeding scenarios. However, eggs collected from wild-type females can be fertilised by the sperm of *fdx1b*$^{-/-}$ mutant males by IVF. The testes of *fdx1b*$^{-/-}$ mutant males are disorganised 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 behaviour, thus enabling further elucidation of the physiological consequences of endocrine disruption in vertebrates.

3.1 - 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 signalling contribute to the endocrine disruption of reproductive physiology and other sex-specific characteristics (Liew and Orban, 2014).

Mitochondrial cytochrome P450 enzymes are crucial for steroidogenesis, and their activity requires electron transfer from cofactors including Ferredoxin 1 (FDX1) (**Figure 1.1**) (Midzak and Papadopoulos, 2016). 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) (Midzak and Papadopoulos, 2016). FDX1, and the associated ferredoxin reductase, act as sequential electron donors, facilitating transfer of electrons from NADPH to cytochrome P450 enzymes (Midzak and Papadopoulos, 2016). 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, catalyse the conversion of cholesterol to pregnenolone, while Cyp11c1, the zebrafish homolog of CYP11B1, catalyses the 11β-hydroxylation of 11-deoxycortisol to produce cortisol (Griffin et al., 2016). Cyp11c1 may also facilitate conversion of the mineralocorticoid 11-deoxycorticosterone to corticosterone in teleost fish (Jiang et al., 1998). Unlike in mammals, 11β-hydroxylase is postulated to play a crucial role in gonadal androgen synthesis in zebrafish, namely the 11β-hydroxylation of androgen precursors (**Figure 1.1**). 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 (Griffin et al., 2016). 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 signalling is pivotal in regulation of the development of male secondary sex characteristics, gonads, and reproductive behaviour. 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 disorganised seminiferous tubules, and are infertile (Crowder et al., 2017; Tang et al., 2018). Mutation of the AR has also been linked to impaired courtship behaviour (Yong et al., 2017) and defective spermatogenesis (Yu et al., 2018). Exposure of zebrafish to the androgen trenbolone during development causes robust masculinisation (Larsen and Baatrup, 2010; Morthorst et al., 2010), whilst anti-androgen treatment causes a shift towards female development and delayed testes maturation (Lor et al., 2015).

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

3.2 - Results

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

Morphological secondary sex characteristics in zebrafish include body and fin 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 (Borg, 1994). 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*$^{-/-}$ mutant 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 3.1**). All *fdx1b*$^{-/-}$ mutant 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 3.1**). We observed an absence of dark golden pigmentation in the anal fins of *fdx1b*$^{-/-}$ mutant 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*$^{-/-}$ mutant 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*$^{-/-}$ mutant males were larger than wild-type sibling males, and both length and weight were significantly increased. *Fdx1b*$^{-/-}$ mutant females were found to be significantly heavier than wild-type female siblings, but no difference in length was recorded (**Figure 3.2**).

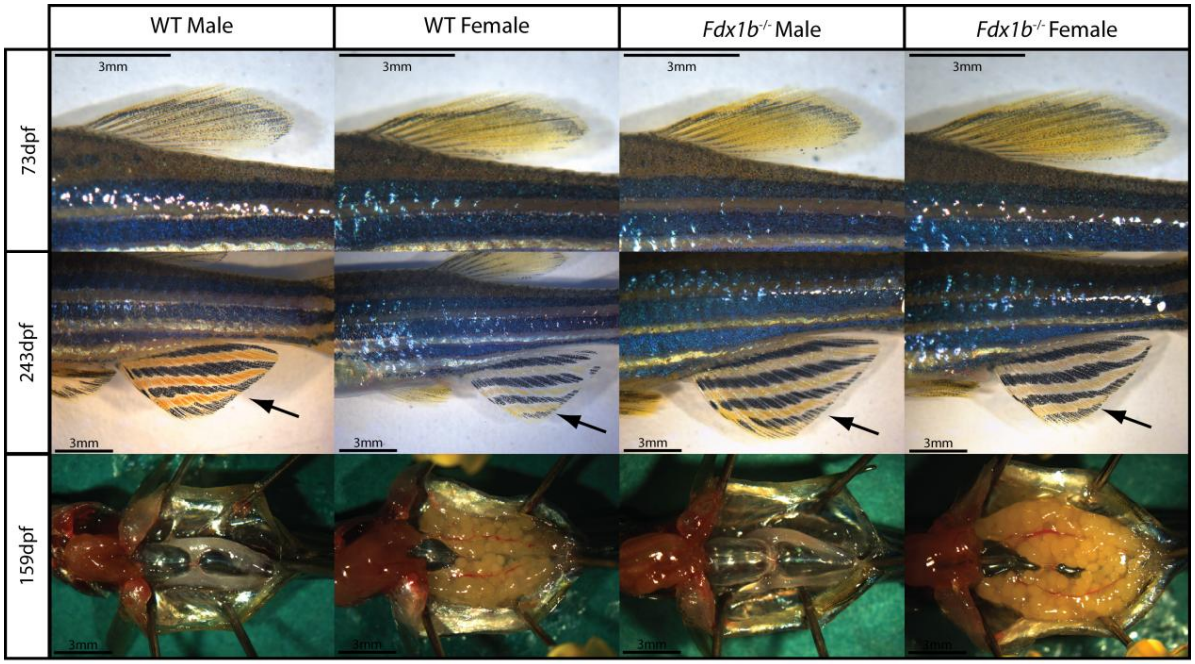

Figure 3.1. Feminisation of secondary sex characteristics in *fdx1b*$^{-/-}$ mutant zebrafish. Wild-type male adult zebrafish exhibit a pale dorsal fin and a blue and golden striped anal fin. Anal fin indicated by arrows. Wild-type female adult zebrafish exhibit a yellow-green pigmented dorsal fin and lack the strong stripes of gold pigment seen in the male anal fin. Mutant male adult zebrafish exhibited female type coloration of the dorsal and anal fins throughout adulthood. No obvious difference in macroscopic gonadal morphology was observed.

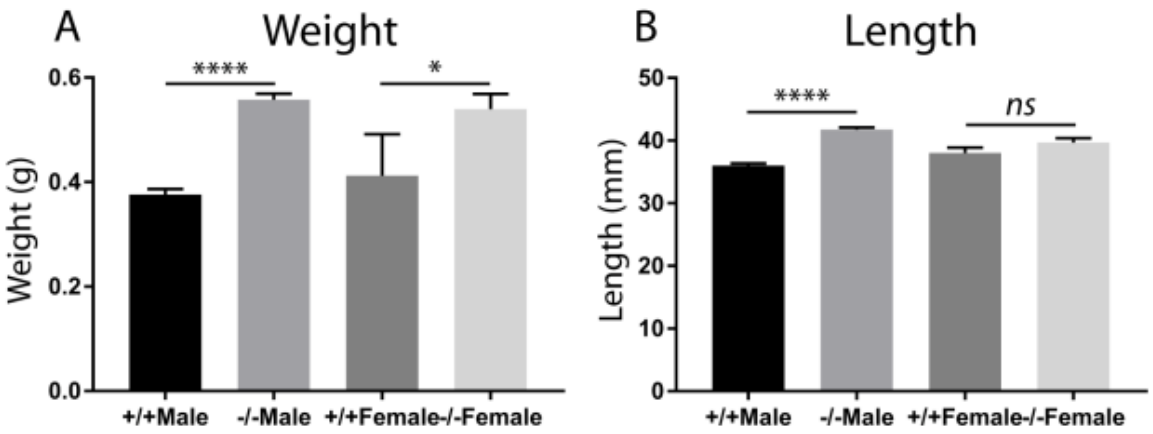

Figure 3.2. Increased length and weight in adult *fdx1b*$^{-/-}$ mutant male zebrafish. A: *Fdx1b*$^{-/-}$ mutant zebrafish (males n=15, females n=15) were on average heavier than wild-type siblings (males n=11, females n=4) (ANOVA with multiple comparisons, p=<0.0001) despite identical feeding regimens and maintenance at similar stocking densities. Male *fdx1b*$^{-/-}$ mutant zebrafish (n=16) were also longer than wild-type sibling males (n=9), no difference in length was observed between female *fdx1b*$^{-/-}$ mutant zebrafish (n=15) and wild-type sibling females (n=4) (ANOVA with multiple comparisons, p=<0.0001). **** p<0.0001, * p<0.05.

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

Fdx1b is a cofactor for the steroidogenic enzymes Cyp11a1, Cyp11a2 and Cyp11c1. Cyp11a1 and Cyp11a2 catalyse the first stage in steroid biosynthesis: conversion of cholesterol to pregnenolone (Parajes et al., 2013). Cyp11c1 is required for the final step of cortisol biosynthesis, as well as 11β-hydroxylation of androstenedione in the androgen biosynthetic pathway (Tokarz et al., 2015). Cyp11c1 may also be required for 11β-hydroxylation of testosterone in the alternative pathway to 11KT production (**Figure 1.1**).

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 (**Figures 3.3+3.4**). Significantly decreased concentrations of 11KT and cortisol were measured in *fdx1b*$^{-/-}$ mutant 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 3.3**). 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 (Griffin et al., 2016). 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 3.3**). Very similar results with comparable fold changes were recorded in a replicate experiment, confirming disruption of steroidogenesis in Fdx1b deficient zebrafish (**Figure 3.4**). Analysis of steroid concentrations in female *fdx1b*$^{-/-}$ mutant zebrafish

can be found in chapter 6 and a comparison of steroid hormone concentrations in male and female, wild-type and *fdx1b*$^{-/-}$ mutant zebrafish, can be found in **Appendix I**.

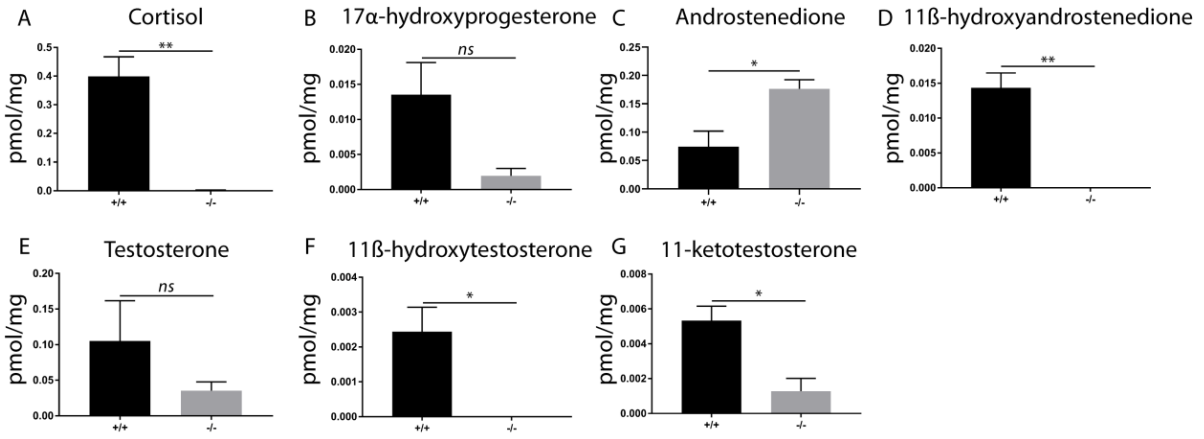

Figure 3.3. Profoundly altered glucocorticoid and androgen steroid profiles in male *fdx1b*$^{-/-}$ mutant zebrafish. The concentration of steroids was measured in samples prepared from whole fish bodies. The concentration of cortisol (**A**) was profoundly reduced in *fdx1b*$^{-/-}$ mutant male zebrafish (n=3, p=0.0044). The concentration of 17α-hydroxyprogesterone (**B**) was not significantly decreased (n=3, p=0.069). The concentration of the sex steroid precursor androstenedione (**C**) was significantly increased in *fdx1b*$^{-/-}$ mutant male zebrafish (n=3, p=0.033); however, testosterone (**E**) concentrations were unchanged (n=3, p=0.30). The presence of 11β-hydroxyandrostenedione (**D**) (n=3, p=0.0026) and 11β-hydroxytestosterone (**F**) (n=3, p=0.025) was undetectable and the concentration of 11KT (**G**) was significantly decreased (n=3, p=0.022) in *fdx1b*$^{-/-}$ mutants. All results were analysed using unpaired *t*-tests. ** p<0.01, * p<0.05.

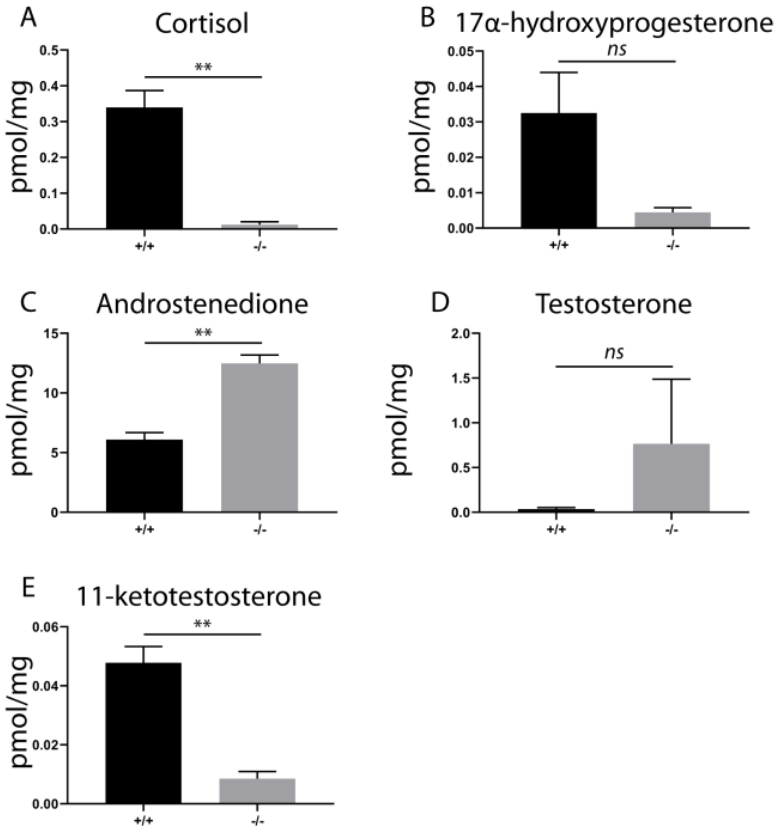

Figure 3.4. Decreased 11-ketotestosterone and cortisol concentrations in *fdx1b*$^{-/-}$ mutant male zebrafish are reproducible. Steroid measurements were repeated in replicate samples of *fdx1b*$^{-/-}$ mutant and wild-type sibling male zebrafish and comparable results were observed. Results analysed by unpaired *t*-test, n=3, cortisol p=0.0024 (**A**), 17α-hydroxyprogesterone p=0.0712 (**B**), androstenedione p=0.0022 (**C**), testosterone p=0.3689 (**D**), 11KT p=0.0029 (**E**). ** p<0.01.

In addition to direct measurement of steroid hormones, systemic deficiency of glucocorticoids and androgens was confirmed by measuring the expression of established steroid responsive genes in liver tissue (**Figure 3.5**). *Fkbp5* and *pck1* are known to be robust glucocorticoid-responsive genes in zebrafish (Eachus et al., 2017; Griffin et al., 2016), with *FKBP5* expression in humans and mice also influenced by androgen receptor transactivation (Magee et al., 2006). *Fkbp5* expression is also inducible by exogenous androgens in zebrafish larvae (Fetter et al., 2015). *Cyp2k22* was used as a well-established androgen-responsive gene in zebrafish (Fetter et al., 2015; Siegenthaler et al., 2017). The expression of all three genes was significantly decreased in *fdx1b*$^{-/-}$ mutant males compared to wild-type siblings (**Figure 3.5**). *Apoeb* expression has been shown to be robustly downregulated in the gonads of zebrafish treated with androgen receptor antagonists (Martinovic-Weigelt et al., 2011). *Apoeb* was significantly upregulated in the livers of *fdx1b*$^{-/-}$ mutant males compared to wild-type siblings, indicating that its expression is also influenced by androgen signalling in this tissue (**Figure 3.5**). Analysis of steroid responsive gene expression in the livers of female *fdx1b*$^{-/-}$ mutant zebrafish can be found in chapter 6. A comparison of steroid responsive gene expression in the livers of male and female, wild-type and *fdx1b*$^{-/-}$ mutant zebrafish, can be found in **Appendix I**.

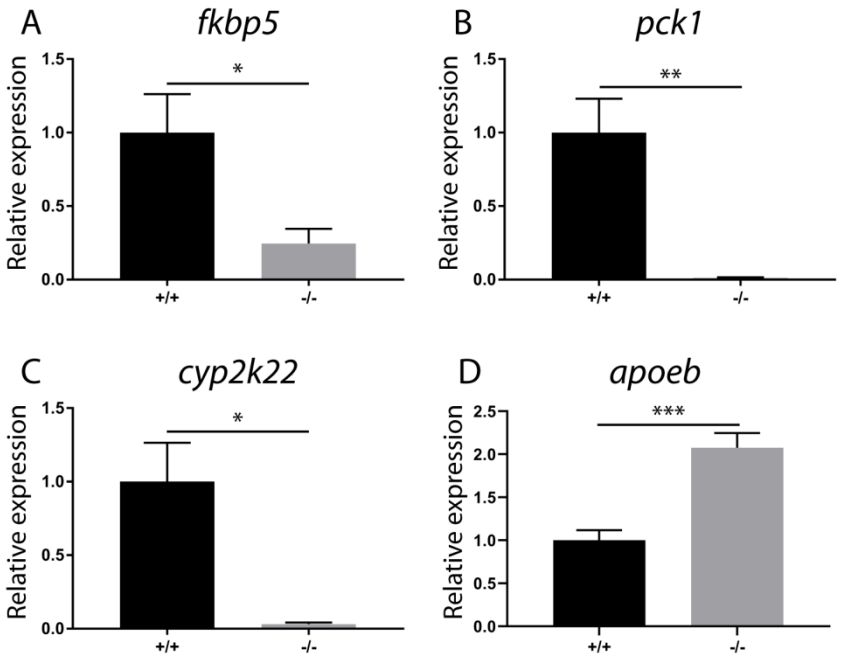

Figure 3.5. Altered expression of glucocorticoid and androgen responsive genes in *fdx1b*$^{-/-}$ mutant male zebrafish. The expression of glucocorticoid responsive genes *fkbp5* (**A**) (n=8, p=0.015) and *pck1* (**B**) (wild-type n=8, mutant n=5, p=0.0064) and of androgen responsive gene *cyp2k22* (**C**) (wild-type n=6, mutant n=5, p=0.013) was significantly decreased in *fdx1b*$^{-/-}$ mutant male zebrafish. Expression of *apoeb* (**D**) (n=5, p=0.0008) was significantly increased in the livers of *fdx1b*$^{-/-}$ mutant male zebrafish. All results were analysed using unpaired *t*-tests. *** p<0.001, ** p<0.01, * p<0.05.

As cortisol and androgen biosynthesis appear to be impaired in *fdx1b*$^{-/-}$ mutant male zebrafish (**Figures 3.3+3.4**), it is possible that precursors for these steroids may accumulate and enter into the oestrogen biosynthetic pathway (**Figure 1.1**). Indeed, the concentration of androstenedione, which is the principal oestrogen precursor, is significantly increased in *fdx1b*$^{-/-}$ mutant male zebrafish in comparison to wild-type sibling males (**Figures 3.3+3.4**). We have attempted to assess the impact of Fdx1b deficiency on oestrogen biosynthesis by using qPCR to measure the expression of oestrogen responsive genes *vtg2* and *esr1* in the liver (Chandrasekar et al., 2010; Henry et al., 2009; Lam et al., 2011). The expression of these genes was considerably higher in female fish than male fish; however no effect of Fdx1b deficiency on expression of these genes was evident (**Figure 3.6**).

In order to further characterize the impact of loss of Fdx1b function and decreased androgen and cortisol concentrations on testicular 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 3.7**). The expression levels of *star* and *hsd17b3* also suggested increased expression in *fdx1b*$^{-/-}$ mutant testes*,* however these changes did not achieve statistical significance (**Figure 3.7**). No changes in the expression of *cyp17a1,* which is crucial for gonadal sex steroid production (**Figure 1.1**), or the oestrogenic enzyme *cyp19a1a* were detected (**Figure 3.7**).

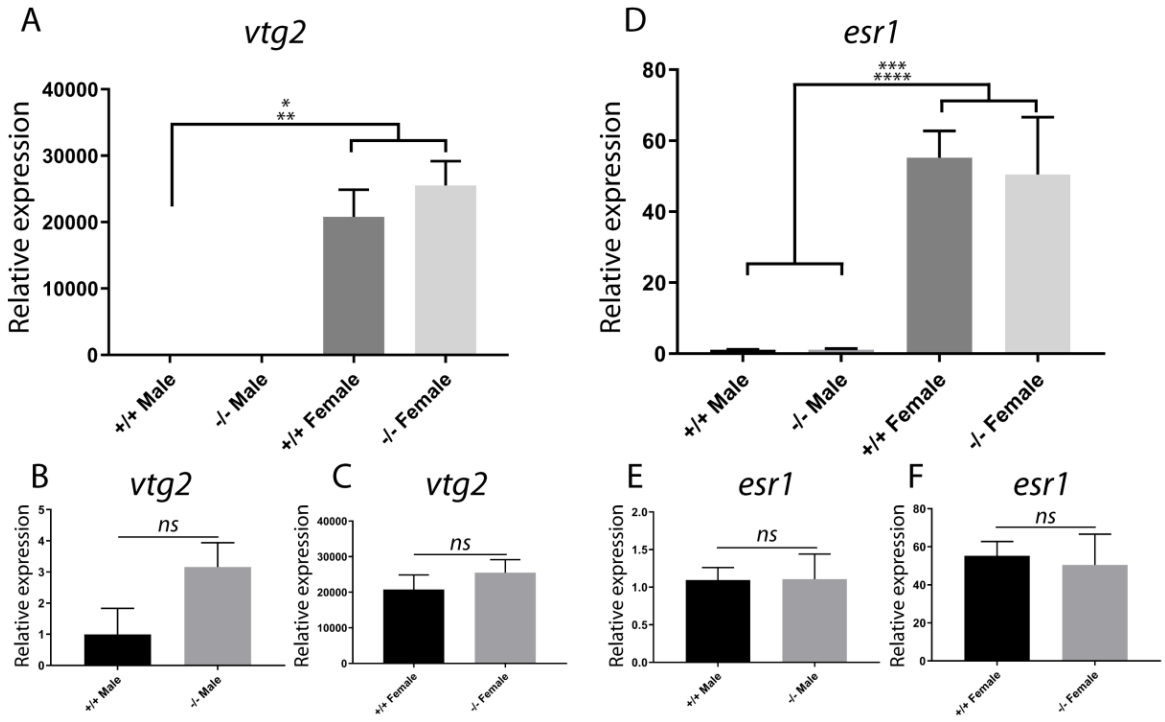

Figure 3.6. Expression of oestrogen responsive genes in the livers of *fdx1b*$^{-/-}$ mutant and wild-type, male and female zebrafish. Expression of oestrogen responsive genes *vtg2* and *esr1* in liver tissue was analysed by qPCR. All data is displayed as a fold-change relative to expression in wild-type male zebrafish. The expression of both genes was considerably higher in female fish compared to male fish, regardless of genotype (*vtg2,* wild-type male n=6, *fdx1b*$^{-/-}$ male n=8, wild-type female n=4, *fdx1b*$^{-/-}$ male n=4, Kruskal-Wallis test p=0.001; *esr1,* wild-type male n=7, *fdx1b*$^{-/-}$ male n=8, wild-type female n=4, *fdx1b*$^{-/-}$ male n=4, one-way ANOVA p<0.0001). Due to the large difference in expression between zebrafish sexes, the expression of *vtg2* and *esr1* in male and female zebrafish was also analysed separately. No significant differences were found in any comparison, though an apparent increase in expression of *vtg2* in *fdx1b*$^{-/-}$ mutant male zebrafish compared to wild-type sibling males approached significance (Mann-Whitney test, p=0.0593). **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05.

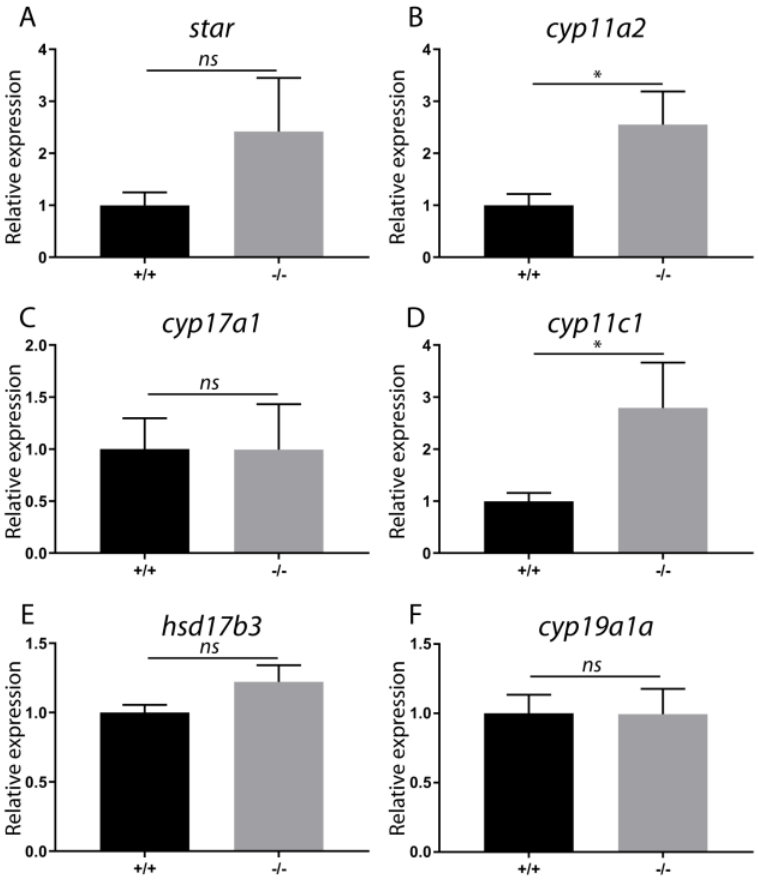

Figure 3.7. Expression profile of steroidogenic enzymes and *star* in the testes of *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings. The expression of *cyp11a2* (**B**) (n=7, p=0.04) and *cyp11c1* (**D**) (wild-type n=7, mutant n=4, p=0.0249) was significantly increased in the testes of *fdx1b*$^{-/-}$ mutant 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 analysed using unpaired *t*-tests. * $p<0.05$.

3.2.3 - *Fdx1b*$^{-/-}$ mutant 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 (Westerfield, 2000). Tanks of *fdx1b*$^{-/-}$ mutant zebrafish or wild-type siblings containing a mixture of males and females were marbled weekly for four weeks. Fertilised embryos were obtained from groups of wild-type siblings in all four trials. However, groups of *fdx1b*$^{-/-}$ mutant fish failed to produce fertilised embryos in any trial and no unfertilised embryos were observed either. To investigate whether this apparent infertility was due to a defect in male or female reproductive physiology, or both, *fdx1b*$^{-/-}$ mutants 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 fertilised embryos in 70% and 75% of trials respectively. *Fdx1b*$^{-/-}$ mutant males (n=5) and females (n=5) were unsuccessful in all trials (**Table 3.1**).

To investigate whether sperm from *fdx1b*$^{-/-}$ mutant 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 zebrafish and 2 out of 5 *fdx1b*$^{-/-}$ mutant zebrafish successfully fertilised eggs collected from wild-type females, indicating that at least some *fdx1b*$^{-/-}$ mutant male zebrafish can produce functional sperm.

Table 3.1. Outcrossing of *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings.

Genotype and sex	Number of fish	Total number of trials*	Trials in which fertilised 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.

3.2.4 - *Fdx1b*$^{-/-}$ mutant male zebrafish exhibit reduced breeding behaviours

Steroid hormones are known to exert powerful impacts on the vertebrate brain and a wide range of behaviours. To investigate whether reproductive behaviours 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 behaviours previously reported in zebrafish: intimate contacts between fish (Zhai et al., 2018) and chasing (Darrow and Harris, 2004). Both of these behaviours were significantly reduced in *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 3.8**).

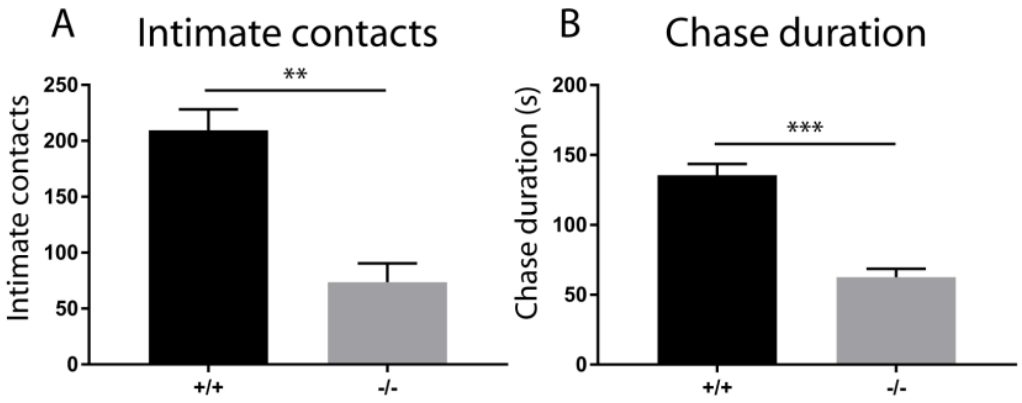

Figure 3.8. Stereotypical breeding behaviours are reduced in *fdx1b*$^{-/-}$ mutant males. A: Intimate contacts, defined as incidences of touching or crossing, were significantly reduced in pairings of *fdx1b*$^{-/-}$ mutant males and wild-type females, compared to pairings of wild-type males and females (n=5, Mann-Whitney Test, p=0.0079). **B:** The total duration of time spent chasing was also significantly reduced compared to time spent chasing by wild-types (n=5, unpaired *t*-test, p=0.0007). *** p<0.001, ** p<0.01.

It was noted that *fdx1b*$^{-/-}$ mutant zebrafish appeared to exhibit slower swimming than wild-type siblings, and a freezing behaviour, were the fish would remain stationary in the tank for extended periods of time. This apparent reduction in locomotion may influence breeding behaviours. To investigate this possibility of reduced locomotion we subjected *fdx1b*$^{-/-}$ mutant and wild-type male zebrafish to open field tests to determine the impact of *fdx1b* mutation on swim speed and duration of freezing behaviours. Open field tests were carried out in triplicate, with at least three days between replicate experiments. Total distance swam, fast swim distance and fast swim duration were all consistently reduced in *fdx1b*$^{-/-}$ mutant male zebrafish compared to wild-types, however these differences rarely achieved statistical significance (**Figure 3.9**). Fast swim distance and duration were significantly reduced in *fdx1b*$^{-/-}$ mutant fish in the first replicate experiment only. Freezing duration was also consistently greater over the three replicate trials, however none of these comparisons achieved significance, probably due to the extremely high variation exhibited by *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 3.9**).

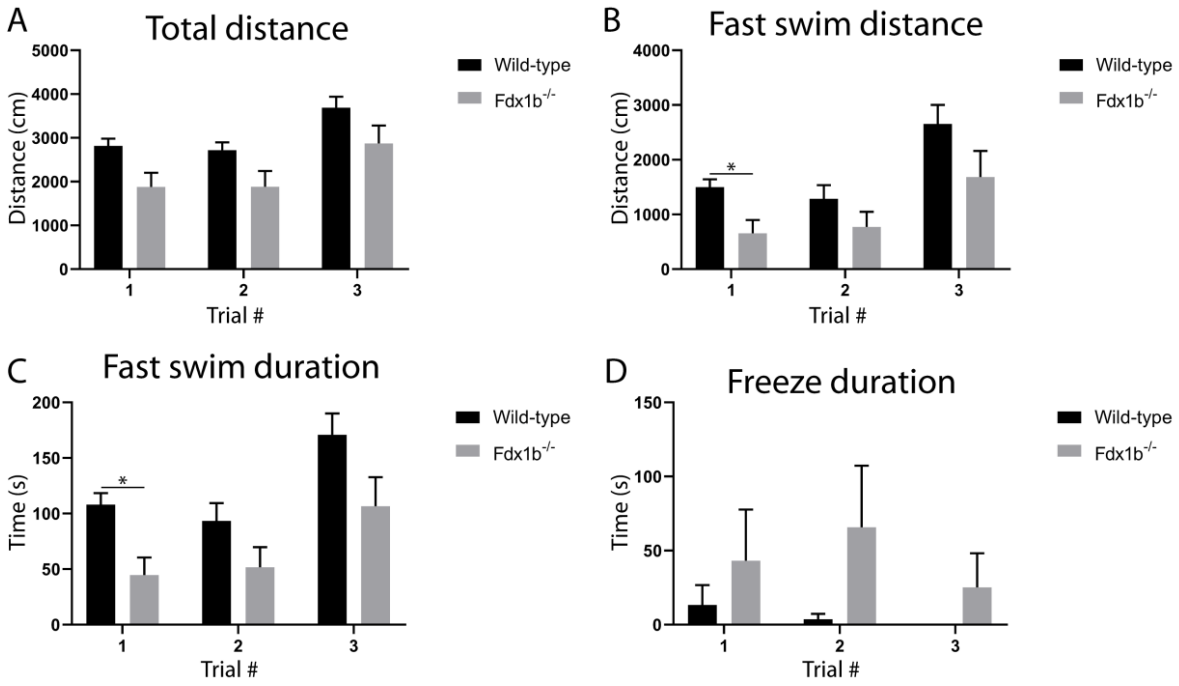

Figure 3.9. Response of *fdx1b*$^{-/-}$ mutant zebrafish to open field tests. *Fdx1b*$^{-/-}$ mutant and wild-type sibling zebrafish were exposed to three replicate open field tests and the total distance swam, fast swim distance, fast swim duration and freeze duration were recorded (n=6). Total distance, fast swim distance and fast swim duration were consistently reduced in *fdx1b*$^{-/-}$ mutant zebrafish, however only the first replicate experiments for fast swim distance and duration produced statistically significant results (p=0.04, p=0.02 respectively). Freezing duration was consistently increased in *fdx1b*$^{-/-}$ mutant zebrafish however this difference was not statistically significant. Results were analysed using multiple *t*-tests with the Holm-Sidak method, * $p<0.05$.

3.2.5 – The testes of *fdx1b*$^{-/-}$ mutant zebrafish are disorganised and have decreased sperm production

Our findings have shown that *fdx1b*$^{-/-}$ mutant 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 (**Figure 3.10**). 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 were located more peripherally. By contrast, the testes of *fdx1b*$^{-/-}$ mutant zebrafish were considerably disorganised and had much less distinct seminiferous tubules (**Figure 3.10**). The organisation of seminiferous tubules was further investigated by picrosirius red staining for collagen, a component of the basement membrane (Dym, 1994; Junqueira et al., 1979; Mazzoni et al., 2018). This revealed that tubules in *fdx1b*$^{-/-}$ mutant fish were generally smaller in diameter and greater in number (**Figure 3.11**). Despite this disorganisation, developing and mature sperm were present (**Figure 3.10**).

Histological examination also revealed a reduction of mature sperm present in the testes of *fdx1b*$^{-/-}$ mutants compared to wild-type siblings (**Figure 3.10**). To quantify this finding, we employed sperm counting techniques to determine the concentration of sperm in whole testes relative to testicular weight. Sperm concentrations were decreased in *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings when sperm was collected from dissected testes (**Figure 3.10**). 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 3.10**).

The disorganised 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 in 3 out of 10 *fdx1b*$^{-/-}$ mutant samples, indicating a decreased ability to release sperm in *fdx1b*$^{-/-}$ mutant 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 3.10**).

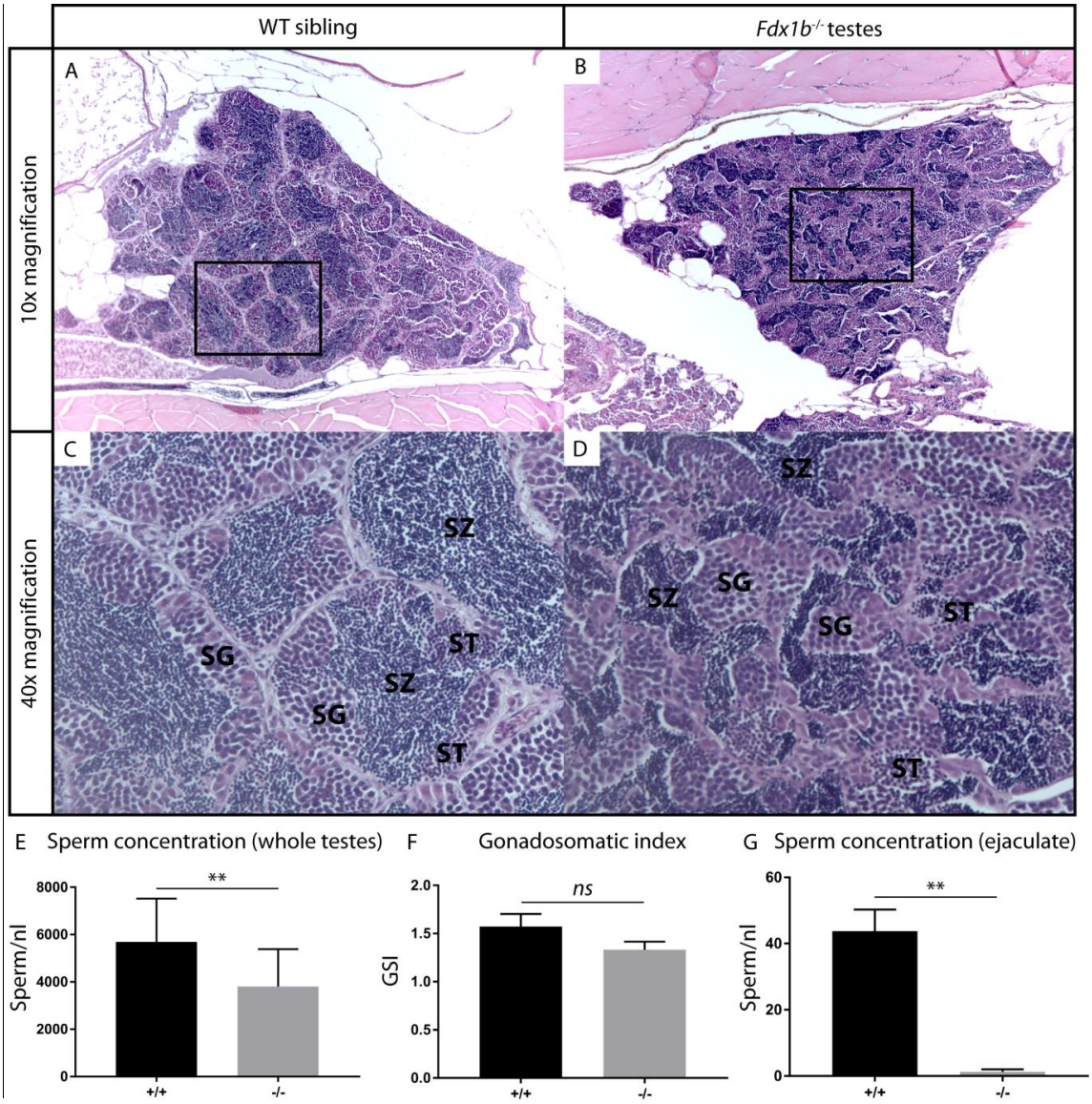

Figure 3.10. Disrupted morphological organisation of the testes and decreased sperm concentration in *fdx1b*$^{-/-}$ mutant zebrafish. A-D: *Fdx1b*$^{-/-}$ mutant zebrafish had disorganised testes and poorly defined seminiferous tubules compared to wild-type males. Developing and mature sperm were observed in both *fdx1b*$^{-/-}$ mutant and wild-type testes. SZ – mature spermatozoa, ST – spermatids, SG – spermatogonia. **E:** Testes dissected from *fdx1b*$^{-/-}$ mutant 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*$^{-/-}$ mutant fish compared to wild-type siblings (unpaired *t*-test, wild-type n=8, mutant n=4, p=0.0012). ** p<0.01.

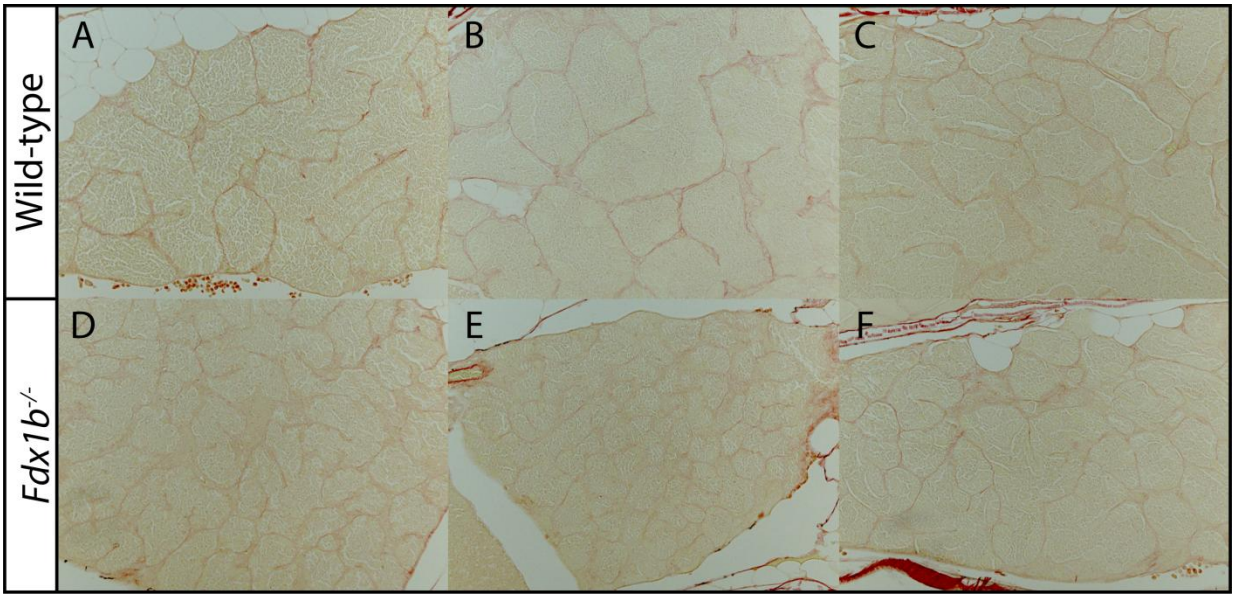

Figure 3.11. Staining for collagen reveals that seminiferous tubules are smaller in diameter and greater in number in *fdx1b*$^{-/-}$ mutants compared to wild-type siblings. Collagen fibres in the testes of wild-type (**A-C**) and *fdx1b*$^{-/-}$ mutant (**D-E**) zebrafish (n=3) were stained by picrosirius red. Tubules in *fdx1b*$^{-/-}$ mutant zebrafish appeared to be generally smaller in diameter and also more numerous.

3.2.6 - Morphological appearance of the spermatic duct is highly variable in *fdx1b*$^{-/-}$ mutant zebrafish

In zebrafish, sperm is conducted from the testes in paired spermatic ducts, these ducts eventually fuse before reaching the urogenital orifice, were sperm is released during breeding (Menke et al., 2011). As sperm release appears to be impaired in *fdx1b*$^{-/-}$ mutant zebrafish, we investigated by H&E staining whether the morphology of the spermatic duct was also compromised (**Figure 3.12**). The spermatic ducts of wild-type sibling male zebrafish (n=3) appeared as epithelium bounded tubules containing mature spermatozoa, and were of a consistent appearance, size and shape (**Figure 3.12A-C**). In contrast to this, the morphology of the spermatic ducts of *fdx1b*$^{-/-}$ mutant zebrafish (n=3) was highly variable (**Figure 3.12D-F**). In one fish the spermatic duct was indistinguishable from wild-type. In another, the spermatic duct appeared hypoplastic and contained fewer mature spermatozoa. In the final *fdx1b*$^{-/-}$ mutant male zebrafish the spermatic duct appeared to be completely absent, or may have constituted a small bundle of tissue immediately posterior to the intestine. Incidentally, this fish also exhibited the most severe morphological disruption of the testes.

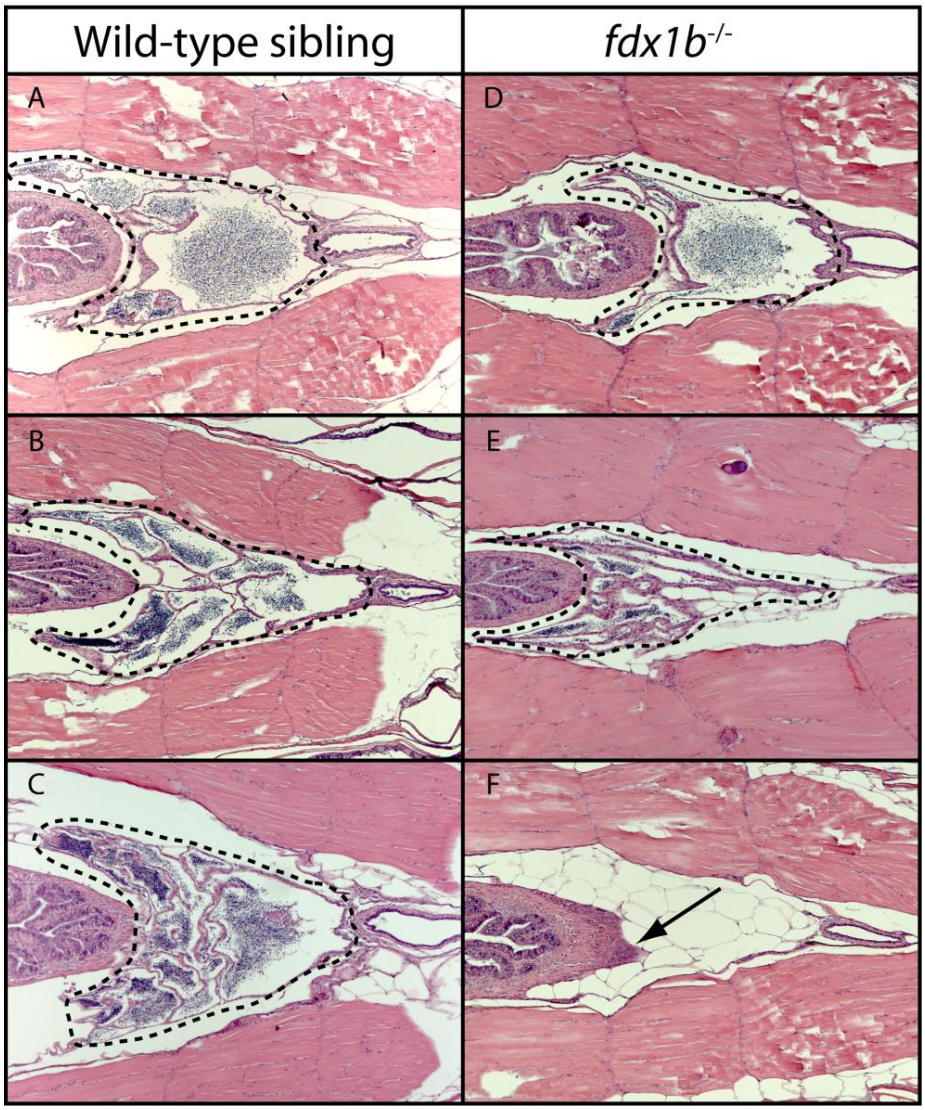

Figure 3.12. Highly variable morphology of the spermatic duct in *fdx1b*$^{-/-}$ mutant zebrafish. The morphological appearance of the spermatic duct in *fdx1b*$^{-/-}$ mutant and wild-type male zebrafish (n=3) was investigated by H&E staining of coronal sections through this structure. Spermatic ducts (**dashed outlines**) were identified directly posterior to the intestine. Spermatic ducts in wild-type male zebrafish (**A-C**) exhibited consistent morphology, characterised by epithelial tubules with mature sperm in the lumina. The spermatic ducts of *fdx1b*$^{-/-}$ mutant males (**D-F**) were highly variable in their morphology, ranging from being indistinguishable from wild-type (**D**), to hypoplastic (**E**), to completely absent (**F**). In the *fdx1b*$^{-/-}$ mutant zebrafish which apparently lacked a spermatic duct a bundle of tissue was observed immediately posterior to the intestine – this may constitute a severely hypoplastic spermatic duct structure (**F**, **arrow**).

3.2.7 - 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 the Sertoli cells of the testes, which has a crucial role in testis differentiation that is conserved throughout most vertebrate species (Rodriguez-Mari et al., 2005; Sun et al., 2013). We observed a significant downregulation of *sox9a* expression in the testes of adult *fdx1b*$^{-/-}$ mutant males compared to wild-type siblings (**Figure 3.13**).

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*$^{-/-}$ mutant males and wild-type siblings (**Figure 3.13**). *Dmrt1* is a transcription factor essential for male development (Webster et al., 2017). Amh is a pro-testis hormone during zebrafish development (Sun et al., 2013) and negatively regulates spermatogenesis in the adult testis (Lin et al., 2017; Morais et al., 2017). The expression of *znrf3* which is a pro-testis gene in mammals was also not affected by mutation of *fdx1b* (Harris et al., 2018).

Igf3 and *insl3* encode spermatogenic signalling 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 (Morais et al., 2017; Nobrega et al., 2015), whereas Insl3 is required only for proliferation and differentiation of type A_{und} spermatogonia (Assis et al., 2016). Expression of *igf3* was downregulated 10-fold, whereas expression of *insl3* was downregulated 25-fold in *fdx1b*$^{-/-}$ mutant testes compared to wild-type (**Figure 3.13**). As both factors are involved in positively regulating the proliferation and differentiation of type A spermatogonia in the early stages of spermatogenesis (Assis et al., 2016; Nobrega et al., 2015), these 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 3.13**). Inhibins exert negative feedback on the hypothalamus-pituitary-gonadal axis and suppress FSH secretion (Gregory and Kaiser, 2004), and this mechanism is also suggested to occur in fish (Poon et al., 2009). In mice, *inha* is important for Sertoli cell proliferation and function and may play a role in spermatogenesis (Cai et al., 2011).

Whereas *sox9a, dmrt1* and *amh* are pro-testis genes during development and also have important roles in testis function, the expression of genes which are considered pro-female or that are involved in ovarian function may also be influenced by altered steroid hormone concentrations due to mutation of *fdx1b. Sox9b* is a paralog of *sox9a* which arose from the teleost-specific whole genome duplication; both genes are orthologs of the mammalian *SOX9*. As *sox9b* is expressed at high levels in the adult ovary compared to adult testes, and as *sox9b* expression has been observed in oocytes during ovarian development, this gene is considered important for ovary development and function (Rodriguez-Mari et al., 2005). *Foxl2a* and *foxl2b* are co-orthologs of the mammalian *FOXL2*, and encode pro-ovary transcription factors. *Foxl2a* and *foxl2b* are least partly functionally redundant, as only knock-out of both genes results in male biased sex differentiation (Yang et al., 2017). No significant difference in the expression of any of these genes was recorded, despite apparent increases in the expression of *sox9b* and *foxl2a* **(Figure 3.13)**. Analysis of gonadal gene expression in wild-type and *fdx1b*$^{-/-}$ mutant female zebrafish can be found in chapter 6. Analysis of the differences in gene expression between male and female, wild-type and *fdx1b*$^{-/-}$ mutant zebrafish, can be found in **Appendix I**.

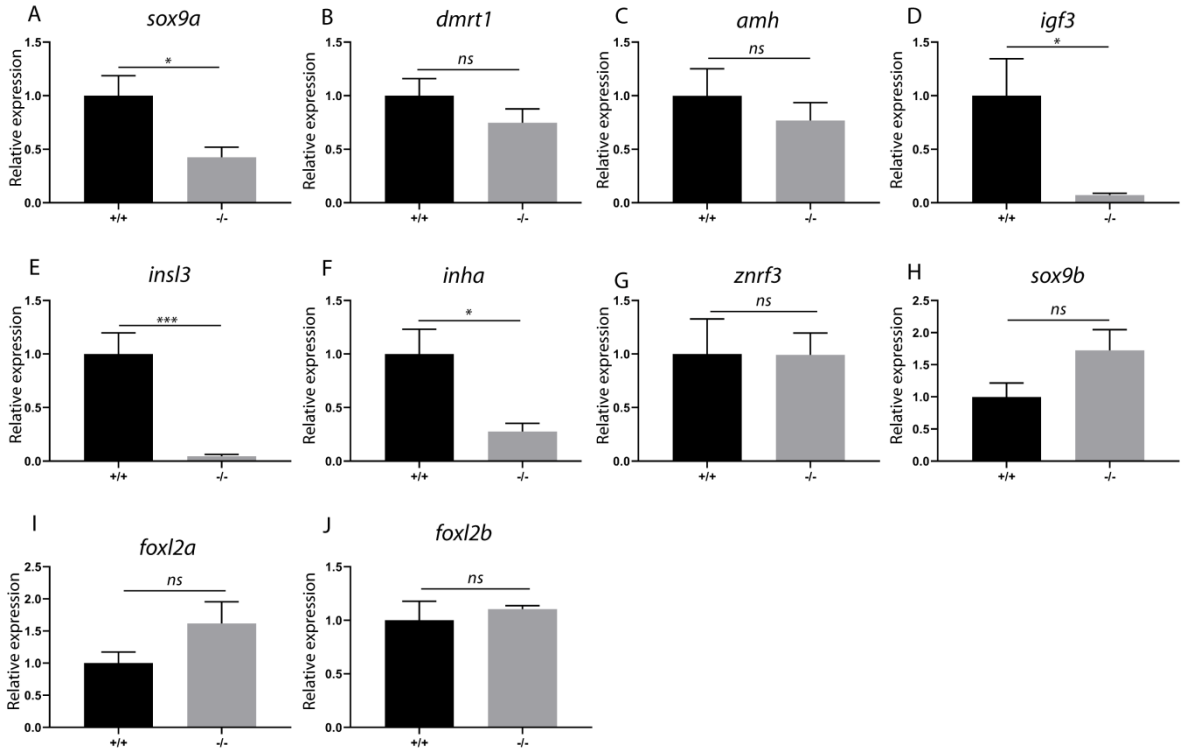

Figure 3.13. Downregulation of pro-testis, spermatogenic and hypothalamic-pituitary-gonadal (HPG) axis regulating genes in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Expression of the conserved pro-testis gene *SRY-box 9a (sox9a)* (wild-type n=10, mutant n=7, p=0.029) (**A**), the spermatogenic 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*$^{-/-}$ mutant 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*. Expression of *znrf3*, considered a pro-male gene in mammals, was also not changed in *fdx1b*$^{-/-}$ mutant testes compared to wild-types (wild-type n=3, mutant n=3, p=0.96) (**G**). Expression of pro-female genes was also investigated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish however no changes in expression were identified. *Sox9b* (**H**) (wild-type n=10, mutant n=7, p=0.0716), *foxl2a* (**I**) (wild-type n=5, mutant n=5, p=0.1040) and *foxl2b* (**J**) (wild-type n=3, mutant n=3, p=0.5969). All results analysed using unpaired *t*-tests. * $p<0.05$, *** $p<0.001$.

3.2.8 - 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 marker genes for germ cells at several stages of spermatogenesis was measured (**Figure 3.14**). Significant upregulation of nanos homolog 2 (*nanos2*) (type A_{und} spermatogonia) (Beer and Draper, 2013; Safian et al., 2016) and piwi-like RNA-mediated gene silencing 1 (*piwil1*) (all type A spermatogonia) (Chen et al., 2013) was observed in *fdx1b*$^{-/-}$ mutant 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 3.14**). No change in expression of deleted in azoospermia-like (*dazl*), expressed mainly in type B spermatogonia (Chen et al., 2013), the spermatocyte marker synaptonemal complex protein 3 (*sycp3*) (Ozaki et al., 2011) or the spermatid marker outer dense fibre of sperm tails 3B (*odf3b*) (Nobrega et al., 2015; Yano et al., 2008) was observed (**Figure 3.14**).

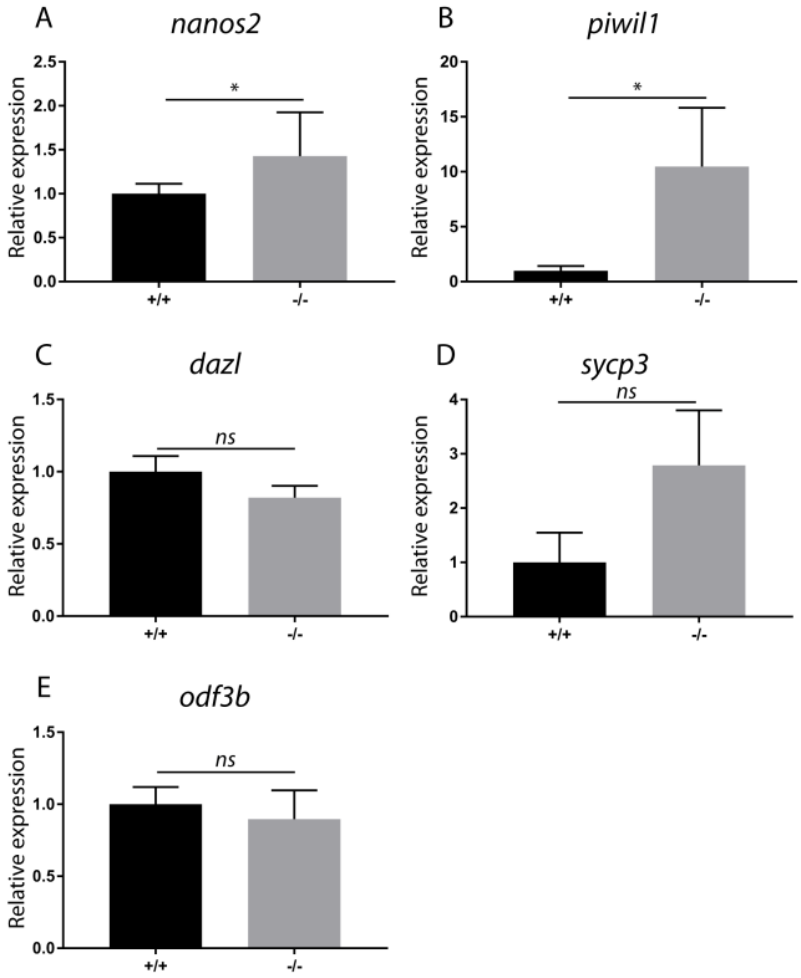

Figure 3.14. Expression of spermatogenesis marker genes in *fdx1b*$^{-/-}$ mutant and wild-type zebrafish testes. *Nanos2, piwil1, dazl, sycp3* and *odf3b* can be used as markers of spermatogenic stages. Expression of *nanos2* (**A**) (wild-type n=4, mutant n=5, p=0.024) and *piwil1* (**B**) (Mann-Whitney 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 analysed using unpaired *t*-tests unless otherwise stated. * $p<0.05$.

3.2.9 - Dysregulation of pituitary genes in the brains of *fdx1b*$^{-/-}$ mutant zebrafish

Release of cortisol from the interrenal is governed by the hypothalamus-pituitary-interrenal (HPI) axis in fish. Cortisol exerts negative feedback on the HPI axis, specifically by inhibiting expression of *pomca*, to attenuate its own release (Alsop and Vijayan, 2009). In addition to this, *inha*, which is down-regulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish, exerts negative feedback upon *fshb* expression (Poon et al., 2009). To assess the impact of Fdx1b deficiency on pituitary gene expression, we used qPCR to measure the expression of *pomca, fshb* and *lhb* in adult zebrafish brains (**Figure 3.15**). *Pomca* was upregulated in the brains of male *fdx1b*$^{-/-}$ mutant zebrafish, indicating dysregulation of the HPI axis due to cortisol deficiency. *Fshb* was also upregulated in *fdx1b*$^{-/-}$ mutant male zebrafish. No change in *lhb* expression was observed in *fdx1b*$^{-/-}$ mutant male zebrafish. Analysis of gonadotropin and *pomc* expression in wild-type and *fdx1b*$^{-/-}$ mutant female zebrafish can be found in chapter 6.

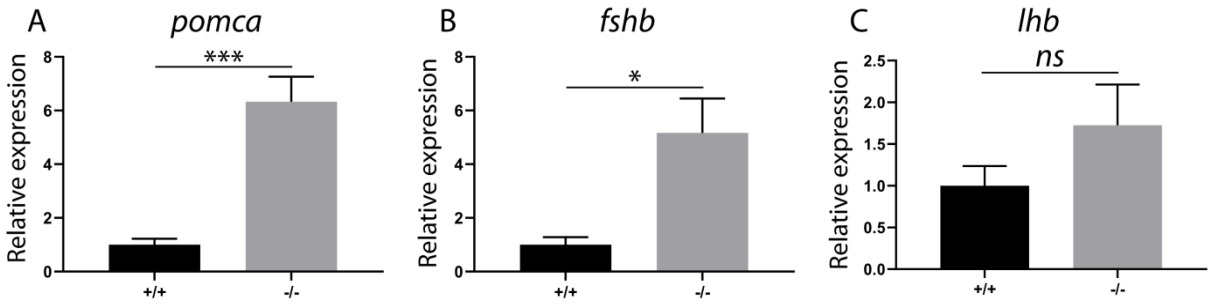

Figure 3.15. Dysregulation of pituitary genes in the brains of *fdx1b*$^{-/-}$ mutant male zebrafish. The expression of *pomca, fshb* and *lhb* was measured in the zebrafish brain by Taqman qPCR. *Pomca* **(A)** was significantly upregulated in the brains of male *fdx1b*$^{-/-}$ mutant zebrafish (n=5, p=0.0005). *Fshb* **(B)** was significantly upregulated in the brains of *fdx1b*$^{-/-}$ mutant male zebrafish (n=5, Mann-Whitney test, p=0.0159). *Lhb* **(C)** was not significantly changed by mutation of *fdx1b* (n=5, p=0.2152). All results analysed by unpaired *t*-tests unless otherwise stated. *** p<0.001, ** p<0.01, * p<0.05.

3.2.10 - Gonadal differentiation proceeds normally in *fdx1b*$^{-/-}$ mutant zebrafish

The crucial period for gonadal differentiation in zebrafish is between 21dpf and 38dpf (Sun et al., 2013). This process is known to susceptible to exogenous steroids and genetic manipulation of steroidogenic enzymes (Andersen et al., 2003; Lau et al., 2016; Zhai et al., 2018). To assess the impact of Fdx1b deficiency on this process, we performed histological analysis of the gonad during the period of gonadal differentiation. The gonads of wild-type and *fdx1b*$^{-/-}$ mutant zebrafish were examined by H&E staining at 25, 31 and 38dpf (**Figure 3.16**).

At 25dpf all fish examined (wild-type n=4, *fdx1b*$^{-/-}$ n=3) were found to possess a gonad containing early oocyte-like cells (**Figure 3.16A-D**), forming a juvenile ovary. As previously described, the extent to which this juvenile ovary developed was highly variable, with many oocyte-like cells seen in some specimens (**Figure 3.16B+D**), whilst others contained comparatively few (**Figure 3.16A+C**) (Wang et al., 2007). The difference in juvenile ovary appearance may be due to variability in the extent to which this structure develops, or may indicate that regression of this structure, and therefore testis differentiation, has already begun in some samples.

By 31dpf (wild-type n=7, *fdx1b*$^{-/-}$ n=6) (**Figure 3.16E-H**), gonadal differentiation has begun in earnest. Several specimens exhibited maintenance of the juvenile ovary structure and consist primarily of oocyte-like cells (**Figure 3.16F+H**). On the other hand, many specimens appeared to have committed to testis differentiation (**Figure 3.16E+G**), as oocyte-like cells were few or absent, stromal cell infiltration was apparent, and residual bodies indicating oocyte degeneration were occasionally present.

At 38dpf (wild-type n=7, *fdx1b*$^{-/-}$ n=8), three gonad types were observed: differentiated ovaries, differentiated testes, and gonads undergoing transformation. Testes were identified by the initiation of spermatogenesis, which was indicated by the presence of spermatids (**Figure 3.16I+K**). Differentiated ovaries appeared similar to the juvenile ovaries seen at 31dpf, however the number

of primary oocytes was increased (**Figure 3.16J+L**). In addition to differentiated gonads, several specimens appeared at various stages of gonadal transformation (**Figure 3.16M-P**), transforming gonads were identified by features including a lack of oocyte-like cells, stromal cell infiltration and presence of residual bodies, although not all examples exhibited all features.

No obvious defect in the process of gonadal differentiation was apparent in *fdx1b*$^{-/-}$ mutant zebrafish, as comparable proportions of specimens were found to exhibit each gonad type at all stages observed.

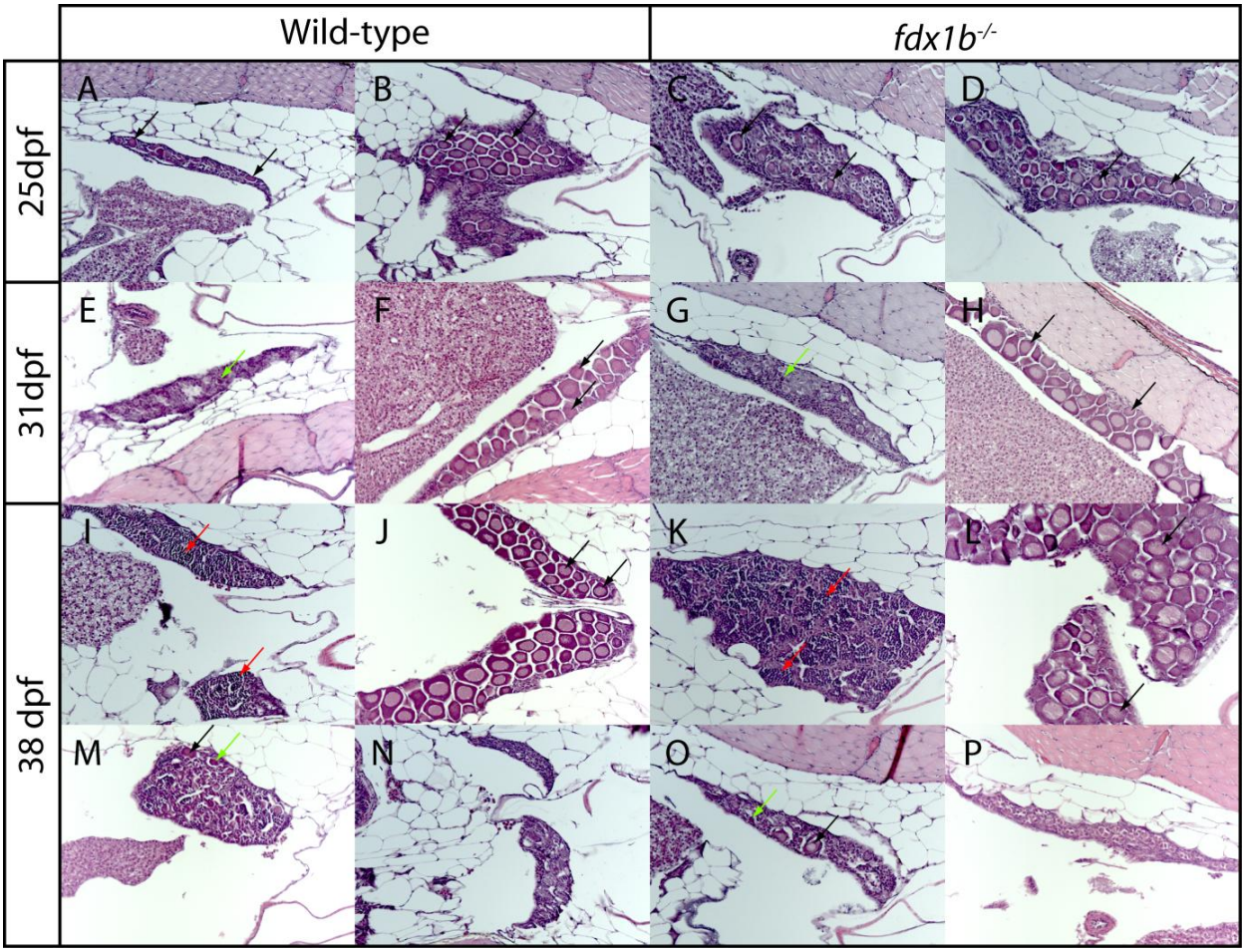

Figure 3.16. Gonadal differentiation appears to proceed normally in *fdx1b*$^{-/-}$ mutant zebrafish. The developing gonads of wild-type and *fdx1b*$^{-/-}$ mutant zebrafish were examined throughout the period of gonadal differentiation by H&E staining. At 25dpf **(A-D)** gonads containing oocyte-like cells were observed, though the number of these cells present was highly variable. At 31dpf fish either possessed a maintained juvenile ovary structure **(F+H)** or were undergoing gonadal transformation **(E+G)**. At 38dpf, differentiated testes **(I+K)**, differentiated ovaries **(J+L)** and transforming gonads were observed **(M-P)**. No obvious difference in the process of gonadal differentiation was observed between wild-type and *fdx1b*$^{-/-}$ mutant zebrafish. Black arrows: oocyte-like cells or primary oocytes. Red arrows: spermatids. Green arrows: residual bodies.

3.3 - 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 (Griffin et al., 2016) 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 (Tokarz et al., 2015). 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 (Midzak and Papadopoulos, 2016). Fdx1b is thought to be the corresponding cofactor in zebrafish, however, concentrations of 17α-hydroxyprogesterone were not significantly different between *fdx1b*$^{-/-}$ mutant males and wild-type siblings (**Figure 3.3+3.4**). 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 (Griffin et al., 2016).

Expression of the cortisol responsive genes *fkbp5* and *pck1*, and of the androgen responsive gene *cyp2k22*, was significantly down-regulated in the livers of *fdx1b*$^{-/-}$ mutant male zebrafish; thus demonstrating systemic deficiency of these steroid hormones. Fdx1b deficiency could hypothetically produce multiple effects on oestrogen biosynthesis. For example, Fdx1b may be important for side chain cleavage of cholesterol in the first stage of steroid biosynthesis, potentially resulting in a decreased availability of steroid precursors and therefore decreased oestrogen production.

However, as discussed above, Fdx1b appears to be less important for this reaction than those catalysed by Cyp11c1. If side chain cleavage is able to proceed with comparative normality, whilst 11β-hydroxylation of cortisol and androgen precursors is severely diminished, it is foreseeable that steroid precursors would accumulate and potentially enter the oestrogen biosynthetic pathway, leading to increased oestrogen production in male *fdx1b*$^{-/-}$ mutant zebrafish. Measurement of oestrogens by mass spectrometry is notoriously challenging, so to circumvent this problem we measured the expression of genes known to be robustly regulated by oestrogens in the livers of zebrafish, namely *vtg2* and *esr1* (Chandrasekar et al., 2010; Henry et al., 2009; Lam et al., 2011). We observed vastly higher expression of these genes in wild-type and *fdx1b*$^{-/-}$ mutant female zebrafish compared to wild-type and *fdx1b*$^{-/-}$ mutant male zebrafish (**Figure 3.6**). No effect of *fdx1b* mutation was observed for either sex.

In qPCR assays measuring expression of *vtg2* and *esr1* the cycle threshold was usually 32-35 in samples derived from male fish, indicating a low number of initial transcripts for these genes. Our assays could not reproducibly determine cycle thresholds for these genes in male fish, as technical replicates frequently differed by >0.5 cycles, which is the usual limit for inclusion in further analysis. As such, these assays may not be sufficiently accurate to determine small changes in gene expression within wild-type and *fdx1b*$^{-/-}$ mutant male zebrafish. However, these results do demonstrate that the expression levels of *vtg2* and *esr1* in *fdx1b*$^{-/-}$ mutant males are not elevated to levels remotely close to those seen in female zebrafish (expression levels of *vtg2* and *esr1* were 6579- and 50-fold higher in wild-type females than in *fdx1b*$^{-/-}$ mutant males) (**Figure 3.6**). Overall, this indicates that the impact of *fdx1b*$^{-/-}$ mutation and disrupted cortisol and androgen biosynthesis on oestrogen concentrations might be minimal, although small differences between wild-type and *fdx1b*$^{-/-}$ mutant male zebrafish cannot be ruled out.

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 (Tang et al., 2018; Yu et al., 2018).

Both AR (Crowder et al., 2017; Tang et al., 2018) and Fdx1b deficient male zebrafish exhibit feminised secondary sex characteristics, including coloration and abdominal shape, however genital papilla prominence is not affected. These results indicate that androgen signalling plays a role in pigment cell fate specification, differentiation, or organization in adult zebrafish, but does not control the development of the genital papilla. The possibility for *fdx1b*$^{-/-}$ mutant zebrafish to undergo testis differentiation, supports previous findings that androgen signalling is dispensable for this process (Crowder et al., 2017). This notion is further supported by the finding that gonadal differentiation appears to proceed normally in Fdx1b deficient zebrafish (**Figure 3.16**).

In contrast to male AR deficient zebrafish, which have a female-like rounded abdomen (Crowder et al., 2017), 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*$^{-/-}$ mutant males had increased length and weight compared to wild-type siblings (**Figure 3.2**), 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 (Yu et al., 2018) and no change reported in another AR mutant line (Tang et al., 2018). 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 signalling, *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 (de Waal et al., 2008; Hossain et al., 2008; Jorgensen et al., 2007); 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 signalling is abolished in AR mutant zebrafish, whereas the AR may still be transactivated by testosterone to some degree in Fdx1b deficient zebrafish (de Waal et al., 2008).

As with AR mutant zebrafish (Crowder et al., 2017; Tang et al., 2018), 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 (Yong et al., 2017) was able to induce egg laying. However, whether or not these eggs were fertilised is unclear, and it has been speculated that this AR mutant represents a hypomorph due to translation of an alternate transcript (Crowder et al., 2017), rather than a complete null allele. *Fdx1b* mutant zebrafish have disorganised testes with poorly defined seminiferous tubules, which may also be smaller and more numerous, and contain fewer sperm than their wild-type siblings (**Figure 3.12**). Zebrafish and mouse AR mutants also exhibit disorganised seminiferous tubules (Crowder et al., 2017; Yeh et al., 2002; Yu et al., 2018) and seminiferous tubule dysgenesis is seen in some cases of complete androgen insensitivity syndrome (Hannema et al., 2006). 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, indeed, hypoplasia of the spermatic duct was observed in some *fdx1b*$^{-/-}$ mutant male zebrafish (**Figure 3.12**). 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 behaviour (**Figure 3.8**) may

also contribute to this phenotype by the absence of behaviours that stimulate release of gametes of either male or female origin. Both possibilities have been suggested in AR mutant zebrafish (Crowder et al., 2017; Yong et al., 2017), and androgen deficient *cyp17a1* mutant zebrafish also exhibit impaired breeding behaviour (Zhai et al., 2018).

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 (Rodriguez-Mari et al., 2005; Sun et al., 2013), 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 (Nakamoto et al., 2005). 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 in *fdx1b*$^{-/-}$ mutant male testes. Both *igf3* and *insl3* are downregulated in AR mutant zebrafish testes (Tang et al., 2018) as well as in fish exhibiting oestrogen-induced androgen insufficiency (de Castro Assis et al., 2018). Expression of *igf3* was upregulated in the testes of Atlantic Salmon following treatment with 11KT (Melo et al., 2015).Decreased availability of spermatogenic factors Igf3 and Insl3 may result in accumulation of type A spermatogonia (**Figure 3.17**), 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 (Cai et al., 2011; Gregory and Kaiser, 2004; Poon et al., 2009) and zebrafish (Morais et al., 2017). 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. *Fshb* expression was increased in the brains of *fdx1b*$^{-/-}$ mutant male zebrafish compared to wild-type siblings (**Figure 3.15**), a possible consequence of decreased *inha* expression in the testes (Poon et al., 2009). Fshb can stimulate spermatogonial proliferation and differentiation via Igf3 (Nobrega et al., 2015), however this signalling pathway appears to also require androgen signalling for normal function, as *igf3* expression was decreased and spermatogonial differentiation appears to be impaired in *fdx1b*$^{-/-}$ mutant zebrafish. 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 a result of androgen deficiency, as well as compromised Sertoli cell function, contribute to decreased spermatogenesis in Fdx1b deficient zebrafish (**Figure 3.17**).

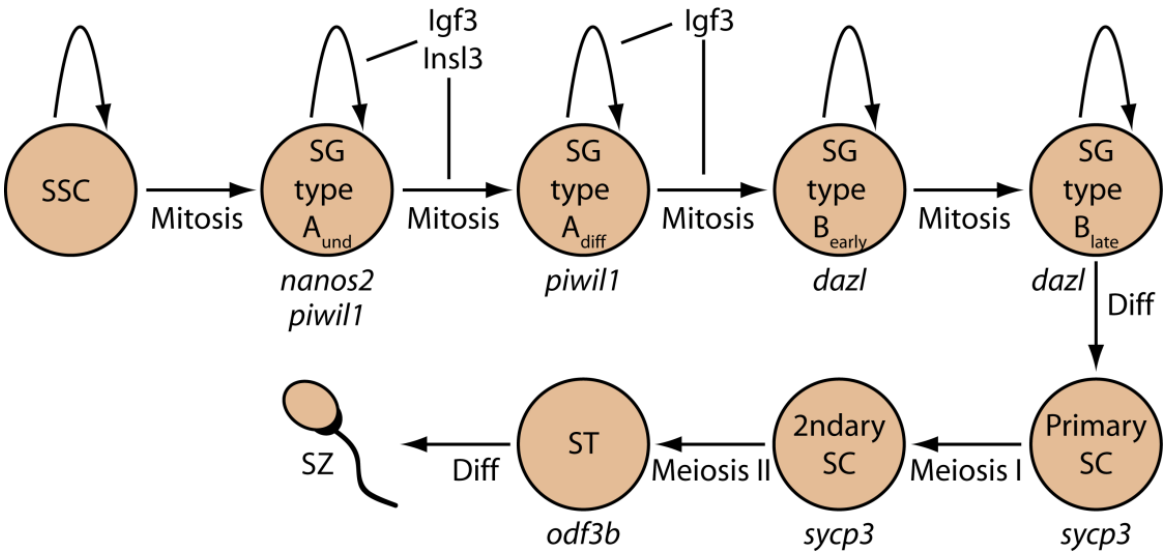

Figure 3.17. Germ cell markers and the roles of Igf3 and Insl3 in zebrafish spermatogenesis. Spermatogonial stem cells undergo several rounds of mitotic division and differentiation, eventually resulting in production of primary spermatocytes. During mitosis, spermatogonia retain some capacity for self-renewal. Primary spermatocytes enter several cycles of meiosis and differentiation, eventually maturing as spermatozoa. Igf3 and Insl3 are important for differentiation and proliferation of type A spermatogonia. *Nanos2, piwil1, dazl, sycp3* and *odf3b* can be used as markers of different stages of spermatogenesis.

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 (Cool et al., 2012), and Sox9 signalling is required for juvenile ovary to testis transformation in zebrafish (Sun et al., 2013). 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 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 disorganisation, as well as impaired breeding behaviour. 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.

Chapter 4 - Steroid 11β-hydroxylase deficiency results in impaired steroidogenesis, feminisation of secondary sex characteristics, infertility, impaired spermatogenesis and hypoplasia of the spermatic duct in zebrafish

Summary

The contribution of androgens to male reproductive development and function in zebrafish are poorly understood. In order to investigate this topic we have employed CRISPR-Cas9 to generate *cyp11c1* (11β-hydroxylase) mutant zebrafish lines. *Cyp11c1*$^{-/-}$ mutant zebrafish displayed predominantly female secondary sex characteristics, but could possess either ovaries or testes. In addition, *cyp11c1*$^{-/-}$ mutant male zebrafish were profoundly androgen and cortisol deficient. These results reinforce the theory that androgens are dispensable for testis differentiation in zebrafish, as has been previously demonstrated in androgen-deficient and resistant zebrafish lines. Herein, we show that the testes of *cyp11c1*$^{-/-}$ mutant zebrafish exhibit a disorganised tubular structure, with smaller and more numerous seminiferous tubules. Furthermore, we show for the first time that the spermatic duct, which connects the testes and urogenital orifice, is severely hypoplastic in androgen-deficient zebrafish. These results indicate important roles for androgens in the correct development and organisation of the male zebrafish reproductive anatomy, after the normal period of testis differentiation. Furthermore, we find that spermatogenesis and the incidence of characteristic breeding behaviours are impaired in *cyp11c1*$^{-/-}$ mutant zebrafish. Expression of *nanos2*, a marker of type A spermatogonia, was significantly increased in the testes of Cyp11c1 deficient male zebrafish, whereas the expression of markers for later stages of spermatogenesis were significantly decreased, suggesting that the differentiation of type A spermatogonia is a highly androgen dependent process in zebrafish.

4.1 - Introduction

The specific roles of androgens in zebrafish sex differentiation, development of male sexual characteristics, and the maintenance and function of the adult testes remain unclear. Laboratory strains of zebrafish lack sex chromosomes and exhibit polygenic sex determination (Liew et al., 2012). Prior to sex differentiation, all zebrafish develop a juvenile ovary; in presumptive female fish this structure is maintained, whereas presumptive male fish undergo juvenile ovary-to-testis transformation (Uchida et al., 2002; Wang et al., 2007). This process may be influenced by environmental factors (Liew and Orban, 2014) and is highly sensitive to sex steroids. Treatment of developing fish with oestrogens results in feminisation (Andersen et al., 2003; Brion et al., 2004; Orn et al., 2016); whilst mutation of *cyp19a1a*, crucial for oestrogen production, causes robust masculinisation (Lau et al., 2016; Yin et al., 2017). Conversely, treatment of developing zebrafish with androgens results in robust masculinisation (Larsen and Baatrup, 2010; Lee et al., 2017; Morthorst et al., 2010). Intuition would suggest that androgen deficiency or resistance should cause feminisation of the gonad and secondary sex characteristics; however, this is not the case.

Recent studies have characterised the phenotypes of androgen-deficient and androgen-resistant zebrafish (Crowder et al., 2017; Oakes et al., 2019; Zhai et al., 2018). These fish share similar phenotypes, exhibiting primarily female secondary sex characteristics. Despite outward appearance, these fish may possess either ovaries or testes, indicating that androgens are in fact dispensable for testis differentiation. Disrupted androgen signalling may affect sex ratios in zebrafish, however results are inconsistent between different zebrafish lines. Androgen-deficient or resistant male zebrafish are infertile when exposed to standard breeding scenarios, however their sperm may fertilise eggs collected from wild-type females in IVF experiments. A number of factors appear to contribute to this phenotype, including disorganised testicular structure, impaired breeding behaviour and impaired spermatogenesis (Crowder et al., 2017; Oakes et al., 2019; Yong et al., 2017).

Steroid 11β-hydroxylase (CYP11B1) is crucial for conversion of 11-deoxycortisol to cortisol in the production of glucocorticoids in humans (Miller and Auchus, 2011). In zebrafish, this final stage of glucocorticoid biosynthesis is catalysed by the zebrafish homolog of 11β-hydroxylase, Cyp11c1 (**Figure 4.1**) (Tokarz et al., 2015). Unlike in mammals, Cyp11c1 is also thought to play an important role in gonadal androgen synthesis in zebrafish (**Figure 4.1**) (Oakes et al., 2019); it is expressed in the steroidogenic cells of the gonad, as well as in certain germ cell stages (Caulier et al., 2015). The principal active androgens in humans are testosterone and 5α-dihydrotestosterone, whereas, in contrast, the principal zebrafish androgen is 11-ketotestosterone (11KT) (Tokarz et al., 2015). This difference in androgen production is due to the fact that zebrafish favour production of 11-oxygenated androgens from androstenedione, rather than conversion of androstenedione to testosterone (de Waal et al., 2008).

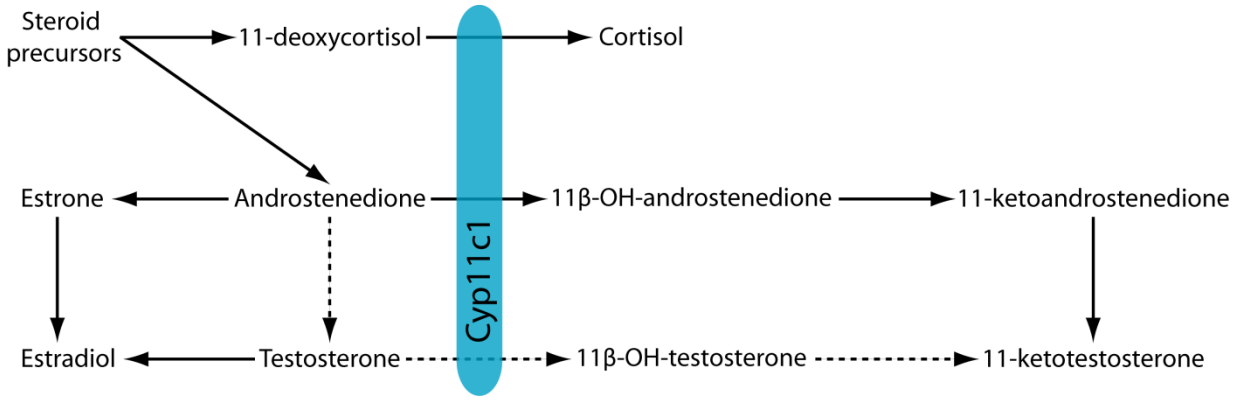

Figure 4.1. The roles of Cyp11c1 (11β-hydroxylase) in androgen and glucocorticoid biosynthesis in zebrafish. Cyp11c1 is required for the final stage of glucocorticoid biosynthesis: the conversion of 11-deoxycortisol to cortisol. 11-ketotestosterone, the principal zebrafish androgen, may be produced via two pathways beginning with androstenedione. The major pathway (solid arrows) involves 11β-hydroxylation of androstenedione to produce 11β-hydroxyandrostenedione, whereas the minor pathway (dashed arrows) requires 11β-hydroxylation of testosterone to produce 11β-hydroxytestosterone.

The activity of Cyp11c1 is thought to depend on electron transfer from the steroidogenic cofactor ferredoxin 1b (Fdx1b) (Griffin et al., 2016). The previous chapter introduced Fdx1b deficient zebrafish as a model of androgen and cortisol deficiency. Fdx1b deficient zebrafish are infertile and exhibit disorganised testis structure and impaired spermatogenesis, as well as reduced stereotypical breeding behaviours (Oakes et al., 2019).

Herein, we present Cyp11c1 deficient zebrafish lines as novel models for research into the roles of steroids in sex differentiation and adult reproductive processes. Cyp11c1 deficient zebrafish exhibit reduced cortisol and androgen concentrations, due to failed 11β-hydroxylation of glucocorticoid and androgen precursors. The resultant phenotype comprises feminisation of secondary sex characteristics, infertility, disorganised testis morphology, impaired breeding behaviour and impaired spermatogenesis. We have also demonstrated, for the first time, that androgens are crucial for development of the spermatic duct, the structure linking the testes and urogenital orifice.

4.2 - Results

4.2.1 - Generation of *cyp11c1* mutant alleles by CRISPR-Cas9

Exon 2 of *cyp11c1* was targeted by injection of 1-cell stage zebrafish embryos with a specific crRNA, tracrRNA and Cas9 protein (see section 2.3). The resultant F0 fish were outcrossed at ~10wpf, and heritable mutations were identified in the F1 progeny. Several out of frame mutations were identified including 11bp and 47bp out-of-frame deletions, fish carrying these mutations were raised and subsequently outcrossed to produce the F2 generation. Inheritance of *cyp11c1* mutant alleles did not significantly deviate from expected Mendelian ratios. The 11bp deletion (c.312_322del) (allele number SH548) allele introduces a stop codon 33 codons downstream of the mutation site and is predicted to produce a truncated protein of 136 amino acids (p.Glu105Profs*33). The 47bp deletion (c.285_331del) (allele number SH547) allele introduces a stop codon 30 codons downstream of the mutation site and is predicted to produce a truncated protein of 124 amino acids (p.Met96Hisfs*30) (wild-type 518 amino acids) (**Figure 4.2**). Both *cyp11c1* mutations described here are very likely to result in null alleles as they are expected to cause severe truncation of the protein to ~25% of the wild-type protein length. This includes loss of conserved residues with important roles in heme binding, enzyme stability and aromatic substrate binding (Khattab et al., 2017).

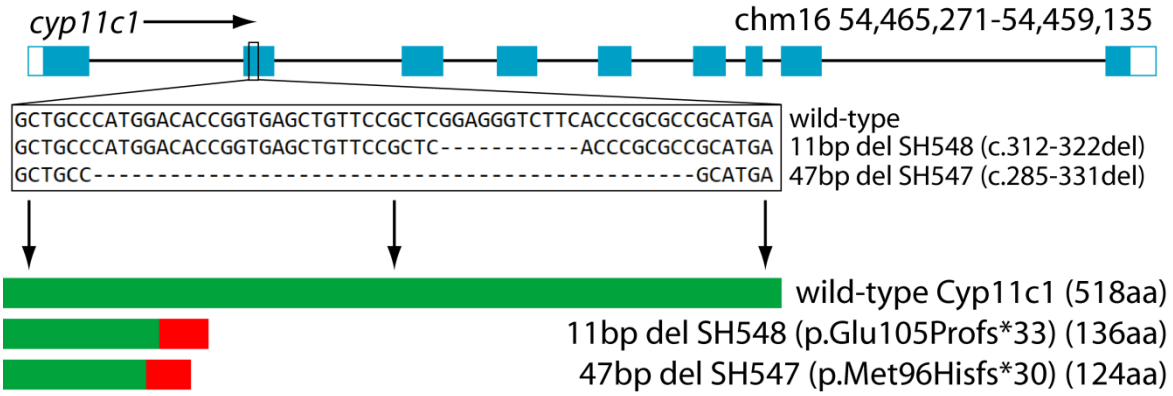

Figure 4.2. Mutations introduced into *cyp11c1* by CRISPR/Cas9. We used a CRISPR/Cas9 strategy to introduce mutations into *cyp11c1*, resulting in production of several stable *cyp11c1* mutant zebrafish lines. *Cyp11c1* is located on chromosome 16 and our CRISPR target site was in exon 2. Two mutant alleles were utilised in this study: an 11bp deletion allele (SH548) (c.312-322del) and a 47bp deletion allele (SH547) (c.285-331del). Wild-type Cyp11c1 is 518 amino acids in length, our 11bp and 47bp deletion alleles are predicted to produce protein products truncated to 136 amino acids (p.Glu105Profs*33) and 124 amino acids (p.Met96Hisfs*30) in length respectively. Protein products are depicted as green bars, the red portion of the mutant proteins comes after the mutation site and does not align to the wild-type protein product.

4.2.2 - Impaired visual background adaptation and decreased expression of glucocorticoid responsive genes in *cyp11c1*$^{-/-}$ mutant larvae

The ability of zebrafish larvae to adjust their pigmentation in response to varying light conditions is termed visual background adaptation (VBA). VBA is impaired in glucocorticoid deficient zebrafish larvae (Eachus et al., 2017; Griffin et al., 2016) and can therefore be used as a screening tool to identify such fish. *Cyp11c1*$^{+/-}$ adult zebrafish were incrossed and the resulting progeny were subjected to VBA sorting at 4 days post fertilisation (dpf), followed by genotyping (**Table 4.1**). Fish identified as having an intact VBA response (VBA+) were exclusively wild-type or heterozygous for *cyp11c1* mutation. The majority of larvae identified as VBA- were found to be homozygous mutants, indicating a failure of *de novo* cortisol biosynthesis in these fish. Occasionally, fish identified as VBA- were found to be wild-types or heterozygotes indicating that this technique cannot identify homozygous mutants with 100% accuracy (**Table 4.1**).

Table 4.1. VBA sorting of *cyp11c1*$^{+/-}$ incross larvae.

Allele	VBA result	+/+ or +/-	-/-
11bp	**VBA+**	12/12 (100%)	0/12 (0%)
	VBA-	2/12 (17%)	10/12 (83%)
47bp	**VBA+**	12/12 (100%)	0/12 (0%)
	VBA-	3/12 (25%)	9/12 (75%)

Table 4.1. The progeny of an incross of adult *cyp11c1* heterozygotes carrying either the 11bp or 47bp deletion allele were subjected to VBA typing at 4dpf and subsequently genotyped. 100% of VBA+ larvae were found to be wild-types or heterozygotes and the majority of VBA- fish were found to be mutants, indicating an impaired VBA response in these fish. Fisher's exact test: 11bp p=<0.0001, 47bp p=0.0003.

In order to further characterise the impact of disrupted Cyp11c1 function on steroid hormone production, pooled samples of VBA sorted larvae were collected. Subsequently, RNA was extracted and the expression of well established glucocorticoid responsive genes *fkbp5* and *pck1* (Eachus et al., 2017; Griffin et al., 2016) was determined by qPCR. Expression of both genes was significantly decreased in VBA- samples, indicating decreased glucocorticoid concentrations in VBA- larvae, the majority of which are *cyp11c1*$^{-/-}$ (**Figure 4.3**).

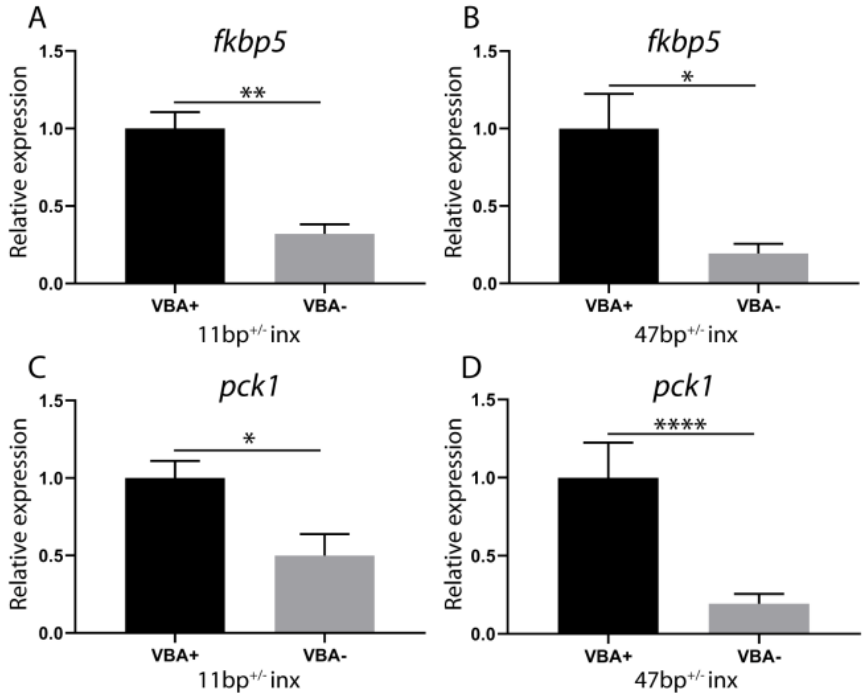

Figure 4.3. Decreased expression of glucocorticoid responsive genes in VBA- larvae. VBA- larvae were identified from the progeny of an incross of *cyp11c1*$^{+/-}$ adult zebrafish. VBA typed larvae were pooled, RNA extracted, and the expression of glucocorticoid responsive genes *fkbp5* and *pck1* was determined by qPCR. Expression of both *fkbp5* (**A+B**) (11bp: VBA+ n=4, VBA- n=3, p=0.0039; 47bp: VBA+ n=8, VBA- n=5, p=0.0183) and *pck1* (**C+D**) (11bp: VBA+ n=4, VBA- n=3, p=0.0349; 47bp: VBA+ n=8, VBA- n=5, p<0.0001) was significantly decreased in VBA- samples from both alleles indicating decreased glucocorticoid production in VBA- larvae. Results analysed using unpaired *t*-tests, * p<0.05, ** p<0.01, **** p<0.0001.

4.2.3 - *Cyp11c1*$^{-/-}$ mutant zebrafish display predominantly female secondary sex characteristics but may possess ovaries or testes

Morphological secondary sex characteristics in zebrafish include body shape, fin and body pigmentation, and genital papilla prominence. Male zebrafish are generally streamlined in shape, whereas females tend to have a more rounded abdomen. Female zebrafish have green-yellow pigmented dorsal fins and little orange pigmentation in the anal fin, the opposite is true in male zebrafish which have little green-yellow pigmentation in the dorsal fin but strong orange pigmentation in the anal fin (**Figure 4.4**). Female zebrafish have a large and prominent genital papilla, in males this structure is much smaller and mainly hidden from view.

Upon raising the progeny of *cyp11c1*$^{+/-}$ incrosses it was immediately apparent that all homozygous mutant fish displayed predominantly female morphological secondary sex characteristics (**Figure 4.4**). Closer inspection revealed that some *cyp11c1*$^{-/-}$ mutant fish had prominent genital papillae, whereas others had small hidden genital papillae reminiscent of those seen in wild-type males. Dissection of *cyp11c1*$^{-/-}$ mutant fish revealed that they could possess either testes or ovaries, and this was accurately predicted by the presence or absence of a prominent genital papilla. The testes of *cyp11c1*$^{-/-}$ mutant male zebrafish appeared pale or translucent in comparison to the testes of wild-type males which were bright white and opaque. The ratio of males to females in populations of wild-type and *cyp11c1*$^{-/-}$ mutant zebrafish did not significantly differ, based on the presence or absence of testes in more than 50 adult fish.

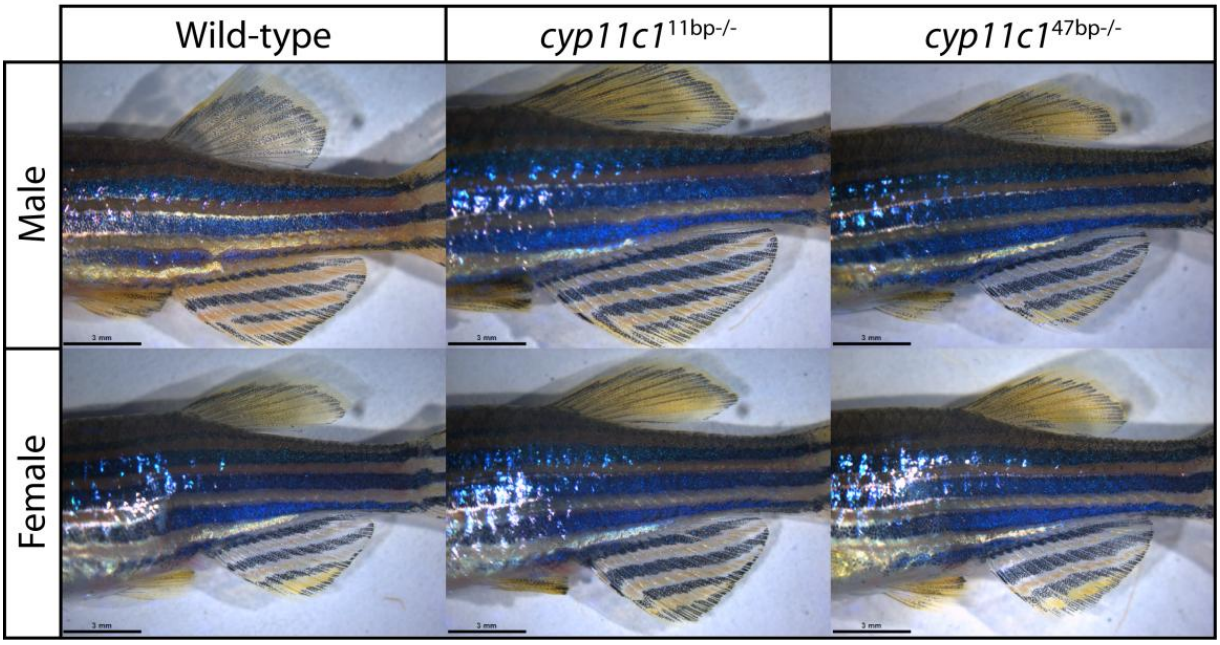

Figure 4.4. Feminisation of secondary sex characteristics in *cyp11c1*$^{-/-}$ mutant male zebrafish. *Cyp11c1*$^{-/-}$ mutant male zebrafish exhibited fin pigmentation patterns more commonly seen in female zebrafish. Wild-type male zebrafish exhibited pale dorsal fins and strongly orange striped anal fins. *Cyp11c1*$^{-/-}$ mutant male zebrafish exhibited reduced orange pigmentation in the anal fin and green-yellow pigmentation in the dorsal fin, similar to that seen in wild-type females. 11bp wild-type male n=10, wild-type female n=5, mutant male n=8, mutant female n=5; 47bp wild-type male n=10, wild-type female n=3, mutant male n=8, mutant female n=3.

In addition to analysis of secondary sex characteristics, biometric data was also collected. Males from both *cyp11c1*$^{-/-}$ mutant lines were significantly longer and heavier than their wild-type siblings (**Figure 4.5**). Dissection of *cyp11c1*$^{-/-}$ mutant male zebrafish revealed increased adipose tissue in the abdominal cavity in comparison to wild-type sibling males, and this may contribute to the increased weight of mutant fish.

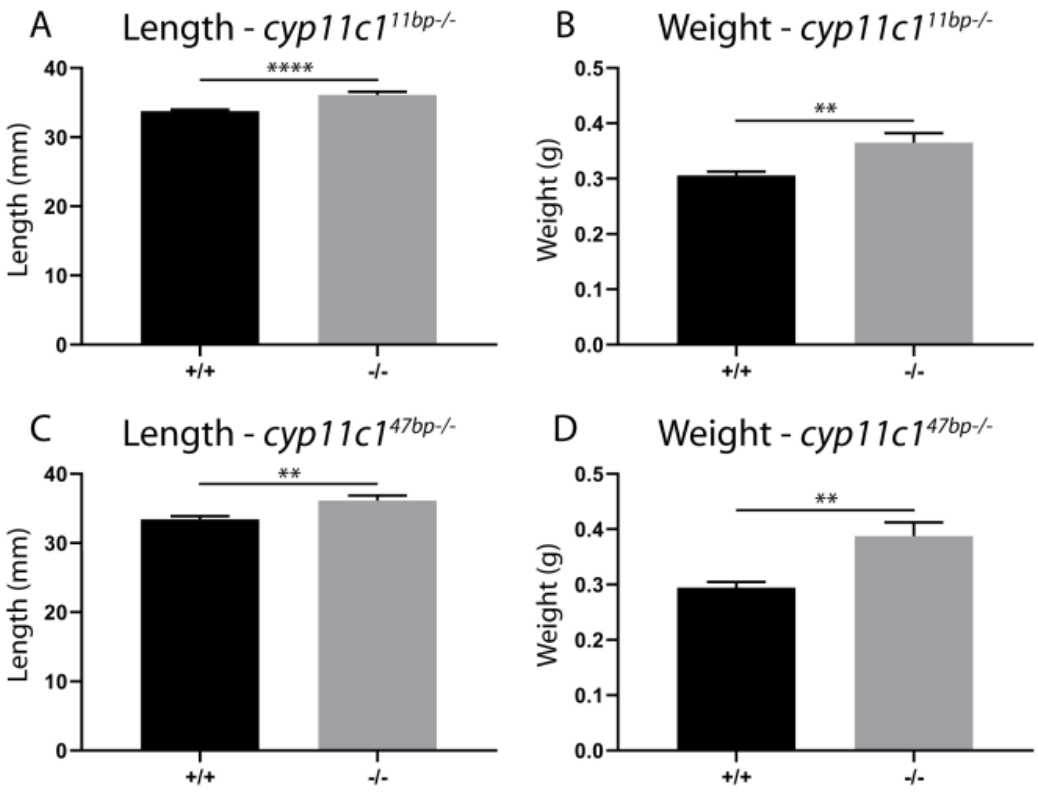

Figure 4.5. Increased weight and length in *cyp11c1*$^{-/-}$ mutant male zebrafish. Length and weight were significantly increased in homozygous mutants from both *cyp11c1* mutant alleles. (11bp deletion allele: wild-type n=23, mutant n=19, length p=<0.0001 weight p=0.0016. 47bp deletion allele: wild-type n=16, mutant n=14, length p=<0.0034 weight p=0.0010). Statistical analysis was by unpaired *t*-tests, **** p<0.0001, ** p<0.01.

4.2.4 - Adult *cyp11c1*$^{-/-}$ mutant male zebrafish exhibit profound cortisol and 11-ketotestosterone deficiency

In order to assess the impact of *cyp11c1* mutation on interrenal and testicular steroidogenesis, we employed LC-MS/MS to quantify steroid hormone concentrations in samples prepared from whole adult zebrafish males. Cortisol concentrations were profoundly decreased by mutation of *cyp11c1* (**Figure 4.6**), whereas concentrations of its direct precursor, 11-deoxycortisol, were significantly increased. Thus, we have demonstrated the *in vivo* importance of Cyp11c1 function for the conversion of 11-deoxycortisol to cortisol in glucocorticoid biosynthesis. Concentrations of the sex steroid precursor androstenedione were significantly increased in *cyp11c1*$^{-/-}$ mutant male zebrafish, probably due to shunting of glucocorticoid precursors in to the sex steroid pathway, as well as blockage of the androgen synthesis pathway. Blockage of the androgen synthesis pathway is evidenced by undetectable concentrations of 11-ketotestosterone and of its precursor 11-ketoandrostenedione (**Figure 4.6**). Concentrations of testosterone were not affected by mutation of *cyp11c1* (**Figure 4.6**). A comparison of steroid hormone concentrations between male and female, wild-type and *cyp11c1*$^{-/-}$ mutant zebrafish, can be found in **Appendix I**.

In order to assess to the systemic consequences of profound cortisol and 11-ketotestosterone deficiency, we used qPCR to measure the expression of steroid hormone responsive genes in the livers of *cyp11c1*$^{-/-}$ mutant males zebrafish and wild-type siblings (**Figure 4.7**). The expression of glucocorticoid responsive genes *fkbp5* and *pck1* (Eachus et al., 2017; Griffin et al., 2016) was significantly reduced in *cyp11c1*$^{-/-}$ mutant male zebrafish livers compared to wild-type siblings. An apparent decrease in the expression of the proposed androgen responsive gene *cyp2k22* (Fetter et al., 2015; Siegenthaler et al., 2017) in the livers of *cyp11c1*$^{-/-}$ mutant zebrafish did not achieve statistical significance (**Figure 4.7**).

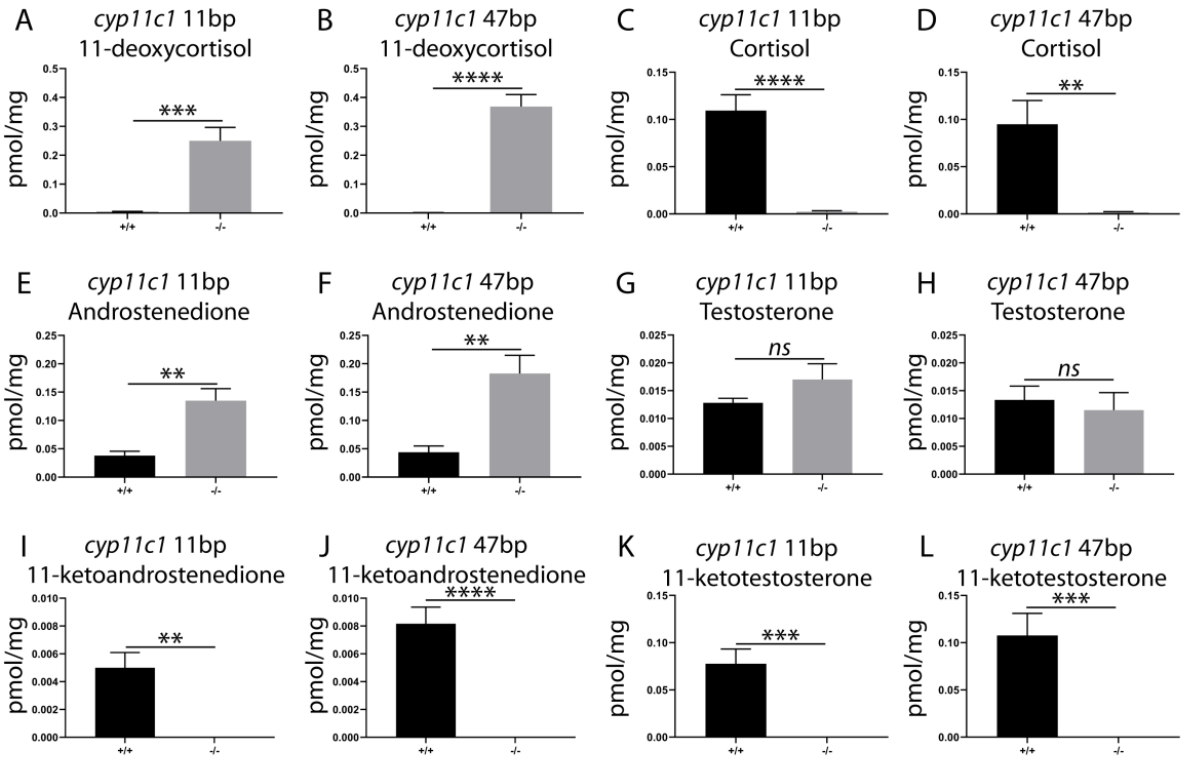

Figure 4.6. Steroid profile of adult male *cyp11c1*$^{-/-}$ mutant zebrafish is consistent with 11β-hydroxylase deficiency. Steroid hormone concentrations were quantified in whole adult zebrafish (n=6) by LC-MS/MS. Concentrations of cortisol (**C+D**) (11bp p<0.0001, 47bp p=0.0039) were profoundly reduced in *cyp11c1*$^{-/-}$ mutant zebrafish, whereas concentrations of the cortisol precursor 11-deoxycortisol (**A+B**) (11bp p=0.0003, 47bp p<0.0001) were significantly increased. Concentrations of the sex steroid precursor androstenedione (**E+F**) (11bp p=0.0017, 47bp p=0.0021) were significantly increased in *cyp11c1*$^{-/-}$ mutant zebrafish whereas concentrations of 11-ketotestosterone (**K+L**) (11bp p=0.0006, 47bp p=0.0010) and the intermediate steroid, 11-ketoandrostenedione (**I+J**) (11bp p=0.0010, 47bp p<0.0001), were undetectable in mutant zebrafish. Concentrations of testosterone (**G+H**) (11bp p=0.1895, 47bp p=0.6581) were not affected by mutant of *cyp11c1.* All results analysed using unpaired *t*-tests, ** p<0.01, *** p<0.001, **** p<0.0001.

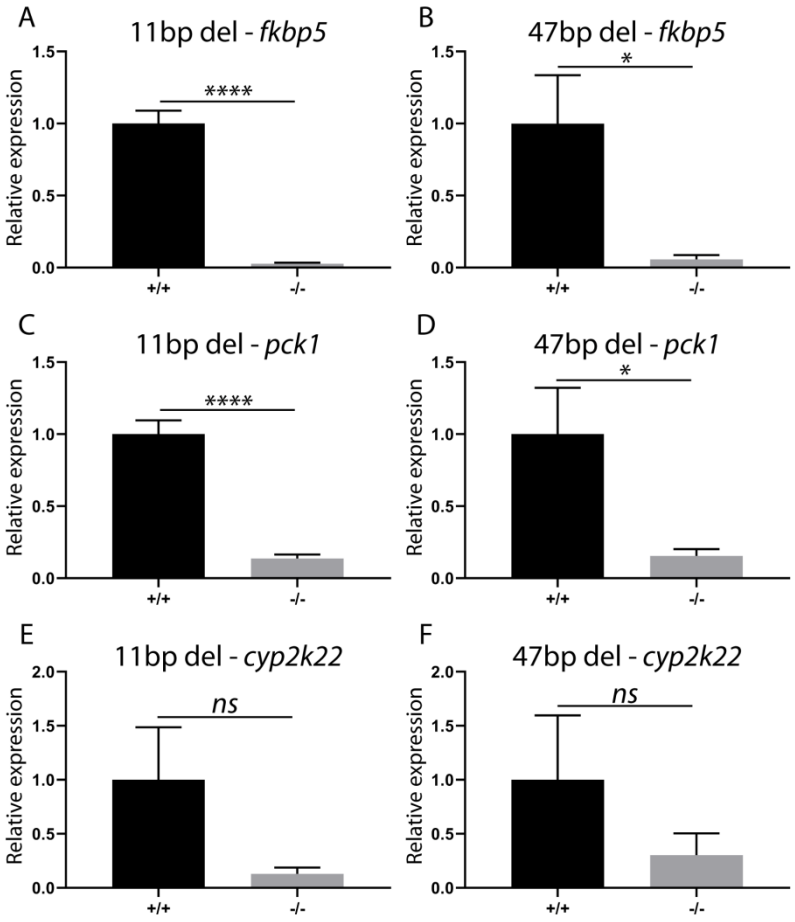

Figure 4.7. Decreased expression of glucocorticoid responsive genes in the livers of Cyp11c1 deficient male zebrafish. Expression of steroid responsive genes in *cyp11c1*$^{-/-}$ mutant male zebrafish and wild-type siblings was quantified by qPCR with *ef1a* as a reference gene. Expression of robustly glucocorticoid responsive genes *fkbp5* (11bp wild-type n=8, mutant n=8, p<0.0001; 47bp wild-type n=5, mutant n=5, p=0.0230) and *pck1* (11bp wild-type n=8, mutant n=8, p<0.0001, 47bp wild-type n=8, mutant n=8, p=0.0212) was significantly reduced in the livers of *cyp11c1*$^{-/-}$ mutant male zebrafish. Quantification of the expression of *cyp2k22* (11bp wild-type n=8, mutant n=8, p=0.2345, 47bp wild-type n=7, mutant n=7, p=0.6200, Mann-Whitney tests), a proposed androgen responsive gene, revealed an apparent reduction in expression in *cyp11c1*$^{-/-}$ mutant male zebrafish, however this did not achieve statistical significance. Data analysed by unpaired *t*-test unless otherwise stated.

4.2.5 - Disruption of *cyp11c1* results in infertility and impaired breeding behaviour in male zebrafish

Androgen resistant and androgen deficient (Crowder et al., 2017; Oakes et al., 2019) male zebrafish are infertile and the incidence of stereotypical breeding behaviours is decreased (Yong et al., 2017). To investigate the impact of *cyp11c1* disruption on breeding behaviour in male zebrafish, we analysed two well characterised behaviours during in the first five minutes of breeding. Four wild-type sibling and four mutant males from each allele were outcrossed with wild-type females on three separate occasions, and behaviour was video recorded for later analysis. Outcrosses of wild-type females and wild-type sibling males from both the 11bp and 47bp alleles produced fertilised embryos in 92% and 66% of crosses respectively. No fertilised embryos were observed in any crosses of *cyp11c1*$^{-/-}$ mutant zebrafish with wild-type females (**Table 4.2**). Analysis of breeding behaviour revealed that the number of intimate contacts, where fish touched, or crossed one another's direction of travel, was significantly reduced in both the 11bp and 47bp deletion *cyp11c1*$^{-/-}$ mutant lines compared to wild-type siblings on all three occasions (**Figure 4.8**). The duration of chasing, where one fish closely followed the other around the tank, was also significantly reduced for both alleles on all three occasions (**Figure 4.8**). Despite exhibiting infertility in normal breeding scenarios, the sperm of *cyp11c1*$^{-/-}$ mutant zebrafish was able to fertilise eggs collected from wild-type females by IVF (**Table 4.3**).

Table 4.2. Infertility in *cyp11c1*$^{-/-}$ mutant male zebrafish.

Allele	Genotype	Total number of crosses	# crosses resulting in fertilised eggs
11bp	**+/+ (n=4)**	12	11 (92%)
	-/- (n=4)	12	0 (0%)
47bp	**+/+ (n=4)**	12	8 (66%)
	-/- (n=4)	12	0 (0%)

Table 4.2. *Cyp11c1*$^{-/-}$ mutant (n=4) and wild-type sibling males (n=4) were outcrossed with wild-type females on three separate occasions. No crosses involving *cyp11c1*$^{-/-}$ mutant males from either the 11bp or 47bp deletion alleles produced any fertilised embryos; their wild-type siblings produced fertilised embryos in 92% and 66% of crosses respectively.

Table 4.3. Proportion of *cyp11c1*$^{-/-}$ mutant and wild-type sibling sperm samples producing fertilised embryos in IVF experiments

Genotype	Proportion producing fertilised embryos
cyp11c1$^{11bp+/+}$ (n=4)	4/4 (100%)
cyp11c1$^{11bp-/-}$ (n=4)	3/4 (75%)
cyp11c1$^{47bp+/+}$(n=4)	4/4 (100%)
cyp11c1$^{47bp-/-}$ (n=4)	4/4 (100%)

Table 4.3. Testes were dissected from male zebrafish and homogenised in a 50x mass:volume dilution of Hank's buffered salt solution. Sperm solutions were used to fertilise eggs collected from wild-type females.

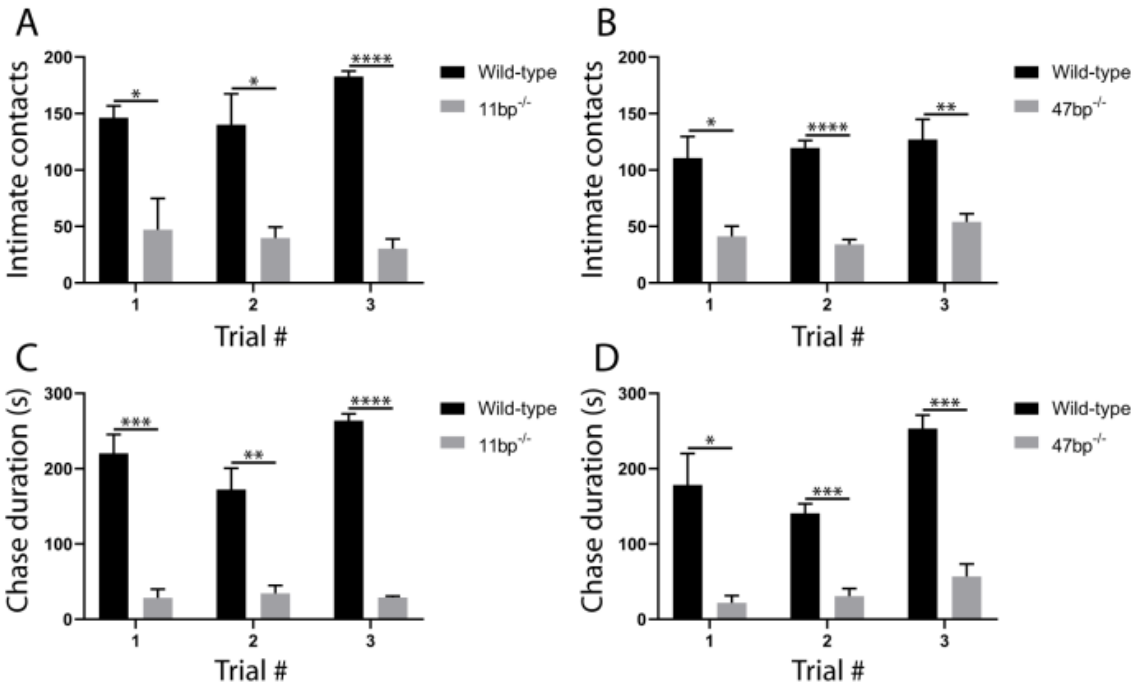

Figure 4.8. Cyp11c1 deficient male zebrafish exhibit reduced stereotypical breeding behaviours in parings with wild-type females. *Cyp11c1*$^{-/-}$ mutant (n=4) and wild-type sibling male (n=4) zebrafish were paired with wild-type females and behaviour was analysed during the first five minutes of breeding. This procedure was repeated on three separate occasions. Intimate contacts (**A+B**) (11bp: 1 p=0.0270, 2 p=0.0270, 3 p<0.0001; 47bp: 1 p=0.0184, 2 p=0.0001, 3 p=0.0184) and chasing duration (**C+D**) (11bp: 1 p=0.0009, 2 p=0.0038, 3 p<0.0001; 47bp: 1 p=0.0109, 2 p=0.0011, 3 p=0.0006) were significantly reduced in all trials and in both 11bp and 47bp deletion alleles. Results were analysed using multiple *t*-tests with the Holm-Sidak method. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Whilst conducting breeding experiments on *cyp11c1*$^{-/-}$ mutant zebrafish it was noted that they appeared to exhibit reduced locomotor activity. In order to quantify this, mutant and wild-type male zebrafish were exposed to an open field test. This revealed that the total distance swam over the duration of the trial, as well as duration of fast swimming, was significantly and consistently reduced in *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 4.9**). Freezing duration, or the duration for which the fish was stationary in the tank, was also recorded. Freezing duration was consistently greater in trials involving mutant zebrafish, however these results were not statistically significant, presumably due to the extremely high variability with which the mutant fish expressed this phenotype (**Figure 4.9**).

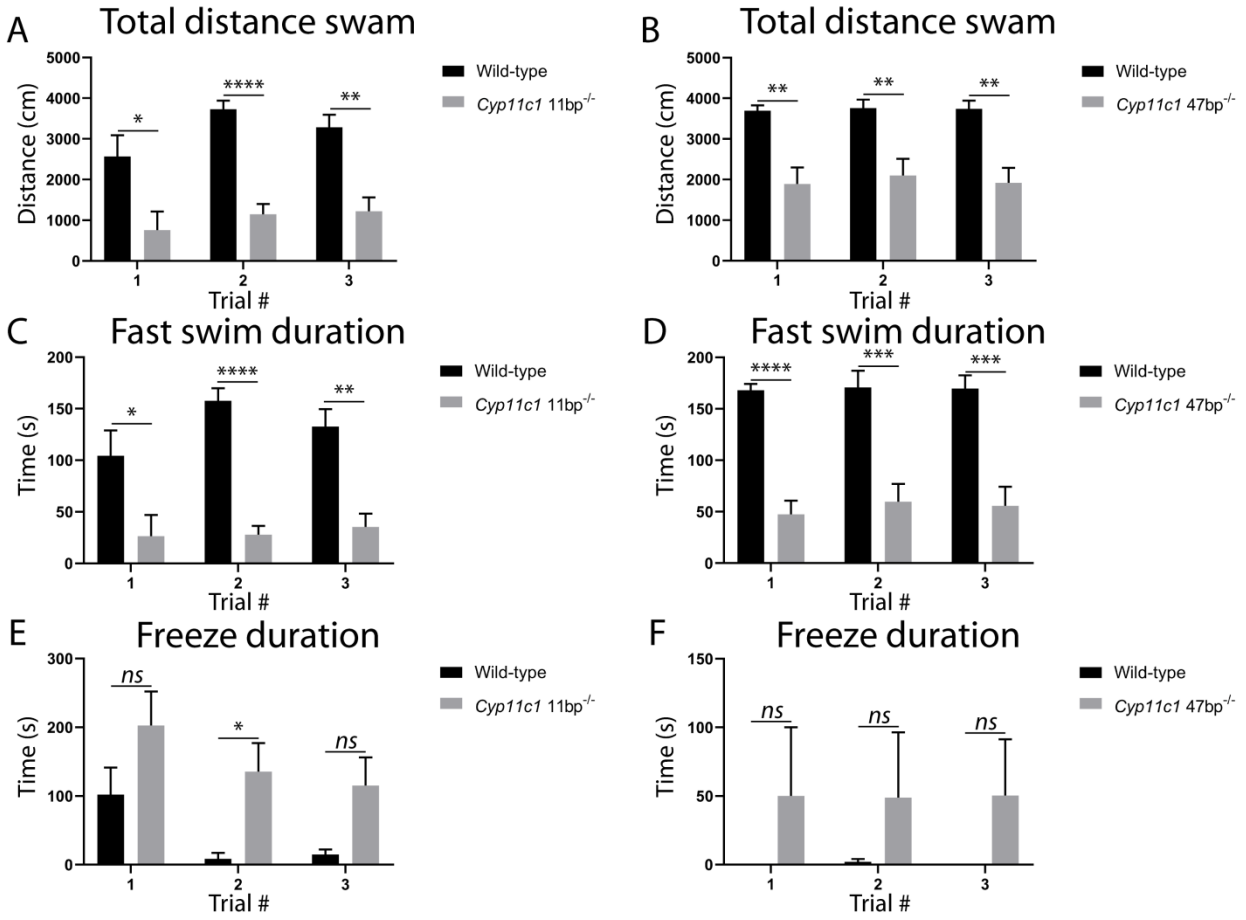

Figure 4.9. Cyp11c1 deficient male zebrafish exhibit decreased locomotor activity. In order to assess locomotion, *cyp11c1*$^{-/-}$ mutant male zebrafish and wild-type siblings (n=6) were exposed to open field tests. Tests were repeated on three occasions, and total distance swam, fast swimming duration and freezing duration were recorded. Total distance swam (**A+B**) (11bp: 1 p=0.0265, 2 p<0.0001, 3 p=0.0021; 47bp: 1 p=0.0045, 2 p=0.0050, 3 p=0.0045) and fast swim duration (**C+D**) (11bp: 1 p=0.0348, 2 p<0.0001, 3 p=0.0021; 47bp: 1 p<0.0001, 2 p=0.0009, 3 p=0.0009) were consistently reduced in both *cyp11c1*$^{-/-}$ mutant alleles across all trials. Freeze duration (**E+F**) (11bp: 1 p=0.1418, 2 p=0.0402, 3 p=0.0708; 47bp: 1 p=0.5740, 2 p=0.5740, 3 p=0.5740) was consistently increased in *cyp11c1*$^{-/-}$ mutant male zebrafish, however this was only significant in one trial (**E**). Results were analysed using multiple *t*-tests with the Holm-Sidak method, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.2.6 - *Cyp11c1* disruption results in testicular disorganisation and reduced spermatogenesis

In order to examine the impact of *cyp11c1* mutation on testis morphology we collected coronal sections through whole adult zebrafish and performed haematoxylin and eosin (H&E) staining. The testes of wild-type sibling males (n=3 per allele) appeared to be well organised, with a defined tubular structure frequently in evidence (**Figure 4.10**). In contrast, the structure of *cyp11c1*$^{-/-}$ mutant testes (n=5 per allele) was generally disorganised, with a defined tubule structure very rarely in evidence (**Figure 4.10**). Picrosirius red staining for collagen, a component of the seminiferous tubule basement membrane, gave a clearer representation of tubule morphology in *cyp11c1*$^{-/-}$ mutant testes. In comparison to wild-type testes, *cyp11c1*$^{-/-}$ mutant testes contained seminiferous tubules with a smaller diameter, as well as an apparent increase in the total number of tubules (**Figure 4.11**). The seminiferous tubules of wild-type testes comprised clusters of developing spermatogonia, spermatocytes and spermatids lining the perimeter, with mature spermatozoa in the central lumen (**Figure 4.10**). The testes of *cyp11c1*$^{-/-}$ mutant zebrafish also contained all stages of spermatogenesis; however, the proportion of developing germ cells to mature sperm appeared to be much greater. This was accompanied by an apparent reduction in the quantity of mature sperm.

The reduction in the sperm quantity suggested by histological examination was confirmed by sperm counting; both *cyp11c1* mutant alleles exhibited significantly lower sperm counts compared to wild-type siblings (**Figure 4.12**). No difference in gonadosomatic index, the percentage contribution of the gonads to overall body weight, was observed for either allele.

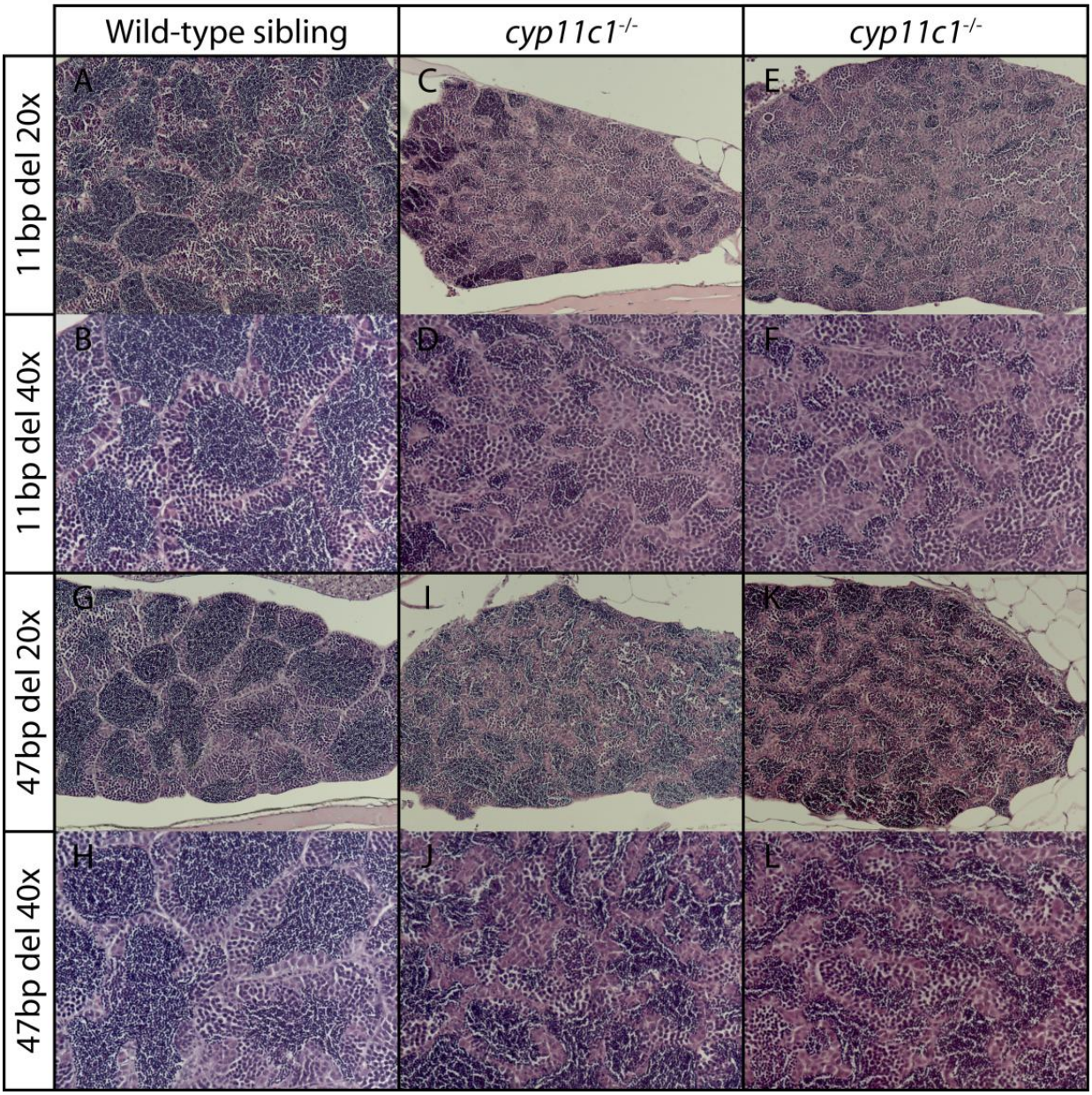

Figure 4.10. Mutation of *cyp11c1* results in disorganised seminiferous tubules and reduced spermatozoa. The morphology and composition of *cyp11c1*$^{-/-}$ mutant and wild-type sibling testes was assessed by H&E staining. The testes of wild-type sibling fish **(A,B,G,H)** contained defined seminiferous tubules whereas *cyp11c1*$^{-/-}$ mutant testes **(C-F, I-L)** contained poorly defined seminiferous tubules and comparatively fewer mature sperm. 11bp wild-type n=3, mutant n=5; 47bp wild-type n=3, mutant n=5.

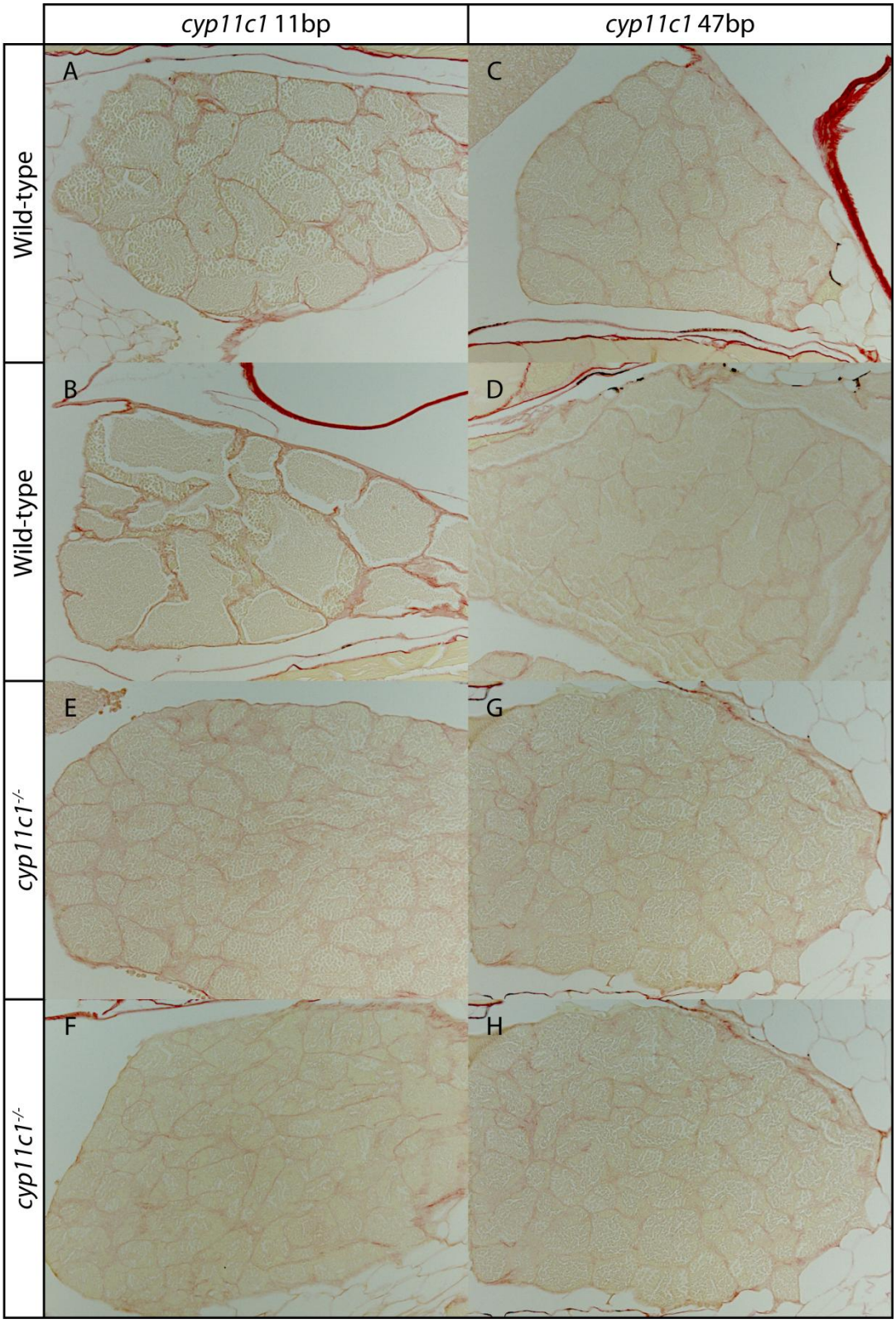

Figure 4.11. Picrosirius red staining of *cyp11c1*$^{-/-}$ mutant testes. In comparison to wild-types (**A-D**) the seminiferous tubules of *cyp11c1*$^{-/-}$ mutant testes (**E-H**) appeared smaller in diameter and also greater in number. 11bp wild-type n=3, mutant n=5; 47bp wild-type n=3, mutant n=5.

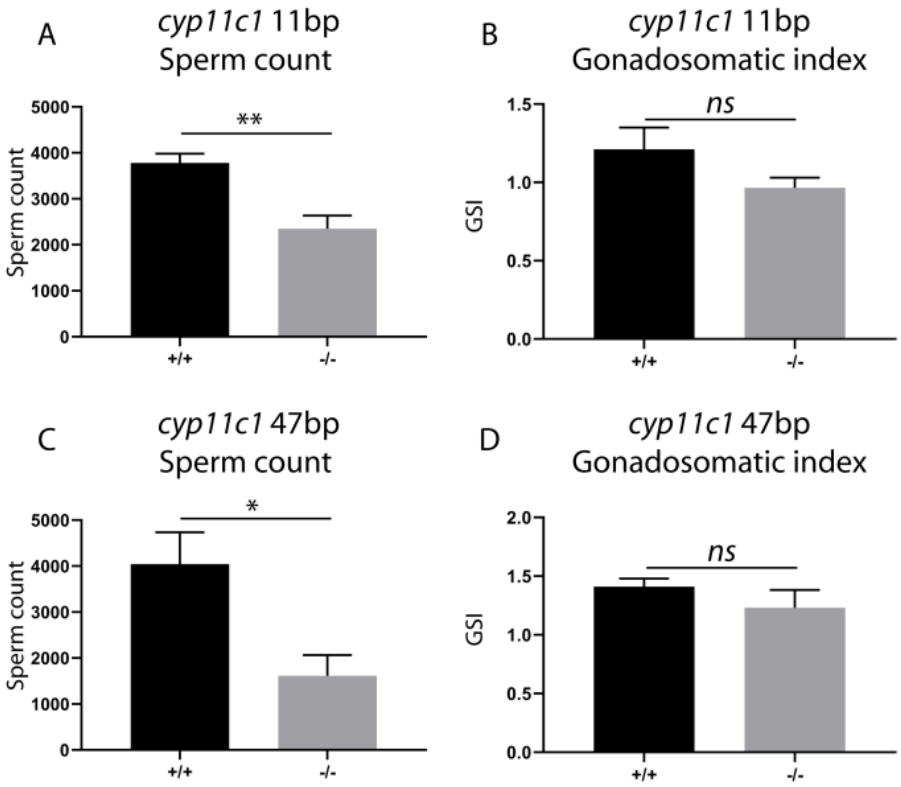

Figure 4.12. Cyp11c1 deficient zebrafish exhibit a decreased sperm count. Testes were dissected from *cyp11c1* mutant or wild-type sibling zebrafish and briefly homogenised in a 50x mass:volume dilution of Hank's buffered salt solution. This was followed by sperm counting. Cyp11c1 mutant zebrafish exhibited significantly reduced sperm counts compared to wild-type siblings (11bp mutant n=6, wild-type n=8, p=0.0023; 47bp mutant n=7, wild-type n=6, p=0.0167). No change in GSI was observed (11bp mutant n=7, wild-type n=8, p=0.1201; 47bp mutant n=7, wild-type n=7, p=0.3046). Results analysed by unpaired *t*-tests, * $p<0.05$, ** $p<0.01$.

4.2.7 - Androgens are crucial for development of the spermatic duct

Cyp11c1$^{-/-}$ mutant male zebrafish were infertile in conventional breeding scenarios, but their sperm could fertilise eggs collected from wild-type females by IVF. This led us to hypothesize that sperm release could be impaired. In zebrafish, sperm is conducted from the testes to the urogenital orifice via the spermatic duct (Menke et al., 2011). As testicular tubule structure was found to be disorganised in *cyp11c1*$^{-/-}$ mutant zebrafish, we investigated the possibility that the spermatic duct may also exhibit impaired development or maintenance.

The structure of the spermatic duct was examined ventral to spermatogenic tissue of the testes and dorsal to the genital orifice, and was found to occupy the region posterior to the intestine and anterior to the renal collecting duct (**Figure 4.13**). The spermatic ducts of wild-type sibling zebrafish appeared as extensive tubular structures, with tubules containing mature spermatozoa (**Figure 4.13A+D**). In contrast, the spermatic ducts of *cyp11c1*$^{-/-}$ mutant zebrafish appeared as severely hypoplastic structures immediately posterior to the intestine. *Cyp11c1*$^{-/-}$ mutant spermatic ducts either contained no sperm (11bp deletion: 4/5, 47bp deletion: 2/5) (**Figure 4.13B+E**), or existed as a slightly more extensively developed structure containing some mature spermatozoa (**Figure 4.13C+F**).

Sperm samples may be collected from zebrafish by manual gamete expression (Westerfield, 2000). In order to determine if hypoplasia of the spermatic duct resulted in impaired sperm release, we subjected *cyp11c1*$^{-/-}$ mutant zebrafish and wild-type siblings to this procedure. Cyp11c1 deficient zebrafish exhibited profoundly impaired sperm release, although sperm was observed in samples obtained from some *cyp11c1*$^{47bp-/-}$ zebrafish in one of two trials (**Table 4.4**). These *cyp11c1*$^{47bp-/-}$ mutant sperm samples contained significantly decreased sperm concentrations compared to wild-type siblings.

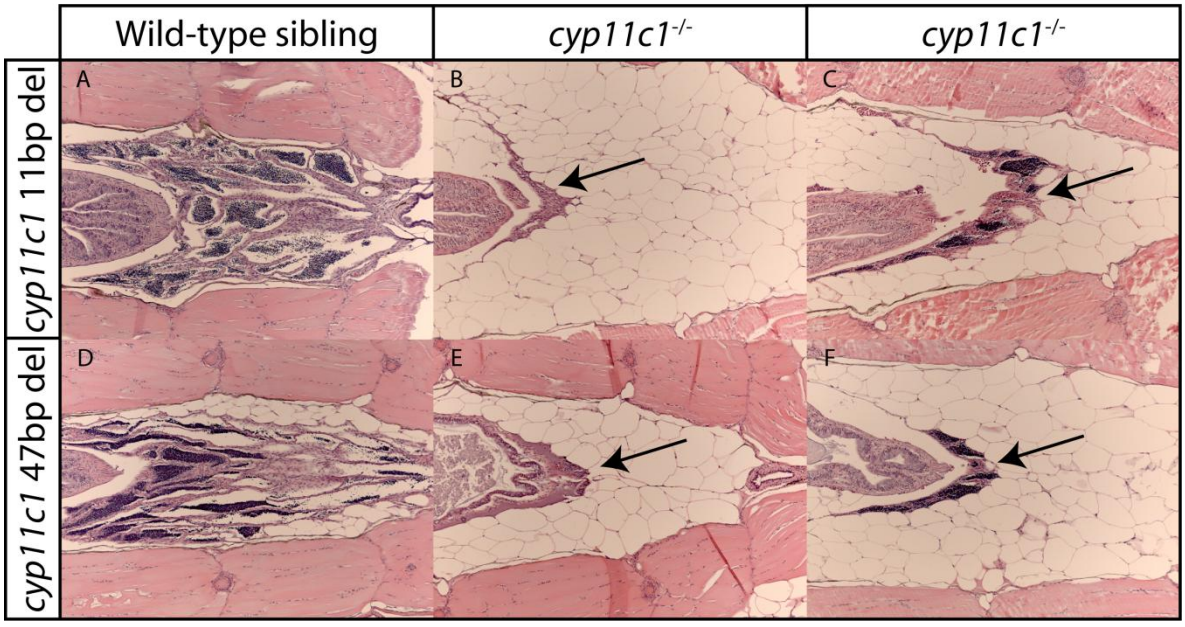

Figure 4.13. The spermatic ducts of *cyp11c1*$^{-/-}$ mutant zebrafish are hypoplastic. A+D: Spermatic ducts of wild-type sibling zebrafish appear as an extensive network of tubules containing mature spermatozoa, situated between the intestine and renal collecting duct. **B+C+E+F:** Spermatic ducts of *cyp11c1*$^{-/-}$ mutant zebrafish were comparatively smaller compared to those of wild-type siblings. *Cyp11c1*$^{-/-}$ mutant spermatic ducts (arrows) appeared immediately posterior to the intestine and were severely underdeveloped, though some contained mature sperm. Images captured at 10x magnification. 11bp wild-type n=3, mutant n=5; 47bp wild-type n=3, mutant n=5.

Table 4.4. Proportion of fish producing sperm samples during gamete expression experiments

Genotype	**# of fish producing sperm sample**	
	First trial	**Second trial**
cyp11c1$^{11bp+/+}$	6/6	8/8
cyp11c1$^{11bp-/-}$	0/6	0/7
cyp11c1$^{47bp+/+}$	6/6	6/6
cyp11c1$^{47bp-/-}$	6/9*	0/6

*2/6 samples from fish which produced sperm contained negligible sperm numbers.

4.2.8 - Reduced expression of pro-male and spermatogenic genes in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish

Androgens, and other steroid hormones, act via their cognate nuclear receptors to influence gene expression (de Waal et al., 2008). In order to understand the impact of altered steroid hormone concentrations on gene expression in the testes, and gain insight into the molecular mechanisms underlying the observed phenotype, we have used qPCR to measure to expression of genes related to sex and gonadal function.

Igf3 and Insl3 are important factors in zebrafish spermatogenesis, specifically involved in the proliferation and differentiation of type A spermatogonia (Assis et al., 2016; Morais et al., 2017; Nobrega et al., 2015). Both genes encoding these factors were significantly down-regulated in *cyp11c1*$^{-/-}$ mutant zebrafish, potentially suggesting impairment of early stages of spermatogenesis in these mutants (**Figure 4.14**).

The expression of *dmrt1* and *sox9a*, both of which play important roles in male sex differentiation (Sun et al., 2013; Webster et al., 2017) was unaffected by mutation of *cyp11c1* (**Figure 4.14**). *Amh* also plays a role in male sex differentiation (Rodriguez-Mari et al., 2005; Wang and Orban, 2007), this gene was upregulated in the 11bp deletion *cyp11c1* mutant but unaffected in the 47bp deletion mutant.

Expression of the androgen receptor, *ar*, via which 11-ketotestosterone exerts its effects on gene expression, was significantly upregulated in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 4.14**). This indicates a potential compensatory mechanism involving increased androgen receptor expression to scavenge for reduced androgens.

Inhibins exert negative feedback on the hypothalamus-pituitary-gonadal axis, and may also play a role in Sertoli cell proliferation and spermatogenesis in vertebrates (Cai et al., 2011; Gregory and Kaiser, 2004; Poon et al., 2009). We observed significant down-regulation of *inha* in our 47bp

deletion *cyp11c1* mutant zebrafish, however the decrease observed in our 11bp deletion mutant did not achieve statistical significance (**Figure 4.14**).

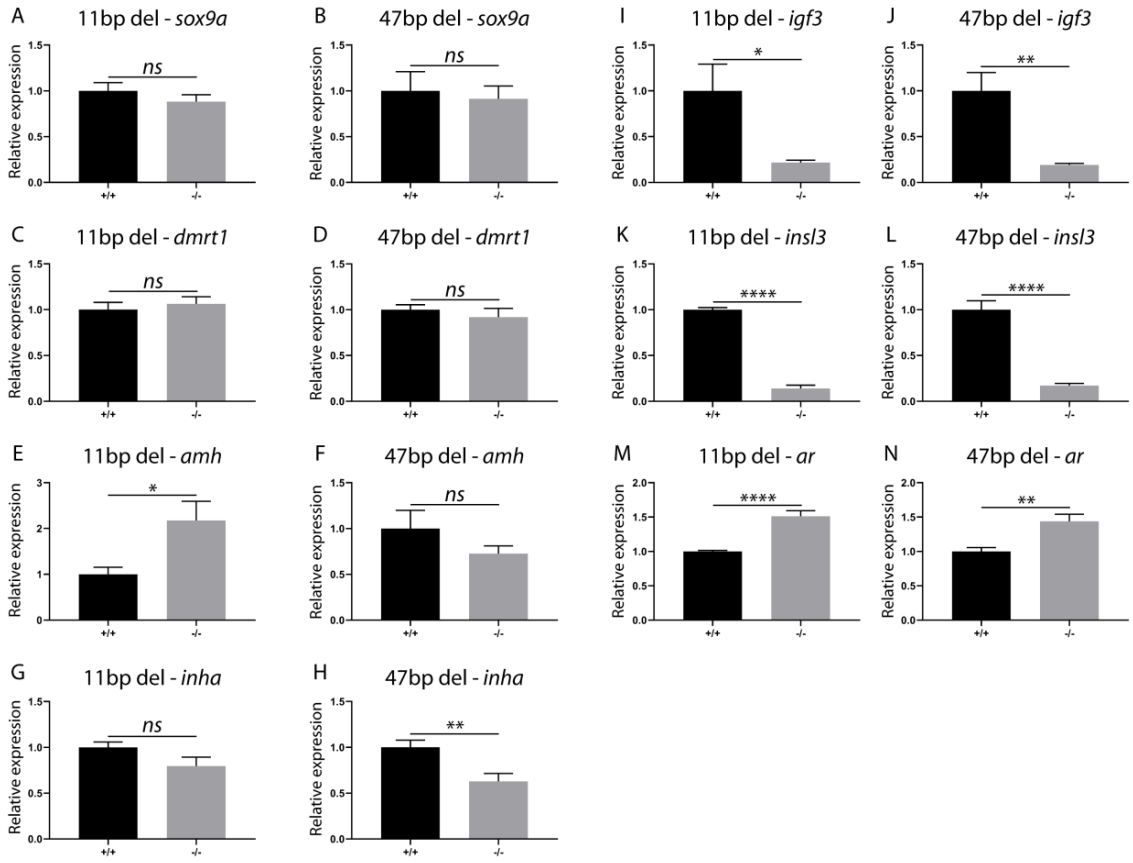

Figure 4.14. Expression of pro-male and spermatogenic genes in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish. The expression pro-male transcription factors *sox9a* (11bp deletion n=7, p=0.3321; 47bp deletion n=8, p=0.7337) and *dmrt1* (11bp deletion n=8, p=0.5821; 47bp deletion n=8, p=0.4756) was unchanged in Cyp11c1-deficient zebrafish. Expression of the pro-male anti-Müllerian hormone was upregulated in the testes of *cyp11c1*$^{-/-}$ mutant fish carrying the 11bp deletion allele (n=8, p=0.0198), but not changed in those carrying the 47bp deletion allele (n=8, p=0.2253). Expression of the hypothalamus-pituitary-gonadal axis regulator inhibin alpha *(inha)* was unchanged in *cyp11c1*$^{-/-}$ mutant fish carrying the 11bp deletion allele (wild-type n=7, mutant n=8, p=0.1067) but downregulated in fish homozygous for the 47bp deletion allele (n=8, p=0.0062). Expression of the spermatogenic factors *igf3* (11bp deletion n=8, p=0.0183; 47bp deletion wild-type n=8, mutant n=7, p=0.0024) and *insl3* (11bp deletion wild-type n=7, mutant n=8, p<0.0001; 47bp deletion n=8, p<0.0001) was profoundly reduced in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish. Expression of the androgen receptor *(ar)* was significantly upregulated in *cyp11c1*$^{-/-}$ mutant zebrafish testes (11bp deletion wild-type n=7, mutant n=8, p<0.0001; 47bp deletion n=8, p=0.0020).

4.2.9 - Expression of spermatogenesis marker genes suggests a crucial role for androgens in the differentiation of type A spermatogonia in zebrafish

Having observed a reduction in the numbers of mature spermatozoa by histology and sperm counting, we endeavoured to deepen our understanding of the impact of androgen deficiency on spermatogenesis. Spermatogenesis comprises a series of cell division and differentiation events, whereupon spermatogonial stem cells give rise to mature spermatozoa. To this end, we measured the expression of marker genes for several intermediate stages of spermatogenesis (**Figure 4.15**). *Nanos2* and *piwil1* are expressed in type A spermatogonia (Beer and Draper, 2013; Chen et al., 2013; Safian et al., 2016). Significantly increased expression of *nanos2* was observed in *cyp11c1*$^{-/-}$ mutant zebrafish. Increased expression of *piwil1* was observed in *cyp11c1*$^{-/-}$ mutant zebrafish carrying the 11bp deletion allele, but not in those carrying the 47bp deletion allele (**Figure 4.15**). Taken together, these results indicate an accumulation of type A spermatogonia and blockade in spermatogenesis at the differentiation of type A spermatogonia. No change in expression of the type B spermatogonia marker *dazl* (Chen et al., 2013) was observed, however, expression of the spermatocyte marker *sycp3* (Ozaki et al., 2011) and spermatid marker *odf3b* (Nobrega et al., 2015; Yano et al., 2008) was significantly reduced in *cyp11c1*$^{-/-}$ mutant zebrafish testes, indicating a reduced proportion of cells at the later stages of spermatogenesis (**Figure 4.15**).

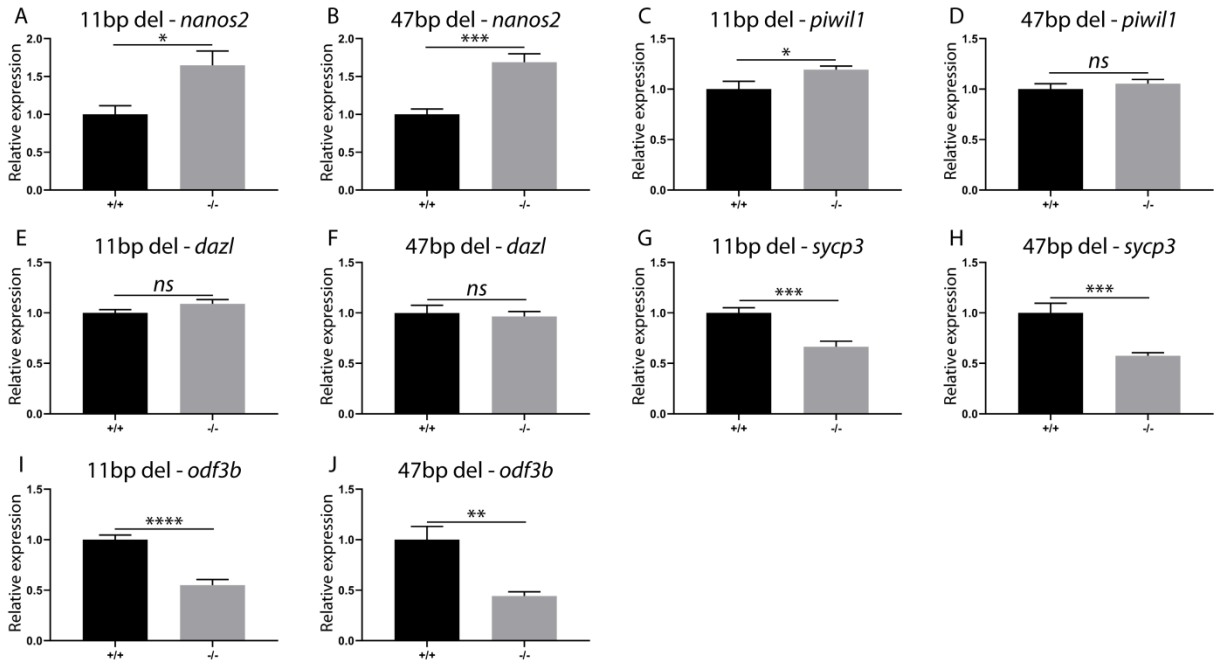

Figure 4.15. Expression of spermatogenic marker genes in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish. qPCR was employed to measure the expression of marker genes for several spermatogenic stages. The expression of *nanos2*, a marker of type A undifferentiated spermatogonia, was increased in *cyp11c1*$^{-/-}$ mutant zebrafish (11bp deletion n=8, p=0.0112; 47bp deletion n=8, p=0.0001). The expression of *piwil1*, a marker of both undifferentiated and differentiating type A spermatogonia was significantly increased in zebrafish homozygous for the 11bp deletion allele (n=8 , p=0.0405), but was not changed in those carrying the 47bp deletion allele (n=8 , p=0.4385). Expression of the type B spermatogonia marker *dazl* was not affected by mutation of *cyp11c1* (11bp deletion n=8, p=0.1213; 47bp deletion n=8, p=0.7009). Expression of the spermatocyte and spermatid markers *sycp3* and *odf3b* was significantly reduced in the testes of *cyp11c1*$^{-/-}$ zebrafish. (*sycp3*: 11bp deletion n=8, p=0.0006; 47bp deletion n=8, p=0.0008. *odf3b*: 11bp deletion n=8, p<0.0001; 47bp deletion n=8, p=0.0013). Results analysed by unpaired *t*-tests. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4.2.10 - Expression of gonadotropins and pomca in the brains of *cyp11c1*$^{-/-}$ mutant male zebrafish

Expression of *pomca* and *fshb* was significantly increased in the brains of *fdx1b*$^{-/-}$ mutant male zebrafish, possibly due to loss of negative feedback from cortisol and reduced expression of the HPG axis regulator *inha* (Alsop and Vijayan, 2009; Poon et al., 2009). An apparent increase in the expression of *pomca* in the brains of *cyp11c1*$^{-/-}$ mutant males was only significant in the 47bp allele, though the result from the 11bp allele was approaching the significance threshold (p=0.0991) (**Figure 4.16**). Expression of *fshb* was significantly increased in 47bp deletion allele mutant males but unchanged in the 11bp allele. No change in *lhb* expression was recorded for either allele.

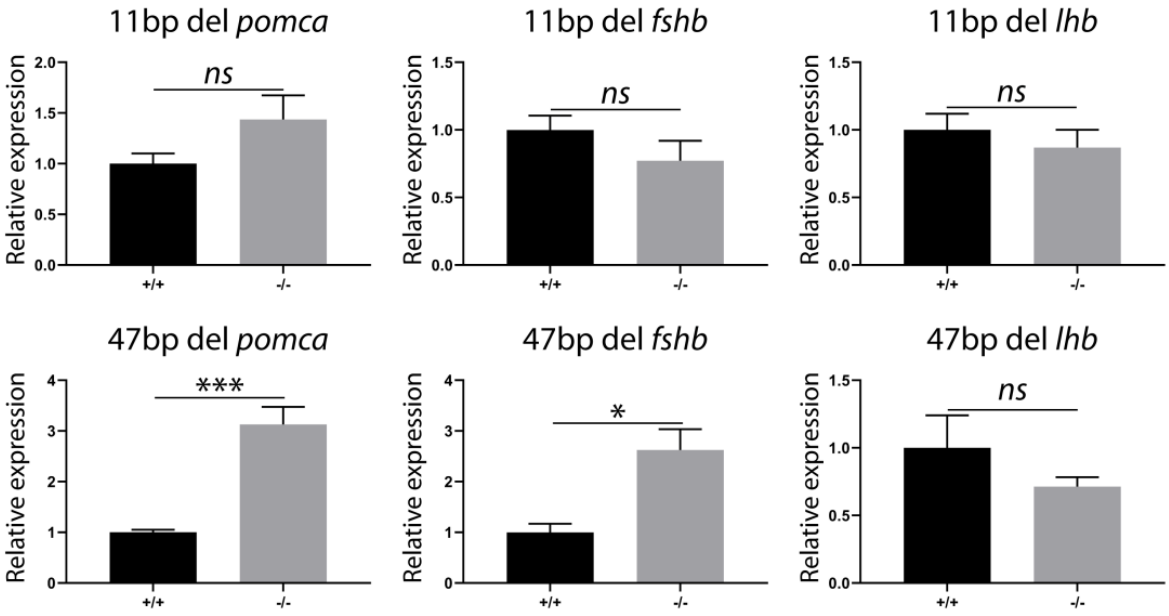

Figure 4.16. Expression of *pomca* and gonadotropins in the brains of *cyp11c1*$^{-/-}$ mutant male zebrafish. An apparent increase in the expression of *pomca* the brains of *cyp11c1*$^{-/-}$ mutant male zebrafish was only significant for the comparison in the 47bp allele (11bp deletion, wild-type n=8, mutant n=7, p=0.0991; 47bp deletion, wild-type n=5, mutant n=8, p=0.0006). Expression of *fshb* was significantly increased the brains of *cyp11c1*$^{-/-}$ mutant male carrying the 47bp allele, but not in those carrying the 11bp allele (11bp deletion, wild-type n=8, mutant n=7, Mann-Whitney test, p=0.2228; 47bp deletion, wild-type n=6 mutant n=7, Mann-Whitney test, p=0.0221). No change in *lhb* expression was observed for either allele (11bp deletion, wild-type n=8, mutant n=7, p=0.4749; 47bp deletion, wild-type n=6, mutant n=8, p=0.2188). Statistical analysis was by unpaired *t*-test unless otherwise stated. * p<0.05, ***p<0.001.

4.3 - Discussion

The specific roles of androgens in testicular function in zebrafish are poorly understood. Herein, we have described the phenotype of androgen and cortisol deficient *cyp11c1*$^{-/-}$ mutant zebrafish, paying particular attention to the roles of steroids in the development, maintenance and function of the male reproductive system.

Cyp11c1 is important for production of cortisol and 11-ketotestosterone, the principal zebrafish androgen (de Waal et al., 2008; Tokarz et al., 2015). We have demonstrated that Cyp11c1 deficient zebrafish exhibit profound cortisol and 11-ketotestosterone deficiency, confirming the crucial roles of this enzyme in steroidogenesis (**Figure 4.6**). Concentrations of testosterone were unaffected by mutation of *cyp11c1*, however the contribution of testosterone to androgen signalling in fish is likely to be negligible as only small amounts of this steroid are produced (de Waal et al., 2008; Mindnich et al., 2005). Concentrations of the sex steroid precursor androstenedione were increased in *cyp11c1* mutant zebrafish, presumably due to blockage of the androgen biosynthetic pathway as well as shunting of precursors from the cortisol biosynthetic pathway. Like 11-ketotestosterone and testosterone, androstenedione also has androgen activity; however, it has a relatively low affinity for the androgen receptor and is considered a weaker androgen and is unlikely to be an important androgen *in vivo* (de Waal et al., 2008).

An accumulation of androstenedione could conceivably result in increased oestrogen production, as the excess precursor may enter the oestrogen biosynthetic pathway. Production of oestrogens requires ovarian aromatase (Cyp19a1a), expression of *cyp19a1a* is comparatively low in the testes (Crowder et al., 2017; Sawyer et al., 2006) making it unlikely that excess androstenedione is converted to oestrogens.

Decreased cortisol concentrations were reflected by decreased expression of the glucocorticoid responsive genes *fkbp5* and *pck1* (Eachus et al., 2017; Griffin et al., 2016) in both *cyp11c1*$^{-/-}$ mutant

male adult liver tissue and in whole 5 days post fertilisation larvae (**Figures 4.3+4.7**), demonstrating the importance of Cyp11c1 for production of this steroid throughout the zebrafish lifecourse. *Cyp2k22* is proposed as an androgen-responsive gene in zebrafish (Fetter et al., 2015; Siegenthaler et al., 2017), and is robustly downregulated in the livers of androgen deficient *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 3.5**) (Oakes et al., 2019). An apparent reduction in the expression of *cyp2k22* in the livers of *cyp11c1*$^{-/-}$ mutant zebrafish was not significant (**Figure 4.7**). The high variability in the expression of this gene, particularly in wild-type fish, is a likely explanation for this finding, and suggests that it may also be regulated by other factors in addition to androgen signalling.

As with other zebrafish lines carrying mutations resulting in impaired androgen signalling, *cyp11c1*$^{-/-}$ mutant zebrafish exhibit primarily female pigmentation patterns (Crowder et al., 2017; Oakes et al., 2019; Zhai et al., 2018). These results suggest that androgens may induce expression of genes important for colour patterning in the fins. A recent study found that there is no difference in the expression of colour patterning genes in the caudal fins of wild-type adult zebrafish males and females (Hosseini et al., 2019). However, the caudal fin has a less sexually dimorphic appearance than the dorsal and anal fins, which were changed in appearance by mutation of *cyp11c1* in the present study. Androgens clearly play a role in sexually dimorphic pigment patterning of the fins in zebrafish, however the mechanism underlying this effect is unclear and may involve promotion of male-type pigment patterning or suppression of female-type patterning.

Cyp11c1 deficient adult male zebrafish were found to be infertile by conventional breeding methods (**Table 4.2**), however their sperm was able to fertilise eggs collected from wild-type female zebrafish by IVF (**Table 4.3**). This suggests that *cyp11c1*$^{-/-}$ mutant zebrafish are able to produce mature sperm but are infertile due to another factor, such as impaired breeding behaviour, decreased spermatogenesis, or morphological aberration of the testes or male reproductive tract resulting in impaired sperm release.

Androgen signalling has previously been implicated in the regulation of breeding behaviours, as these are decreased in both androgen resistant and androgen deficient fish (Oakes et al., 2019; Yong et al., 2017). Here we have replicated the phenotype we previously described in *fdx1b*$^{-/-}$ mutant zebrafish in our new *cyp11c1*$^{-/-}$ mutant zebrafish lines (**Figure 4.8**) (Oakes et al., 2019). In addition to this it was found that *cyp11c1*$^{-/-}$ mutant zebrafish exhibit decreased locomotor activity compared to wild-type siblings (**Figure 4.9**). This decrease in locomotor activity may affect our readouts of breeding behaviours. For example, fish which swim more slowly may have less opportunity for intimate contacts with their tank mate and may not be able to participate effectively in chasing behaviour. Locomotor activity and freezing behaviours have been linked to stress and glucocorticoid signalling in zebrafish; glucocorticoid receptor mutants are known to exhibit freezing behaviour and slower average swim velocity when exposed to the open field test (Ziv et al., 2013). This indicates that cortisol deficiency is likely to be responsible for the impaired locomotor behaviour seen in *cyp11c1*$^{-/-}$ mutant zebrafish. Overall, a combination of cortisol and 11-ketotestosterone deficiency is likely to be responsible for impaired breeding behaviours in *cyp11c1*$^{-/-}$ mutant zebrafish; as impaired locomotor behaviour, probably due to cortisol deficiency, must play a role, however mutation of the androgen receptor also produces a similar phenotype (Yong et al., 2017).

As previously described in androgen deficient and resistant zebrafish lines, we have here shown that androgen signalling is dispensable for testicular differentiation, with male and female zebrafish present in similar proportions in *cyp11c1*$^{-/-}$ mutant and wild-type sibling populations. However, histological examination of *cyp11c1*$^{-/-}$ mutants revealed considerable disorganisation of the testes; seminiferous tubules appeared to be smaller and more numerous, and the quantity of mature sperm appeared to be decreased (**Figures 4.10-4.12**). The histological appearance of *cyp11c1*$^{-/-}$ mutant testes was reminiscent of the phenotype described in androgen resistant and other androgen deficient zebrafish lines (Crowder et al., 2017; Oakes et al., 2019), thus providing further confirmation of the role of androgens in correct organisation and morphological development or maintenance of this structure. The formation of the tubular structure of the testes appears to occur

during the latter stages of, or after, the period of testicular differentiation in zebrafish, as tubules are not clearly visible until after the gonad is committed to testis development (van der Ven and Wester, 2003). As such, it appears that the crucial role of androgens in the development of the testis tubules is during the period of maturation, between gonadal differentiation and adulthood.

We have previously postulated that Sertoli cell dysfunction may be responsible for the testicular phenotype observed in androgen deficient zebrafish. Several Sertoli cell expressed genes, such as *sox9a* and *inha*, were downregulated in *fdx1b*$^{-/-}$ mutant zebrafish, which exhibit a similar phenotype to that described in the present study (Oakes et al., 2019). *Sox9a* may be of importance in testis tubulogenesis (Oakes et al., 2019), as a role in this process has been proposed in a related teleost (Nakamoto et al., 2005). However, *sox9a* expression was unaffected by mutation of *cyp11c1*, and downregulation of *inha* was only significant in one of the two mutant lines examined (**Figure 4.14**). Therefore, the mechanisms by which androgens control appropriate testis tubule morphogenesis or maintenance remain enigmatic, and are exciting topics for further study.

The decrease in sperm abundance observed in *cyp11c1*$^{-/-}$ mutant testes by histology was subsequently confirmed by sperm counting (**Figure 4.12**). As mentioned above, the abdominal cavities of *cyp11c1*$^{-/-}$ mutant fish typically contained more adipose tissue than those of their wild-type siblings. This excess tissue was frequently firmly bonded to the testes and may have resulted in a small overestimation of testes weight in mutant fish, which in turn may have caused an underestimation of sperm count. However, the impact of this is likely to be minimal, and when combined with histological evidence, our results provide compelling evidence for impaired spermatogenesis in this mutant line.

We have observed significant down-regulation of *igf3* and *insl3* in *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 4.14**), both genes are important in the differentiation and proliferation of type A spermatogonia (Assis et al., 2016; Morais et al., 2017; Nobrega et al., 2015). This was reflected by increased expression of *nanos2*, a marker for type A spermatogonia (Beer and Draper, 2013; Safian et al.,

2016), and decreased expression of *sycp3* and *odf3b*, markers of later stages in spermatogenesis (**Figure 4.15**) (Nobrega et al., 2015; Ozaki et al., 2011; Yano et al., 2008). This pattern of gene expression reinforces the theory that the differentiation of type A spermatogonia is a highly androgen dependent process in zebrafish. The specific roles of androgens in regulating spermatogonial differentiation in mammals is poorly characterised, however, retinoic acid signalling plays an important role in this process in both mammals and fish, and interplay between these systems has been suggested (Crespo et al., 2019; Smith and Walker, 2014).

In addition to impaired spermatogonial differentiation, entry of type B spermatogonia into meiosis may also be disrupted in *cyp11c1*$^{-/-}$ mutant zebrafish. Expression of the type B spermatogonia marker *dazl* was unchanged by mutation of *cyp11c1.* In contrast, expression of the spermatocyte marker *sycp3* was downregulated, as was the spermatid marker *odf3b* (**Figure 4.15**). Spermatogenic arrest during meiosis has previously been reported in androgen receptor mutant zebrafish (Yu et al., 2018). Sycp3 is a synaptonemal complex component; this structure has important roles during meiotic prophase including regulation of chromosome recombination (Page and Hawley, 2004; Syrjänen et al., 2014).

Attempts to manually collect semen from *cyp11c1*$^{-/-}$ mutant zebrafish revealed that sperm release is impaired. However, this technique may not accurately replicate the ejaculatory mechanism in natural breeding, and therefore the possibility of successful sperm release in natural breeding conditions cannot be ruled out. Histological examination revealed that the spermatic duct was severely hypoplastic in *cyp11c1*$^{-/-}$ mutant zebrafish. Little is known about the development of the spermatic duct, however we have shown here for the first time that its development is highly steroid hormone dependent, and is likely to be mediated by 11-ketotestosterone. This structure may be thought of as comparable to Wolffian duct structures in mammals, however these structures are of different embryological origins and may be analogous in function alone (Matthews et al., 2018; Shaw and Renfree, 2014). Nevertheless, both structures appear to be highly dependent on androgens, as

abnormal Wolffian duct structures are frequently seen in complete androgen insensitivity syndrome (Barbaro et al., 2007; Hannema et al., 2006). In addition to this Wolffian duct structures are also absent in AR knock-out mice (Yeh et al., 2002).

When producing homozygous mutant zebrafish lines it is important to consider the possibility of severely reduced genetic variance in the region surrounding the mutated target gene. This reduction in variation arises from the fact that the region surrounding the target gene is inherited along with the mutation of interest. This may result in unintended deleterious effects due to homozygosity in the region surrounding the gene of interest. This possibility should be considered, especially when unexpected phenotypes are observed; for example, in this study expression of *amh* appeared to be elevated in one *cyp11c1* allele but not the other. This problem may simply be an artefact of multiple testing and further qPCR experiments using cDNA from additional incrosses could be used to eliminate this source of error. Alternatively, compound heterozygotes (*cyp11c1*$^{11bpdel/47bpdel}$) could be produced to ensure normal genomic variation in the region surrounding the gene of interest.

Herein, we have described novel zebrafish lines carrying mutation of *cyp11c1*, which is crucial for the biosynthesis of 11-ketotestosterone and cortisol. These mutant zebrafish exhibit a phenotype characteristic of androgen deficiency and represent a novel tool for the investigation of the roles of androgens in male reproductive development and function. The discovery that androgens are essential for spermatic duct morphogenesis in zebrafish is a particularly exciting finding, and will pave the way for further research into this poorly characterised structure.

Chapter 5 - Exploring the implications of androgen and cortisol deficiency on the zebrafish testis transcriptome in *fdx1b*$^{-/-}$ mutant zebrafish

5.1 - Introduction

Several models of androgen deficiency or resistance in zebrafish have been published (Crowder et al., 2017; Li et al., 2019b; Tang et al., 2018; Yu et al., 2018). Androgen deficient or resistant male zebrafish exhibit infertility, due to a combination of decreased spermatogenesis, impaired breeding behaviour and impaired sperm release. The testicular phenotype in zebrafish with impaired androgen signalling comprises disorganised structure and impaired spermatogenesis. Histological examination of the testes reveals an accumulation of germ cells at early stages of spermatogenesis, and this is mirrored by upregulation of spermatogonia marker genes (**Figures 3.15+4.15**) (Li et al., 2019b; Tang et al., 2018). Some progress has been made towards understanding the molecular mechanisms underpinning impaired spermatogonial differentiation: *igf3* and *insl3,* genes crucial for this process, are profoundly downregulated in the testes of androgen deficient or resistant fish (**Figures 3.13+4.14**) (Tang et al., 2018). In contrast, evidence for perturbed molecular pathways resulting in testis disorganisation is scarce. *Sox9a* is suggested to play a role in testis tubulogenesis in Medaka (Nakamoto et al., 2005), and a small downregulation of this gene was observed in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. However, this was not recapitulated in *cyp11c1*$^{-/-}$ or *ar*$^{-/-}$ mutant zebrafish lines (**Figure 4.14**) (Crowder et al., 2017; Yu et al., 2018). In order to identify pathways affected by androgen deficiency in the zebrafish testes we have performed mRNA sequencing of the testes of *fdx1b*$^{-/-}$ mutant and wild-type sibling zebrafish.

In addition to mRNA sequencing, we have performed small RNA sequencing of the same samples. Micro RNAs (miRNAs) are short RNA molecules (~22nt) which exert post-transcriptional repression on gene expression by preventing translation. Most mRNAs are suggested to be potential miRNA targets, indicating that miRNAs influence most, if not all, biological processes (Bartel, 2018). miRNAs have known roles in the development and function of the testes, particularly in spermatogenesis

(Mobasheri and Babatunde, 2019). For example, miRNAs are essential for spermatogenesis in mice, as germ cell knockout of Dicer, which plays a role in miRNA biogenesis, resulted in spermatogenic arrest during early stages of spermatogenesis (Hayashi et al., 2008). Furthermore, androgens appear to regulate some miRNAs in the testes, as suppression of androgen signalling in mice resulted in differential expression of 28 miRNAs in Sertoli cells, indicating a potential role for miRNAs in the androgen regulation of spermatogenesis and testis function (Panneerdoss et al., 2012). Several miRNAs play important roles in regulation of spermatogonial differentiation, a process which is impaired in *fdx1b*$^{-/-}$ mutant zebrafish (Mobasheri and Babatunde, 2019).

Paternal stress can influence the miRNA content of mature spermatozoa. Chronic stress causes upregulation of nine miRNAs in the sperm of male mice, and the offspring of these mice exhibit a reduced corticosterone response to stress (Rodgers et al., 2013). The phenotype displayed by the offspring can be recapitulated by zygotic microinjection of the nine upregulated miRNAs (Rodgers et al., 2015). These studies indicate that sensitivity of the stress axis can be programmed by a paternally inherited epigenetic signature, which could potentially be brought about by increased glucocorticoid exposure as a result of stress.

Fdx1b$^{-/-}$ mutant zebrafish are glucocorticoid and androgen deficient. We hypothesised that deficiency of these steroid hormones would result in differential expression of mRNAs and miRNAs in the testes, some of which may be important in regulation of spermatogenesis or participate in transgenerational inheritance.

5.2 - Methods

5.2.1 - RNA isolation

Testis tissue was obtained by dissection of humanely euthanized 131dpf adult male zebrafish. Testes were stored in RNA later (Thermo Fisher Scientific, California, United States) until RNA extraction. Total RNA extraction was performed using Trizol reagent (Ambion, Texas, United States) as per the manufacturer's instructions, except that precipitation was performed overnight at -20°C with addition of 10µg of glycogen (Roche, Basel, Switzerland). RNA integrity was checked on a ~1.2% TAE agarose gel and bioanalyser.

5.2.2 - RNA library preparation and sequencing

RNA library preparation and sequencing was performed by the Deep Sequencing Facility at Technische Universität Dresden. mRNA pulldown was achieved with the NEBNext poly(A) mRNA magnetic isolation module with 300ng total RNA input, and libraries were produced using the NEBNext ultra II directional RNA library prep kit for Illumina (NEB, Ipswich, Massachusetts, United States). Small RNA libraries were prepared using the Nextflex small RNA seq kit v3 with 50µg total RNA input (Biooscientific, Austin, Texas, United States). Small RNA and mRNA libraries were 75bp single end sequenced on a NextSeq 500 sequencing system (Illumina, San Diego, California, United States) to a depth of ~10 million and ~30 million reads respectively.

5.2.3 - mRNA sequencing data analysis

Fastq.gz files produced by RNA sequencing were first trimmed using Trimmomatic version 0.39 (ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) followed by removal of rRNAs by SortmeRNA version 2.1. Quality

control was performed using FastQC. Trimmed reads were aligned to the zebrafish genome (GRCz11) using STAR. DESeq2 was used in R-studio for normalisation of counts and differential expression analysis. Principal component analysis and heat map production was performed in R studio by the plotPCA and pheatmap packages respectively. Code used for mRNA sequencing analysis can be found in **Appendix II**.

5.2.4 - Gene ontology term overrepresentation and KEGG pathway analysis

Genes detected as significantly differentially expressed by RNA sequencing were filtered to remove genes with a base mean count of <300 and a fold change of <+/-1.5 (**Additional file 1**). Statistical overrepresentation analysis of gene ontology terms associated with differentially expressed genes (DEGs) was performed using GOrilla (Eden et al., 2009), with the GO biological processes gene annotation set. Analysis for enrichment of biological pathways was accomplished using the DAVID functional annotation tool to find KEGG pathways overrepresented in our list of DEGs (Huang da et al., 2009). The background gene list (**Additional file 1**) contained all genes detected in our RNA seq data with a base mean count of >40, this approach is suggested to reduce biological bias by excluding genes not expressed, or those only expressed at low levels, in the tissue of interest (Timmons et al., 2015).

5.2.5 - Small RNA sequencing data analysis

Initially, FastQC (Andrews, 2010) was used to perform quality assurance on small RNA sequencing files. Small RNA sequencing data were analysed using the Oasis 2.0 web interface (Rahman et al., 2018). .fastq.gz files were uploaded to the web interface and processed using the following parameters: *Danio rerio* (zebrafish) genome assembly GRCz10 (danRer10), customer adapter string NNNNTGGAATTCTCGGGTGCCAAGG, mismatches 5% of read length, minimum length 15, maximum

length 50. The Oasis 2.0 web interface utilises the tools listed in **Table 5.1** and aligns reads to the databases listed in **Table 5.2**.

Differential expression analysis was also performed using the Oasis web interface. Count files were uploaded to the Oasis 2.0 differential expression analysis interface and the reference genome was set to *Danio rerio* (zebrafish) genome assembly GRCz10 (danRer10). miRNA and piRNA counts were analysed separately.

Table 5.1. Tools utilised by the Oasis 2.0 web interface

Tool	**Version**
Cutadapt	1.7.1
FastQC	v0.10.1
STAR	2.4.1d
featureCounts	v1.4.6
miRDeep2	2.0.0.5

Table 5.2. Databases used for read alignment by Oasis 2.0

Species	**Database Version**
miRNA	miRBase version 21
piRNA	piRNAbank V.2
snRNA	Ensembl v84
snoRNA	Ensembl v84
rRNA	Ensembl v84

5.3 - Results

5.3.1 - *Fdx1b*$^{-/-}$ mutant and wild-type sibling mRNA sequencing quality control

Quality control was performed by FastQC and MultiQC both before (not shown) and subsequent to rRNA removal and trimming (**Figure 5.1**) (Andrews, 2010; Ewels et al., 2016), and revealed excellent sequence quality (**Figure 5.1B-D**). All samples failed the per sequence GC content module and triggered warning for the sequence duplication level module, however only slight left skewing of the GC content chart and reasonably low duplication levels were evident (**Figure 1E+F**). All samples showed biased base composition in the first 12bp of reads, this is normal for RNA seq libraries and is not thought to affect downstream analysis (**Figure 5.1G**). No overrepresented sequences were detected in any samples and all samples passed the adapter content module. All samples failed the kmer content module (**Figure 5.1H**) – this is likely to be due to the random primed nature of the library causing kmer bias near the start of reads.

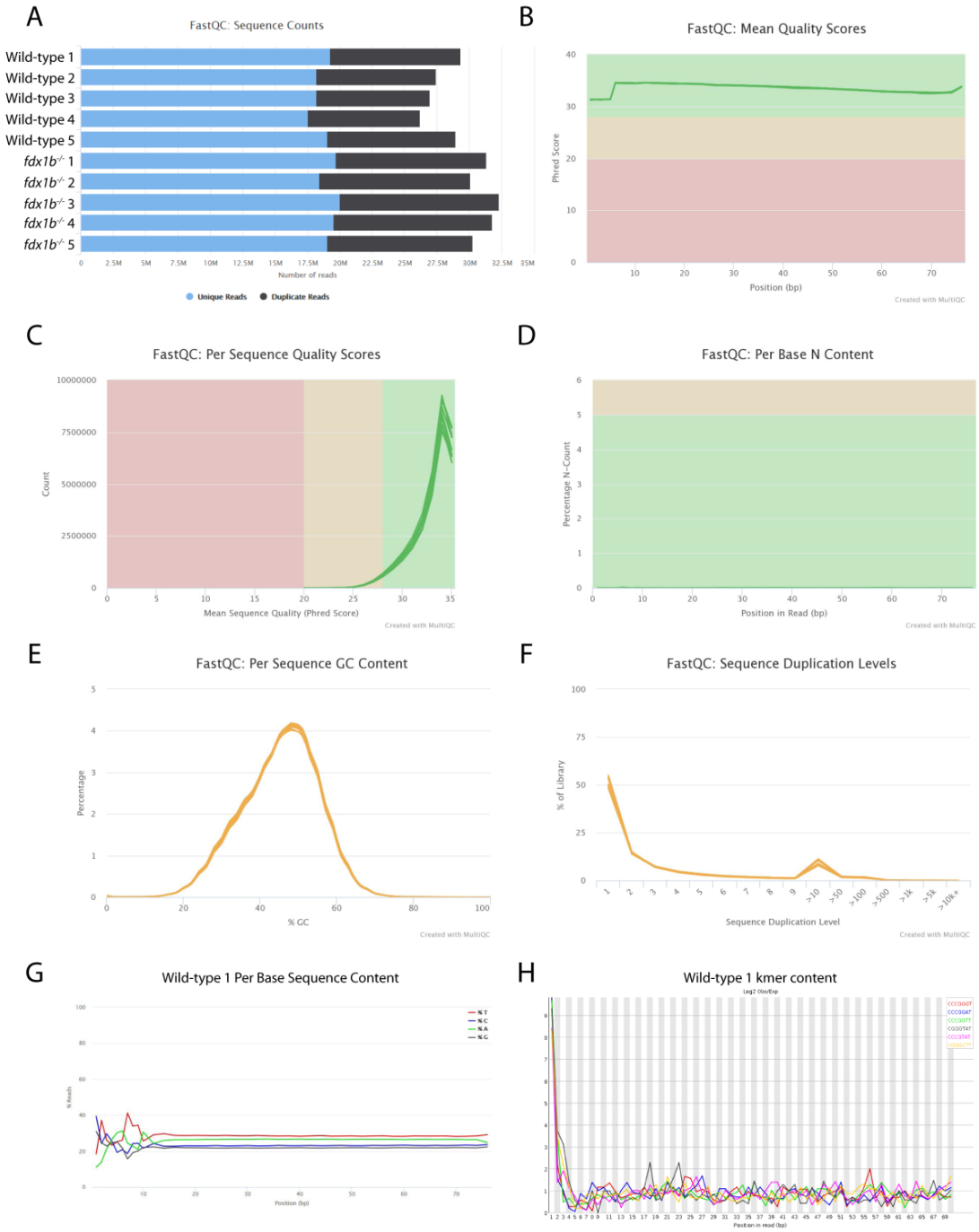

Figure 5.1. Quality control of RNA sequencing reads after initial processing. Quality control was performed by FastQC/MultiQC after trimming reads using Trimmomatic and removing rRNA reads using SortmeRNA. **A:** Samples were sequenced to a depth of ~26-32 million reads. **B-D:** Quality scores and N content indicate very high sequence quality. **E:** Per sequence GC content showed slight left skew. **F:** Sequencing data contained a reasonably low duplication level. **G+H:** Example charts for per base sequence content and kmer content, all other samples produced charts with a similar profile.

5.3.2 - mRNA sequencing reveals differentially expressed genes in the testes of *fdx1b*$^{-/-}$ mutant zebrafish vs. wild-type siblings

Fdx1b$^{-/-}$ mutant zebrafish are androgen and cortisol deficient, and as a result of this exhibit disorganisation of the testes and reduced spermatogenesis. Several genes, including *igf3, insl3, sox9a* and *inha* are dysregulated in the testes of these mutant fish, indicating an impact of androgen or cortisol deficiency on the gonadal transcriptome. To further understand this impact, and to gain further insight into the molecular mechanisms underpinning the phenotype observed, we performed RNA sequencing of the testis transcriptome.

PCA (**Figure 5.2A**) revealed clear segregation of *fdx1b*$^{-/-}$ mutant and wild-type samples by the first and second principal components, however considerable variation within each group was also observed. Heatmaps of normalised count data also revealed generally robust differences between the two samples groups (**Figure 5.2B**).

Differential expression analysis revealed a total of 9549 DEGs between *fdx1b*$^{-/-}$ mutant and wild-type sibling testes, this was reduced to 2128 DEGs after filtering genes with <300 reads and <+/-1.5-fold change. The 2128 DEGs was comprised of 1594 down-regulated and 534 up-regulated genes (**Additional file 1**). A comparison of gene expression changes measured by qPCR and RNA sequencing revealed reasonable replication of results (**Table 5.3**). The results for the majority of genes with the largest fold changes were comparable for the two methods, for example *cyp11c1, inha, igf3* and *insl3*. Expression of the glucocorticoid responsive gene *fkbp5* was significantly downregulated ~7-fold (p= 0.0001) in *fdx1b*$^{-/-}$ mutant testes compared to wild-type siblings (**Additional file 1**).

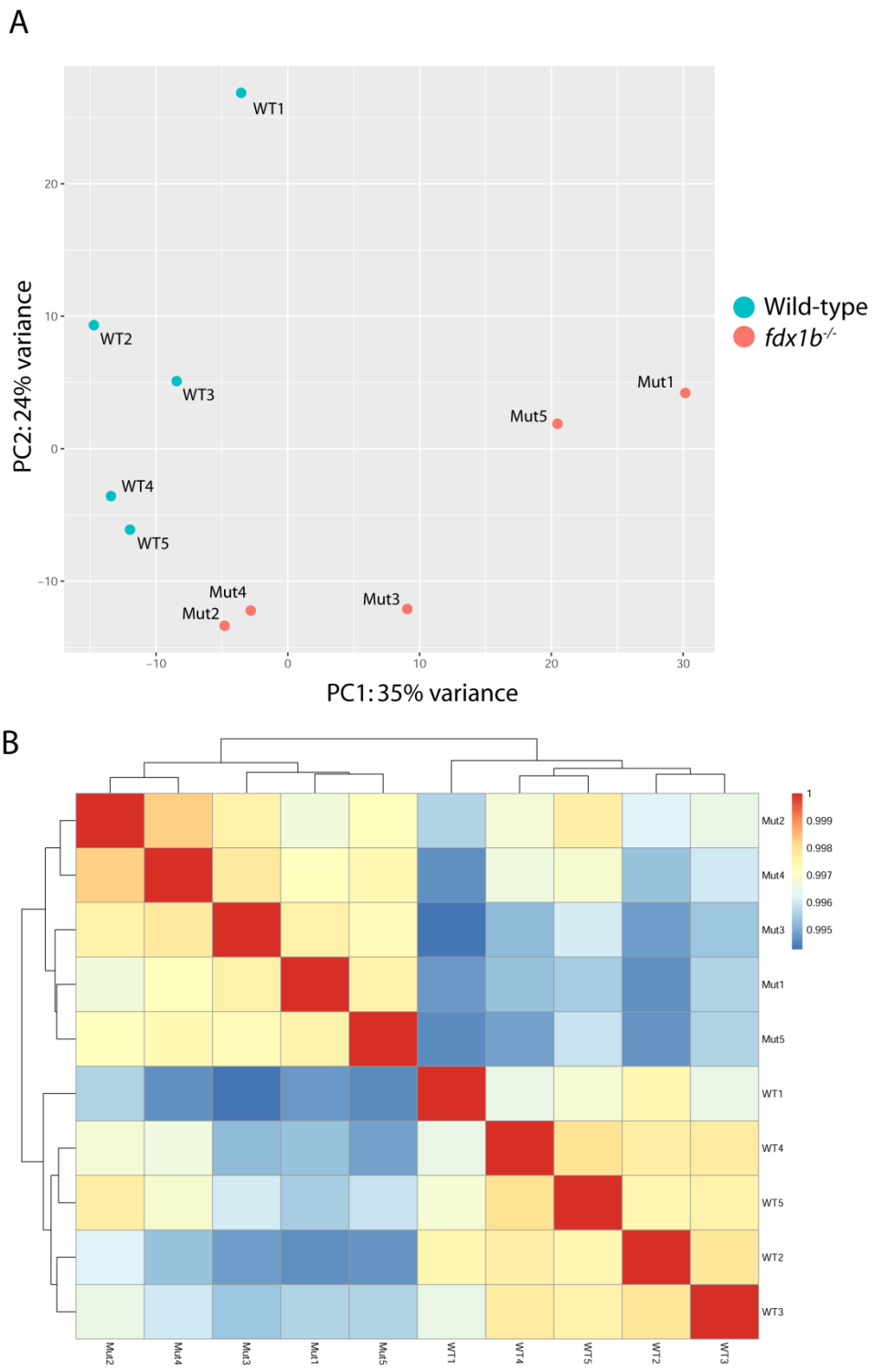

Figure 5.2. Principal component and heatmap analysis reveals variation between the *fdx1b*$^{-/-}$ mutant and wild-type testis transcriptomes. A: PCA analysis (ntop=500): although *fdx1b*$^{-/-}$ mutant and wild-type samples were not highly clustered, the two groups were clearly segregated by the first and second principal components. **B:** Heatmap generated from the same data.

Table 5.3. Comparison of changes in expression of key genes measured by qPCR and RNA sequencing

Gene	qPCR fold change	Significance	RNA-seq fold change	Significance
star	2.42	ns	2.72	**
cyp11a2	2.55	*	1.66	ns
cyp17a2	1.00	ns	1.75	*
cyp11c1	2.80	*	3.01	****
hsd17b3	1.22	ns	0.86	ns
cyp19a1a	0.99	ns	2.60	**
sox9a	0.42	*	1.10	ns
dmrt1	0.75	ns	0.78	***
amh	0.77	ns	1.12	ns
igf3	0.07	*	0.12	****
insl3	0.05	***	0.05	****
inha	0.28	*	0.52	ns
znrf3	0.99	ns	1.22	ns
sox9b	1.72	ns	0.96	ns
foxl2a	1.62	ns	3.00	ns
foxl2b	1.10	ns	0.68	*
nanos2	1.43	*	2.33	****
piwil1	10.48	*	0.91	ns
dazl	0.82	ns	0.81	*
sycp3	2.80	ns	0.39	****
odf3b	0.90	ns	1.25	ns

Analysis of the most significantly up- and downregulated genes (**Tables 5.4+5.5**) revealed several with roles in testicular function. *Npas2* expression was reduced ~4-fold in *fdx1b*$^{-/-}$ mutant testes compared to wild-type siblings. NPAS2 is a transcription factor and in the brain plays an important role in maintenance of circadian rhythms (Kelleher et al., 2014). Mutations in *NPAS2* have been linked to non-obstructive azoospermia in humans (Ramasamy et al., 2015). Interestingly, NPAS2 is hypothesised to be a positive regulator of StAR, however testosterone concentrations appeared to be within the normal range in non-obstructive azoospermia patients with mutation of *NPAS2* (Ramasamy et al., 2015).

EPAS1 is another transcription factor with roles in spermatogenesis. *Epas1* disruption in mice resulted in male infertility with a 50% reduction in testis size. Histological examination of the testes revealed reduced spermatid and spermatozoa numbers, as well as reduced seminiferous tubule diameter and number. Finally, *Epas1* mutant mice exhibited disrupted seminiferous tubule basement membranes and abnormal tight junctions, resulting in a compromised blood-testis barrier (Gruber et al., 2010). The zebrafish ortholog of *EPAS1*, *epas1a*, was downregulated ~4-fold in *fdx1b*$^{-/-}$ mutant testes compared to wild-type siblings. In contrast, the paralog of *epas1a, epas1b,* was upregulated 1.4-fold in *fdx1b*$^{-/-}$ mutant testes.

CFTR is a cAMP activated chloride and bicarbonate channel; mutations in this gene are the cause of cystic fibrosis. Mutations in this gene, or reduced gene expression, can result in congenital bilateral absence of vas deferens and sperm abnormalities. As such, this gene is considered an important regulator of male fertility (Chen et al., 2012). Expression of *cftr* was reduced ~8-fold in the testes of *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings. Expression of *cftr* in the gills of the teleost *Fundulus heteroclitus* could be induced by injection of cortisol and the *cftr* promoter was found to contain glucocorticoid responsive elements (Singer et al., 2008).

Several members of the solute carrier gene family were amongst the most significantly downregulated genes in *fdx1b*$^{-/-}$ mutant testes, including *slc12a2* (**Table 5.4**). *Slc12a2*$^{-/-}$ mutant mice

are smaller in size than their wild-type littermates at two weeks of age and exhibited poor motor function. *Slc12a2*$^{-/-}$ mutant males are infertile and exhibit delayed seminiferous tubule lumen formation, in addition to this, the testes of mature *slc12a2*$^{-/-}$ mutant mice were morphologically abnormal and spermatogenesis was severely disrupted (Pace et al., 2000). Expression of *slc12a2* was reduced ~2-fold in *fdx1b*$^{-/-}$ mutant testes in comparison to wild-type.

In addition to the above described downregulated genes, several of the most significantly upregulated genes also play important roles in testicular function (**Table 5.5**). *Nanos1* was upregulated nearly 5-fold in the testes of *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings. Mutations in the human homolog of this gene (*NANOS1*) can result in azoospermia (Kusz-Zamelczyk et al., 2013) and may play a role in self-renewal or maintenance of germline stem cells, as it does in *Drosophila* ovaries (Wang and Lin, 2004). In zebrafish embryos, Nanos1 plays an important role in primordial germ cell migration (Köprunner et al., 2001).

GATA4 is a highly conserved transcription factor with varied roles in embryo morphogenesis, in humans it is expressed in the testes during foetal development and post-puberty. *GATA4* expression coincides with stages of high androgen production and is expressed at low levels in patients with androgen resistance, leading to speculation that GATA4 may interact with steroidogenesis and androgen production (Ketola et al., 2000). This idea is supported by the finding that several steroidogenic proteins are downregulated in murine Leydig cells treated with a *Gata4* targeting siRNA (Schrade et al., 2015). GATA4 may also play roles in regulating Sertoli cell function and spermatogenesis. Conditional loss of *Gata4* expression in the Sertoli cells of adult mice results in progressive testicular atrophy accompanied by reduced fertility and spermatogenesis, blood-testis barrier permeability was also increased (Kyrönlahti et al., 2011). siRNA knock-down of *Gata4* expression in mouse Sertoli cells also found that a variety of tight junction related genes and processes were dysregulated (Schrade et al., 2016). We observed a 2.6-fold upregulation of *gata4* in

fdx1b$^{-/-}$ mutant testes compared to wild-type siblings (**Table 5.5**). Upregulation of *GATA4* has been observed in the testes of men with non-obstructive azoospermia (Kovac et al., 2013).

Table 5.4. Top 30 most significantly downregulated genes in *fdx1b*$^{-/-}$ mutant testes vs. wild-type siblings

Gene name	Mean counts	Fold change	p-adj
si:dkey-11o18.5	1247.15	0.05	1.54E-130
cftr	1088.02	0.12	1.47E-85
elovl5	671.14	0.05	5.80E-77
tmprss3b	117.11	0.09	1.24E-56
lrata	247.17	0.07	4.74E-53
slc5a8l	717.11	0.04	1.77E-43
zgc:113208	1399.85	0.44	8.75E-42
npas2	339.39	0.27	2.46E-40
idh1	3348.09	0.14	3.81E-40
slc17a7b	469.99	0.02	1.29E-37
oat	1934.95	0.14	1.32E-36
slc9a3.1	571.97	0.17	3.35E-35
zgc:194629	132.45	0.04	1.03E-34
grnb	2271.61	0.57	1.10E-32
slc16a10	3112.64	0.21	4.31E-32
slc12a2	4426.50	0.44	1.77E-31
slc7a9	541.24	0.08	1.92E-31
angptl4	783.67	0.10	4.77E-31
fam167b	1697.11	0.10	6.97E-31
si:ch211-153l6.6	243.09	0.04	1.01E-29
cldn2	1316.52	0.30	1.31E-29
epas1a	855.28	0.28	1.48E-29
tuba7l	47548.99	0.54	6.16E-29
hnrnpd	5189.17	0.65	2.20E-28
bpnt1	842.46	0.30	3.90E-28
slc7a6	3509.92	0.31	6.75E-28
adipor2	5649.79	0.56	1.02E-26
CFAP77	3726.03	0.47	4.13E-26
glulb	3976.46	0.17	4.21E-26
cth	2752.05	0.22	4.21E-26

Table 5.5. Top 30 most significantly upregulated genes in *fdx1b*$^{-/-}$ mutant testes vs. wild-type siblings

Gene name	Mean counts	Fold change	p-adj
si:dkey-27p18.3	163.25	63.07	1.14E-63
id1	2748.66	3.88	5.91E-55
tnfsf10l4	83.84	13.40	1.79E-50
si:dkey-183j2.10	1200.84	27.62	4.77E-31
abi3bpb	696.18	15.73	2.97E-27
BX546499.1	99.33	3.78	8.33E-26
cxxc5b	157.82	3.50	9.90E-24
caskin1	626.94	3.38	1.67E-23
sart3	4188.49	1.76	3.68E-23
si:ch211-195m9.3	471.11	5.85	2.79E-22
noxo1b	572.20	1.69	9.65E-22
nsg2	659.22	2.44	2.00E-21
erap2	450.17	2.20	2.18E-21
scube1	1222.96	4.02	5.94E-21
ghrb	556.99	2.40	5.94E-21
gabra5	164.73	4.32	3.75E-20
cxxc5a	347.58	2.00	1.51E-19
thrb	792.56	1.70	1.69E-19
antxr1c	910.66	2.36	2.51E-19
nanos1	187.00	4.73	3.60E-19
cpeb4a	620.86	1.71	5.53E-19
znf362a	197.01	2.16	3.93E-18
ezh1	386.72	2.08	1.03E-17
sptlc2a	402.05	1.92	2.66E-17
gata4	153.30	2.62	3.04E-17
castor2	1095.68	1.59	3.04E-17
aldh6a1	1035.06	1.96	4.49E-17
c4	5564.94	4.41	6.59E-17
prickle1a	180.51	2.76	8.48E-17
apbb3	152.89	5.61	1.04E-16

5.3.3 – Gene ontology and KEGG pathway enrichment analysis

Statistical overrepresentation analysis for downregulated genes revealed overrepresentation of genes involved in microtubule formation and transport, cilia formation and function, reproduction, and the cell cycle and meiosis (**Table 5.6**). The same analysis, but for genes upregulated in *fdx1b*$^{-/-}$ mutant testes, uncovered overrepresentation of genes involved in steroid metabolism, immune regulation, cytoskeleton organisation and cell migration or motility (**Table 5.7**).

Table 5.6. Selected gene ontology terms overrepresented in genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish.

GO term	Description	Enrichment (N, B, n, b)	P-value	FDR
GO:0007017	microtubule-based process	4.31 (11987,280,1063,107)	3.22E-42	2.54E-38
GO:0060271	cilium assembly	4.51 (11987,145,1063,58)	1.91E-24	3.77E-21
GO:0006996	organelle organization	1.98 (11987,938,1063,165)	6.64E-19	6.53E-16
GO:0003341	cilium movement	7.08 (11987,43,1063,27)	1.89E-18	1.65E-15
GO:0000226	microtubule cytoskeleton organization	3.42 (11987,165,1063,50)	2.31E-15	1.3E-12
GO:0070286	axonemal dynein complex assembly	8.46 (11987,16,1063,12)	2.9E-10	1.04E-7
GO:0001578	microtubule bundle formation	8.46 (11987,16,1063,12)	2.9E-10	1.09E-7
GO:1903047	mitotic cell cycle process	2.61 (11987,186,1063,43)	3.25E-9	1.02E-6
GO:0022414	reproductive process	2.26 (11987,180,1063,36)	2.59E-6	6.59E-4
GO:0007049	cell cycle	2.14 (11987,200,1063,38)	5E-6	1.16E-3
GO:0070192	chromosome organization involved in meiotic cell cycle	5.34 (11987,19,1063,9)	1.34E-5	2.69E-3
GO:0007059	chromosome segregation	3.59 (11987,44,1063,14)	1.55E-5	3.05E-3
GO:0051321	meiotic cell cycle	4.18 (11987,27,1063,10)	5.97E-5	1.02E-2
GO:0007130	synaptonemal complex assembly	9.02 (11987,5,1063,4)	2.86E-4	3.75E-2

N, number genes in background list; B, genes in background annotated with GO term; n, genes in target list; b, genes in target list annotated with GO term. Enrichment = (b/n)/(B/N).

Table 5.7. Selected gene ontology terms overrepresented in genes upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish.

GO term	Description	Enrichment (N, B, n, b)	P-value	FDR
GO:0002376	immune system process	4.43 (11987,255,371,35)	9.19E-14	7.23E-10
GO:0072376	protein activation cascade	12.92 (11987,25,371,10)	1.54E-9	4.05E-6
GO:0019882	antigen processing and presentation	17.62 (11987,11,371,6)	3.42E-7	3.37E-4
GO:0048870	cell motility	2.80 (11987,346,371,30)	3.39E-7	3.82E-4
GO:0040011	locomotion	2.68 (11987,374,371,31)	5.77E-7	5.05E-4
GO:0008207	C21-steroid hormone metabolic process	25.85 (11987,5,371,4)	4.41E-6	2.89E-3
GO:0006959	humoral immune response	8.38 (11987,27,371,7)	1.34E-5	8.09E-3
GO:0060326	cell chemotaxis	5.62 (11987,46,371,8)	7.27E-5	2.6E-2
GO:0030866	cortical actin cytoskeleton organization	7.18 (11987,27,371,6)	1.44E-4	3.91E-2

N, number genes in background list; B, genes in background annotated with GO term; n, genes in target list; b, genes in target list annotated with GO term. Enrichment = (b/n)/(B/N)

Analysis for enrichment of biological pathways was performed using lists of up- and down-regulated significant DEGs (Additional file 1) (DAVID accessed September 2019). Enrichment of biological pathways in the downregulated gene list identified pathways relating to the cell cycle and tight junctions (**Table 5.8**). Enrichment of biological pathways in the upregulated gene list identified two significantly enriched pathways: ECM-receptor interaction and herpes simplex infection (**Table 5.9**).

Table 5.8. KEGG pathways identified by gene set enrichment analysis of genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish.

Term	# of genes in pathway	Fold Enrichment	P-value	Benjamini
Cell cycle	23	2.170669	6.70E-04	**0.029472**
Oocyte meiosis	22	2.277224	4.72E-04	**0.031164**
Tight junction	15	2.601742	0.001389	**0.045505**
Biosynthesis of antibiotics	32	1.910364	4.69E-04	0.06088
p53 signaling pathway	13	2.490424	0.00481	0.121218
Cardiac muscle contraction	13	2.418237	0.006143	0.128561
Progesterone-mediated oocyte maturation	16	2.074385	0.008721	0.154373
Oxidative phosphorylation	19	1.890465	0.010082	0.156114
Metabolic pathways	104	1.211313	0.015355	0.205782
Ubiquitin mediated proteolysis	17	1.653026	0.048123	0.483604

Table 5.9. KEGG pathways identified by gene set enrichment analysis of genes upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish.

Term	# of genes in pathway	Fold Enrichment	P-value	Benjamini
ECM-receptor interaction	11	4.538369	1.16E-04	**0.011739**
Herpes simplex infection	15	2.827942	6.92E-04	**0.034711**
Intestinal immune network for IgA production	5	7.907764	0.002979	0.09646
RIG-I-like receptor signaling pathway	7	3.907366	0.008066	0.186578
Focal adhesion	15	2.023788	0.014634	0.259729
Cell adhesion molecules (CAMs)	8	2.743899	0.024864	0.348206
Regulation of actin cytoskeleton	14	1.888869	0.031596	0.373636
Inositol phosphate metabolism	7	2.729803	0.040882	0.412687

5.3.4 - Upregulation of genes encoding steroidogenic enzymes in the testes of *fdx1b*$^{-/-}$ mutant zebrafish

Genes annotated with the GO term "C21-steroid hormone biosynthetic process" were significantly enriched in the population of genes upregulated in *fdx1b*$^{-/-}$ mutant testes. *StAR, cyp11a1, cyp17a1, cyp17a2* and *cyp11c1* were all significantly upregulated, whereas *cyp11a2, hsd3b1, hsd17b3* and *hsd11b2* expression was not affected. Expression of *nr5a1a (ff1b)*, an important regulator of steroidogenesis, was also significantly upregulated.

5.3.5 - Genes encoding tight junction proteins are downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish

In mammals, the Sertoli cell (blood-testis) barrier consists of tight junctions, ectoplasmic specialisations, desmosomes, and gap junctions, with tight junctions thought to be the most important interaction regulating this structure. Tight junctions form between adjacent Sertoli cells, and claudins and occludins are of great importance in these interactions (Mruk and Cheng, 2010; Mruk and Cheng, 2015). Of 9 claudin genes with counts of >300, 6 were downregulated in *fdx1b*$^{-/-}$ mutant testes, whilst the only occludin gene detected, *oclna,* was also downregulated. Several other tight junction components such as *crumbs (crb3a), α-actinin 3a (actn3a)* and *tight junction protein 2a (tjp2a)* were also downregulated (**Figure 5.3**). *Tjp2b* was significantly upregulated 1.4-fold in *fdx1b*$^{-/-}$ mutant testes compared to wild-type.

5.3.6 - Genes encoding extracellular matrix components are upregulated in *fdx1b*$^{-/-}$ mutant testes

Genes annotated to the KEGG pathway "ECM receptor interactions" were significantly enriched in the population of genes upregulated in *fdx1b*$^{-/-}$ mutant testes (**Figure 5.4**). We have observed

upregulation of genes encoding extracellular matrix components including collagens (*col1a1a, col1a2, col6a4a*), laminins (*lama4, lama5, lamb2*), integrin α1 (*itga1*), *hspg2* (encodes perlecan) and agrin (*agrn).* Several of these proteins are basement membrane constituents (laminins, perlecan, agrin, collagen VI) (Groulx et al., 2011; Pozzi et al., 2017). Basement membranes are extracellular matrices and in the testes cover the basal surfaces of the seminiferous tubules. Type IV collagens are the principal collagen component of basement membranes, however expression of genes encoding these proteins were unaffected by mutation of *fdx1b*. Type VI, and XVIII collagens are also basement membrane components, several genes encoding these proteins (*col6a2, col6a3, col6a4a, col18a1a*) were upregulated in the testes of fdx1b$^{-/-}$ mutant zebrafish. Of the nine laminin genes which surpassed the threshold 300 reads, 4 were upregulated (see above), 1 was downregulated (*lamb1a*), and the remainder were not affected by mutation of *fdx1b* (*lama1, lamb1b, lamc1, lamc2*). Several additional basement membrane components were also upregulated, including *nid2* and *dag1*. Integrins are transmembrane receptors which anchor the cell to extracellular matrix proteins, out of 17 integrins with >300 counts, 6 were significantly upregulated whilst 3 were significantly down regulated.

5.3.7 - Microtubule related processes are enriched in the population of downregulated genes in the *fdx1b*$^{-/-}$ mutant testis

The roles of microtubules within the testes are varied, ranging from maintenance of Sertoli cell shape, mitotic and meiotic cell division and development of spermatozoon structure (O'Donnell and O'Bryan, 2014). Several microtubule related gene ontology terms were enriched in our list of genes downregulated in *fdx1b*$^{-/-}$ mutant testes, including “microtubule-based process”, “cilium assembly” and “chromosome segregation”. Microtubules are an important cytoskeleton component and contribute to maintenance of Sertoli cell shape and polarisation (Vogl et al., 2008). In addition to their role in maintaining Sertoli cell structure, microtubules are also important throughout

spermatogenesis, being involved in mitotic and meiotic cell division, and sperm manchette and flagella development (O'Donnell and O'Bryan, 2014).

5.3.8 - Genes regulating the cell cycle and meiosis are downregulated in *fdx1b*$^{-/-}$ mutant testes

Genes associated with the "cell cycle" KEGG pathway and the GO terms "chromosome organization involved in meiotic cell cycle" and "synaptonemal complex assembly" were enriched in our list of genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. As described above, microtubule dynamics are important in both mitotic and meiotic cell division, however genes identified in the above mentioned KEGG pathway and GO terms may highlight additional stages in these processes affected by mutation of *fdx1b.* In particular, many genes with regulatory roles in anaphase were downregulated such as (*anapc11, bub1bb, mad2l1, anapc10, cdc20, espl1, pttg1, stag1b, smc1b, smc3*) (**Figure 5.5**). During the cell cycle, cells must pass several checkpoints to ensure correct cell division. These processes are dependent on cyclin dependent kinases and their associated cyclins. We have observed downregulation of *cdk1* and *cdk2*, as well as the associated cyclins *ccna2, ccnb1*, *ccnb2* and *ccnb3* (**Figure 5.5**). Misregulation of mitosis or meiosis may be a contributing factor to decreased spermatogenesis in androgen deficient zebrafish.

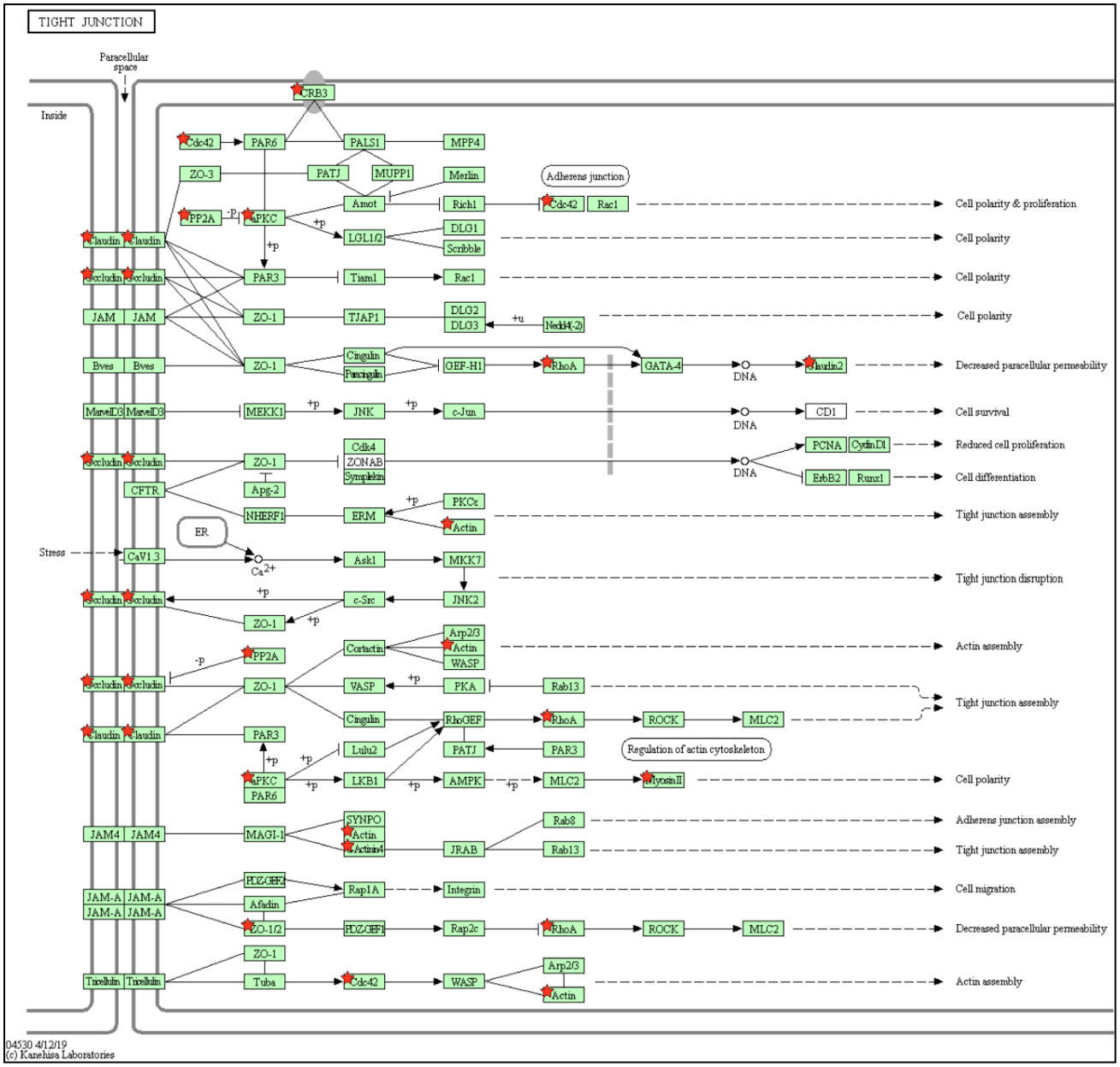

Figure 5.3. Genes annotated to the tight junction KEGG pathway were significantly enriched in genes upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Differentially expressed pathway components are indicated with red stars.

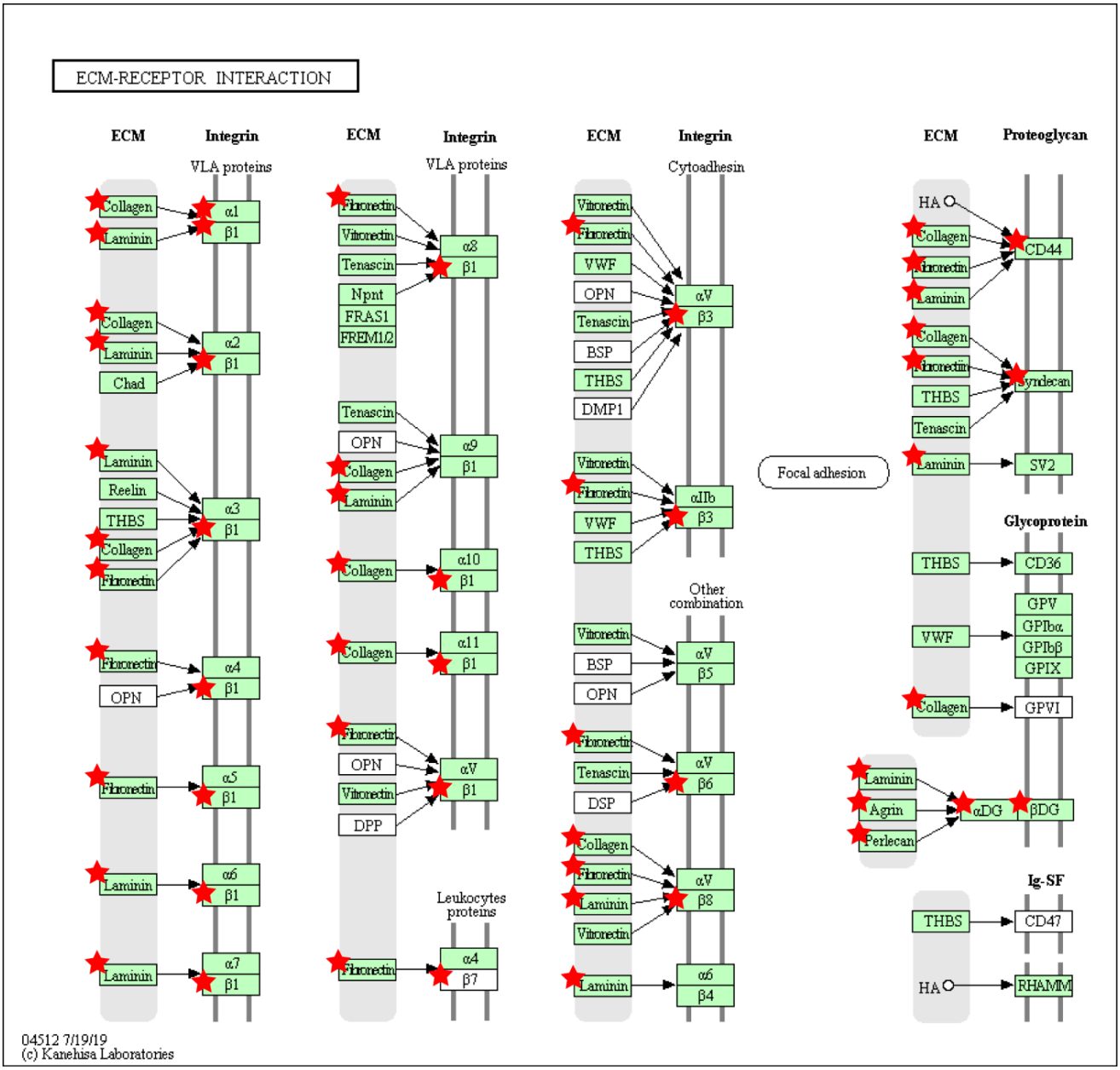

Figure 5.4. Genes annotated to the ECM-receptor interaction KEGG pathway were significantly enriched in genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Differentially expressed pathway components are indicated with red stars.

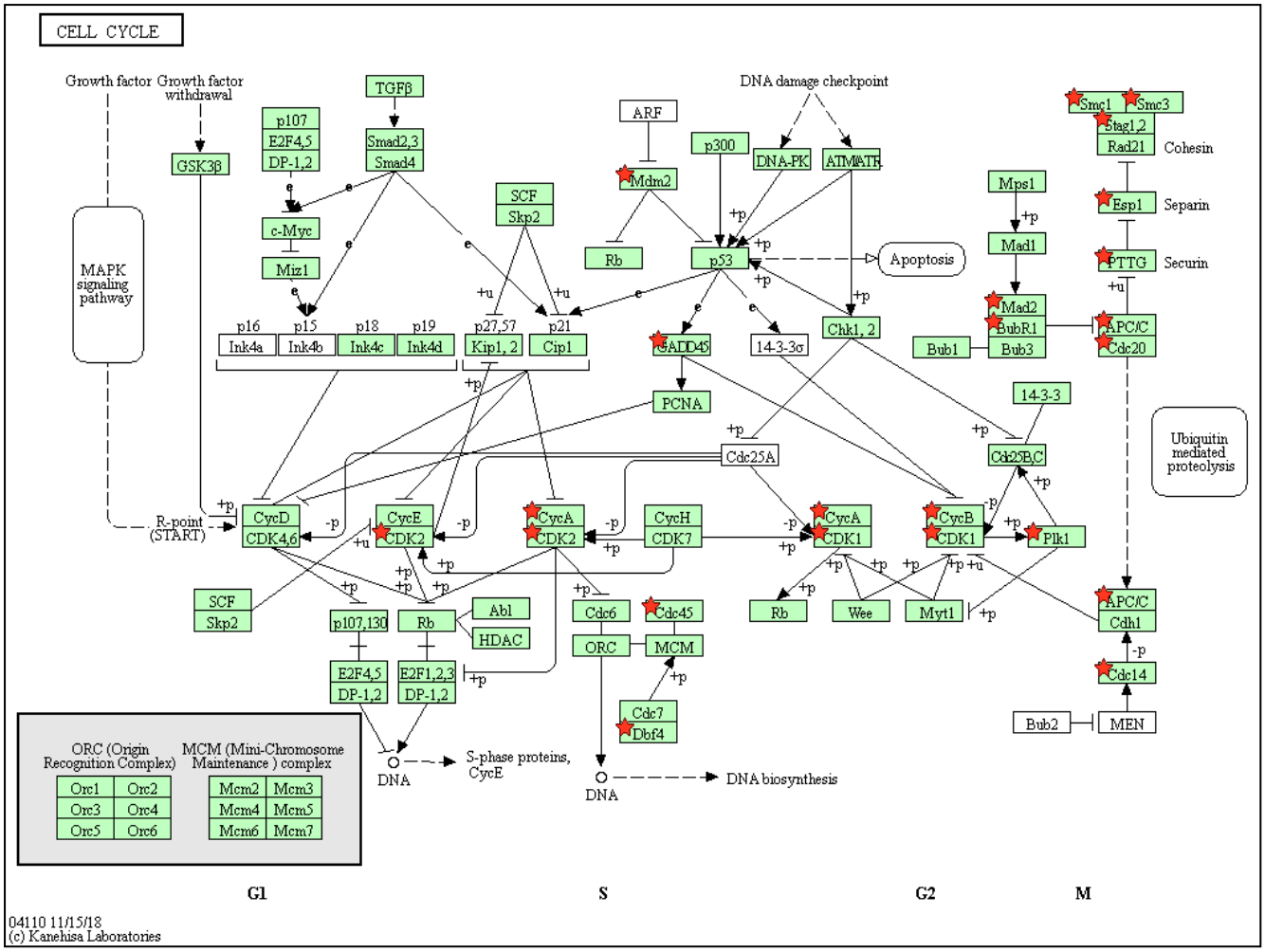

Figure 5.5. Genes annotated to the cell cycle KEGG pathway were significantly enriched in genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Genes with regulatory roles in anaphase were frequently affected. Differentially expressed pathway components are indicated with red stars.

5.3.9 - Initial analysis of the small RNAome of the testes of *fdx1b*$^{-/-}$ mutant zebrafish vs. wild-type siblings

Small RNA libraries prepared from testis RNA (n=5) were sequenced to a depth of ~10 million reads. Quality control by FastQC/MultiQC (Andrews, 2010; Ewels et al., 2016) confirmed high sequence quality, however two metrics, per base sequence content and sequence duplication levels were failed (**Figure 5.6**). Unfragmented small RNA libraries contain high levels of duplicate sequences so failure of this metric is expected. Failure of the per base sequence content metric is likely due to presence of adapter or barcode and is unlikely to affect downstream analysis. Sequence length distribution revealed two distinct peaks representing miRNAs and piRNAs. miRNAs are normally ~22nt in whereas piRNAs are ~26-30nt (Bartel, 2018; Houwing et al., 2007), the peaks seen in Figure 5.7A are shifted by 4nt due to presence of 5' adapter. As would be expected in testes tissue (Houwing et al., 2007) piRNAs constituted the most numerous small RNA species, accounting for ~75-85% of all reads. The second most abundant RNA species were miRNAs, which accounted for ~15-25% of all reads (**Figure 7B**). A handful of snoRNAs and snRNAs were also detected.

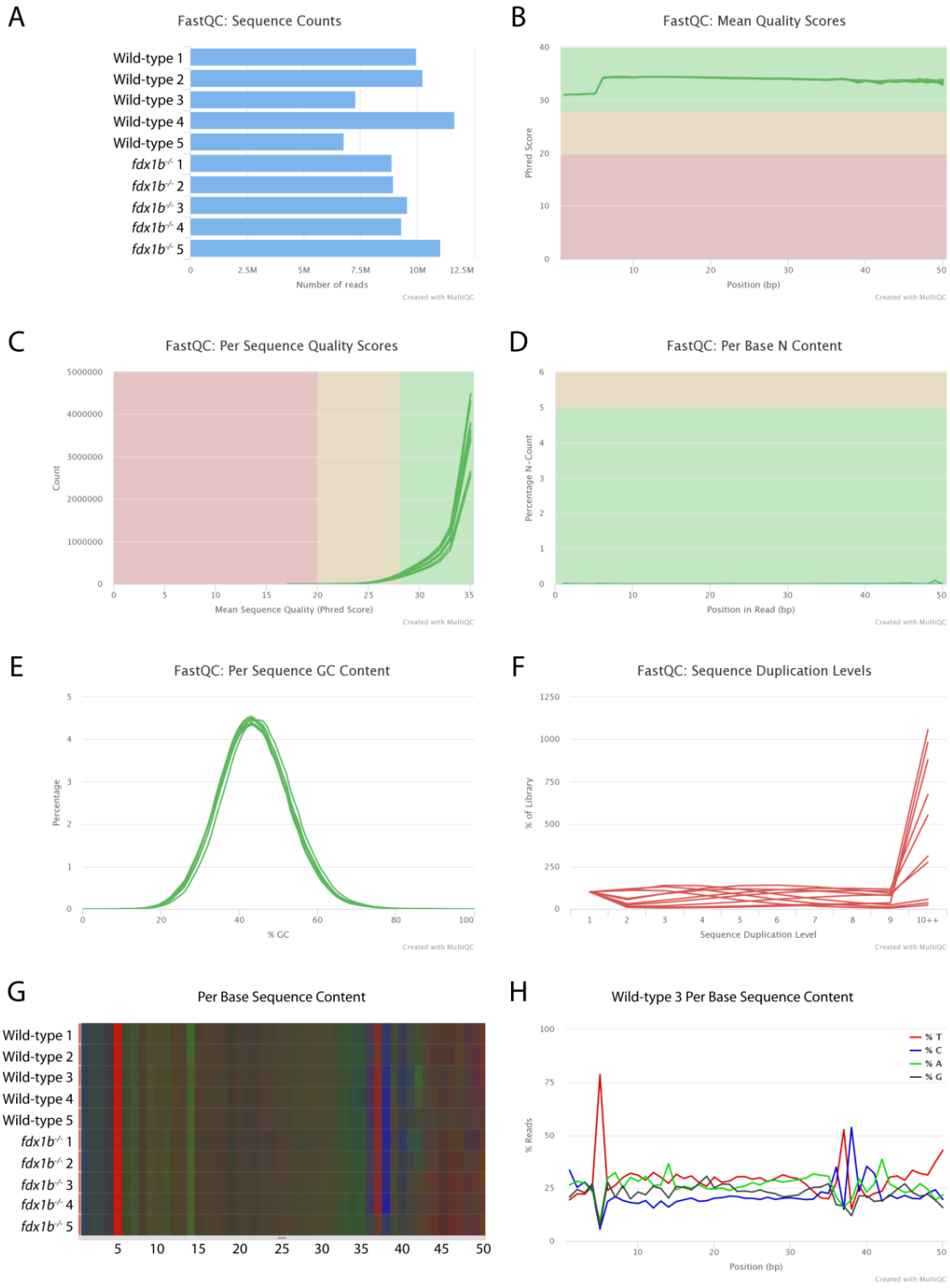

Figure 5.6. FastQC summary for small RNA sequencing datasets. FastQC analyses were combined using the MultiQC tool. Mean quality score, per sequence quality score, per base n content and per sequence GC content metrics (**B-E**) were all passed. All samples failed the sequence duplication and per base sequence content metrics (**F+G**). An example of an individual per base sequence content plot (Wild-type 3) is also provided (**H**).

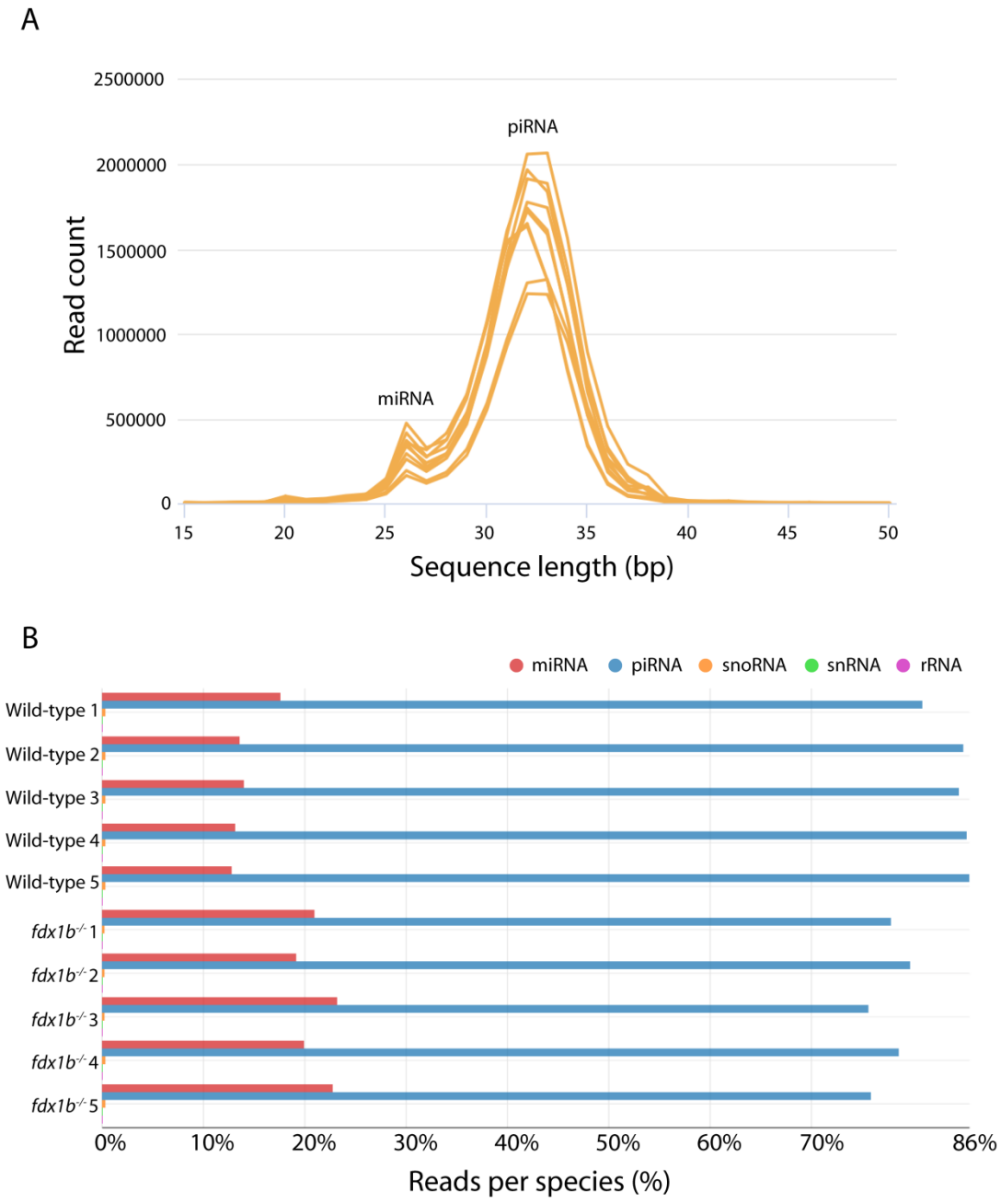

Figure 5.7. Small RNA species distribution in *fdx1b*$^{-/-}$ mutant and wild-type sibling testes. A: Two peaks representing miRNAs and piRNAs were detected. **B:** The most frequently observed species of small RNA were piRNAs (~75-85%), followed by miRNAs (15-25%). A small number of snoRNAs and snRNAs were also detected.

Principal component analysis (PCA) of piRNA counts revealed no clustering of samples (**Figure 5.8A**); however PCA of miRNA counts revealed two distinct clusters of samples representing *fdx1b*$^{-/-}$ mutant or wild-type sibling samples (**Figure 5.8B**). In both PCAs it was clear that two samples, representing one wild-type and one *fdx1b*$^{-/-}$ mutant zebrafish, did not cluster closely with other samples from their groups; these samples were therefore eliminated from further analysis. Given this apparent association between mutation of *fdx1b* and the miRNA transcriptome, and the comparatively extensive annotation of this species in comparison to the piRNAs, the effects of *fdx1b* mutation and steroid deficiency on miRNA expression became the main focus of this investigation.

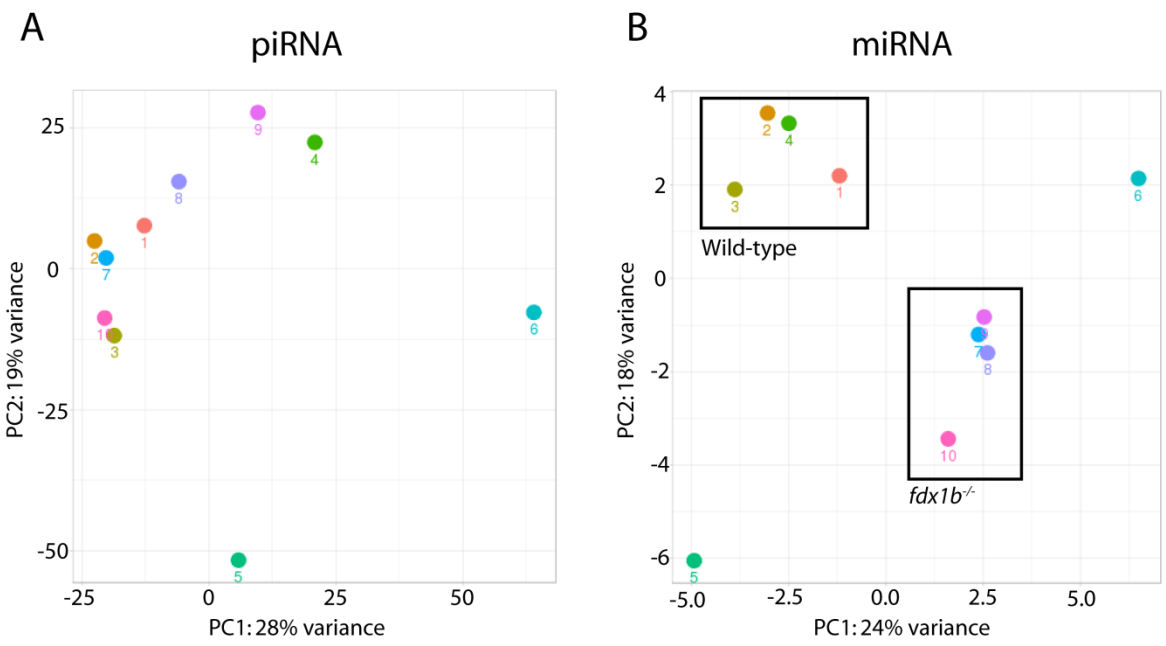

Figure 5.8. Principal component analysis (PCA) of small RNA expression data from *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings. PCA of piRNA expression (**A**) revealed no segregation of wild-types (1-5) and *fdx1b*$^{-/-}$ mutants (6-10), however two samples (5+6) appeared to exhibit a considerably different gene expression profile in comparison to the other samples. PCA plots for miRNA expression (**B**) revealed two distinct groups of samples representing each of the two sample groups. Again, samples 5+6 did not segregate with their respective sample groups.

5.3.10 - Characterisation of differentially expressed miRNAs in the testes of *fdx1b*$^{-/-}$ mutant zebrafish vs. wild-type siblings

A total of 280 previously annotated miRNAs with a mean normalised count of >5 were identified by small RNA sequencing (**Additional file 2**). Of these, 33 were found to be differentially expressed between *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings (**Table 5.10, Figure 5.9**). Amongst these significantly regulated miRNAs, 7 were found to be within the top 30 most abundantly expressed miRNAs across all samples (**Figure 5.10**), indicating that miRNAs important for testis function are misregulated in *fdx1b*$^{-/-}$ mutant zebrafish.

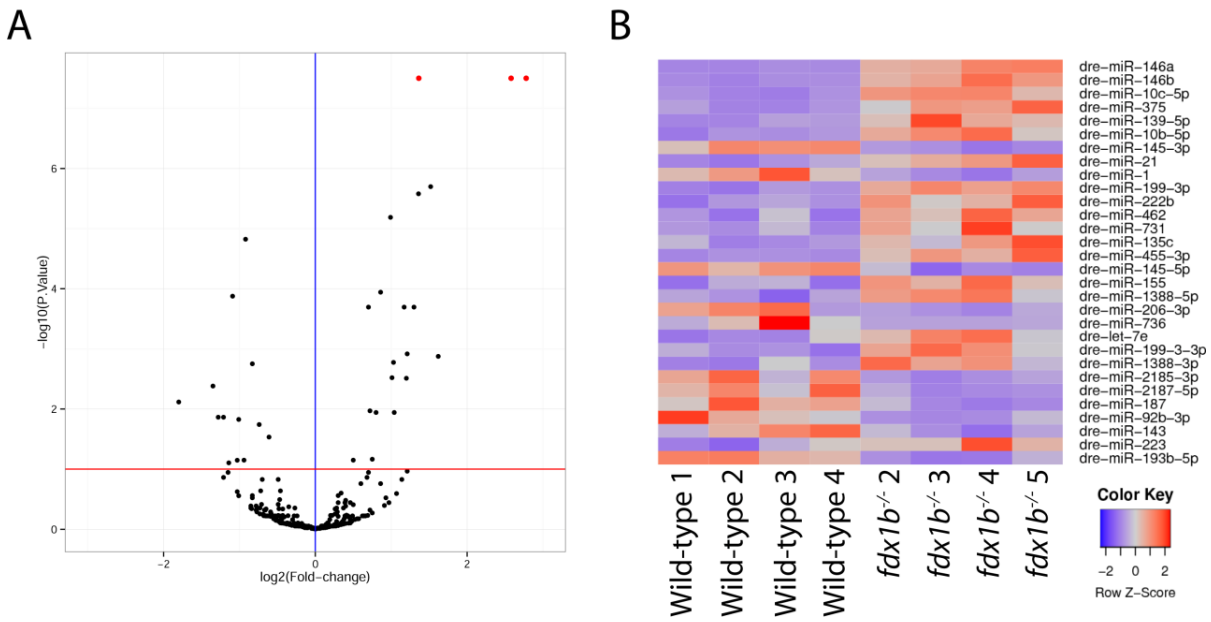

Figure 5.9. 33 miRNAs are differentially expressed in the testes of *fdx1b*$^{-/-}$ mutants compared to wild-type siblings. A: Volcano plot displaying differentially expressed miRNAs, indicating a variety of up- and down-regulated genes, red line indicates p=0.1. P-values were limited to –log10(p) = 4 to fit constraints of plot, affected points indicated by red highlighting. **B:** Heat-map displaying the 30 miRNAs most significantly affected by mutation of *fdx1b.*

Table 5.10. Significantly (p-adj<0.1) differentially expressed miRNAs in the testes of *fdx1b*$^{-/-}$ mutant zebrafish vs. wild-type siblings.

miRNA	p-adj	Wild-type normalised count	*fdx1b*$^{-/-}$ mutant normalised count	log2 fold change
dre-miR-146a	1.94E-40	2578.93	19081.82	2.89
dre-miR-146b	1.01E-26	543.16	3584.85	2.72
dre-miR-135c	1.33E-03	41.75	164.80	1.98
dre-miR-375	2.00E-06	131.09	407.77	1.64
dre-miR-139-5p	2.63E-06	336.86	918.39	1.45
dre-miR-222b	2.02E-04	117.65	316.08	1.43
dre-miR-10c-5p	2.45E-10	379.99	1009.03	1.41
dre-miR-1388-5p	3.08E-03	106.75	270.13	1.34
dre-miR-731	1.21E-03	216.13	544.17	1.33
dre-miR-462	2.02E-04	1212.53	2889.04	1.25
dre-miR-1388-3p	1.14E-02	116.11	259.53	1.16
dre-miR-455-3p	1.68E-03	89.60	192.70	1.10
dre-miR-155	3.01E-03	199.07	421.69	1.08
dre-miR-10b-5p	6.50E-06	1945.18	3963.10	1.03
dre-miR-21	1.14E-04	25929.19	47827.89	0.88
dre-miR-199-3-3p	1.14E-02	179.32	323.23	0.85
dre-miR-223	6.84E-02	291.71	513.23	0.82
dre-let-7e	1.07E-02	6678.17	11291.04	0.76
dre-miR-199-3p	2.02E-04	1290.07	2121.83	0.72
dre-miR-101a	7.07E-02	789.59	1132.28	0.52
dre-miR-143	2.92E-02	25497.31	16441.59	-0.63
dre-miR-92b-3p	1.81E-02	656.05	383.06	-0.78
dre-miR-145-5p	1.77E-03	695.69	382.56	-0.86
dre-miR-145-3p	1.50E-05	1307.21	677.35	-0.95
dre-miR-193b-5p	7.07E-02	72.32	34.38	-1.07
dre-miR-187	1.49E-02	136.62	62.51	-1.13
dre-miR-1	1.33E-04	865.52	388.32	-1.16
dre-miR-183-5p	7.10E-02	64.64	27.85	-1.21
dre-miR-2185-3p	1.37E-02	82.30	30.81	-1.42
dre-miR-96-3p	7.84E-02	119.95	43.69	-1.46
dre-miR-2187-5p	1.37E-02	67.00	23.05	-1.54
dre-miR-206-3p	4.14E-03	172.81	57.58	-1.59
dre-miR-736	7.65E-03	18.51	0.26	-6.17

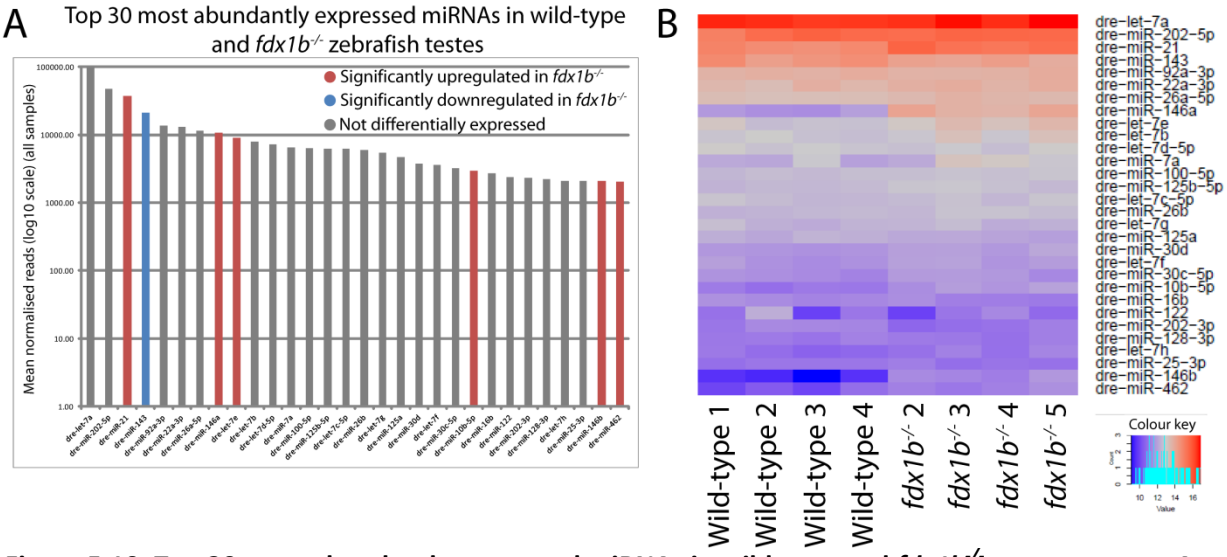

Figure 5.10. Top 30 most abundantly expressed miRNAs in wild-type and *fdx1b*$^{-/-}$ mutant testes. A: Bar chart displaying the relative expression of the top 30 most abundantly expressed miRNAs in wild-type and *fdx1b*$^{-/-}$ mutant testes. Of these genes, seven were differentially expressed; red bars indicate upregulation in *fdx1b*$^{-/-}$ mutant testes, whereas blue bars indicate downregulation. **B:** Heat-map (regularised log transformed counts) of the top 30 most abundantly expressed miRNAs.

5.3.11 - miRNAs with functions in spermatogenesis are differentially expressed in *fdx1b*$^{-/-}$ mutant zebrafish testes

miRNAs are known to play regulatory roles in spermatogenesis (Mobasheri and Babatunde, 2019). Several of the miRNAs found to be differentially expressed in *fdx1b*$^{-/-}$ mutant zebrafish have known roles in spermatogenesis in various species (**Table 5.11**). In particular, upregulation of miRNAs with roles in the early stages of spermatogenesis was evident. miR-146a and miR-146b play important roles in repressing spermatogonial differentiation in mice and both were upregulated ~6-fold in *fdx1b*$^{-/-}$ mutant zebrafish (Huszar and Payne, 2013). miR-21 and miR-222b were also upregulated in *fdx1b*$^{-/-}$ mutant zebrafish, these miRNAs are both thought to play roles in maintaining the spermatogonial stem cell population (Niu et al., 2011; Yang et al., 2013). let-7e was also upregulated in *fdx1b*$^{-/-}$ mutant testes. In contrast to the previously mentioned miRNAs, the let-7 family is thought to promote spermatogonial differentiation (Tong et al., 2011).

Table 5.11. Misregulation of miRNAs with roles in spermatogenesis.

miRNA	log2 fold change	Regulation in *fdx1b*$^{-/-}$ zebrafish	Roles in spermatogenesis
miR-146a	2.89	↑	Repression of spermatogonial differentiation (Huszar and Payne, 2013)
miR-146b	2.72	↑	Repression of spermatogonial differentiation (Huszar and Payne, 2013)
miR-222b	1.43	↑	SSC maintenance (Yang et al., 2013)
miR-21	0.88	↑	SSC maintenance (Niu et al., 2011)
let-7e	0.76	↑	Spermatogonial differentiation (Tong et al., 2011)

5.3.12 - Differential expression of conserved miRNA clusters in *fdx1b*$^{-/-}$ mutant testes

miRNAs are often found in clusters; physically local groups of miRNA genes in the same orientation as one another. We have identified several miRNA clusters in which several members are differentially regulated in *fdx1b*$^{-/-}$ mutant zebrafish.

Both members of the miR-143/miR-145 cluster were significantly down-regulated in *fdx1b*$^{-/-}$ mutant zebrafish (**Table 5.10**). Despite the relative abundance of miR-143 in both zebrafish and mammalian testes, little is known about its function (Huang et al., 2011; Presslauer et al., 2017). However, the miR-143/145 cluster is thought to play roles in inhibiting cell proliferation (Cordes et al., 2009; Sirotkin et al., 2010).

The miR-96/miR-183 cluster was also down-regulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish (**Table 5.10**). miR-96-3p and miR-183-5p were significantly downregulated approximately 2-fold. Expression of a third member, miR-182, was also reduced, however this did not achieve statistical significance. In contrast to the miR-143/miR-145 cluster, this cluster is expressed at very low levels in the zebrafish testes, and is poorly characterised by comparison. Expression of the miR-183-96-182 cluster generally promotes cell proliferation in cancer (Ma et al., 2016), as well as proliferation of granulosa cells in the bovine ovary (Gebremedhn et al., 2016).

miR-462 and -731 are clustered zebrafish miRNAs orthologous to the mammalian miR-191/425 cluster (Schyth et al., 2015). In zebrafish, this miRNA cluster is suggested to play roles in the immune response, hypoxic stress and haematopoiesis (Huang et al., 2015; Huang et al., 2019; Schyth et al., 2015). Both members of the miR-462/731 cluster were upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish (**Table 5.10**).

5.3.13 - The impact of steroid deficiency on the abundance of spermatozoa miRNAs

Several miRNAs previously shown to be abundant in spermatozoa were differentially expressed in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. miR-146a, -462, -21, 199-3p and let-7e were found amongst the top 26 miRNAs expressed in zebrafish spermatozoa and all were upregulated in *fdx1b*$^{-/-}$ mutant testes (Jia et al., 2015). miR-92b-3p and miR-1 are also abundant in zebrafish spermatozoa but were downregulated in *fdx1b*$^{-/-}$ mutant testes (Jia et al., 2015; Presslauer et al., 2017). These findings implicate steroid signalling in correct formation of the spermatozoa small RNA payload.

5.3.14 - Differentially expressed piRNAs in *fdx1b*$^{-/-}$ mutant testes

piRNAs play important roles in transposon silencing, germ cell maintenance and differentiation. We have detected 57 differentially expressed piRNAs in the testes of *fdx1b*$^{-/-}$ mutant zebrafish out of a total of 39103 detected with >5 counts average (**Figure 5.11**) (**Additional file 2**). piRNAs and interacting proteins Piwil1 and Piwil2 are thought to play roles in germ cell maintenance and differentiation, as well as in meiosis (Houwing et al., 2008; Houwing et al., 2007).

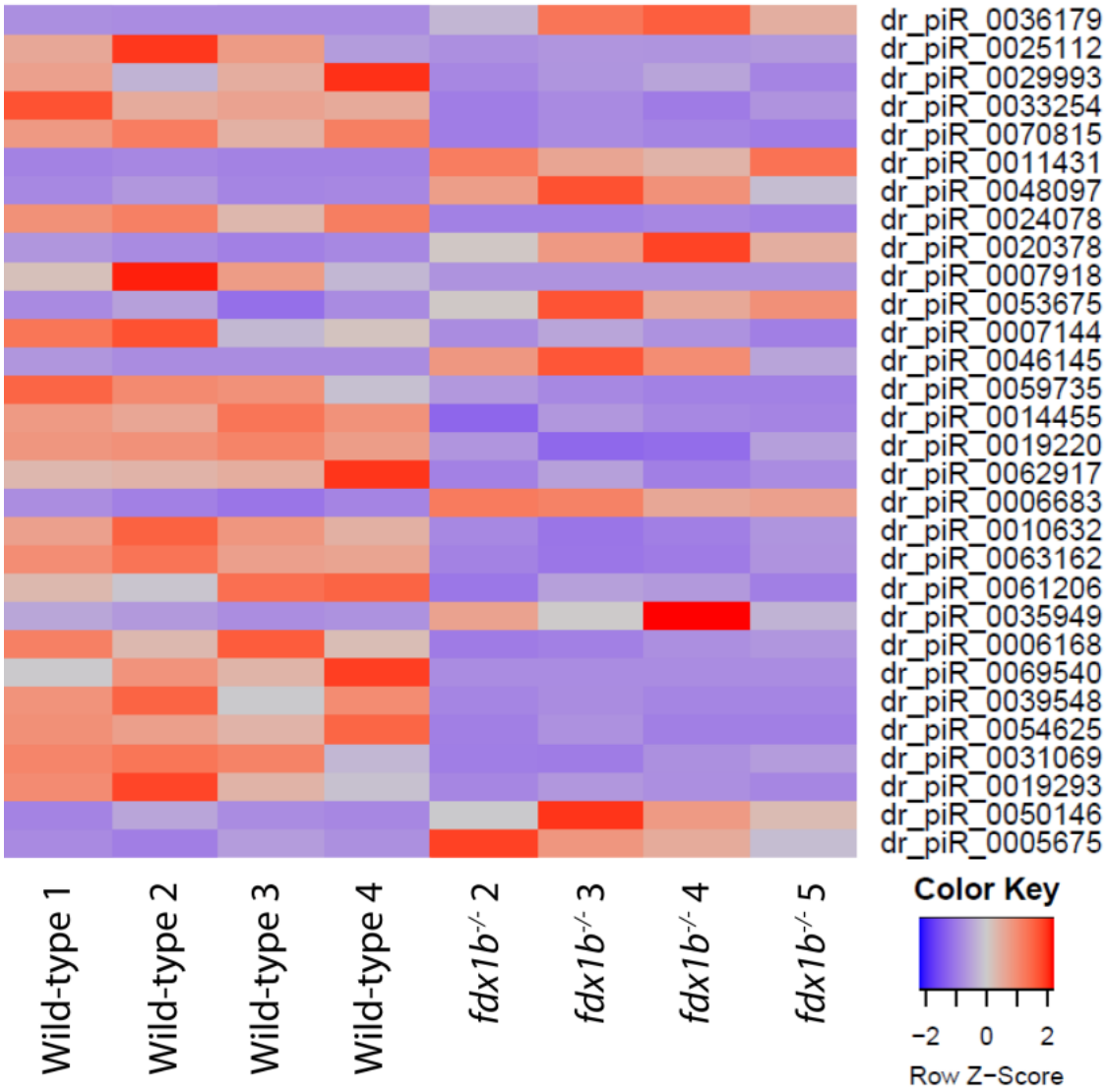

Figure 5.11. Top 30 most significantly differentially expressed piRNAs in *fdx1b*$^{-/-}$ mutant testes.

piRBank accession code indicated on right hand side.

5.4 - Discussion

In order to further explore the molecular mechanisms underlying the reproductive phenotypes of *fdx1b*$^{-/-}$ mutant zebrafish, we have performed RNA sequencing experiments targeting both poly-A tailed transcripts and small RNAs. mRNA sequencing results identified similar dysregulation of genes previously measured by qPCR, including *inha, insl3* and *igf3.* This indicates efficient data analysis and assures accurate representation of the impacts of *fdx1b* mutation on the testis transcriptome.

In chapter 3 it was demonstrated by haematoxylin and eosin staining that the testes of *fdx1b*$^{-/-}$ mutant zebrafish exhibit abnormal organisation of the seminiferous tubules (**Figure 3.10**). In addition, picrosirius red staining indicated that the seminiferous tubules of *fdx1b*$^{-/-}$ mutants may be smaller and greater in number than in wild-type siblings (**Figure 3.11**). A similar phenotype was observed in *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 4.11**), as well as in models of androgen resistance (Crowder et al., 2017; Yu et al., 2018). Despite the consistency of this phenotype in fish exhibiting impaired androgen signalling, no mechanistic perturbation leading to its development has been uncovered. Using mRNA sequencing we have identified aberrant expression of genes related to a number of structural elements in gonad, including microtubules, tight junctions and the basement membrane. Disruption of these elements may contribute to the structural disorganisation observed in the testes of *fdx1b*$^{-/-}$ mutant zebrafish.

The testis basement membrane segregates the germinal and interstitial compartments in both fish and mammals, and thus forms a component of the seminiferous tubule boundary (Uribe et al., 2014). We observed dysregulation of a variety of basement membrane protein encoding genes, including laminins, agrin, perlecan (*hspg2*), nidogen and collagens VI and XVIII. Transmembrane receptors including integrins and dystroglycan (*dag1*) which bind basement membrane proteins were also dysregulated. Type IV collagens are the principal collagen component of basement membranes (Pozzi et al., 2017), however, surprisingly, expression of genes encoding these proteins was unaffected by mutation of *fdx1b*. Literature relating to the effect of basement membrane

protein mutations on testis structure and fertility are scarce, however, mutation of *lama2* in mice results in abnormal testis structure. Laminin α2 deficient mice exhibited seminiferous tubules with small or absent lumina at 26-30dpf and spermatogenesis was impaired (Hager et al., 2005). Mice reach reproductive age at ~6 weeks, *lama2* mutant mice die at 4-5 weeks and therefore fertility was not assessed. *Epas1a* was amongst the most significantly downregulated genes in *fdx1b*$^{-/-}$ mutant testes, mutation of *Epas1* in mice resulted in disruption of the seminiferous tubule basement membrane (Gruber et al., 2010). The evidence presented here supports a role for androgens in regulation of the extracellular matrix and basement membrane in zebrafish testes. Further supporting evidence for this hypothesis is provided by previous studies of the effects of androgen modulation on the gonadal transcriptome in fish. Short term anti-androgen treatment resulted in differential testicular expression of genes annotated with "integrin signalling" in zebrafish (Martinovic-Weigelt et al., 2011), whilst pre-pubertal androgen supplementation in rainbow trout resulted in upregulation of genes annotated with the gene ontology terms "collagen metabolism" and "collagen organisation". Together these findings indicate roles for androgens in extracellular matrix regulation and tubule formation (Rolland et al., 2013).

In mammals, the Sertoli cell barrier separates two regions of the seminiferous tubules: basal and adluminal. Spermatogonia and pre-leptotene spermatocytes reside in the basal compartment whereas cells at more advanced stages are found in the adluminal compartment. The function of the Sertoli cell barrier is to provide an immunologically privileged environment in which spermatogenesis can progress. The Sertoli cell barrier is composed of several types of cell junction, however tight junctions are the most important component (Mruk and Cheng, 2015). We have observed downregulation of genes annotated to the KEGG pathway "tight junction" (**Table 5.8**). In contrast to mammals, the Sertoli cell barrier in fish forms towards the end of, or after, meiosis (Batlouni et al., 2009; Leal et al., 2009; Schulz et al., 2010). Androgens have been shown to regulate the formation and permeability of Sertoli cell tight junctions. In mice expressing an androgen receptor hypomorph allele, *cldn3* expression is reduced and Sertoli cell barrier permeability is

increased (Meng et al., 2005). Sertoli cell-specific androgen receptor knockout mice have an abnormal tight junction ultrastructure and decreased expression of several claudins and tight junction proteins (Chakraborty et al., 2014; Meng et al., 2011). In addition, the immunologically privileged adluminal compartment is compromised (Meng et al., 2011). Testosterone may induce expression of *cldn1* in cultured immature mouse Sertoli cells (Gye, 2003). Dysregulation of genes encoding tight junction proteins in *fdx1b*$^{-/-}$ mutant zebrafish is likely to be a direct consequence of androgen deficiency and may have implications for spermatogenesis and seminiferous tubule structure.

Overrepresentation analysis identified enrichment of genes annotated with microtubule- and microtubule cytoskeleton-related ontology terms within the set of genes downregulated in *fdx1b*$^{-/-}$ mutant testes. Conversely "cortical actin cytoskeleton" annotated genes were enriched in the set of genes upregulated in *fdx1b*$^{-/-}$ mutant testes. Microtubules, along with intermediate filaments and microfilaments, are key cytoskeleton components (Vogl et al., 2008). In mammals, microtubules play an important role in maintaining the columnar structure of Sertoli cells (Vogl et al., 2008), being aligned linearly along the basal-apical axis (O'Donnell and O'Bryan, 2014). Changes in the expression of microtubule associated genes may result in changes to cell shape or polarity, which may in turn have implications for testicular structure. A previous study which investigated differential gene expression in developing zebrafish gonads found that genes annotated with the GO term "cytoskeleton organisation" were enriched in developing testes compared to developing ovaries, indicating a role for this process in maturation of the male gonad (Lee et al., 2017). In addition to their roles in Sertoli cells, microtubules play important roles throughout spermatogenesis including in mitosis, meiosis and manchette and flagellum formation during spermiogenesis (O'Donnell and O'Bryan, 2014). Sperm motility is founded upon the presence of the flagellum, and mutations in genes required for sperm flagellum formation can result in infertility in humans (Lehti and Sironen, 2017). Motile sperm were observed in samples collected from *fdx1b*$^{-/-}$ mutant and wild-type sibling

zebrafish, however in depth analysis of the impact of Fdx1b deficiency on sperm motility was not conducted.

Spermatogonial differentiation is impaired in *fdx1b*$^{-/-}$ mutant zebrafish, as demonstrated by upregulation of *piwil1* and *nanos2* – markers of type A spermatogonia (**Figure 3.15**). Upregulation of *nanos2* was recapitulated in the present RNA-seq dataset (2.3-fold increase, p=3.36E-08); however, surprisingly, no change in *piwil1* expression was detected by RNA-seq. An additional marker of undifferentiated spermatogonia, *thy1* (2.2-fold, *p*-adj= 0.0001), was also expressed more abundantly in *fdx1b*$^{-/-}$ mutant testes in the present RNA-seq experiment, supporting an increased population of these cell types (Huszar and Payne, 2013).

Expression of spermatogenesis marker genes in *cyp11c1*$^{-/-}$ mutant zebrafish testes suggested that entry of spermatogonia into meiosis may also be impaired in androgen deficient zebrafish (**Figure 4.15**). Expression of the spermatocyte marker gene *sycp3* in *fdx1b*$^{-/-}$ testes was found to be unchanged by qPCR (**Figure 3.14**) but downregulated by RNA sequencing (**Additional file 1**), further investigation is required to definitively characterise the effect of *fdx1b* disruption on expression of this gene. Several gene ontology terms relating to meiosis were identified to be enriched in the list of genes downregulated in *fdx1b*$^{-/-}$ testes, including “meiotic cell cycle”, “synaptonemal complex assembly” and “chromosome organization involved in meiotic cell cycle” (**Table 5.6**). Downregulated genes included *sycp3, trip13, rec8b, mns1, mnd1, aurka* and *hormad1* (**Additional file 1**). These findings provide further evidence that entry of spermatogonia into meiosis, as well as spermatogonial differentiation may be impaired in androgen deficient zebrafish.

miR-146a, -146b, 222b and 21 were all upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish, and all have known roles in maintenance of the spermatogonial stem cell population or repression of spermatogonial differentiation in mice and are highly expressed in these cell types (Huszar and Payne, 2013; Niu et al., 2011; Yang et al., 2013). miR-143 and -199a are also highly expressed in undifferentiated spermatogonia in mice (Niu et al., 2011), but have no defined roles in

spermatogonial differentiation. miR-143 was decreased in abundance in *fdx1b*$^{-/-}$ mutant testes whereas abundance of miR-199 was increased.

Increased expression of these miRNAs may simply be a by-product of an increase in the numbers of undifferentiated spermatogonia due to some other mechanism, much like the changes in expression of other marker genes mentioned previously. Alternatively, the increased numbers of undifferentiated spermatogonia observed in *fdx1b*$^{-/-}$ mutant zebrafish may be a direct consequence of increased expression of miRNAs with known roles in repression of spermatogonial differentiation. Several of the miRNAs described above are regulated by retinoic acid, an important inducer of spermatogonial differentiation (Huszar and Payne, 2013; Tong et al., 2011; Yang et al., 2013). Interestingly, interplay between retinoic acid and androgen signalling has been implicated in spermatogonial differentiation in zebrafish (Crespo et al., 2019).

Three miRNA clusters were found to be misregulated in *fdx1b*$^{-/-}$ mutant testes; strikingly, all three have roles in cell proliferation or differentiation processes. However, there currently exists a void in the literature concerning the specific functions of these potentially steroid regulated miRNAs in the testes. As such, these miRNA clusters are prime targets for further elucidation of the roles of miRNAs in the proliferation and differentiation events of spermatogenesis.

In mammals, miRNAs are proposed as a vector for paternal transgenerational inheritance of metabolic phenotypes, cancer risk and stress axis dysregulation (Dupont et al., 2019; Fontelles et al., 2016; Short et al., 2016). For example, exposure of male mice to chronic stress causes upregulation of nine sperm miRNAs and the offspring of these mice exhibit a decreased corticosterone response to stress. This phenotype can be accurately reproduced by zygote microinjection of dysregulated miRNAs (Rodgers et al., 2013; Rodgers et al., 2015). Of these nine miRNAs, only one (miR-375) was significantly upregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Differential expression of the other eight miRNAs in spermatozoa may be masked by expression in immature germ cells or in somatic tissue. miRNA profiling of purified spermatozoa would be required to definitively determine

whether these stress-regulated miRNAs are differentially expressed in the gametes of *fdx1b*$^{-/-}$ mutant male zebrafish.

Sperm miRNAs may play a role in fertilisation and influence the developmental potential of embryos (Yuan et al., 2016). Furthermore, miRNAs are essential for embryonic development in zebrafish (Giraldez et al., 2005), however little is known about the roles, if any, of paternally sourced miRNAs. Analysis of the literature reveals that several miRNAs are abundant in the zebrafish embryo prior to maternal-zygotic transition, as well as in spermatozoa, but absent from mature oocytes (Jia et al., 2015; Presslauer et al., 2017; Yao et al., 2014). These observations suggest that presence of these miRNAs in the early embryo is due to paternal contribution. These miRNAs include miR-199, miR-92b, miR-146a and miR-1, which were differentially expressed in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. In zebrafish, miR-1 has roles in angiogenesis (Lin et al., 2013) and sarcomeric actin organization (Mishima et al., 2009), whilst miR-92 play roles in left-right symmetry and endoderm formation (Li et al., 2011).

We have used RNA sequencing technologies to characterise differentially expressed mRNAs and small RNAs in the testes of androgen and cortisol deficient *fdx1b*$^{-/-}$ mutant zebrafish. Androgen deficient or resistant zebrafish exhibit a disorganised tubular structure in the testes, prior to this study no perturbation leading to this phenotype has been put forward. We have identified dysregulation of genes regulating several testis structural elements: the basement membrane, Sertoli cell barrier and microtubule related pathways. Dysregulation of one or more of these pathways may be responsible for testis disorganisation in fish exhibiting disrupted androgen signalling. Androgen deficient or resistant zebrafish also exhibit impaired spermatogonial differentiation. We have identified upregulation of several miRNAs which in mammals play roles in regulation of spermatogonial differentiation. As such, our work hints at a conserved role for these miRNAs in regulation of spermatogonial differentiation in mammals and fish.

Chapter 6 – Description of an infertility phenotype in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant female zebrafish

6.1 - Introduction

As described in chapter 3, *fdx1b*$^{-/-}$ mutant zebrafish may possess either ovaries or testes. *Fdx1b*$^{-/-}$ mutant female zebrafish and their wild-type siblings exhibit comparable secondary sex characteristics and no obvious difference in gonadal morphology is apparent (**Figure 3.1**). Like Fdx1b deficient male zebrafish, Fdx1b deficient female zebrafish are also heavier than their wild-type siblings, however no difference in length was recorded (**Figure 3.2**). Finally, *fdx1b*$^{-/-}$ mutant female zebrafish are infertile in response to conventional breeding techniques (**Table 3.1**). Similarly to *fdx1b*$^{-/-}$ mutant zebrafish, *cyp11c1*$^{-/-}$ mutant zebrafish could also possess either ovaries or testes (**Chapter 4**). *Cyp11c1*$^{-/-}$ mutant females also exhibited similar secondary sex characteristics to wild-type sibling females (**Figure 4.4**).

Steroids, principally oestrogens, are crucial for the development and maintenance of the ovary in zebrafish. Mutation of ovarian aromatase, which is crucial for oestrogen biosynthesis results in robust masculinisation (Lau et al., 2016). Oestrogen treatment of *cyp19a1a* mutant zebrafish rescued the all male phenotype, however ovaries transformed to testes after treatment was withdrawn, indicating that oestrogens are essential for ovary maintenance throughout the life course (Yin et al., 2017).

Adult zebrafish ovaries are elongated structures consisting of oocytes at various stages of development (Menke et al., 2011). Following oogenesis, which is the transformation of oogonia into primary oocytes (Selman et al., 1993), these cells enter an extensive maturation process to produce mature oocytes. Primary oocytes (stage I) enter meiosis, which arrests at the diplotene stage, before transformation into cortical alveolar oocytes (stage II). Cortical alveolar oocytes are distinguished by the formation of yolk vesicles and thickening of the follicle lining. Subsequently, cortical alveolar

oocytes progress to the vitellogenic stage (stage III), during this stage the oocyte grows in size due to the production of yolk, and eventually enters the final stage of this process: oocyte maturation. During oocyte maturation (stage IV) meiosis recommences, and this is followed by transformation into a mature egg (stage V) (Koc et al., 2008; Selman et al., 1993). Mature eggs are ovulated and collect in the caudal oviduct (van der Ven and Wester, 2003). Oocyte maturation is dependent on and influenced by a myriad of factors, including steroid hormone and gonadotropin signalling (Nagahama and Yamashita, 2008).

In order to further understand the impact of endocrine disruption on female fertility and gonadal function we have employed a range of techniques to explore the impacts of *fdx1b* and *cyp11c1* mutations on steroidogenesis, ovarian morphology and expression of genes important for ovarian function.

6.2 - Results

6.2.1 - Biometric statistics for *cyp11c1*$^{-/-}$ mutant female zebrafish

Although a trend for increased weight and length in female fish was observed for both *cyp11c1*$^{-/-}$ mutant alleles, the differences were small (**Figure 6.1**). The only significant comparison was increased length in *cyp11c1*$^{-/-}$ mutant female zebrafish carrying the 11bp deletion allele versus wild-type female siblings.

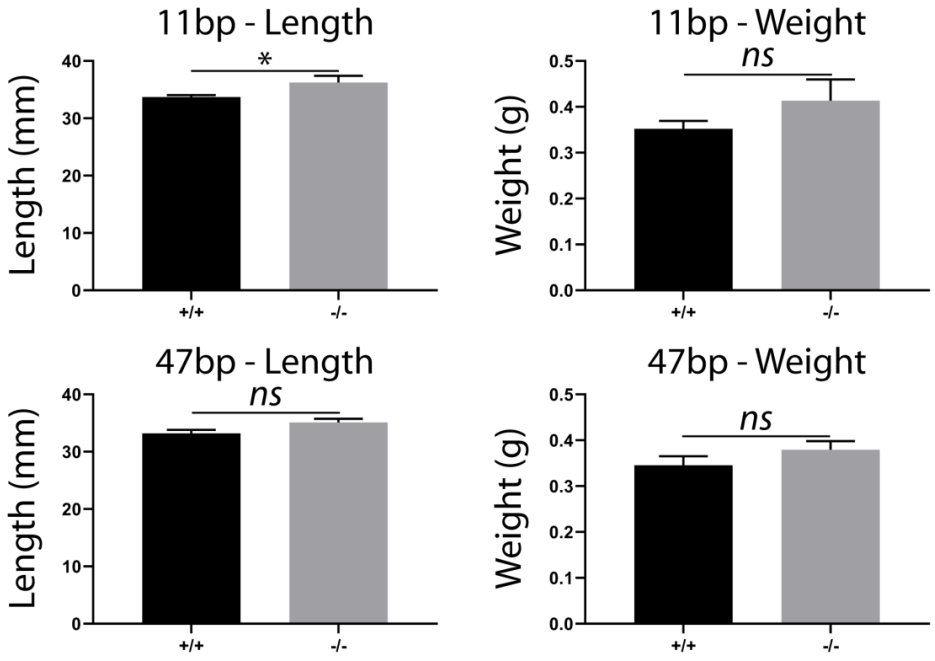

Figure 6.1. Biometric statistics for *cyp11c1*$^{-/-}$ mutant female zebrafish. *Cyp11c1*$^{-/-}$ mutant female zebrafish carrying the 11bp deletion allele were significantly longer than their wild-type siblings, an apparent increase in the length of mutants carrying the 47bp deletion approached significance (11bp, wild-type n=10 mutant n=4, p=0.0138; 47bp, wild-type n=15 mutant n=8, p=0.0583). No difference in the weight of mutant fish was observed for either allele (11bp, wild-type n=10 mutant n=4, p=0.1440; 47bp, wild-type n=15 mutant n=8, p=0.2703). When data were pooled from both alleles length was significantly increased in *cyp11c1*$^{-/-}$ fish (wild-type n=25 mutant n=12, p=0.0041), whereas an apparent increase in weight was not significant (wild-type n=25 mutant n=12, p=0.0764). All results analysed by unpaired *t*-test, * $p<0.05$.

6.2.2 - Cyp11c1 deficient female zebrafish are subfertile

In order to assess the impact of *cyp11c1* mutation on fertility in female zebrafish we exposed *cyp11c1*$^{-/-}$ mutants and wild-type siblings to pair mating with unrelated wild-type males (**Table 6.1**). Crosses of *cyp11c1*$^{-/-}$ mutant female zebrafish carrying the 47bp deletion allele with wild-type males did not produce any fertilised embryos, however outcrosses of female mutants carrying the 11bp deletion allele produced fertilised embryos in 28% of crosses. Outcrosses of 11bp deletion and 47bp deletion wild-type siblings produced fertilised embryos in 80% and 72% of cases respectively. Cyp11c1 deficient are therefore subfertile, as the number of crosses resulting in production of fertilised embryos was considerably lower in outcrosses of mutant fish than outcrosses of wild-type siblings.

Table 6.1. *Cyp11c1*$^{-/-}$ mutant female zebrafish are subfertile

Allele	Genotype	Total number of crosses	# crosses resulting in fertilised eggs
11bp	**+/+ (n=5)**	15	12 (80%)
	-/- (n=6)	18	5 (28%)
47bp	**+/+ (n=6)**	18	13 (72%)
	-/- (n=5)	15	0 (0%)

Table 6.1. *Cyp11c1*$^{-/-}$ mutant and wild-type sibling females were outcrossed with wild-type males on three separate occasions. No crosses involving *cyp11c1*$^{-/-}$ mutant females from 47bp deletion allele produced any fertilised embryos; their wild-type siblings produced fertilised embryos in 72% of crosses. Fertilised embryos were obtained from *cyp11c1*$^{-/-}$ mutant females carrying the 11bp deletion allele in 28% of crosses, their wild-type siblings produced fertilised embryos in 80% of crosses. Fisher's exact test: 11bp $p=0.0049$, 47bp $p<0.0001$.

6.2.3 - Significant disruption of steroidogenesis in Fdx1b and Cyp11c1 deficient female zebrafish

As demonstrated previously, Fdx1b is essential for the activity of Cyp11c1. Cyp11c1 is crucial for the final stage of cortisol biosynthesis. As such, we have observed significantly decreased concentrations of cortisol in *fdx1b*$^{-/-}$ mutant female zebrafish compared to wild-type siblings (**Figure 6.2**). No changes in the concentrations of 17α-hydroxyprogesterone, testosterone or 11-ketotestosterone were detected; however, the concentration of androstenedione was significantly increased in *fdx1b*$^{-/-}$ mutant female zebrafish compared to wild-type siblings, probably due to shunting of cortisol precursors in to the sex steroid pathway (**Figure 1.1**).

In order to demonstrate systemic cortisol deficiency, the expression of glucocorticoid responsive genes *fkpb5* and *pck1* was also quantified (**Figure 6.3**). Expression of both genes was significantly decreased in the livers of *fdx1b*$^{-/-}$ mutant females compared to wild-type siblings, indicating that the cortisol deficiency observed in these fish is sufficient to produce a functional deficit in glucocorticoid regulated gene expression. Expression of the androgen responsive gene *cyp2k22* was not changed by mutations of *fdx1b* in female zebrafish.

The concentration of androstenedione is increased in *fdx1b*$^{-/-}$ mutant female zebrafish, this may result in increased production of oestrogens, of which androstenedione is a precursor (**Figure 1.1**). To assess the impact of Fdx1b deficiency on oestrogen concentrations we measured the expression of oestrogen responsive genes *vtg2* and *esr1*. No change in the expression of these genes was observed, indicating limited effects of Fdx1b deficiency on oestrogen biosynthesis (**Figure 3.6**).

Female Cyp11c1 deficient zebrafish also exhibited significantly reduced cortisol production (**Figure 6.4**). This was in contrast to increased concentrations of 11-deoxycortisol, which can be directly converted to cortisol, a reaction which is catalysed by Cyp11c1. No change in androstenedione concentrations was recorded however testosterone concentrations were significantly reduced in

both alleles. 11β-hydroxyandrostenedione, 11-ketoandrostenedione and 11-ketotestosterone were undetectable in *cyp11c1*$^{-/-}$ mutant female zebrafish or wild-type siblings.

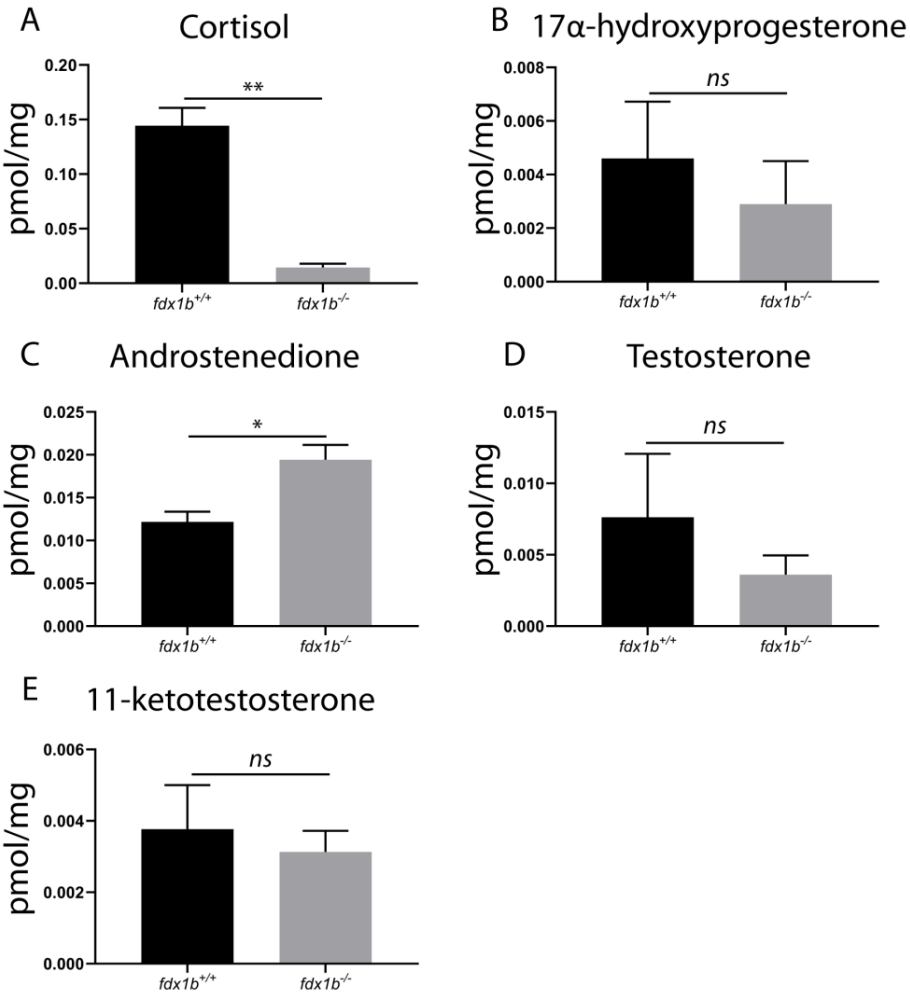

Figure 6.2. Impaired cortisol biosynthesis in *fdx1b*$^{-/-}$ mutant female zebrafish. Steroid concentrations were measured in whole fish bodies using LC-MS/MS (n=3, unless otherwise stated). The concentration of cortisol was significantly decreased in *fdx1b*$^{-/-}$ female zebrafish compared to wild-type siblings (p=0.0015), whereas the concentration of androstenedione was significantly increased (p=0.0255). The concentrations of 17α-hydroxyprogesterone (wild-type n=3, mutant n=2, p=0.6092), testosterone (p=0.4339) and 11-ketotestosterone (p=0.6675) were unchanged by mutation of *fdx1b*. * p<0.05, ** p<0.01.

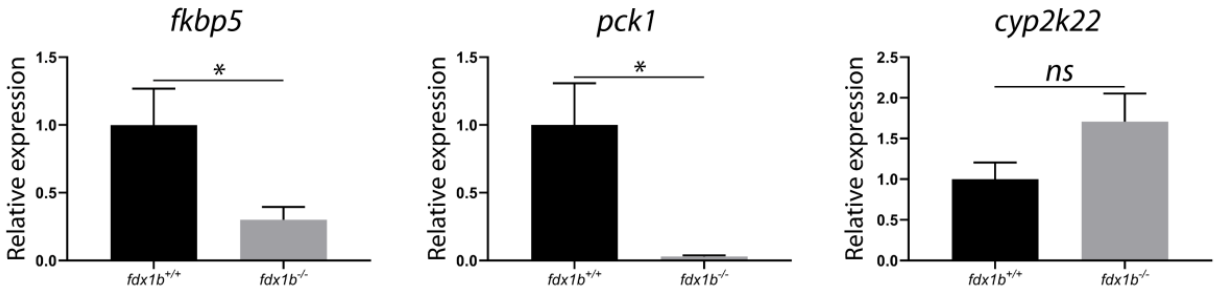

Figure 6.3. Reduced expression of glucocorticoid responsive genes in the livers of *fdx1b*$^{-/-}$ mutant female zebrafish. Expression of the glucocorticoid responsive genes *fkbp5* and *pck1*, and of the androgen responsive gene *cyp2k22*, was quantified by qPCR. The expression of *fkbp5* (wild-type n=8, mutant n=7, p=0.0369) and *pck1* (wild-type n=7, mutant n=6, p=0.0146) was significantly decreased in the livers of *fdx1b*$^{-/-}$ mutant female zebrafish compared to wild-type siblings. No change in the expression of the androgen responsive gene *cyp2k22* was observed (wild-type n=8, mutant n=6, p=0.0878). * p>0.05.

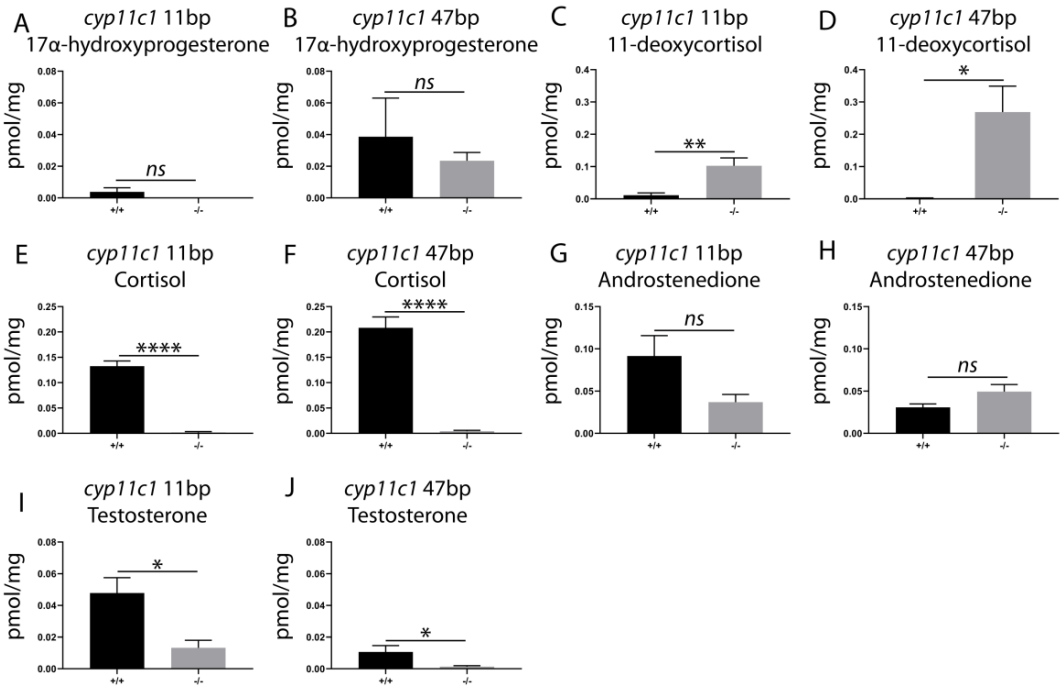

Figure 6.4. Disrupted steroidogenesis in Cyp11c1 deficient female zebrafish. Concentrations of steroid hormones were measured in whole zebrafish bodies by LC-MS/MS (11bp wild-type n=6 mutant n=4; 47bp wild-type n=5 mutant n=6; unless otherwise stated). Concentrations of 17α-hydroxyprogesterone (Mann-Whitney test: 11bp p=0.4667; 47bp p=0.8052) were unaffected by mutation of *cyp11c1*. Concentrations of 11-deoxycortisol were significantly increased in *cyp11c1*$^{-/-}$ mutant females (11bp Mann-Whitney test p=0.0095; 47bp p=0.0146), whereas concentrations of cortisol were significantly reduced (11bp p<0.0001; 47bp p<0.0001). Concentrations of androstenedione (11bp wild-type n=5 mutant n=4 p=0.0961; 47bp p=0.0905) were unaffected by Cyp11c1-deficiency however concentrations of testosterone were significantly reduced (11bp p=0.0259; 47bp Mann-Whitney test p=0.0130).

6.2.4 - Fdx1b-deficient female zebrafish possess larger ovaries and oocyte maturation may be impaired

Fdx1b$^{-/-}$ mutant females are infertile and exhibit disrupted steroidogenesis. To assess the impact of Fdx1b deficiency on ovary structure and oocyte maturation, we undertook histological examination of the adult ovaries of *fdx1b*$^{-/-}$ mutant zebrafish and wild-type sibling females (n=3). Cells at all stages of oocyte maturation were present in both mutant and wild-type zebrafish ovaries (**Figure 6.5**). Strikingly, the ovaries of *fdx1b*$^{-/-}$ mutant zebrafish appeared much larger than those of their wild-type siblings, and this was consistent through several coronal planes (**Figures 6.6+6.7**). However, this increase in size may simply be a product of the larger size of *fdx1b*$^{-/-}$ mutant fish compared to wild-type siblings. In the more ventral coronal plane examined (**Figure 6.6**), 2 of 3 wild-type females exhibited an accumulation of mature oocytes in the caudal oviduct, none of the *fdx1b*$^{-/-}$ mutant zebrafish examined exhibited a comparable accumulation, suggesting a possible reduction in the number of mature oocytes.

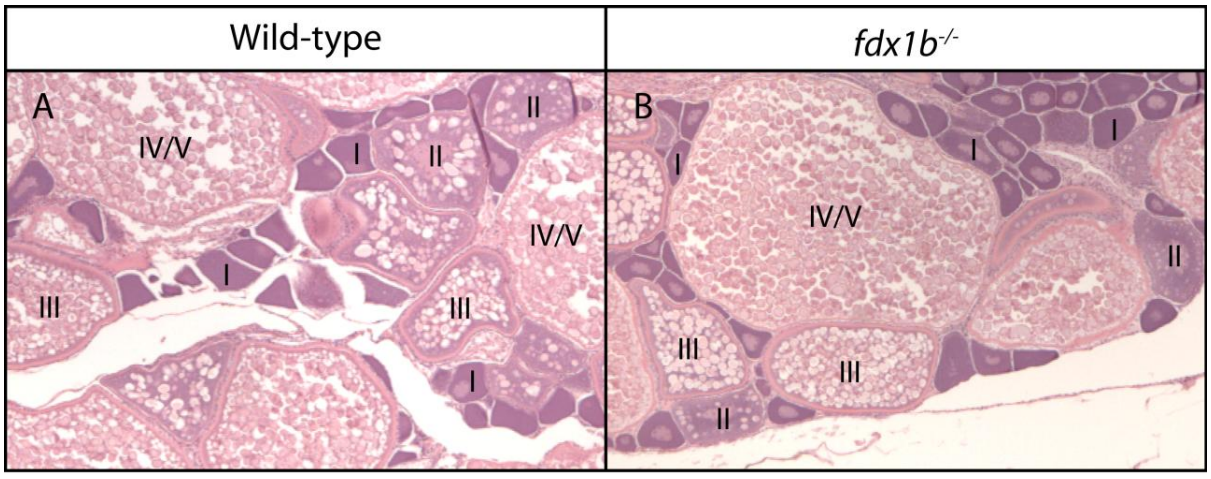

Figure 6.5. All stages of oocyte maturation are represented in the ovaries of *fdx1b*$^{-/-}$ mutant female zebrafish. Primary (stage I), cortical alveolar (stage II), vitellogenic (stage III) and maturing or mature oocytes (stage IV/V) were present in the ovaries of both wild-type (**A**) and *fdx1b*$^{-/-}$ mutant (**B**) zebrafish. Images captured at 5x magnification and are displayed to scale.

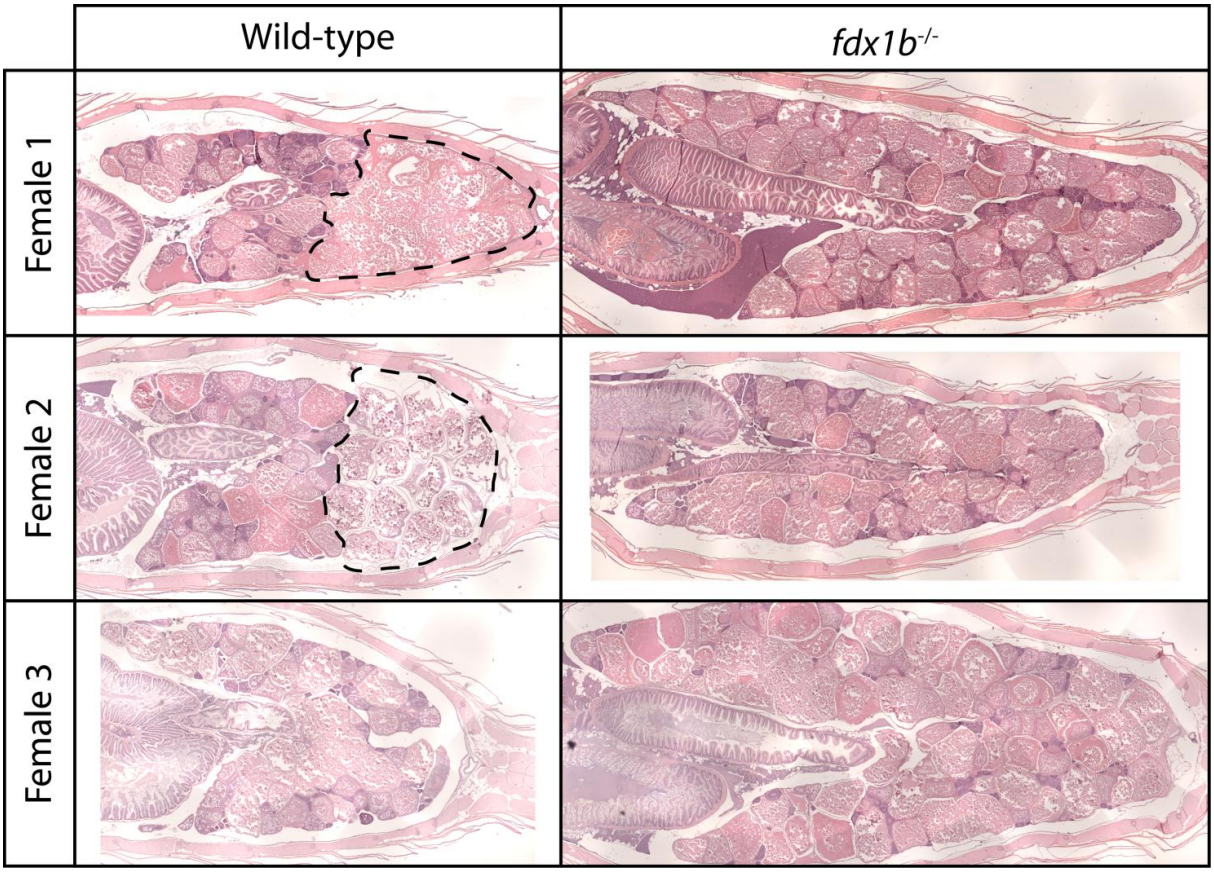

Figure 6.6. Fdx1b deficiency results in increased ovary size and may cause impaired oocyte maturation – ventral coronal plane. Ovaries appeared considerably larger in *fdx1b*$^{-/-}$ mutant zebrafish compared to wild type siblings (n=3). In addition to this, 2 wild-type females (Females 1+2) exhibited an accumulation of mature oocytes in the caudal oviduct (indicated by dashed lines), no such accumulation was observed in any *fdx1b*$^{-/-}$ mutant female. Coronal sections from a more ventral plane through the ovary are displayed here. These sections were deemed to be comparable because of the similar appearance of the intestine and urinary collecting duct in each image. Images captured at 5x magnification and displayed to scale.

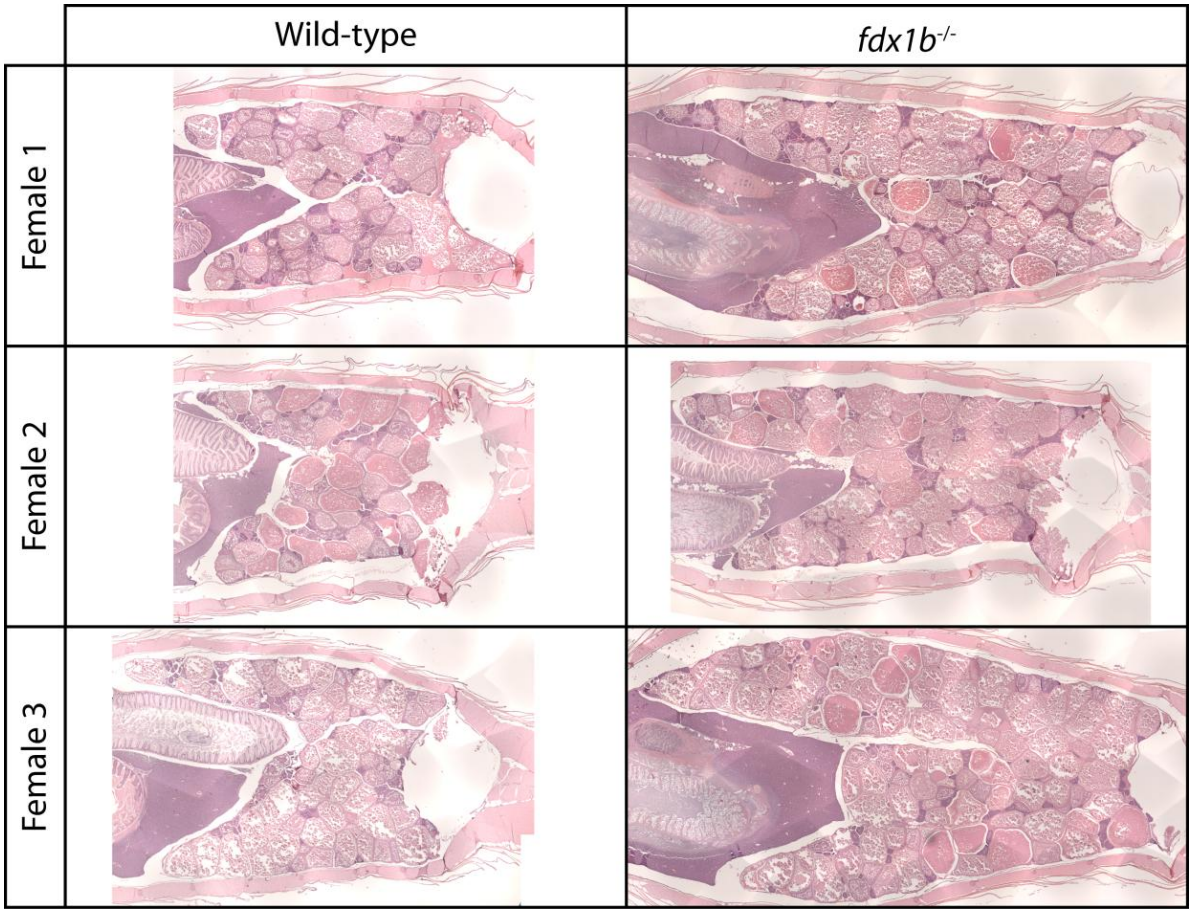

Figure 6.7. Fdx1b deficiency results in increased ovary size and may cause impaired oocyte maturation – dorsal coronal plane. Ovaries appeared considerably larger in *fdx1b*$^{-/-}$ mutant zebrafish compared to wild type siblings (n=3). Coronal sections from a more dorsal plane through the ovary are displayed here. These sections were deemed to be comparable because of the similar appearance of the intestines, liver and swim bladder in each image. Images captured at 5x magnification and displayed to scale.

6.2.5 - Dysregulation of genes associated with sex differentiation and gonadal function in the ovaries of *fdx1b*$^{-/-}$ mutant female zebrafish

qPCR was used to assess the impact of steroid hormone deficiency, as a consequence of homozygous *fdx1b* mutation, on expression of genes with known roles in gonadal differentiation and function (**Figure 6.8**). Expression of two pro-male genes, *sox9a* and *dmrt1*, was significantly increased in the ovaries of *fdx1b*$^{-/-}$ mutant females compared to wild-types. Expression of two pro-female genes, *sox9b* and *foxl2b,* was also significantly increased. An apparent reduction in the expression of *inha* was not significant, and no significant difference was found for the expression of *foxl2a.* Expression of the pro-female ovarian aromatase, which is crucial for oestrogen production, was significantly decreased approximately 7-fold.

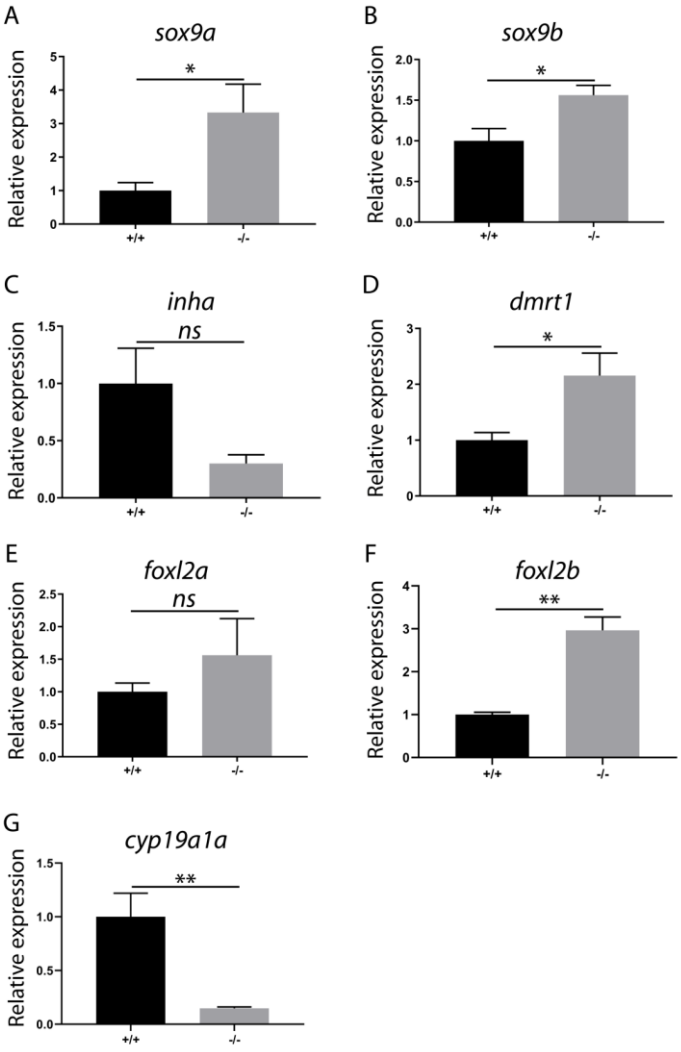

Figure 6.8. Dysregulation of genes associated with sex differentiation and gonadal function in the ovaries of *fdx1b*$^{-/-}$ mutant female zebrafish. The impact of *fdx1b* mutation on ovarian expression of genes known to play important roles in sex differentiation and gonadal function was investigated using qPCR. *Sox9a* (**A**) (wild-type n=8, mutant n=7, p=0.0148), *sox9b* (**B**) (wild-type n=7, mutant n=8, p=0.0108), *dmrt1* (**D**) (wild-type n=6, mutant n=7, p=0.0272) and *foxl2b* (**F**) (wild-type n=3, mutant n=3, p=0.0272) were all significantly upregulated in the ovaries of *fdx1b*$^{-/-}$ mutant females. In contrast no change in the expression of *foxl2a* (**E**) was observed (wild-type n=3, mutant n=3, p=0.3858). An apparent decrease in the expression of *inha* (**C**) was not significant (wild-type n=9, mutant n=6, p=0.0938). The expression of *cyp19a1a* (**G**) was profoundly reduced in ovaries of *fdx1b*$^{-/-}$ mutant females compared to wild-type siblings (wild-type n=7, mutant n=6, p=0.0044). All results analysed by unpaired *t*-test, ** p<0.01, * p<0.05.

6.2.6 - Increased expression of *pomca* in the brains of *fdx1b*$^{-/-}$ mutant female zebrafish indicates dysregulation of the stress axis

Cortisol exerts negative feedback on the stress axis by inhibiting the expression of *pomca* (Alsop and Vijayan, 2009). We have observed increased expression of *pomca* in the brains of Fdx1b deficient female zebrafish (**Figure 6.9**), indicating dysregulation of the stress axis. In contrast to this, no change in the expression of the pituitary gonadotropins *fshb* and *lhb* was detected.

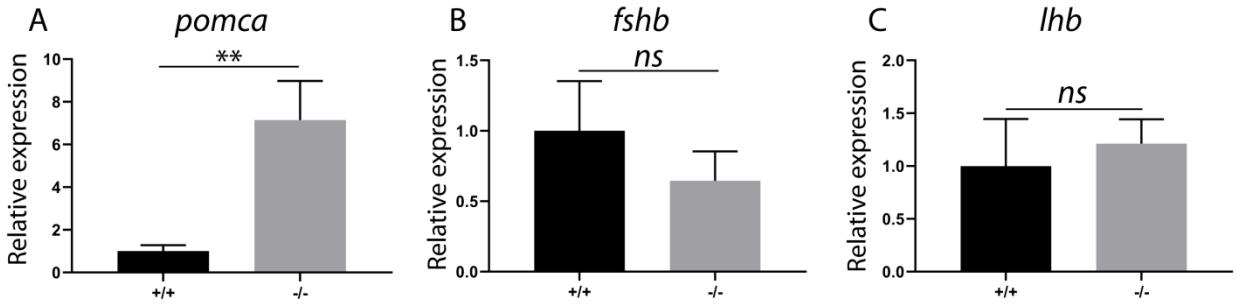

Figure 6.9. Dysregulation of pituitary genes in the brains of *fdx1b*$^{-/-}$ mutant female zebrafish. The expression of *pomca, fshb* and *lhb* was measured in the zebrafish brain by Taqman qPCR. **A:** *Pomca* expression was significantly upregulated in the brains of female *fdx1b*$^{-/-}$ mutant zebrafish (wild-type female n=5, *fdx1b*$^{-/-}$ mutant female n=4, p=0.0072). **B:** *Fshb* expression was unchanged in female *fdx1b*$^{-/-}$ mutants (wild-type female n=6, *fdx1b*$^{-/-}$ female n=4, Mann-Whitney test p=0.6095). **C:** *Lhb* expression was not significantly different in female *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings (wild-type female n=5, *fdx1b*$^{-/-}$ female n=5, p=0.6833). *Fdx1b*$^{-/-}$ mutant data is plotted relative to expression in wild-type fish of the same sex. All results analysed by unpaired *t*-tests unless otherwise stated. ** p <0.01.

6.3 - Discussion

Fdx1b$^{-/-}$ mutant adult zebrafish were found to possess either testes or ovaries. Fdx1b deficient female zebrafish had reduced cortisol concentrations and were found to be infertile when outcrossed with wild-type male zebrafish (**Chapter 3**). Similarly, Cyp11c1 deficient female zebrafish also exhibited decreased cortisol production (**Figure 6.4**) and were subfertile (**Table 6.1**). We employed histological techniques and gene expression assays to further investigate this infertility, as well as the effect of disrupted steroid biosynthesis on gonadal morphology and function.

Fdx1b deficient female zebrafish were found to be infertile (**Table 3.1**) whereas Cyp11c1 deficient females were subfertile. *Cyp11c1*$^{-/-}$ mutant females carrying the 47bp deletion allele did not produce any fertilised embryos in outcrosses whereas around a quarter of females carrying the 11bp deletion allele did produce fertilised embryos in outcrosses (**Table 6.1**). In isolation this result might suggest that the *cyp11c1* 11bp deletion allele is a hypomorph, however when the comparable effects of *cyp11c1* mutations on steroid hormone biosynthesis are considered this seems highly unlikely. *Fdx1b* mutant zebrafish were slightly older when outcrossed than *cyp11c1* mutant fish. The infertility phenotype seen in these fish may be progressive, explaining the discrepancy between Fdx1b and Cyp11c1 deficient female zebrafish. Progressive infertility in female zebrafish due to impaired androgen signalling has previously been demonstrated in androgen receptor mutant zebrafish (Yu et al., 2018). Alternatively, the fact the Fdx1b may be involved in side chain cleavage of cholesterol, the first stage of steroid hormone biosynthesis, whilst Cyp11c1 is not, may affect concentrations of oocyte maturation inducing steroid hormones (MIS) (discussed below).

Histological examination of the ovaries revealed that oocyte maturation was able to proceed to completion, as all oocyte stages were observed in *fdx1b*$^{-/-}$ mutant female zebrafish. Accumulation of ovulated oocytes was observed in the caudal oviduct in 2 out of 3 wild-type female zebrafish, but such an accumulation was not observed in any of the three *fdx1b*$^{-/-}$ mutant female zebrafish examined, suggesting a decrease in the proportion of mature oocytes or reduced ovulation. This

finding could be confirmed by accurate quantification of the number of germ cells at each stage of oocyte development or maturation. Before further discussion of this finding, it must be stressed that the number of replicates used in this experiment was low (n=3); and as such, further experimentation is required to confirm these results. Further histological examination should be undertaken to accurately analyse to possibility perturbed oocyte maturation in Fdx1b and Cyp11c1 deficient zebrafish.

Androgens are able to induce oocyte maturation in a variety of species; androstenedione and testosterone can induce maturation of *Xenopus* oocytes and testosterone induces maturation in mice and zebrafish (Gill et al., 2004; Li et al., 2019a; Lutz et al., 2001; Tokumoto et al., 2011). In addition to this, the anti-androgen flutamide impaired testosterone induced oocyte maturation in *Xenopus* (Lutz et al., 2001). Finally, mutation of the androgen receptor in zebrafish resulted in decreased numbers of mature oocytes as well as decreased fecundity; some *ar* mutant fish were fertile but spawned fewer eggs on average than wild-type females (Crowder et al., 2017).

In zebrafish, maturation of ovarian follicles can be stimulated by maturation inducing steroids (MIS) (Nagahama and Yamashita, 2008). 17α,20β-dihydroxy-4-pregnen-3-one is the major MIS in zebrafish (Tokumoto et al., 2011) and this steroid is synthesised from 17α-hydroxyprogesterone in a reaction catalysed by 20β-hydroxysteroid dehydrogenase (Nagahama, 1997). Production of MIS by ovarian follicles is stimulated by luteinising hormone (Nagahama and Yamashita, 2008). Expression of LH in the brain was unchanged by mutation of *fdx1b* (**Figure 6.9**), and whole body concentrations of 17α-hydroxyprogesterone were not affected by mutation of either *fdx1b* or *cyp11c1* (**Figures 6.2+6.4**); however androgens may also play a role in MIS production. When follicles were simultaneously treated with LH and the steroidal anti-androgen cyproterone acetate (CPA), production of MIS and associated oocyte maturation was significantly impaired (Rime et al., 2015). These effects could not be reproduced when using the non-steroidal anti-androgen flutamide. CPA has wide ranging effects on steroidogenesis, and therefore the results of this study cannot be attributed to anti-androgen

properties of this compound with a high degree of certainty. The impact of Fdx1b deficiency on MIS production could be definitively determined by their direct measurement.

Fdx1b is important for androgen production and given the known roles of androgens in oocyte maturation it seems likely that perturbed androgen signalling is behind the phenotype observed. Testosterone concentrations were significantly reduced in Cyp11c1 deficient females (**Figure 6.4**), however they were unchanged in Fdx1b deficient females (**Figure 6.2**). Concentrations of the principal zebrafish androgen 11-ketotestosterone were very low, or undetectable, in both wild-type and *fdx1b*$^{-/-}$ or *cyp11c1*$^{-/-}$ mutant zebrafish, indicating that this steroid is present in female fish at concentrations below the sensitivity threshold of our LC-MS/MS protocol. Overall, when the previous literature is taken into account, our data suggest that oocyte maturation is impaired in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant female zebrafish as a consequence of androgen deficiency. However, we have not definitively proven that impaired production of androgens was responsible for the decreased fertility observed in these fish.

Altered expression of genes involved in gonadal differentiation and maintenance is very likely to contribute to infertility in Fdx1b and Cyp11c1 deficient female zebrafish. Decreased expression of *cyp19a1a* in the ovaries of *fdx1b*$^{-/-}$ mutant zebrafish suggests decreased oestrogen production; however this is at odds with the expression of oestrogen responsive genes in the livers of these fish, which was unchanged (**Figure 3.6**). It is not unfeasible that oestrogen concentrations could be affected by mutation of *fdx1b* in female zebrafish, and direct measurement of these hormones would be desirable, however this was not possible using our current LC-MS/MS approach.

Dmrt1 and *sox9a* are considered pro-male genes with important roles in testis differentiation (Sun et al., 2013; Webster et al., 2017), and both genes were significantly upregulated in the ovaries of Fdx1b deficient female zebrafish. Two pro-female genes were also significantly upregulated: *sox9b* and *foxl2b* (Rodriguez-Mari et al., 2005; Yang et al., 2017). These juxtaposed gene expression profiles indicate a complex impact of *fdx1b* mutations on gonadal gene expression. Elucidation of the

molecular pathways underlying infertility in these fish would be best achieved by a comprehensive examination of the effects of *fdx1b* mutation on gonadal histology and oocyte maturation, as well as further steroid hormone profiling and ovary transcriptome characterisation.

Chapter 7 – Summary and general discussion

This thesis has described phenotypes resulting from disruption of the steroidogenic enzyme Cyp11c1 and its co-factor Fdx1b in zebrafish. Severe defects in interrenal and gonadal steroidogenesis were characterised and the resultant reproductive phenotype has been described in detail. Disrupted steroidogenesis due to Fdx1b or Cyp11c1 deficiency resulted in infertility in male zebrafish due to a combination of factors including abnormal testis organisation, impaired spermatogenesis, impaired sperm release and perturbed breeding behaviours. The molecular mechanisms underpinning this phenotype were investigated using targeted gene expression analysis as well as transcriptome profiling of *fdx1b*$^{-/-}$ mutant testes. The key findings in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant male zebrafish are summarised in **Table 7.1**. A reproductive phenotype in androgen and cortisol deficient female zebrafish was also described. In this chapter, the results from the previous four chapters will be discussed in the context of the broader scientific landscape surrounding steroid signalling and fertility in zebrafish and other vertebrate species.

7.1 - Mutation of *fdx1b* or *cyp11c1* results in severely disrupted steroidogenesis in zebrafish

Previous work has demonstrated that *fdx1b* is essential for interrenal steroidogenesis in larval zebrafish, as concentrations of cortisol are profoundly decreased in mutant larvae (Griffin et al., 2016). Fdx1b is a cofactor to Cyp11c1, and we have shown here that *cyp11c1*$^{-/-}$ mutant larvae also exhibit impaired glucocorticoid signalling, as they have a delayed VBA response and decreased expression of glucocorticoid responsive genes (**Figure 4.3**).

Fdx1b and Cyp11c1 both play important roles in glucocorticoid and androgen biosynthesis. We have shown that concentrations of cortisol and 11-ketotestosterone, the principal zebrafish androgen, are profoundly reduced in mutant zebrafish, confirming the roles of these proteins in the adult interrenal and gonadal steroidogenic pathways (**Figures 3.3+3.4+4.6**) (Tokarz et al., 2015).

Table 7.1. Comparison of the main findings in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant male zebrafish.

	***fdx1b*$^{-/-}$ mutant male zebrafish**	***cyp11c1*$^{-/-}$ mutant male zebrafish**	
Morphological characterisation	May possess ovaries or testes. No change in genital papilla prominence. Feminisation of secondary sex characteristics: increased green-yellow pigmentation in dorsal fin, reduced orange pigmentation in anal fin. Increased weight and length.		
Whole body steroid concentrations and expression of steroid responsive genes (liver tissue)	17α-hydroxyprogesterone ↓ (ns) Cortisol ↓ Androstenedione ↑ 11β-hydroxyandrostenedione Testosterone ↓ (ns) 11β-hydroxytestosterone ↓ 11-ketotestosterone ↓ *fkbp5, pck1, cyp2k22* ↓ *vtg2, esr1* (-)	11-deoxycortisol↑ Cortisol ↓ Androstenedione ↑ Testosterone (-) 11-ketoandrostenedione ↓ 11-ketotestosterone ↓ *fkbp5, pck1, cyp2k22* (ns) ↓	
Fertility and behavioural analysis	Infertile by conventional breeding techniques. IVF successful. Breeding behaviours reduced. Locomotion reduced in open field test (*fdx1b*$^{-/-}$ mainly ns). Sperm release by manual gamete expression reduced. Sperm count from whole testes preparation reduced.		
Histology	Disorganised arrangement of seminiferous tubules. Seminiferous tubules smaller and more numerous. Qualitatively fewer mature spermatozoa and a greater proportion of germ cells at earlier stages of spermatogenesis. Appearance of spermatic duct highly variable in *fdx1b*$^{-/-}$ mutant males, ranging from normal to severely hypoplastic or absent. Spermatic duct severely hypoplastic or absent in *cyp11c1*$^{-/-}$.		
Gonadal gene expression (testis tissue)	*sox9a* ↓ *dmrt1* (-) small decrease ns *amh* (-) small decrease ns *igf3* ↓ *insl3* ↓ *inha* ↓ *foxl2a* ↑ (ns) *foxl2b* (-) *sox9b* ↑ (ns)	**11bp**$^{-/-}$ *sox9a* (-) *dmrt1* (-) *amh* (-) *igf3* ↓ *insl3* ↓ *inha* ↓ (ns) *ar* ↑	**47bp**$^{-/-}$ *sox9a* (-) *dmrt1* (-) *amh* (-) *igf3* ↓ *insl3* ↓ *inha* ↓ *ar* ↑
Spermatogenesis marker gene expression (testis tissue)	*nanos2* ↑ *piwil1* ↑ *dazl* (-) *sycp3* (-) *odf3b* (-)	*nanos2* ↑ *piwil1* ↑ *dazl* (-) *sycp3* ↓ *odf3b* ↓	*nanos2* ↑ *piwil1* (-) *dazl* (-) *sycp3* ↓ *odf3b* ↓
Pituitary gene expression (brain tissue)	*pomca* ↑ *fshb* ↑ *lhb* (-)	*pomca* ↑ (ns) *fshb* (-) *lhb* (-)	*pomca* ↑ *fshb* ↑ *lhb* (-)

ns=non-significant, (-)=no change

7.2 - Androgens are dispensable for testis differentiation

Adult populations of *fdx1b* or *cyp11c1* mutant zebrafish appear at the first glance to be comprised of only female individuals: body and fin pigmentation are indicative of female sex and male pigmentation patterns are reduced or absent (**Figures 3.1+4.4**). Closer inspection revealed that some fish had a rounded abdomen associated with presence of an ovary whereas others had a more streamlined profile characteristic of male zebrafish. It was also noted that some fish possessed a large and protruding genital papilla characteristic of female zebrafish, whereas in other fish this structure was small or hidden. Subsequent dissection of these fish determined that *fdx1b* or *cyp11c1* mutant zebrafish could in fact develop as testis or ovary bearing individuals. Mutant males exhibited feminised pigmentation patterns but could be identified by streamlined body shape and absence of a prominent genital papilla. These findings indicate that androgens are important for development of male pigmentation patterns but are dispensable for testis differentiation.

Androgens are powerful factors in controlling development of male secondary sex characteristics in many vertebrates. The stoplight parrotfish (*Sparisoma viride*) undergoes sex change from female to male as an adult and this involves a dramatic colour change. During colour change, concentrations of 11-ketotestosterone increase rapidly whereas oestrogen concentrations decrease, thus implicating the sex steroid profile with the pigmentation phenotype (Cardwell and Liley, 1991). A related phenomenon occasionally occurs in chickens; at embryonic stages hens have two ovaries however typically only the left ovary develops in to a functional gonad, damage to the left ovary can cause the right gonad to develop into an ovotestis with the capacity to produce androgens. This results in the development of male phenotypic traits (Jacob, 2015).

The finding that androgens are dispensable for testis differentiation is consistent with findings in other models of impaired androgen signalling in zebrafish (Crowder et al., 2017; Li et al., 2019b; Zhai et al., 2018). Histological examination of the gonads during gonadal differentiation in *fdx1b*$^{-/-}$ mutant zebrafish revealed that this process is able to proceed relatively normally (**Figure 4.17**).

7.3 - Androgen and cortisol deficient male zebrafish are infertile due to a variety of reproductive phenotypes

Outcrossing of male *fdx1b* or *cyp11c1* mutant male zebrafish with unrelated wild-type females revealed that they were infertile under natural conditions (**Tables 3.1+4.2**), however, sperm collected from these fish could fertilise wild-type oocytes in IVF experiments (**Chapter 3, Table 4.3**). This led us to formulate several hypotheses relating to infertility in these fish:

1. Androgen and cortisol deficient zebrafish exhibit abnormal structure of the reproductive tract resulting in infertility due to impaired sperm release.
2. Spermatogenesis is impaired in androgen and cortisol deficient zebrafish resulting in reduced numbers of mature sperm and infertility.
3. Courtship behaviours are reduced in androgen and cortisol deficient male zebrafish resulting in failed breeding.

These hypotheses were systematically investigated and it was revealed that abnormal testicular structure, impaired spermatogenesis and reduced breeding behaviours are all likely to contribute to infertility in androgen and cortisol deficient zebrafish.

Two well characterised courtship behaviours were robustly decreased in both *fdx1b* and *cyp11c1* mutant male zebrafish (Darrow and Harris, 2004; Zhai et al., 2018). The roles of androgens in regulating mating behaviour in fish are well known; mutation of the androgen receptor also results in impaired courtship behaviours in zebrafish (Yong et al., 2017). In addition to this, anti-androgen treatment of the three-spined stickleback (*Gasterosteus aculeatus*) also caused a reduction in courtship behaviours (Sebire et al., 2008).

The nature and molecular mechanisms underlying structural disorganisation of the testes and impaired spermatogenesis were investigated in more detail and will be discussed forthwith.

7.4 - Insights into the molecular pathways underlying testis disorganisation in androgen and cortisol deficient zebrafish

Abnormal tubule structure in the testes is a common phenotype in fish exhibiting androgen deficiency or resistance (Crowder et al., 2017; Li et al., 2019b; Yu et al., 2018). However, previous studies have made few inroads into understanding the molecular pathways leading to this phenotype. *Sox9a* is postulated to play a role testis tubulogenesis in Medaka (*Oryzias latipes*) (Nakamoto et al., 2005) and this gene was downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 3.13**). This finding was not recapitulated in *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 4.14**) or *ar* mutants (Crowder et al., 2017; Yu et al., 2018), casting doubt on its contribution to this phenotype. In chapter 5, we performed RNA sequencing to characterise the testis transcriptome in *fdx1b*$^{-/-}$ mutant zebrafish, this revealed several pathways which are good targets for further investigation of the molecular mechanisms underlying testis disorganisation.

Upregulation of genes annotated to the pathway ECM receptor interactions was of particular interest as many of these genes are basement membrane components. Collagen is a major basement membrane component and we had previously performed staining for collagen fibres in the testes by picrosirius red staining (**Figures 3.11+4.11**). This revealed that seminiferous tubules in *fdx1b* and *cyp11c1* mutant zebrafish were smaller in diameter and apparently more numerous than in wild-type siblings; further characterisation with a quantitative methodology is required to confirm this finding. Increased production of basement membrane proteins may be a contributing factor to this phenotype. Reduced germ cell numbers or decreased seminiferous tubule fluid production could contribute to reduced tubule diameter but this does not explain the apparent increase in tubule number.

A role for androgens in regulating seminiferous tubule diameter is not unprecedented. Seminiferous tubules of Sertoli cell-specific and peritubular myoid cell-specific androgen receptor knockout mice both exhibit reduced tubule diameter in comparison to wild-type siblings (De Gendt et al., 2004;

Welsh et al., 2009). Androgen deficiency in rats causes a decrease in epididymal tubule diameter (Vidal and Whitney, 2014).

Genes annotated to the 'tight junction' KEGG pathway were significantly enriched in the group of genes downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish. Tight junction dysfunction may also contribute to the seminiferous tubule disorganisation seen in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant zebrafish, as mutation of the tight junction component claudin-11 in mice results in a narrowing of the seminiferous tubules (Jiang et al., 2014).

7.5 - Androgens play an important role in spermatogonial differentiation in zebrafish

A variety of genes regulating spermatogonial differentiation were differentially expressed in the testes of *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 7.1**). Targeted gene expression analysis by qPCR revealed that *igf3* and *insl3* were profoundly downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 3.13**). Both these genes play important roles in regulating early stages of spermatogenesis. As such, we looked at the expression of marker genes for different germ cell stages and found that markers of type A spermatogonia were upregulated in the testes of *fdx1b*$^{-/-}$ mutants, indicating an increased population of these cells (**Figure 3.15**). This finding was in agreement with histological analysis of the testes which also suggested a greater proportion of cells at early stages of spermatogenesis (**Figure 3.10**). These findings were recapitulated in *cyp11c1*$^{-/-}$ mutant zebrafish and in RNA sequencing data from *fdx1b*$^{-/-}$ mutant testes (**Chapters 4+5**). Future investigations should consider morphometric evaluation of histological samples to quantify the proportions of germ cell types. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) staining to quantify apoptosis of germ cells throughout spermatogenesis should also be considered.

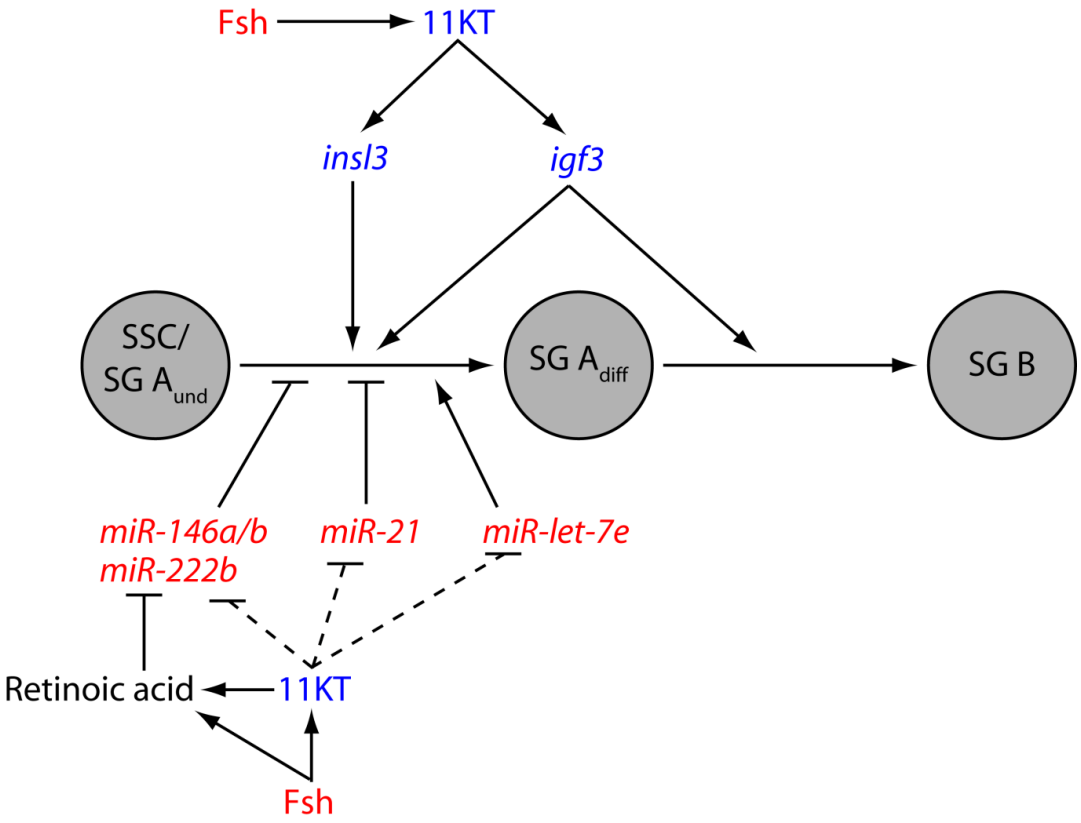

Figure 7.1. Perturbed signalling pathways regulating spermatogonial differentiation in androgen deficient zebrafish. Spermatogonial differentiation was impaired in *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant zebrafish. Several genes regulating this process were differentially expressed in the testes of these fish. In general stimulatory factors were downregulated whilst inhibitory factors were upregulated. Downregulated genes are displayed in blue text whilst upregulated genes are in red. 11KT concentrations were also decreased in mutant fish and this is also displayed in blue text. *Igf3* and *insl3* are growth factors which stimulate spermatogonial differentiation in zebrafish (Assis et al., 2016; Nobrega et al., 2015). Fsh is known to stimulate 11KT and retinoic acid synthesis in zebrafish and is an upstream regulator of spermatogonial differentiation (Crespo et al., 2019). Several miRNAs known to regulate spermatogonial differentiation in mammals were downregulated in the testes of *fdx1b*$^{-/-}$ mutant zebrafish, including miR-146a/b, miR-222b, miR-21 and miR-let-7e (Huszar and Payne, 2013; Niu et al., 2011; Tong et al., 2011; Yang et al., 2013). These miRNAs may be regulated by 11KT. Pathways which have been demonstrated in the literature are signified by solid arrows, potential interactions suggested by the results of this study are indicated by dashed arrows.

In addition to regulation by 11KT, *igf3* is also thought to be regulated by Fsh; indeed, Nobrega *et al* (2015) showed that Fsh was able to induce expression of *igf3* and spermatogonial differentiation independent of androgen signalling in *ex vivo* model systems. We have observed decreased 11KT production and decreased expression of *igf3*. *Fshb* expression in the brain was increased in *fdx1b*$^{-/-}$ mutant male zebrafish and in one of our *cyp11c1*$^{-/-}$ mutant lines. These results suggest that Fsh is not able to induce sufficient expression of *igf3* to stimulate spermatogonial differentiation, and that expression of *igf3* is dependent on androgen signalling *in vivo.*

Small RNA sequencing of *fdx1b*$^{-/-}$ mutant testes also revealed differential expression of several miRNAs with known roles in spermatogonial differentiation. MiR146a/b, miR222b and miR-21 all function in maintaining the spermatogonial stem cell or undifferentiated type A spermatogonia population by preventing differentiation (**Figure 7.1**), and all were upregulated in *fdx1b*$^{-/-}$ mutant testes (Huszar and Payne, 2013; Niu et al., 2011; Yang et al., 2013). It is possible that in normal spermatogenesis androgens suppress expression of these miRNAs to allow spermatogonial differentiation to proceed, however, there is no evidence in the literature that these miRNAs are regulated by androgens. It is also possible that these miRNAs are upregulated simply because they are principally expressed in undifferentiated spermatogonia, a cell population which appears to be increased in mutant testes compared to wild-types.

A recent study has highlighted interactions between androgen, retinoic acid and Fsh signalling in regulation of spermatogonial differentiation in zebrafish (Crespo et al., 2019). The role of retinoic acid in spermatogonial differentiation is well known in mammalian species but has now been demonstrated in zebrafish, this is interesting as *miR146a/b* and *miR-222b* are negatively regulated by this compound – a mechanism which plays a role in retinoic acid stimulated spermatogonial differentiation in mammals (Huszar and Payne, 2013; Yang et al., 2013). No evidence for regulation of these miRNAs by retinoic acid or androgens exists in the literature and the role of miRNAs in the regulation of spermatogenesis in fish is an exciting topic for further investigation. 11KT is postulated

to support retinoic acid production in zebrafish as 11KT treatment increased *aldh1a2* expression *in vivo;* an increase in *cyp26a1* expression was only significant in an *ex vivo* system (Crespo et al., 2019). Aldh1a2 plays a role in retinoic acid production whereas Cyp26a1 is involved in its catabolism. In this study, no difference in the testicular expression of *aldh1a2* was found by RNA-seq, though expression of *cyp26a1* was significantly increased. No change in the expression of the retinoic acid responsive genes *sall4* and *rec8a* was observed, however *rec8b* was significantly downregulated (**Additional file 1**).

Overall, we have observed downregulation of factors promoting spermatogonial differentiation (*igf3, insl3*) and upregulation of factors which inhibit this process (*miR146a/b*, *miR-222b, miR-21*). A notable exception to this is *fshb*. Our results indicate that increased Fshb expression was not able to stimulate normal levels of spermatogonial differentiation in the absence of 11KT.

Disorganised testis structure is likely to have a negative impact on spermatogenesis. Future studies utilising models of androgen deficiency to investigate spermatogenesis in zebrafish should consider using androgen replacement during development, to ensure correct organisation of the testes. This would allow researchers to identify the molecular events resulting directly from androgen deficiency which regulate spermatogenesis in fish.

The discrepancy in our findings relating to expression of *fshb* in the brains of male *cyp11c1*$^{-/-}$ mutant zebrafish is likely a consequence of the difficulty in obtaining pituitary tissue. Several methods of dissecting the brain were trialled to improve quantification of gonadotropin and *pomca* expression. Firstly, the whole brain was removed from the skull. qPCR readings from these samples indicated very low expression, leading to suspicion that the pituitary was lost during dissection. Further dissections revealed that the pituitary became detached from the brain and was retained in the sella turcica of the sphenoid bone. Secondly the pituitary was collected separately from neural tissue, unfortunately insufficient RNA could be obtained to facilitate cDNA synthesis and target gene quantification. Finally a method which involved removing the brain with the sphenoid still attached

was devised which circumvented the above described problems. Whilst providing good RNA yields and retaining the pituitary this method is not without limitations. It is difficult to standardise the amount of non-brain tissue collected and this introduces error in to comparisons of transcript quantification. All tissue collected expresses *ef1a*, the reference gene selected for this experiment, however *pomca* and gonadotropin expression is very low outside of the pituitary. If the proportion of pituitary to non-pituitary tissue is not consistent between samples this may result inconsistent gene expression quantification between samples. This problem could be circumvented by utilising an RNA isolation kit designed for low tissue input, by dissecting out the pituitary and pairing this with a known mass of brain tissue for RNA extraction, or by use of an alternative technique such as *in situ* hybridisation.

7.6 - Comparison of androgen deficiency and resistance phenotypes in zebrafish and mammals

We have shown that testicular structure in *fdx1b* and *cyp11c1* mutant zebrafish is disorganised and spermatogenesis is impaired. Some similarities may be drawn between zebrafish, mouse and human phenotypes associated with deficient androgen signalling. A reduction in seminiferous tubule size, as well as thickening of the tubule membrane has been reported in complete androgen insensitivity syndrome, which is caused by mutations affecting androgen receptor function (Cools et al., 2005). Increased thickness of the seminiferous tubule basement membrane has also been reported in 5α-reductase deficiency type 2 and HSD17B3 deficiency (Cai et al., 1994; Longo et al., 1975). 5α-reductase is required for the conversion of testosterone to the more potent dihydrotestosterone in target tissues whereas HSD17B3 is required for the conversion of androstenedione to testosterone (Miller and Auchus, 2011). These phenotypic features in humans have similarities with those seen in our zebrafish models of androgen deficiency. For example, the seminiferous tubules of *fdx1b*$^{-/-}$ and *cyp11c1*$^{-/-}$ mutant appeared narrower than those of wild-type siblings (**Figures 3.11+4.11**). Although thickness of the seminiferous tubule membrane was not investigated in this study, we did observe

upregulation of genes encoding many basement membrane components in the testes of *fdx1b*$^{-/-}$ mutant zebrafish by RNA sequencing (**Chapter 5**).

AR knockout mice exhibit profoundly reduced testis size, feminisation of external genitalia and disrupted spermatogenesis (Yeh et al., 2002). When the AR was specifically disrupted in mouse peritubular myoid cells, decreased expression of tight junction related genes was reported (Zhang et al., 2006). Reduced prevalence of tight junctions has been observed in human HSD17B3 deficiency (Longo et al., 1975). The prevalence of tight junctions in our zebrafish models of androgen deficiency was not investigated; however decreased expression of tight junction related genes in *fdx1b*$^{-/-}$ mutant zebrafish testes was detected by RNA sequencing (**Chapter 5**).

Our studies have identified two stages of zebrafish spermatogenesis which appear to be particularly dependent on androgen signalling: spermatogonial differentiation and meiotic entry. These stages of spermatogenesis appear to be disrupted in human disorders of androgen signalling as well as in mouse models of disrupted androgen signalling. Testis histology in complete androgen insensitivity syndrome reveals that spermatogenesis is incomplete and that seminiferous tubules contain mainly spermatogonia with only occasional spermatocytes (Hannema et al., 2006; Islam et al., 2019). Clusters of spermatogonia and spermatocytes can be found in HSD17B3 deficiency, however no germ cells beyond this stage were observed (Longo et al., 1975). Total testis AR knock-out mice exhibit abnormal spermatogonia, some spermatocytes but no spermatids or spermatozoa (Yeh et al., 2002), whereas Sertoli cell only AR knock-out mice exhibit spermatogenic arrest during or prior to meiosis (Chang et al., 2004; Willems et al., 2015).

These comparable features of disrupted androgen signalling in humans, mice and zebrafish suggest that some androgen regulated processes in testis morphological development and function may be conserved in these vertebrates. Androgen deficient zebrafish may be useful for further exploration of the molecular pathways underlying these phenotypes and may increase our understanding of pathological mechanisms in disorders of androgen signalling.

7.7 - Limitations and other considerations

Steroid hormones are involved in regulation of many phenotypes and pathways relating to reproductive fitness. Whilst many of these phenotypes were investigated in the present study, some remained unexplored. For example, the testes are a site of steroid glucuronide production, these hormones are male pheromones in fish and can induce ovulation in females (van den Hurk et al., 1987). Perturbed production of these pheromones could contribute to infertility in these fish.

Male zebrafish possess structures called breeding tubercles on their pectoral fins, these are important for stimulating egg release during breeding (McMillan et al., 2015). Regeneration of breeding tubercles is dependent on androgen signalling and these structures are absent in *ar* mutant zebrafish (McMillan et al., 2013; Yu et al., 2018). Perturbed breeding tubercle development could also contribute to failed breeding in androgen deficient zebrafish.

In zebrafish, to date, 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 signalling results in a similar phenotype to that which we have described (Crowder et al., 2017). In addition to this, androgen insufficiency and androgen receptor mutations cause similar changes in gene expression, including downregulation of *igf3* and *insl3* (de Castro Assis et al., 2018; Tang et al., 2018). These observations suggest 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 masculinising effects in zebrafish (Ribas et al., 2017b) 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 (Milla et al., 2009). Cortisol treatment of an *ex vivo* zebrafish testis culture system found that cortisol could stimulate spermatogonial differentiation, whist also promoting meiosis and increasing spermatozoa numbers (Tovo-Neto et al.,

2020). However the importance of glucocorticoid signalling to spermatogenesis *in vivo* remains unclear. No feminisation of secondary sex characteristics or breeding difficulties were reported in a glucocorticoid receptor mutant zebrafish, indicating that absent signalling by cortisol is unlikely to play a major role in the phenotype we have observed (Griffiths et al., 2012; Ziv et al., 2013). Several approaches could be considered to dissect the individual roles of glucocorticoids and androgens in sex development and spermatogenesis in zebrafish. Glucocorticoid treatment could be used to replace cortisol in *fdx1b*$^{-/-}$ or *cyp11c1*$^{-/-}$ mutant zebrafish, allowing the study of purely androgen deficient zebrafish. However, physiological glucocorticoid replacement, accurately mimicking daily rhythms, is likely to be extremely challenging. Another approach would be to investigate sex development and gonadal function in a zebrafish line purely deficient in glucocorticoid signalling. As mentioned above, no sex development or reproductive phenotype has been reported in glucocorticoid receptor mutant zebrafish (Griffiths et al., 2012; Ziv et al., 2013), but these studies did not undertake in depth analysis of testicular function. This could be achieved using models of glucocorticoid resistance such as *gr* mutant lines, or models of glucocorticoid deficiency, such as the *cyp21a2* mutant line (Eachus et al., 2017). *Cyp21a2* is not involved in androgen biosynthesis, and mutant larvae are cortisol deficient, however, in adult zebrafish glucocorticoid precursors could be shunted in to the sex steroid biosynthetic pathway – potentially increasing concentrations of these steroids, as happens in congenital adrenal hyperplasia (Kamrath et al., 2013). Alternatively, knock-out of genes upstream of interrenal steroidogenesis could be employed to reduce interrenal cortisol production. Mutation of *pomca*, encoding Acth, which stimulates cortisol production, or the gene encoding its receptor, *mc2r,* would be likely to produce cortisol deficient zebrafish, with no impairment or enhancement of sex steroid biosynthesis. Future studies wishing to focus solely on androgen deficiency could employ targeted disruption of *hsd17b3*, which only has roles in androgen synthesis, to produce a purely androgen deficient mutant.

A limitation of this study is the lack of integrated bioinformatic analysis of mRNA and miRNA sequencing data. Subsequent investigations of a similar nature should attempt to define the impacts

of differentially expressed miRNAs by determination of the effect on target transcript abundance. This would also help to determine the biological relevance of differential miRNA expression.

The above described RNA sequencing experiments have identified pathways in the testes which are affected by mutation of *fdx1b*, and have shed new light on molecular mechanisms potentially underpinning the generally comparable phenotypes observed in various androgen deficient or resistant zebrafish lines. Of particular interest is the finding that genes involved in cell and tissue structure, such as basement membrane, cytoskeleton and tight junction components, are dysregulated, thus allowing for future in depth exploration of the roles of steroid signalling in the formation and maintenance of these structures.

Appendix I – Comparison of steroid hormone concentrations and gene expression between male and female, wild-type and mutant zebrafish

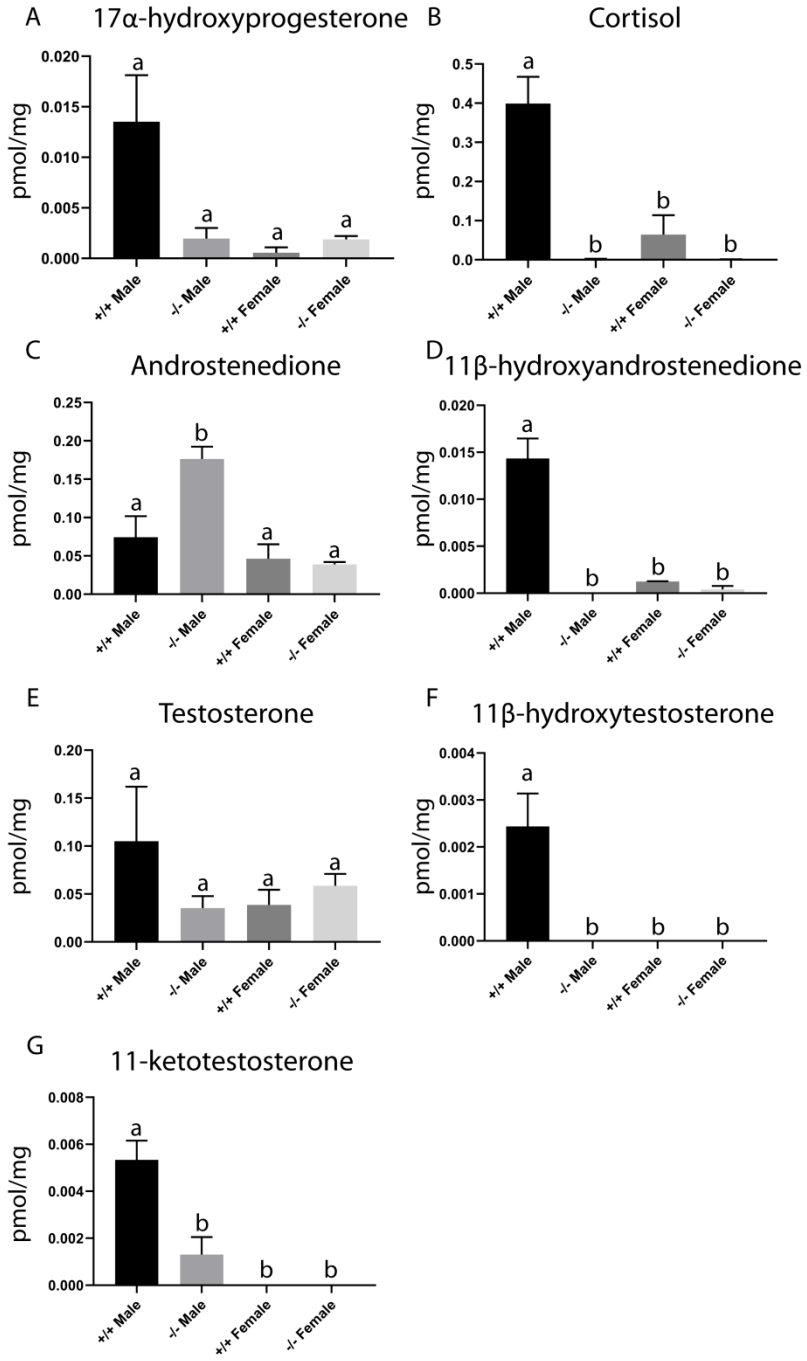

Figure A1.1. Profoundly altered glucocorticoid and androgen biosynthesis steroid profiles in *fdx1b*$^{-/-}$ mutant zebrafish. The concentration of steroids in male and female *fdx1b*$^{-/-}$ mutants and wild-type sibling whole fish bodies was measured by LC-MS/MS (wild-type male n=3, *fdx1b*$^{-/-}$ mutant male n=3,

wild-type female n=2, *fdx1b*$^{-/-}$ mutant female n=3). **A:** The concentration of 17α-hydroxyprogesterone (ANOVA p=0.0298) appeared to be higher in wild-type males compared to *fdx1b*$^{-/-}$ mutant males, wild-type females and *fdx1b*$^{-/-}$ mutant females, but these comparisons were not statistically significant (p=0.052, 0.054 0.053 respectively). **B:** The concentration of cortisol (ANOVA p=0.0005) was significantly higher in wild-type males than in all other groups. An apparent decrease in cortisol concentrations in *fdx1b*$^{-/-}$ mutant females compared to wild-type sibling females was not significant (p=0.7511). **C:** Androstenedione (ANOVA p=0.0040) concentrations were significantly higher in *fdx1b*$^{-/-}$ mutant males compared to wild-type males and wild-type and mutant female fish. No effect of *fdx1b*$^{-/-}$ mutation on androstenedione concentrations in female fish was observed. **D+F+G:** The concentrations of 11β-hydroxyandrostenedione (ANOVA p=0.0001), 11β-hydroxytestosterone (ANOVA p=0.0058) and 11-ketotestosterone (ANOVA p=0.0013) were all significantly decreased in *fdx1b*$^{-/-}$ mutant males compared to wild-type siblings. These steroids were all significantly lower (or undetected) in wild-type and *fdx1b*$^{-/-}$ mutant females compared to wild-type males. **E:** Concentrations of testosterone (ANOVA p=0.4659) were unaffected by mutation of *fdx1b*. Testosterone concentrations appeared slightly higher in wild-type males compared to other group however this difference was not significant. All results were analysed using one-way ANOVA with multiple comparisons. Statistical significance of multiple comparisons is indicated by letters above each bar – different letters indicates a statistically significant result.

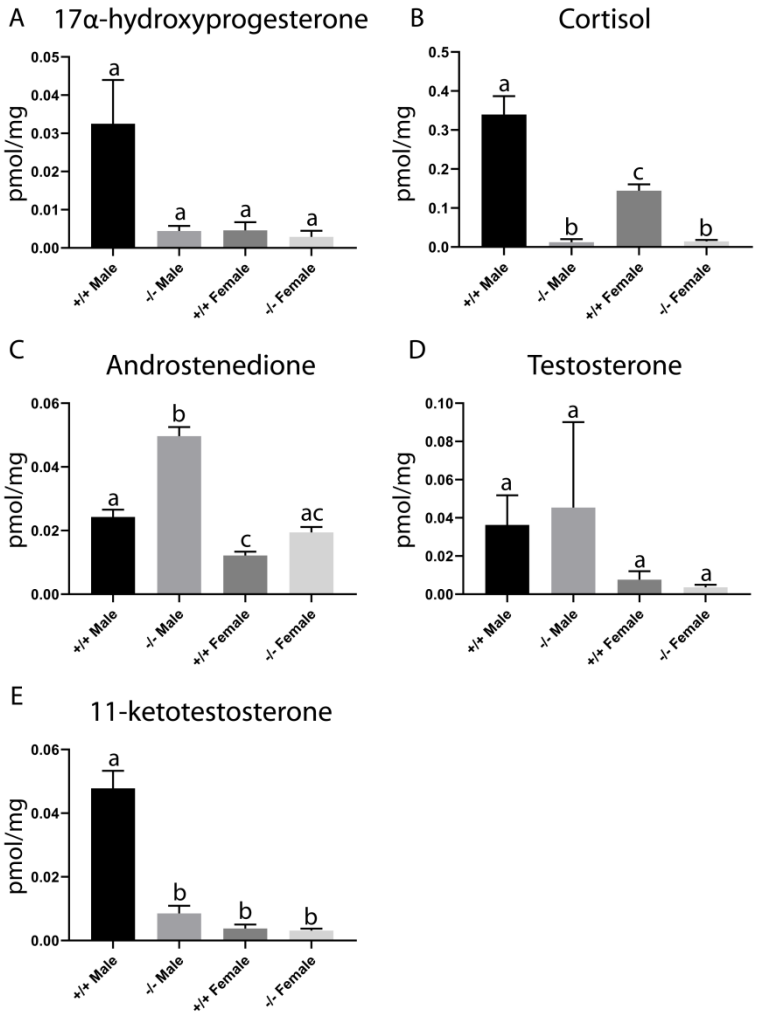

Figure A1.2. Altered steroid hormone concentrations in *fdx1b*$^{-/-}$ mutant zebrafish are reproducible. The concentration of steroids in male and female *fdx1b*$^{-/-}$ mutants and wild-type sibling whole fish bodies was measured by LC-MS/MS. Wild-type male n=3, *fdx1b*$^{-/-}$ mutant male n=3, wild-type female n=2, *fdx1b*$^{-/-}$ mutant female n=3, unless otherwise stated. **A:** Concentrations of 17α-hydroxyprogesterone appeared to be higher in wild-type males compared to all other groups but these comparisons were not statistically significant. **B:** Cortisol concentrations were significantly lower in both male and female *fdx1b*$^{-/-}$ mutant zebrafish compared to wild-type siblings of the same sex. In addition to this, cortisol concentrations were significantly lower in wild-type female fish compared to wild-type male fish. **C:** Androstenedione concentrations were significantly higher in *fdx1b*$^{-/-}$ mutant males compared to wild-type sibling males. Concentrations of androstenedione were

lower in wild-type females than in wild-type males. No effect of *fdx1b*$^{-/-}$ mutation on androstenedione concentrations was observed between *fdx1b*$^{-/-}$ mutant females and wild-type siblings. **D:** No effect of *fdx1b*$^{-/-}$ mutation on testosterone was observed. **E:** Concentrations of 11KT were significantly lower in wild-type and *fdx1b*$^{-/-}$ mutant female fish compared to wild-type males. Mutation of *fdx1b* in male fish resulted in a significant reduction in 11KT concentrations compared to wild-type male siblings, to a level only slightly higher than that observed on wild-type female fish. All results were analysed using one-way ANOVA with multiple comparisons. Statistical significance of multiple comparisons is indicated by letters above each bar – different letters indicates a statistically significant result.

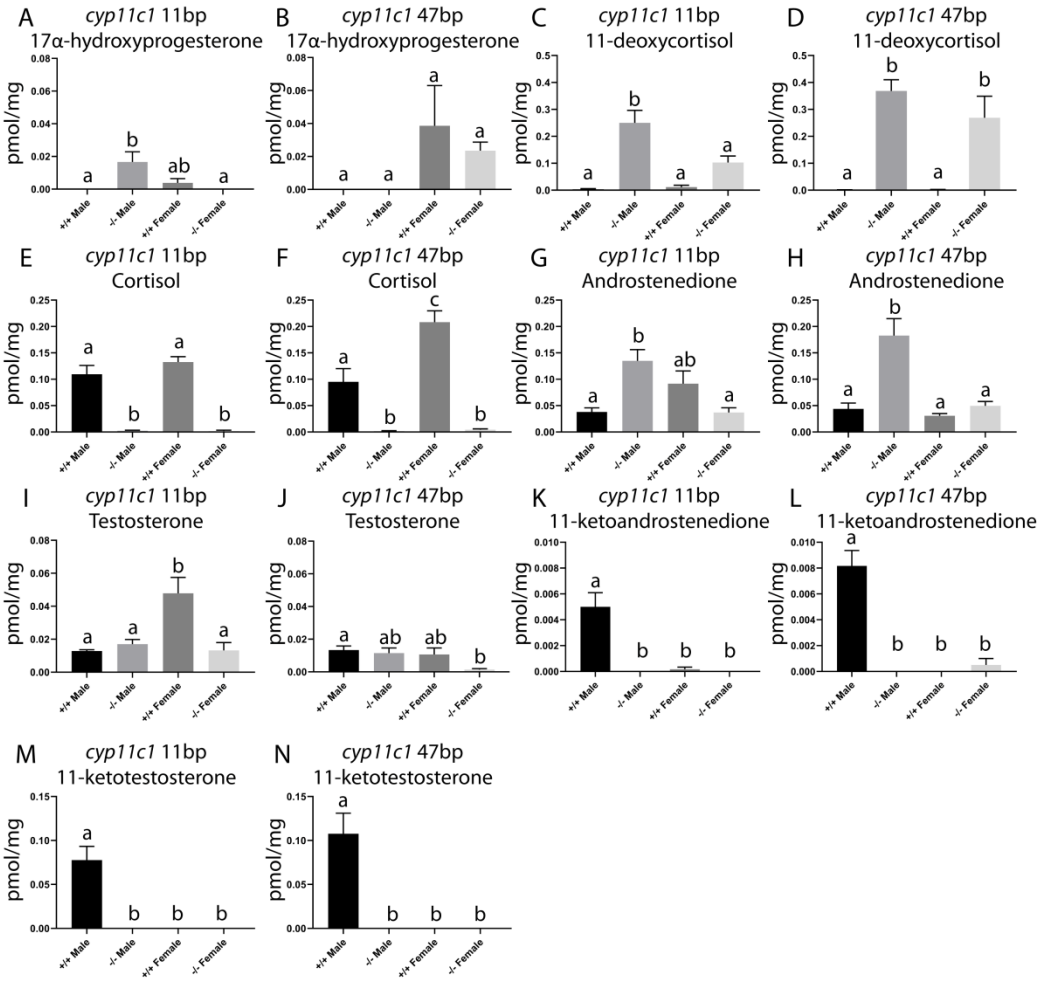

Figure A1.3. Disrupted steroid hormone biosynthesis in male and female *cyp11c1*$^{-/-}$ mutant zebrafish. Whole body steroid hormone concentrations were determined by LC-MS/MS. **A+B:** Concentrations of 17α-hydroxyprogesterone were highly variable and not reproducible between the two *cyp11c1*$^{-/-}$ mutant alleles, therefore these measurements should be treated with caution. **C+D:** Concentrations of 11-deoxycortisol were higher in *cyp11c1*$^{-/-}$ mutant zebrafish compared to wild-type siblings of the same sex. All comparisons were significant with the exception of *cyp11c1*$^{-/-}$ 11bp deletion allele female mutants vs. wild-type sibling females (p=0.1589). **E+F:** Concentrations of cortisol were significantly and profoundly reduced in *cyp11c1*$^{-/-}$ mutant zebrafish compared to wild-type siblings of the same sex, indicating abolished cortisol production. **G+H:** Androstenedione concentrations were significantly higher in *cyp11c1*$^{-/-}$ mutant male zebrafish compared to wild-type siblings. No effect of *cyp11c1* disruption on androstenedione concentrations was observed in female fish. **I+J:** Testosterone concentrations were not affected by *cyp11c1*$^{-/-}$ mutations in male fish.

Testosterone concentrations appeared to be decreased in *cyp11c1*$^{-/-}$ mutant female zebrafish compared to wild-type sibling females, however this was only significant for the 11bp allele. **K+L+M+N:** Concentrations of 11-ketoandrostenedione and 11-ketotestosterone were undetectable, or occasionally present at extremely low concentrations, in *cyp11c1*$^{-/-}$ mutant males and females and wild-type sibling females. These steroid hormones were detected at comparably high concentrations in wild-type male zebrafish, indicating abolished 11-ketotestosterone production in *cyp11c1*$^{-/-}$ mutant males. All results were analysed using one-way ANOVA with multiple comparisons. Statistical significance of multiple comparisons is indicated by letters above each bar – different letters indicates a statistically significant result.

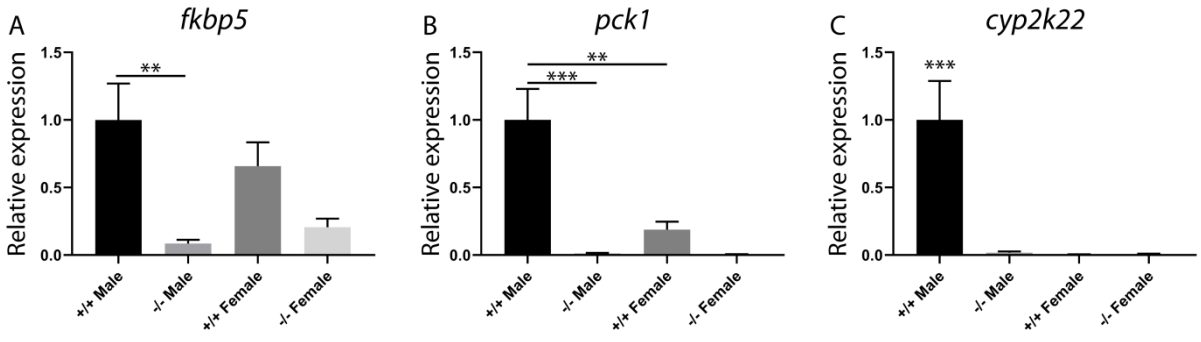

Figure A1.4. Decreased expression of glucocorticoid and androgen responsive genes in *fdx1b*$^{-/-}$ mutant male zebrafish. The expression of glucocorticoid or androgen responsive genes was measured in the livers of *fdx1b*$^{-/-}$ mutant zebrafish and wild-type siblings by qPCR. The expression of glucocorticoid responsive genes *fkbp5* (**A**) (wild-type male n=8, *fdx1b*$^{-/-}$ mutant male n=6, wild-type female n=8, *fdx1b*$^{-/-}$ mutant female n=7, ANOVA p=0.057) and *pck1* (**B**) (wild-type male n=8, *fdx1b*$^{-/-}$ mutant male n=5, wild-type female n=7, *fdx1b*$^{-/-}$ mutant female n=6, ANOVA p<0.0001) was significantly reduced in *fdx1b*$^{-/-}$ mutant male zebrafish compared to wild-type male siblings. An apparent reduction in the expression of *fkbp5* (**A**) and *pck1* (**B**) in *fdx1b*$^{-/-}$ mutant females compared to wild-type sibling females was not significant. *Pck1* expression was also significantly lower in wild-type females compared to wild-type males (**B**). Expression of the androgen responsive gene *cyp2k22* (**C**) (wild-type male n=6, *fdx1b*$^{-/-}$ mutant male n=5, wild-type female n=8, *fdx1b*$^{-/-}$ mutant female n=6, ANOVA p<0.0001) was significantly decreased in *fdx1b*$^{-/-}$ mutant male zebrafish, as well as in wild-type and *fdx1b*$^{-/-}$ mutant female zebrafish compared to wild-type sibling males. All results were analysed using one-way ANOVA with multiple comparisons. *** p<0.001, ** p<0.01.

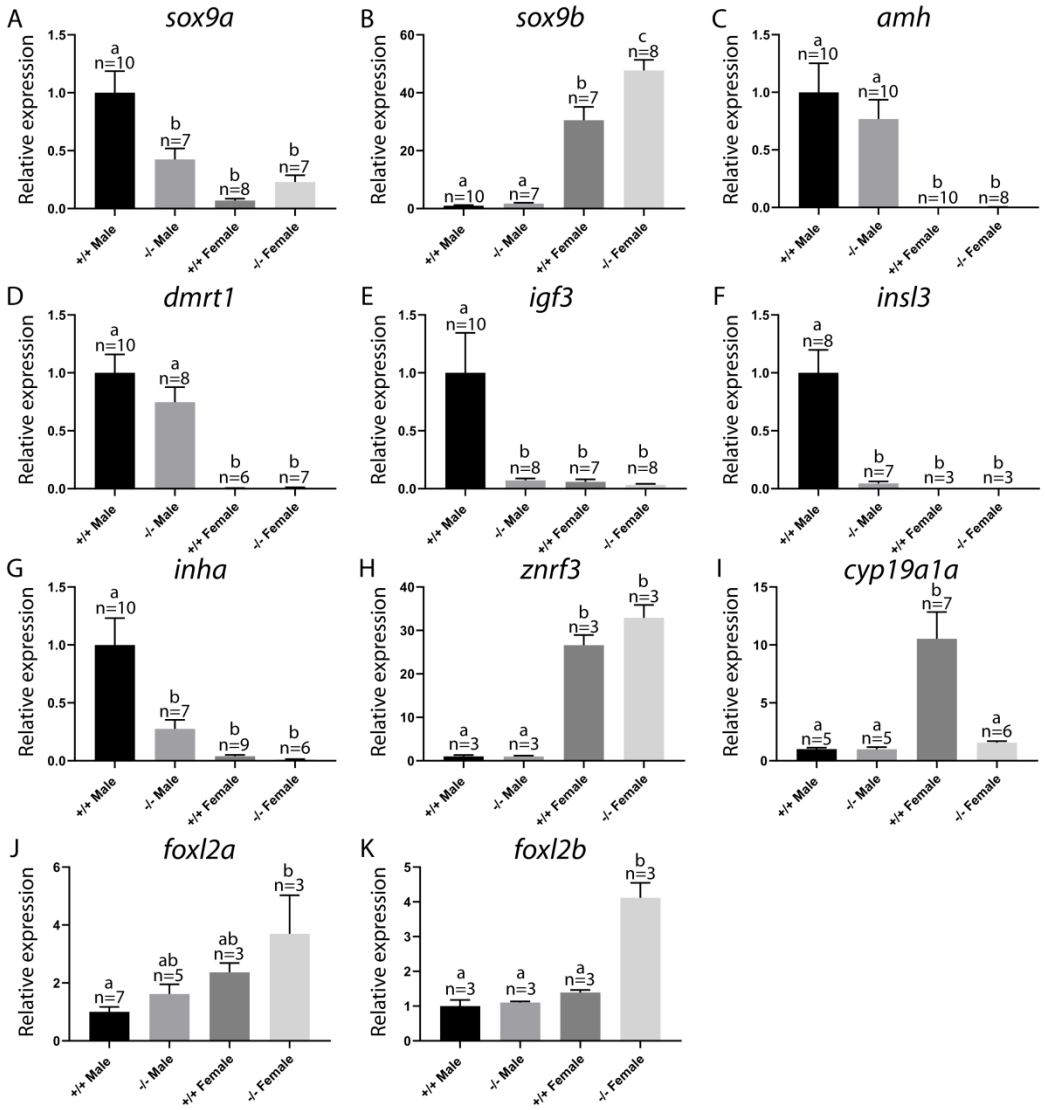

Figure A1.5. Comparison of the gonadal gene expression profile in male and female, wild-type and *fdx1b*$^{-/-}$ mutant zebrafish. Ovary and testis gene expression was quantified by qPCR. **A:** *Sox9a* (ANOVA p<0.0001) expression was significantly higher in wild-type male testes than in *fdx1b*$^{-/-}$ mutant testes and both wild-type and *fdx1b*$^{-/-}$ mutant ovaries. **B:** *Sox9b* (ANOVA p<0.0001) expression, in contrast to *sox9a* expression, was significantly higher in ovaries compared to testes. *Sox9b* expression was significantly higher in *fdx1b*$^{-/-}$ mutant ovaries compared to wild-type ovaries. **C+D:** *Amh* (ANOVA p<0.0001) and *dmrt1* (ANOVA p<0.0001) expression was nearly undetectable in ovary samples but both genes were robustly expressed in the testes. No difference in *amh* or *dmrt1* expression was detected between wild-type and *fdx1b*$^{-/-}$ mutant testes. **E+F:** *Igf3* (ANOVA p=0.0037)

was expressed at very low levels whereas *insl3* (ANOVA p=0.0002) expression was nearly undetectable in ovary samples; both genes were expressed at comparably high levels in wild-type testes. *Fdx1b*$^{-/-}$ mutation resulted in profoundly reduced expression of *igf3* and *insl3*, to a similar level as ovary expression. **G:** *Inha* (ANOVA p<0.0001) was expressed at low levels in ovaries compared to testes. Mutation of *fdx1b* resulted in significantly reduced testicular expression of *inha.* **H:** *Znrf3* (ANOVA p<0.0001) was expressed at low levels in testes compared to ovaries. No effect of *fdx1b*$^{-/-}$ mutation on *znrf3* expression was detected. **I:** *Cyp19a1a* (ANOVA p=0.0001) was expressed at high levels in the wild-type ovary compared to wild-type and *fdx1b*$^{-/-}$ mutant testes. Mutation of *fdx1b* resulted in a significant reduction of ovarian *cyp19a1a* expression, to approximately the same level as testicular expression. **J:** *Foxl2a* (ANOVA p<0.0128) appeared to exhibit higher expression in ovaries than testes, however comparisons were generally not significant. Expression of *foxl2a* appeared to be higher in *fdx1b*$^{-/-}$ mutant gonads compared to wild-type gonads of the same sex, however these comparisons were not statistically significant. **K:** *Foxl2b* (ANOVA p<0.0001) expression was comparable in wild-type testes and ovaries, as well as *fdx1b*$^{-/-}$ mutant testes. However, mutation of *fdx1b* resulted in significantly increased *foxl2b* expression in ovaries. All results analysed by one-way ANOVA with multiple comparisons. The number of replicates in each category is indicated above the respective bar. Statistical significance of multiple comparisons is indicated by letters above each bar – different letters indicates a statistically significant result.

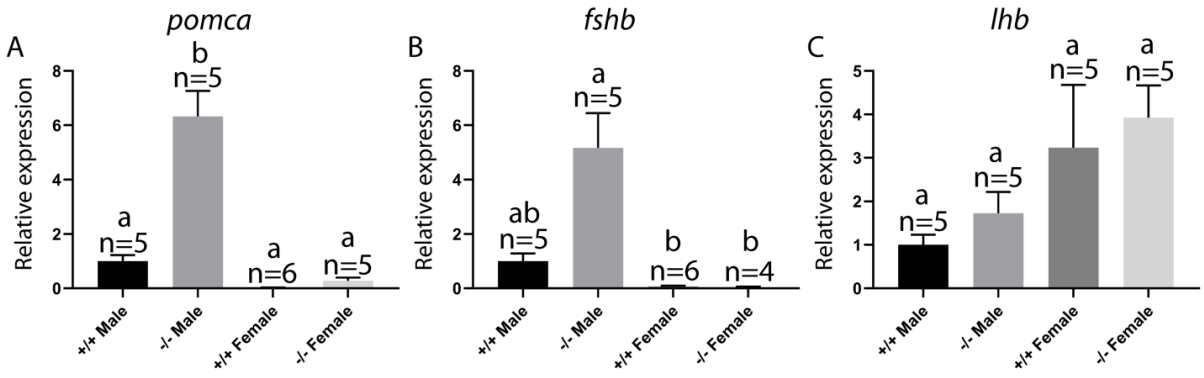

Figure A1.6. Comparison of the pituitary gene expression profile in male and female, wild-type and *fdx1b*$^{-/-}$ mutant zebrafish. Expression of gonadotropins and *pomca* in the brains of adult zebrafish was determined by Taqman qPCR. **A:** *Pomca* (ANOVA p<0.0001) expression was significantly higher in *fdx1b*$^{-/-}$ mutant males compared to all other groups. **B:** *Fshb* (Kruskal-Wallis test p=0.0012) expression was significantly higher in *fdx1b*$^{-/-}$ mutant males compared to wild-type and *fdx1b*$^{-/-}$ mutant females. Increased expression of *fshb* in the brains of *fdx1b*$^{-/-}$ mutant males compared to wild-type male siblings did not achieve statistical significance in this test. **C:** No significant difference in *lhb* (ANOVA p=0.10) expression was identified between any group.

Appendix II – Code for RNA sequencing analysis

```
#fdx1b RNA seq analysis

#connect to server in filezilla host:sharc.shef.ac.uk, username, password,
port 22
#Upload raw data to /fastdata/mdq15jao/raw_data

#connect to server in command prompt

ssh -X mdq15jao@sharc.shef.ac.uk

#enter a node

qrsh

#get more RAM

qrsh -l rmem=16G

#use gunzip to unzip fastq.gz files, * indicates all files in folder

cd /fastdata/mdq15jao/raw_data/
gunzip *


#########################################################################


#FASTQC

cd /fastdata/mdq15jao

wget
http://www.bioinformatics.babraham.ac.uk/projects/fastqc/fastqc_v0.11.5.zip

unzip fastqc_v0.11.5.zip

module load apps/java

java -version

#enter FastQC folder using cd, must be in folder that fastqc command is
saved in

cd FastQC/

chmod 755 fastqc

export PATH=$PATH:/fastdata/mdq15jao/FastQC

fastqc /fastdata/mdq15jao/raw_data/*


#########################################################################


#MultiQC

#check python version must be 2.7+, 3.4+ or 3.5+
```

```
python --version

cd /fastdata/mdq15jao/

#install miniconda

wget https://repo.anaconda.com/miniconda/Miniconda2-latest-Linux-x86_64.sh

bash Miniconda2-latest-Linux-x86_64.sh

#answer 'yes' when prompted

#create python environment

conda create --name py3.7 python=3.7

conda activate py3.7

#install MultiQC

conda install -c bioconda -c conda-forge multiqc

#run MultiQC

#change to directory containing FastQC reports

multiqc .

#########################################################################

#SortmeRNA, removes rRNA

cd /fastdata/mdq15jao/

wget https://github.com/biocore/sortmerna/archive/2.1.zip

unzip 2.1.zip

cd sortmerna-2.1/

bash ./build.sh

make install

export PATH=$PATH:/fastdata/mdq15jao/sortmerna-2.1

indexdb_rna -h

./indexdb_rna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
```

```
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db

#example code for running sortmerna on 1 fastq file
./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40169_Track-78805_R1_WT1.fastq --aligned
WT1_rrna  --other WT1_sortmerna --fastx --num_alignments 1 --log --sam -v

#run all samples using nano

nano

#samples run as two scripts

#########################################################################

#$ #WT sortmerna script

#$ #!/bin/bash

#$ -l h_rt=90:00:00

#$ -l rmem=32G

#$ -m bea

#$ -M jaoakes1@sheffield.ac.uk

#$ -j y

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
```

```
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40169_Track-78805_R1_WT1.fastq --aligned
WT1_rrna  --other WT1_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40170_Track-78806_R1_WT2.fastq --aligned
WT2_rrna  --other WT2_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40171_Track-78807_R1_WT3.fastq --aligned
WT3_rrna  --other WT3_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
```

```
/fastdata/mdq15jao/raw_data/L40172_Track-78808_R1_WT4.fastq --aligned
WT4_rrna  --other WT4_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40173_Track-78809_R1_WT5.fastq --aligned
WT5_rrna  --other WT5_sortmerna --fastx --num_alignments 1 --log --sam -v


#save as wtscript-qsubber-sh

#####################################################################

#$ #Mut sortmerna script

#$ #!/bin/bash

#$ -l h_rt=90:00:00

#$ -l rmem=16G

#$ -m bea

#$ -M jaoakes1@sheffield.ac.uk

#$ -j y

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40174_Track-78810_R1_Mut1.fastq --aligned
Mut1_rrna  --other Mut1_sortmerna --fastx --num_alignments 1 --log --sam -v
```

```
./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40175_Track-78811_R1_Mut2.fastq --aligned
Mut2_rrna  --other Mut2_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40176_Track-78812_R1_Mut3.fastq --aligned
Mut3_rrna  --other Mut3_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/L40177_Track-78813_R1_Mut4.fastq --aligned
Mut4_rrna  --other Mut4_sortmerna --fastx --num_alignments 1 --log --sam -v

./sortmerna --ref /fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-
5.8s-database-id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5.8s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/rfam-5s-database-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/rfam-5s-
```

```
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-16s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-arc-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-arc-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-16s-
id90.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-16s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-bac-23s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-bac-23s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-18s-
id95.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-18s-
db:/fastdata/mdq15jao/sortmerna-2.1/rRNA_databases/silva-euk-28s-
id98.fasta,/fastdata/mdq15jao/sortmerna-2.1/index/silva-euk-28s-db --reads
/fastdata/mdq15jao/raw_data/Mut5a+bmerge.fastq --aligned Mut5_rrna  --other
Mut5_sortmerna --fastx --num_alignments 1 --log --sam -v


#save as mutscript-qsubber-sh


#########################################################################

#run scripts

qsub wtscript-qsubber-sh

qsub mutscript-qsubber-sh

#check status of jobs

qstat


#########################################################################


#trimmomatic

cd /fastdata/mdq15jao/

wget
http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/Trimmomatic-
0.39.zip

unzip Trimmomatic-Src-0.39.zip

module load apps/java

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/WT1_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/WT1_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/WT2_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/WT2_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/WT3_sortmerna.fastq
```

```
/fastdata/mdq15jao/Trimmomatic-0.39/WT3_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/WT4_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/WT4_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/WT5_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/WT5_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut1_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut1_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut2_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut2_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut3_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut3_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut4_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut4_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut5a_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut5a_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

java -jar Trimmomatic-0.39/trimmomatic-0.39.jar SE -phred33
/fastdata/mdq15jao/sortmerna-2.1/sortmernaoutputs/Mut5b_sortmerna.fastq
/fastdata/mdq15jao/Trimmomatic-0.39/Mut5b_sortmerna_trimmed.fastq
ILLUMINACLIP:Trimmomatic-0.39/adapters/TruSeq3-SE.fa:2:30:10 LEADING:3
TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36


######################################################################


#merge mutant 5 files

cat Mut5a_sortmerna_trimmed.fastq Mut5b_sortmerna_trimmed.fastq >
Mut5_merge_after_sort+trim.fastq
```

```
#FastQC/MultiQC on trimmed fastq files

#enter FastQC folder using cd, must be in folder that fastqc command is
saved in

chmod 755 fastqc

export PATH=$PATH:/fastdata/mdq15jao/FastQC

cd ..

fastqc Trimmomatic-0.39/*


#MultiQC

conda activate py3.7

multiqc .


########################################################################


#STAR

git clone https://github.com/alexdobin/STAR.git

#install

cd STAR/source

make STAR

export PATH=$PATH:/fastdata/mdt16j/STAR/bin/Linux_x86_64_static/

mkdir genome

#get genome

wget ftp://ftp.ensembl.org/pub/release-
97/fasta/danio_rerio/dna/Danio_rerio.GRCz11.dna.primary_assembly.fa.gz

#get annotations

wget ftp://ftp.ensembl.org/pub/release-
96/gtf/danio_rerio/Danio_rerio.GRCz11.96.gtf.gz

gunzip Danio_rerio.GRCz11.dna.primary_assembly.fa.gz

gunzip Danio_rerio.GRCz11.96.gtf.gz

qrsh -l rmem=32G

#run STAR in genome folder

cd /fastdata/mdt16j/STAR/genome

export PATH=$PATH:/fastdata/mdt16j/STAR/bin/Linux_x86_64_static/
```

```
STAR --runThreadN 8 --runMode genomeGenerate --genomeDir
/fastdata/mdq15jao/STAR/genome --genomeFastaFiles
/fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.dna.primary_assembly.fa --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --
sjdbOverhang 100

#run STAR as nano scripts

nano

#########################################################################

#$ #!/bin/bash

#$ -l h_rt=94:00:00

#$ -l rmem=32G

#$ -m bea
#$ -M jaoakes1@sheffield.ac.uk

#$ -j y

export PATH=$PATH:/fastdata/mdq15jao/STAR/bin/Linux_x86_64_static/

export PATH=$PATH:/fastdata/mdq15jao/STAR/genome

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/WT1_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_WT1 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/WT2_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_WT2 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/WT3_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_WT3 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/WT4_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_WT4 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/WT5_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_WT5 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic
```

```
#save as wt-star-qsubber-sh

#####################################################################

#$ #!/bin/bash

#$ -l h_rt=94:00:00

#$ -l rmem=32G

#$ -m bea
#$ -M jaoakes1@sheffield.ac.uk

#$ -j y

export PATH=$PATH:/fastdata/mdq15jao/STAR/bin/Linux_x86_64_static/

export PATH=$PATH:/fastdata/mdq15jao/STAR/genome

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/Mut1_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_Mut1 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/Mut2_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_Mut2 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/Mut3_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_Mut3 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/Mut4_sortmerna_trimmed.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_Mut4 --outSAMunmapped Within --quantMode
TranscriptomeSAM GeneCounts --twopassMode Basic

STAR --runThreadN 1 --genomeDir /fastdata/mdq15jao/STAR/genome/ --
sjdbGTFfile /fastdata/mdq15jao/STAR/Danio_rerio.GRCz11.96.gtf --readFilesIn
/fastdata/mdq15jao/Trimmomatic-0.39/Mut5_merge_after_sort+trim.fastq --
outSAMtype BAM SortedByCoordinate Unsorted --outFileNamePrefix
/fastdata/mdq15jao/STAR/star_Mut5_merge_after_sort_trim --outSAMunmapped
Within --quantMode TranscriptomeSAM GeneCounts --twopassMode Basic

#save as mut-star-qsubber-sh

#####################################################################

#run scripts
```

```
qsub wt-star-qsubber-sh

qsub mut-star-qsubber-sh

qstat

#########################################################################


R-Studio script - deseq2

#Download RStudio
#Download R 3.6

if (!requireNamespace("BiocManager", quietly = TRUE))
+     install.packages("BiocManager")
BiocManager::install("DESeq2")

library(DESeq2)

#Download ReadsPerGene.out.tab files
#Open in notepad
#Paste lists into excel - first column no title contains gene IDs,
subsequent columns final column of reads.per.gene.tab file
#Save them as fdx1b_RNAseq_counts.txt

cts <- read.table("C:/Users/James
Oakes/Downloads/RNA_seq/fdx1b_RNAseq_counts.txt")

#make metadata table in excel. Column 1 'no title', WT1, WT2... Column 2
'genotype', WT or mut.
#save as fdx1b_metadata.txt

coldata <- read.table("C:/Users/James
Oakes/Downloads/RNA_seq/fdx1b_metadata.txt")

#check tables

coldata

head(cts)

#sort rows with zero transcripts

dds <- DESeqDataSetFromMatrix(countData = cts,colData = coldata,design =
~genotype)

> dds <- DESeq(dds)

results(dds,alpha = 0.05)

fdx1b_testes <- results(dds,alpha = 0.05)

head(fdx1b_testes)

setwd('C:/Users/James Oakes/Downloads/RNA_seq/')

write.csv(as.data.frame(fdx1b_testes), file = "fdx1b_testes.csv")

fdx1b_testes_sig <- subset(fdx1b_testes,padj<0.05)
```

```
write.csv(as.data.frame(fdx1b_testes_sig), file = "fdx1b_testes_sig.csv")

#PCA and heatmap

fdx1b_testes_dds <- DESeqDataSetFromMatrix(countData = cts,colData =
coldata,design = ~genotype)

fdx1b_testes_log <- rlog(dds, blind = TRUE)

plotPCA(fdx1b_testes_log, intgroup="genotype")

install.packages("pheatmap")

library(pheatmap)

fdx1b_testes_logmatrix <- assay(fdx1b_testes_log)

fdx1b_testes_cor <- cor(fdx1b_testes_logmatrix)

pheatmap(fdx1b_testes_cor)
```

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