White Rose Research Online

eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/165743/

Version: Accepted Version

Article:
Oakes, J.A., Barnard, L., Storbeck, K.-H. et al. (2020) 11β-hydroxylase loss disrupts steroidogenesis and reproductive function in zebrafish. Journal of Endocrinology, 247 (2). pp. 197-212. ISSN: 0022-0795

https://doi.org/10.1530/joe-20-0160

This manuscript has been accepted for publication in Journal of Endocrinology, but the version presented here has not yet been copy-edited, formatted or proofed. The definitive version is now freely available at https://doi.org/10.1530/JOE-20-0160 2020.

Reuse
Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

11β-hydroxylase loss disrupts steroidogenesis and reproductive function in zebrafish

James A Oakes[1,2], Lise Barnard[3], Karl-Heinz Storbeck[3], Vincent T Cunliffe[2], Nils P Krone[1,2,4].

[1]Department of Oncology & Metabolism, School of Medicine, University of Sheffield, Sheffield, S10 2TH, United Kingdom

[2]The Bateson Centre, Department of Biomedical Science, Firth Court, University of Sheffield, Western Bank, Sheffield, S10 2TN, United Kingdom

[3]Department of Biochemistry, Stellenbosch University, Stellenbosch, 7602, Matieland, South Africa

[4]Department of Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Fetscherstrasse 74, 01307 Dresden, Germany

Corresponding author / reprint requests:

Nils P Krone MD FRCPCH, Academic Unit of Child Health, Department of Oncology & Metabolism, University of Sheffield, Sheffield Children's Hospital, Western Bank, SHEFFIELD, S10 2TH, UNITED KINGDOM
Email: n.krone@sheffield.ac.uk

Short title: Cyp11c1 and reproductive function in zebrafish

Key words: zebrafish, testes, sperm, androgen, cyp11c1

#VCT and NPK contributed equally to this work.

Word count: 5276

Abstract

The roles of androgens in male reproductive development and function in zebrafish are poorly understood. To investigate this topic, we employed CRISPR/Cas9 to generate *cyp11c1* (11β-hydroxylase) mutant zebrafish lines. Our study confirms recently published findings from a different cyp11c1$^{-/-}$ mutant zebrafish line, and also reports novel aspects of the phenotype caused by loss of Cyp11c1 function. We report that Cyp11c1-deficient zebrafish display predominantly female secondary sex characteristics, but may possess either ovaries or testes. Moreover, we observed that *cyp11c1*$^{-/-}$ mutant male zebrafish are profoundly androgen- and cortisol-deficient. These results provide further evidence that androgens are dispensable for testis formation in zebrafish, as has been demonstrated previously in androgen-deficient and androgen-resistant zebrafish. Herein, we show that the testes of *cyp11c1*$^{-/-}$ mutant zebrafish exhibit a disorganised tubular structure; and for the first time demonstrate that the spermatic ducts, which connect the testes to the urogenital orifice, are severely hypoplastic in androgen-deficient zebrafish. Furthermore, we show that spermatogenesis and characteristic breeding behaviours are impaired in *cyp11c1*$^{-/-}$ mutant zebrafish. Expression of *nanos2*, a type A spermatogonia marker, was significantly increased in the testes of Cyp11c1-deficient zebrafish, whereas expression of markers for later stages of spermatogenesis was significantly decreased. These observations indicate that in zebrafish, production of type A spermatogonia is androgen-independent, but differentiation of type A spermatogonia is an androgen-dependent process. Overall, our results demonstrate that whilst androgens are not required for testis formation, they play important roles in determining secondary sexual characteristics, proper organisation of seminiferous tubules, and differentiation of male germ cells.

Introduction

The roles of androgens in zebrafish sex differentiation, development of male sexual characteristics, and maintenance and function of the adult testes are poorly understood. Laboratory strains of zebrafish lack sex chromosomes and exhibit polygenic sex determination (Liew et al., 2012). Prior to gonadal differentiation zebrafish develop a juvenile ovary; this is maintained in presumptive females and continues to develop, whereas presumptive males undergo juvenile ovary-to-testis transformation (Uchida et al., 2002, Wang et al., 2007). This process 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, Morthorst et al., 2010, Lee et al., 2017). These findings suggest that androgen-deficiency or androgen-resistance might cause robust feminisation; however this is not the case.

Recent studies have characterised androgen-deficient and -resistant zebrafish (Yong et al., 2017, Crowder et al., 2017, Tang et al., 2018, Oakes et al., 2019, Li et al., 2019). These fish share similar phenotypes, exhibiting primarily female secondary sex characteristics. Despite their appearance, these fish may possess either ovaries or testes, indicating that androgens are dispensable for testis differentiation. Androgen-deficient or -resistant male zebrafish are infertile in standard breeding scenarios, however their sperm may fertilise eggs collected from wild-type females in IVF experiments. Several factors appear to contribute to this phenotype, including disorganised testicular structure and impaired breeding behaviour and spermatogenesis.

Steroid 11β-hydroxylase (CYP11B1) is crucial for conversion of 11-deoxycortisol to cortisol in the final stage of glucocorticoid biosynthesis in humans (Miller and Auchus, 2011). CYP11B subfamily enzymes are located at the inner-mitochondrial membrane, where they are supplied with electrons by NADPH via ferredoxin and ferredoxin reductase, to allow substrate hydroxylation (Schiffer et al.,

2015). In zebrafish, the final stage of glucocorticoid biosynthesis is catalysed by the zebrafish homolog of 11β-hydroxylase, Cyp11c1 (**Figure 1**) (Tokarz et al., 2015). Genomic analysis of CYP11 genes suggests that the CYP11C genes in fish and the CYP11B genes in terrestrial mammals are orthologous, which is consistent with studies on the evolution of adrenal and sex steroidogenic enzymes, reviewed in Baker et al (2015). Unlike the situation in mammals, Cyp11c1 is thought to play an important role in gonadal androgen synthesis in zebrafish (**Figure 1**) (Oakes et al., 2019). In the zebrafish testes Cyp11c1 is found in the steroidogenic Leydig cells, as well as in certain germ cell stages (Caulier et al., 2015). The principal androgens in humans are testosterone and 5α-dihydrotestosterone, whereas in zebrafish the principal androgen is 11-ketotestosterone (Tokarz et al., 2015). This 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).

Cyp11c1 activity depends on electron transfer from the steroidogenic cofactor Fdx1b (Griffin et al., 2016). Our recent work has established Fdx1b-deficient zebrafish as a model of combined 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).

Almost simultaneously with the submission of this manuscript, another paper was published describing a different zebrafish *cyp11c1*-mutant line (Zhang et al., 2020). This study focussed primarily on the phenotypic characteristics of *cyp11c1*-mutant zebrafish during development. Herein, we present *cyp11c1*-mutant zebrafish lines as novel models for research into the roles of steroid deficiency on sex differentiation and adult reproductive processes. Our study confirms key findings of the previously published *cyp11c1*-mutant line and also presents several novel, previously undescribed phenotypes. Our results confirm that Cyp11c1-deficient zebrafish are cortisol and androgen deficient, exhibit infertility and impaired breeding behaviour, as well as reduced spermatogenesis. Novel findings in our study include characterisation of steroid precursor

concentrations, disorganised testis morphology in adult mutants and impaired locomotor behaviour. In addition to this, we have performed in depth molecular investigation into the nature of impaired spermatogenesis in *cyp11c1*$^{-/-}$ mutant zebrafish. We also demonstrate, for the first time, that androgens are crucial for development or maintenance of key anatomical reproductive structures such as the spermatic duct, the structure linking the testes and urogenital orifice.

Methods

Zebrafish husbandry and ethics

Adult zebrafish were maintained in a recirculating system (ZebTECTM, Tecniplast®, Kettering, UK) at 28.5°C on a 10:14hr dark/light photoperiod. Zebrafish were bred from an AB wild-type background. Fish were aged between 96-154 days post fertilisation (dpf) at the time of experimentation. Fish were humanely euthanised by administration of the anaesthetic tricaine mesylate (Pharmaq, Fordingbridge, UK). All experiments with animals were performed under licence from the UK Home Office and approved by the University of Sheffield Animal Welfare and Ethical Review Body (AWERB).

Targeted genetic disruption of *cyp11c1* by CRISPR/Cas9

Mutation of *cyp11c1* was achieved using the SygRNA system (Sigma, St. Louis, Missouri, USA). A crRNA was designed to target exon 2 of *cyp11c1* (ENSDART00000185978.1). ~1nl of a 4µl mixture containing 0.1µM crRNA, 0.1µM tracrRNA (Sigma), 1µl phenol red and 1µl Cas9 (NEB, Ipswich, Massachusetts, USA) was injected into 1-cell stage embryos. The Cas9 cut site overlapped a BslI restriction site, allowing screening for mutant alleles lacking sensitivity to BslI (**Supplementary figure 1**). CRISPR/Cas9-injected embryos were raised and outcrossed to unrelated wild-type fish. The resulting progeny were screened for disruption of *cyp11c1,* and out-of-frame mutations were identified by DNA sequencing.

Steroid quantification by LC-MS/MS

Adult zebrafish were humanely euthanised, transferred to a silanized test tube, and snap-frozen on dry ice. Sample preparation was as previously described except for omission of the final Phree

column elution (Oakes et al., 2019). Steroids were separated and quantified using an Acquity UPLC System (Waters, Milford, Connecticut, USA) coupled to a Xevo TQ-S tandem mass spectrometer (Waters) as previously described (O'Reilly et al., 2017).

Fertility and behavioural analysis, IVF and sperm release

Cyp11c1$^{-/-}$ mutant male zebrafish were outcrossed with unrelated wild-type females on three separate occasions using the pair mating technique, breeding was deemed successful if fertilised eggs were produced (Westerfield, 2000). Breeding behaviour was analysed as previously described (Oakes et al., 2019). For open field tests fish were transferred individually to an opaque test tank and movement was tracked for 5 minutes using Zebralab software (Viewpoint, Lyon, France), this was repeated on three occasions with at least three days between trials. Fish were deemed to exhibit fast swimming behaviour at speeds of greater than 10cm/s.

For *in vitro* fertilisation (IVF) and sperm counting, testes were dissected and lightly homogenised in a 50x mass:volume dilution of Hank's balanced salt solution (HBSS). Eggs were obtained by gentle abdominal palpation of anaesthetised wild-type female fish. 50µl of sperm solution was added to a clutch of eggs, followed by 400µl of aquarium water; after two minutes a further 2ml of aquarium water was added (Westerfield, 2000). Fertilisation was confirmed under a dissecting microscope. For sperm counting, 10µl of sperm solution was transferred to each chamber of an improved Neubauer haemocytometer (Hawksley, Sussex, UK). A minimum of 200 sperm were counted in each chamber and the number of sperm/nl was multiplied by the dilution factor (50x) to obtain sperm counts. Gonadosomatic index was calculated using the formula GSI=[gonad weight/total tissue weight]×100.

To assess sperm release, *cyp11c1*$^{-/-}$ mutant and wild-type sibling zebrafish were anaesthetised, and semen was collected by stroking the abdomen with blunt ended forceps (Millipore, Burlington, Massachusetts, USA), followed by aspiration of expelled fluid into a microcapillary tube and transfer

to 25µl of ice cold HBSS (Westerfield, 2000). Presence of mature sperm was confirmed by visualisation under a 20x objective.

Histology

Preparation of samples and H+E staining was performed as previously described (Oakes et al., 2019).

Gene expression analysis by quantitative PCR (qPCR)

For larval gene expression analysis, the progeny of a *cyp11c1*$^{+/-}$ incross were sorted by visual background adaption (VBA) at 4-5dpf (Griffin et al., 2016). Larvae were housed in dark conditions for 1hr, followed by a 20 minute light exposure. Larvae were subsequently sorted into lightly (VBA+) and darkly (VBA-) pigmented groups. Sorted larvae were pooled into groups of 20 and snap frozen on dry ice. For adult gene expression analysis, fish were humanely euthanised, dissected, and organs collected by snap freezing on dry ice.

Total RNA was isolated using Trizol (Ambion, Texas, USA). cDNA was prepared using SuperScript II (Thermo Fisher Scientific, California, USA) with 20mer oligo(dT) primers (IDT, Coralville, Iowa, USA) and 1µg of RNA. GoTaq qPCR master mix (Promega, Madison, Wisconsin, USA) was utilised in reactions containing 1µl cDNA synthesis product and specific primers (**Supplementary table 1**) at 1000nM. Reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Data were analysed by the Livak method (Livak and Schmittgen, 2001) with *ef1a* as reference gene. Fold changes in gene expression are displayed relative to expression in wild-type male tissue.

Statistical methods

Statistical analysis was performed in Graphpad Prism (GraphPad Software, San Diego, California, USA). Data normality was assessed using inbuilt tests. Normally distributed biometric and qPCR data was analysed using unpaired *t*-tests, non-normal data was analysed by Mann-Whitney tests. Behavioural data was analysed using multiple *t*-tests (Holm-Sidak method). Scatter plot error bars represent the standard error of the mean. Statistical significances are reported using asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns=not significant.

Results

Generation of *cyp11c1* mutant alleles by CRISPR/Cas9

Exon 2 of *cyp11c1* was targeted using the SygRNA two-part system (see methods). CRISPR-injected fish were outcrossed at ~10wpf, and heritable mutations were identified in their progeny. Several out-of-frame mutations were identified, including 11bp (c.312_322del, p.Glu105Profs*33, allele number SH548) and 47bp (c.285_331del, p.Met96Hisfs*30, allele number SH547) deletions. Both are predicted to produce a truncated protein ~25% the size of the wild-type isoform (**Supplementary figure 2**). Inheritance of *cyp11c1* mutant alleles did not significantly deviate from expected Mendelian ratios.

Cortisol-deficient zebrafish larvae exhibit impaired visual background adaptation (VBA) – the ability to adapt pigmentation to light conditions. We found that VBA was impaired in *cyp11c1*$^{-/-}$ mutant larvae at 5dpf, and expression of glucocorticoid responsive genes *fkbp5* and *pck1* was significantly decreased, suggesting reduced cortisol production due to loss of Cyp11c1 function (**Supplementary table 2, Supplementary figure 3**).

Cyp11c1$^{-/-}$ mutant zebrafish exhibit female secondary sex characteristics but may possess either ovaries or testes

Morphological secondary sex characteristics in zebrafish include body shape, fin and body pigmentation, and genital papilla prominence. Male zebrafish are streamlined in shape, have palely pigmented dorsal fins, and orange striped anal fins. Female fish have a more rounded abdomen, green-yellow pigmented dorsal fins and little orange pigmentation in the anal fin (**Figure 2**). Female zebrafish have a large and prominent genital papilla; in males this structure is much smaller and mostly hidden from view.

Upon raising the progeny of *cyp11c1*$^{+/-}$ incrosses, it was apparent that all homozygous mutant fish displayed predominantly female secondary sex characteristics (**Figure 2**). Close inspection revealed that some *cyp11c1*$^{-/-}$ mutant fish had prominent genital papillae like wild-type females, whereas others had small hidden genital papillae like those of 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 ratio of males:females (testes:ovary) in populations of wild-type and *cyp11c1*$^{-/-}$ zebrafish did not significantly differ.

Additionally, biometric data was also collected. Males from both *cyp11c1*$^{-/-}$ mutant lines were significantly longer and heavier than wild-type siblings (**Figure 2**).

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 concentrations in samples prepared from whole adult zebrafish males. Cortisol concentrations were profoundly decreased by mutation of *cyp11c1* (**Figure 3**), whereas concentrations of its 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 blocking of the androgen synthesis pathway; shunting of glucocorticoid precursors into the sex steroid pathway may also contribute to increased androstenedione concentrations. Blockade of the androgen synthesis pathway was evidenced by undetectable concentrations of 11-ketotestosterone and its precursor 11-ketoandrostenedione (**Figure 3**). Concentrations of testosterone were not affected by mutation of *cyp11c1* (**Figure 3**).

Expression of the glucocorticoid-responsive genes *fkbp5* and *pck1* (Griffin et al., 2016, Eachus et al., 2017) was significantly reduced in *cyp11c1*$^{-/-}$ mutant male zebrafish livers compared to wild-type siblings, thus demonstrating the systemic consequences of glucocorticoid deficiency. An apparent decrease in the expression of the proposed androgen-responsive gene *cyp2k22*, which is postulated to play a role in androgen metabolism, (Fetter et al., 2015, Siegenthaler et al., 2017) did not achieve statistical significance (**Figure 4**).

Disruption of *cyp11c1* results in infertility and impaired breeding behaviour

Androgen-resistant and androgen-deficient male zebrafish are infertile (Crowder et al., 2017, Oakes et al., 2019) and the incidence of stereotypical breeding behaviours is decreased (Yong et al., 2017). To investigate the impact of Cyp11c1-deficiency on breeding behaviour in male zebrafish, we analysed two well-characterised breeding behaviours. In all trials, the number of intimate contacts, where fish touch or cross one another, and the duration of chasing, where one fish closely follows the other, were significantly reduced in *cyp11c1*$^{-/-}$ mutant lines compared to wild-type siblings. (**Figure 5**). The proportion of trials resulting in production of fertilised embryos was also recorded. Outcrosses of wild-type females and wild-type sibling males from the 11bp and 47bp alleles produced fertilised embryos in 92% and 66% of crosses respectively. No fertilised embryos were observed in any crosses with *cyp11c1*$^{-/-}$ mutant zebrafish (**Table 1**). Despite exhibiting infertility in normal breeding scenarios, the sperm of *cyp11c1*$^{-/-}$ mutant zebrafish were able to fertilise eggs collected from wild-type females by IVF (**Table 2**).

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 open field tests. This revealed that the total distance swam, as well as duration of fast swimming, was significantly and consistently reduced in *cyp11c1*$^{-/-}$ mutant males

(**Figure 6**). Freezing duration, the duration for which the fish was stationary in the tank, was also recorded. Freezing duration was consistently greater in trials involving *cyp11c1*$^{-/-}$ mutant zebrafish; however, these results were not statistically significant, presumably due to the extremely high variability with which fish express this phenotype (**Figure 6**).

Cyp11c1 disruption results in testicular disorganisation and reduced spermatogenesis

In order to examine the impact of *cyp11c1* mutations on testis morphology, we collected coronal sections through whole adult zebrafish and performed H&E staining. The testes of wild-type males appeared to be well organised, with defined seminiferous tubule structures clearly visible (**Figure 7**). In contrast, the structure of *cyp11c1*$^{-/-}$ mutant testes was generally disorganised, with defined seminiferous tubule structures rarely in evidence. 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 7**). The testes of *cyp11c1*$^{-/-}$ mutant zebrafish also contained cells at all stages of spermatogenesis; however, the proportion of developing germ cells to mature sperm appeared to be much greater. This was accompanied by a qualitative reduction in the amount of mature spermatozoa; this finding was later quantified by sperm counting (**Figure 7**). No difference in gonadosomatic index, the percentage contribution of the gonads to body weight, was recorded for either *cyp11c1*$^{-/-}$ mutant allele.

Cyp11c1 is crucial for development of the spermatic duct

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 the 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 8**). The spermatic ducts of wild-type zebrafish comprised an extensive tubular structure, with tubules containing spermatozoa (**Figure 8A+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 8B+E**) or existed as a slightly more extensive structure containing some mature spermatozoa (**Figure 8C+F**).

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 manual gamete expression (Westerfield, 2000). Cyp11c1-deficient zebrafish exhibited profoundly impaired sperm release, although spermatozoa were observed in samples obtained from some *cyp11c1*$^{47bp-/-}$ mutant zebrafish in one of two trials (**Table 3**).

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

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

Igf3 and Insl3 are important factors in zebrafish spermatogenesis; specifically, they are involved in regulating the proliferation and differentiation of type A spermatogonia (Nobrega et al., 2015, Assis et al., 2016, Morais et al., 2017). Both *igf3* and *insl3* were significantly down-regulated in *cyp11c1*$^{-/-}$ mutant zebrafish, potentially suggesting impairment of early stages of spermatogenesis in these mutants (**Figure 9**).

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 Cyp11c1-deficiency (**Figure 9**). Expression of the androgen receptor, via which 11-ketotestosterone exerts its effects on gene expression, was significantly upregulated in the testes of *cyp11c1*$^{-/-}$ mutant zebrafish (**Figure 9**). 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 (Gregory and Kaiser, 2004, Poon et al., 2009, Cai et al., 2011). We observed significant down-regulation of *inha* in Cyp11c1-deficient zebrafish (**Figure 9**).

Expression of spermatogenesis marker genes suggests a crucial role for androgens in the differentiation of type A spermatogonia into meiotic spermatocytes

Spermatogenesis comprises a series of cell division and differentiation events whereupon spermatogonial stem cells give rise to primary spermatocytes, which then enter meiosis, and eventually give rise to mature haploid spermatozoa. 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. To this end, we measured the expression of marker genes for several stages of spermatogenesis (**Figure 10**). *Nanos2* and *piwil1* are expressed in type A spermatogonia (Chen et al., 2013, Beer and Draper, 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 10**). No change in the 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* (Yano et al., 2008, Nobrega et al., 2015) was significantly reduced in *cyp11c1*$^{-/-}$ mutant zebrafish, indicating

320 a reduced proportion of cells at the later stages of spermatogenesis (**Figure 10**). Taken together,
321 these results indicate an accumulation of type A spermatogonia in *cyp11c1*$^{-/-}$ mutant testes, due to
322 the blockade or impairment of the transformation of spermatogonia into spermatocytes and
323 subsequently spermatozoa.

324

Discussion

Herein, we described the phenotype of androgen- and cortisol-deficient *cyp11c1* mutant zebrafish, paying particular attention to the roles of these steroids in the development, maintenance and function of the male reproductive system. We produced *cyp11c1*$^{-/-}$ mutant alleles using CRISPR/Cas9 to target exon 2 of ENSDART00000185978.1 (**Supplementary figure 2**), whereas the previously published *cyp11c1*-mutant zebrafish line used CRISPR to target exon 3 of this transcript (Zhang et al., 2020). These mutations are predicted to produce similar truncated and functionally inactive protein products**.**

Cyp11c1 is important for production of cortisol and 11-ketotestosterone (11KT), the principal zebrafish androgen (de Waal et al., 2008). Cyp11c1-deficient zebrafish exhibit profound deficiencies of both steroids, confirming the crucial role of Cyp11c1 in steroidogenesis (**Figure 3**) (Zhang et al., 2020). In addition to measuring cortisol and 11KT, we measured the concentrations of several intermediate steroid hormone precursors. We observed accumulation of 11-deoxycortisol and androstenedione (**Figure 3**); precursors that may enter other steroidogenic pathways such as the oestrogen biosynthetic pathway. The phenotypic impact of the shunting of steroid precursors into alternative pathways remains unknown. Unchanged concentrations of testosterone in Cyp11c1-deficient zebrafish suggest that excess androstenedione was not converted to testosterone; this provides *in vivo* evidence for previous *in vitro* findings indicating that conversion of androstenedione to testosterone, followed by 11β-hydroxylation of testosterone by Cyp11c1 to produce the 11KT precursor 11β-hydroxytestosterone, is a minor pathway to 11KT production in zebrafish (de Waal et al., 2008).

Decreased cortisol concentrations were reflected in decreased expression of the glucocorticoid responsive genes *fkbp5* and *pck1* (Griffin et al., 2016, Eachus et al., 2017) in *cyp11c1*$^{-/-}$ mutant male liver tissue, demonstrating systemic glucocorticoid deficiency (**Figure 4**). *Cyp2k22* has been 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 (Oakes et al., 2019). An apparent reduction in the expression of *cyp2k22* in the livers of *cyp11c1*$^{-/-}$ mutant zebrafish was not significant (**Figure 4**). 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, Zhai et al., 2018, Oakes et al., 2019). Feminisation of anal fin pigmentation appears to be to more pronounced in our study compared to Zhang et al (2020). This variability may arise from differences in time of analysis and age of fish. In addition to analysis of the anal fin, we have also described feminisation of dorsal fin pigmentation in Cyp11c1-deficient males, this was not formally assessed in the study of Zhang et al, but appeared to be the case in the representative fish presented in their paper. Overall, these findings suggest that androgens may induce expression of genes important for fin colour patterning during development.

Cyp11c1-deficient adult male zebrafish were infertile in normal breeding scenarios (**Table 1**); nevertheless, we observed that their sperm could fertilise eggs collected from wild-type female zebrafish by IVF (**Table 2**). These findings confirm the results of Zhang et al (2020), and indicate that Cyp11c1-deficient zebrafish are able to produce mature sperm but are infertile due to another factor, such as impaired breeding behaviour or spermatogenesis, or morphological disruption of the testes or male reproductive tract resulting in impaired sperm release.

Breeding behaviours are decreased in both androgen-deficient and androgen-resistant zebrafish (Yong et al., 2017, Oakes et al., 2019). In this regard, the phenotype of our new *cyp11c1*$^{-/-}$ mutant zebrafish lines closely resembles that of *fdx1b*$^{-/-}$ mutant zebrafish (**Figure 5**) (Oakes et al., 2019). This finding also confirms similar results obtained by Zhang et al (2020), although different behavioural assays were used. A key difference is our experimental design, in which behavioural trials were

repeated several times with the same fish to control for novelty and habituation. Importantly, our results were similar irrespective of trial number, indicating that the phenotype remains the same despite habituation to the technique (**Figure 5**).

In addition to analysis of breeding behaviour, we have demonstrated altered locomotor behaviour in Cyp11c1-deficient adult zebrafish. To our knowledge this is the first time adult androgen- and glucocorticoid-deficient zebrafish have been revealed to exhibit such a behavioural phenotype. Cyp11c1-deficient male zebrafish exhibited decreased locomotor activity compared to wild-type siblings (**Figure 6**). Decreased locomotor activity may affect readouts of multiple breeding behaviours; for example, slow swimming fish 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 velocities (Ziv et al., 2013). Cortisol deficiency may be responsible for the impaired locomotor behaviour seen in Cyp11c1-deficient zebrafish, but may not fully explain the reduction in breeding behaviours, as mutation of the androgen receptor also produces a similar phenotype (Yong et al., 2017). Overall, the behavioural phenotype of Cyp11c1-deficient zebrafish is likely to result from disruption of both glucocorticoid- and androgen-regulated processes.

As previously described in androgen-deficient and androgen-resistant zebrafish lines, we show that androgen signalling is dispensable for definitive testicular differentiation (Crowder et al., 2017, Oakes et al., 2019). In the other recently reported *cyp11c1*$^{-/-}$ mutant zebrafish line, histological examination was restricted to the stage when gonadal differentiation is taking place. Zhang et al (2020) showed that Cyp11c1-deficiency resulted in prolonged juvenile ovary-to-testis transformation, with degenerating oocytes present long after the normal period of testis differentiation. In contrast to Zhang et al, we examined the histological testicular phenotype of adult *cyp11c1*$^{-/-}$ mutant male zebrafish. Our histological examination of adult *cyp11c1*$^{-/-}$ mutants revealed

that their testes were highly disorganised: seminiferous tubules were poorly defined, and the quantity of spermatozoa was reduced (**Figures 7**). The histological appearance of *cyp11c1*$^{-/-}$ mutant testes was similar to that described in other zebrafish models of disrupted androgen signalling (Oakes et al., 2019, Crowder et al., 2017), thus providing further confirmation that androgens are required for correct organisation and morphological development or maintenance of the testes. Tubular structure formation in 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 well after the gonad is committed to testis development (van der Ven and Wester, 2003). Overall, it appears that the crucial roles for androgens in zebrafish testicular development are temporal control of gonadal differentiation (Zhang et al., 2020), and subsequent formation and maintenance of correct seminiferous tubule organisation in the juvenile and adult testis.

We 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, as a role in this process has been proposed for this gene in a related teleost (Nakamoto et al., 2005). *Sox9a* expression was unaffected by mutation of *cyp11c1*, whereas *inha* was significantly downregulated (**Figure 9**). The mechanism by which androgens control appropriate testis tubule morphogenesis or maintenance remains elusive, and is an exciting topic for further study.

Zhang et al (2020) described that, despite their infertility, Cyp11c1-deficent zebrafish could produce morphologically normal spermatozoa. However, when Cyp11c1-deficient males were subjected to manual gamete expression, a reduced volume of semen was produced in comparison to wild-types, indicating impaired spermatogenesis or sperm release in the *cyp11c1*$^{-/-}$ mutants (Zhang et al., 2020). In our Cyp11c1-deficient zebrafish, whole-testes sperm counts were significantly lower than in wild-

type siblings, also indicating impaired spermatogenesis (**Figure 7**). To further investigate impaired spermatogenesis we performed novel and in-depth characterisation of spermatogenic defects in Cyp11c1-deficient zebrafish by measuring the expression of spermatogenic factors and spermatogenic stage-specific marker genes. We observed significant down-regulation of *igf3* and *insl3* in Cyp11c1-deficient zebrafish (**Figure 9**); both genes are important for the differentiation and proliferation of type A spermatogonia (Nobrega et al., 2015, Assis et al., 2016, Morais et al., 2017). 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 10**) (Yano et al., 2008, Ozaki et al., 2011, Nobrega et al., 2015). Histological examination also suggested a qualitative increase in the proportion of developing sperm to mature sperm in the testes (**Figure 7**). Taken together these findings support the proposition that, whilst production of type A spermatogonia is normal in *cyp11c1*$^{-/-}$ mutant testes, the subsequent differentiation of type A spermatogonia is highly androgen-dependent.

In addition to impaired spermatogonial differentiation, entry of type B spermatogonia into meiosis may also be disrupted in *cyp11c1*$^{-/-}$ mutant zebrafish. Whilst expression of the type B spermatogonia marker *dazl* was unchanged by mutation *cyp11c1,* expression of the spermatocyte marker *sycp3* was downregulated, as was that of the spermatid marker *odf3b* (**Figure 10**). Spermatogenic arrest or delay during meiosis has previously been reported in androgen receptor mutant zebrafish (Yu et al., 2018). Sycp3 is a component of the synaptonemal complex, which plays important roles during meiotic prophase, including regulation of chromosome recombination (Page and Hawley, 2004, Syrjänen et al., 2014). Thus, reduced expression of *sycp3* in *cyp11c1*$^{-/-}$ mutant spermatocytes could contribute to the impairment of meiosis.

Expression of a small number of genes was characterised at the adult stage in the previously published *cyp11c1*$^{-/-}$ mutant zebrafish line (Zhang et al., 2020). Both the previous mutant and the mutant described herein exhibited decreased expression of the spermatogenic factor *insl3*. In

contrast to observations described by Zhang et al, no change of *dmrt1* expression was observed in our *cyp11c1*$^{-/-}$ mutants (**Figure 9**), which is consistent with our previously reported findings in androgen- and cortisol-deficient *fdx1b*$^{-/-}$ mutants (Oakes et al., 2019).

Attempts to manually collect semen from Cyp11c1-deficient zebrafish revealed that sperm release may be impaired. However, this technique may not accurately replicate natural ejaculation and therefore successful sperm release in natural breeding conditions cannot be ruled out. As sperm release appeared to be impaired, we investigated the structure of the spermatic duct and found that it to be severely hypoplastic in Cyp11c1-deficient zebrafish. Little is known about the development of this structure; however, we have shown here for the first time that its development is highly steroid dependent, and this is likely to be mediated by 11-ketotestosterone. This structure may be comparable to Wolffian duct structures in mammals, however these structures are of different embryological origins and may be analogous in function alone (Shaw and Renfree, 2014, Matthews et al., 2018). Nevertheless, both structures appear to be highly dependent on androgens for their development, as abnormal Wolffian duct structures are frequently seen in complete androgen insensitivity syndrome (Hannema et al., 2006, Barbaro et al., 2007). Wolffian duct structures are also absent in AR knock-out mice (Yeh et al., 2002).

Herein, we have described novel zebrafish lines carrying mutation of *cyp11c1*, which is crucial for 11-ketotestosterone and cortisol biosynthesis. In addition to confirming several results described in a recently published *cyp11c1* zebrafish mutant (Zhang et al., 2020), our study describes novel phenotypes, including testicular disorganisation, hypoplastic spermatic ducts, and impaired locomotor function, as well as characterising spermatogenic defects through the measurement of marker gene expression. As such, our work represents a significant and novel contribution to the literature regarding the roles of steroids in regulation of zebrafish reproduction. *Cyp11c1*$^{-/-}$ 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.

Declaration of interest and funding

Declaration of interest: The authors have no conflicts of interest to declare.

Funding: This work was supported by the International Fund Congenital Adrenal Hyperplasia 2017 research grant (to NPK and VTC) and the Deutsche Forschungsgemeinschaft (KR 3363/3-1).

Author contribution statement: VTC and NPK contributed equally to this work.

References

ANDERSEN, L., HOLBECH, H., GESSBO, A., NORRGREN, L. & PETERSEN, G. I. 2003. Effects of exposure to 17alpha-ethinylestradiol during early development on sexual differentiation and induction of vitellogenin in zebrafish (Danio rerio). *Comp Biochem Physiol C Toxicol Pharmacol,* 134**,** 365-74.

ASSIS, L. H. C., CRESPO, D., MORAIS, R. D. V. S., FRANÇA, L. R., BOGERD, J. & SCHULZ, R. W. 2016. INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (Danio rerio). *Cell and Tissue Research,* 363**,** 579-588.

BAKER, M. E., NELSON, D. R. & STUDER, R. A. 2015. Origin of the response to adrenal and sex steroids: Roles of promiscuity and co-evolution of enzymes and steroid receptors. *J Steroid Biochem Mol Biol,* 151**,** 12-24.

BARBARO, M., OSCARSON, M., ALMSKOG, I., HAMBERG, H. & WEDELL, A. 2007. Complete androgen insensitivity without Wolffian duct development: the AR-A form of the androgen receptor is not sufficient for male genital development. *Clin Endocrinol (Oxf),* 66**,** 822-6.

BEER, R. L. & DRAPER, B. W. 2013. nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. *Dev Biol,* 374**,** 308-18.

BRION, F., TYLER, C. R., PALAZZI, X., LAILLET, B., PORCHER, J. M., GARRIC, J. & FLAMMARION, P. 2004. Impacts of 17beta-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (Danio rerio). *Aquat Toxicol,* 68**,** 193-217.

CAI, K., HUA, G., AHMAD, S., LIANG, A., HAN, L., WU, C., YANG, F. & YANG, L. 2011. Action Mechanism of Inhibin α-Subunit on the Development of Sertoli Cells and First Wave of Spermatogenesis in Mice. *PLOS ONE,* 6**,** e25585.

CAULIER, M., BRION, F., CHADILI, E., TURIES, C., PICCINI, B., PORCHER, J. M., GUIGUEN, Y. & HINFRAY, N. 2015. Localization of steroidogenic enzymes and Foxl2a in the gonads of mature zebrafish (Danio rerio). *Comp Biochem Physiol A Mol Integr Physiol,* 188**,** 96-106.

CHEN, S. X., BOGERD, J., SCHOONEN, N. E., MARTIJN, J., DE WAAL, P. P. & SCHULZ, R. W. 2013. A progestin (17alpha,20beta-dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *Gen Comp Endocrinol,* 185**,** 1-9.

CROWDER, C. M., LASSITER, C. S. & GORELICK, D. A. 2017. Nuclear Androgen Receptor Regulates Testes Organization and Oocyte Maturation in Zebrafish. *Endocrinology,* 159**,** 980-993.

DE WAAL, P. P., WANG, D. S., NIJENHUIS, W. A., SCHULZ, R. W. & BOGERD, J. 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (Danio rerio) testis. *Reproduction,* 136**,** 225-34.

EACHUS, H., ZAUCKER, A., OAKES, J. A., GRIFFIN, A., WEGER, M., GURAN, T., TAYLOR, A., HARRIS, A., GREENFIELD, A., QUANSON, J. L., STORBECK, K. H., CUNLIFFE, V. T., MULLER, F. & KRONE, N. 2017. Genetic Disruption of 21-Hydroxylase in Zebrafish Causes Interrenal Hyperplasia. *Endocrinology,* 158**,** 4165-4173.

FETTER, E., SMETANOVA, S., BALDAUF, L., LIDZBA, A., ALTENBURGER, R., SCHUTTLER, A. & SCHOLZ, S. 2015. Identification and Characterization of Androgen-Responsive Genes in Zebrafish Embryos. *Environ Sci Technol,* 49**,** 11789-98.

GREGORY, S. J. & KAISER, U. B. 2004. Regulation of gonadotropins by inhibin and activin. *Semin Reprod Med,* 22**,** 253-67.

GRIFFIN, A., PARAJES, S., WEGER, M., ZAUCKER, A., TAYLOR, A. E., O'NEIL, D. M., MULLER, F. & KRONE, N. 2016. Ferredoxin 1b (Fdx1b) Is the Essential Mitochondrial Redox Partner for Cortisol Biosynthesis in Zebrafish. *Endocrinology,* 157**,** 1122-34.

HANNEMA, S. E., SCOTT, I. S., RAJPERT-DE MEYTS, E., SKAKKEBAEK, N. E., COLEMAN, N. & HUGHES, I. A. 2006. Testicular development in the complete androgen insensitivity syndrome. *J Pathol,* 208**,** 518-27.

LARSEN, M. G. & BAATRUP, E. 2010. Functional behavior and reproduction in androgenic sex reversed zebrafish (Danio rerio). *Environ Toxicol Chem,* 29**,** 1828-33.
LAU, E. S., ZHANG, Z., QIN, M. & GE, W. 2016. Knockout of Zebrafish Ovarian Aromatase Gene (cyp19a1a) by TALEN and CRISPR/Cas9 Leads to All-male Offspring Due to Failed Ovarian Differentiation. *Sci Rep,* 6**,** 37357.
LEE, S. L. J., HORSFIELD, J. A., BLACK, M. A., RUTHERFORD, K., FISHER, A. & GEMMELL, N. J. 2017. Histological and transcriptomic effects of 17alpha-methyltestosterone on zebrafish gonad development. *BMC Genomics,* 18**,** 557.
LI, N., OAKES, J. A., STORBECK, K.-H., CUNLIFFE, V. T. & KRONE, N. 2019. The P450 side chain cleavage enzyme Cyp11a2 facilitates steroidogenesis in zebrafish. *Journal of Endocrinology***,** JOE-19-0384.
LIEW, W. C., BARTFAI, R., LIM, Z., SREENIVASAN, R., SIEGFRIED, K. R. & ORBAN, L. 2012. Polygenic sex determination system in zebrafish. *PLoS One,* 7**,** e34397.
LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.
MATTHEWS, J. L., MURPHY, J. M., CARMICHAEL, C., YANG, H., TIERSCH, T., WESTERFIELD, M. & VARGA, Z. M. 2018. Changes to Extender, Cryoprotective Medium, and In Vitro Fertilization Improve Zebrafish Sperm Cryopreservation. *Zebrafish,* 15**,** 279-290.
MENKE, A. L., SPITSBERGEN, J. M., WOLTERBEEK, A. P. & WOUTERSEN, R. A. 2011. Normal anatomy and histology of the adult zebrafish. *Toxicol Pathol,* 39**,** 759-75.
MILLER, W. L. & AUCHUS, R. J. 2011. The Molecular Biology, Biochemistry, and Physiology of Human Steroidogenesis and Its Disorders. *Endocrine Reviews,* 32**,** 81-151.
MORAIS, R., CRESPO, D., NOBREGA, R. H., LEMOS, M. S., VAN DE KANT, H. J. G., DE FRANCA, L. R., MALE, R., BOGERD, J. & SCHULZ, R. W. 2017. Antagonistic regulation of spermatogonial differentiation in zebrafish (Danio rerio) by Igf3 and Amh. *Mol Cell Endocrinol*.
MORTHORST, J. E., HOLBECH, H. & BJERREGAARD, P. 2010. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. *Aquat Toxicol,* 98**,** 336-43.
NAKAMOTO, M., SUZUKI, A., MATSUDA, M., NAGAHAMA, Y. & SHIBATA, N. 2005. Testicular type Sox9 is not involved in sex determination but might be in the development of testicular structures in the medaka, Oryzias latipes. *Biochem Biophys Res Commun,* 333**,** 729-36.
NOBREGA, R. H., MORAIS, R. D., CRESPO, D., DE WAAL, P. P., DE FRANCA, L. R., SCHULZ, R. W. & BOGERD, J. 2015. Fsh Stimulates Spermatogonial Proliferation and Differentiation in Zebrafish via Igf3. *Endocrinology,* 156**,** 3804-17.
O'REILLY, M. W., KEMPEGOWDA, P., JENKINSON, C., TAYLOR, A. E., QUANSON, J. L., STORBECK, K. H. & ARLT, W. 2017. 11-Oxygenated C19 Steroids Are the Predominant Androgens in Polycystic Ovary Syndrome. *J Clin Endocrinol Metab,* 102**,** 840-848.
OAKES, J. A., LI, N., WISTOW, B. R. C., GRIFFIN, A., BARNARD, L., STORBECK, K. H., CUNLIFFE, V. T. & KRONE, N. P. 2019. Ferredoxin 1b deficiency leads to testis disorganization, impaired spermatogenesis and feminization in zebrafish. *Endocrinology*.
ORN, S., HOLBECH, H. & NORRGREN, L. 2016. Sexual disruption in zebrafish (Danio rerio) exposed to mixtures of 17alpha-ethinylestradiol and 17beta-trenbolone. *Environ Toxicol Pharmacol,* 41**,** 225-31.
OZAKI, Y., SAITO, K., SHINYA, M., KAWASAKI, T. & SAKAI, N. 2011. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. *Gene Expr Patterns,* 11**,** 309-15.
PAGE, S. L. & HAWLEY, R. S. 2004. The genetics and molecular biology of the synaptonemal complex. *Annu Rev Cell Dev Biol,* 20**,** 525-58.
POON, S. K., SO, W. K., YU, X., LIU, L. & GE, W. 2009. Characterization of inhibin alpha subunit (inha) in the zebrafish: evidence for a potential feedback loop between the pituitary and ovary. *Reproduction,* 138**,** 709-19.

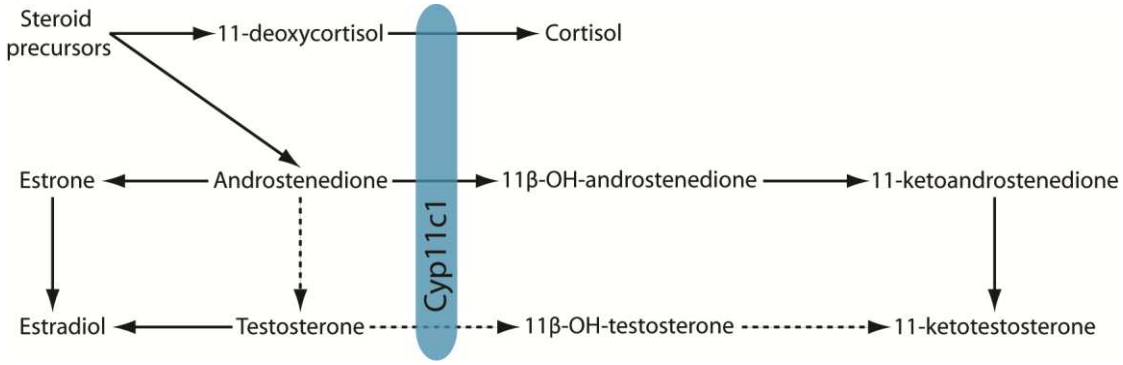
584 SAFIAN, D., MORAIS, R. D., BOGERD, J. & SCHULZ, R. W. 2016. Igf Binding Proteins Protect
585 Undifferentiated Spermatogonia in the Zebrafish Testis Against Excessive Differentiation.
586 *Endocrinology,* 157**,** 4423-4433.
587 SCHIFFER, L., ANDERKO, S., HANNEMANN, F., EIDEN-PLACH, A. & BERNHARDT, R. 2015. The CYP11B
588 subfamily. *J Steroid Biochem Mol Biol,* 151**,** 38-51.
589 SHAW, G. & RENFREE, M. B. 2014. Wolffian duct development. *Sex Dev,* 8**,** 273-80.
590 SIEGENTHALER, P. F., ZHAO, Y., ZHANG, K. & FENT, K. 2017. Reproductive and transcriptional effects
591 of the antiandrogenic progestin chlormadinone acetate in zebrafish (Danio rerio). *Environ*
592 *Pollut,* 223**,** 346-356.
593 SUN, D., ZHANG, Y., WANG, C., HUA, X., ZHANG, X. A. & YAN, J. 2013. Sox9-related signaling controls
594 zebrafish juvenile ovary-testis transformation. *Cell Death Dis,* 4**,** e930.
595 SYRJÄNEN, J. L., PELLEGRINI, L. & DAVIES, O. R. 2014. A molecular model for the role of SYCP3 in
596 meiotic chromosome organisation. *eLife,* 3**,** e02963.
597 TANG, H., CHEN, Y., WANG, L., YIN, Y., LI, G., GUO, Y., LIU, Y., LIN, H., CHENG, C. H. K. & LIU, X. 2018.
598 Fertility impairment with defective spermatogenesis and steroidogenesis in male zebrafish
599 lacking androgen receptor. *Biol Reprod,* 98**,** 227-238.
600 TOKARZ, J., MOLLER, G., HRABE DE ANGELIS, M. & ADAMSKI, J. 2015. Steroids in teleost fishes: A
601 functional point of view. *Steroids,* 103**,** 123-44.
602 UCHIDA, D., YAMASHITA, M., KITANO, T. & IGUCHI, T. 2002. Oocyte apoptosis during the transition
603 from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J Exp Biol,*
604 205**,** 711-8.
605 VAN DER VEN, L. & WESTER, P. 2003. *Histology and Histopathology Atlas of the Zebrafish,*
606 Netherlands.
607 WANG, X. G., BARTFAI, R., SLEPTSOVA-FREIDRICH, I. & ORBAN, L. 2007. The timing and extent of
608 'juvenile ovary' phase are highly variable during zebrafish testis differentiation. *Journal of*
609 *Fish Biology,* 70**,** 33-44.
610 WEBSTER, K. A., SCHACH, U., ORDAZ, A., STEINFELD, J. S., DRAPER, B. W. & SIEGFRIED, K. R. 2017.
611 Dmrt1 is necessary for male sexual development in zebrafish. *Dev Biol,* 422**,** 33-46.
612 WESTERFIELD, M. 2000. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).
613 4th ed. Eugene: Univ. of Oregon Press, .
614 YANO, A., SUZUKI, K. & YOSHIZAKI, G. 2008. Flow-cytometric isolation of testicular germ cells from
615 rainbow trout (Oncorhynchus mykiss) carrying the green fluorescent protein gene driven by
616 trout vasa regulatory regions. *Biol Reprod,* 78**,** 151-8.
617 YEH, S., TSAI, M.-Y., XU, Q., MU, X.-M., LARDY, H., HUANG, K.-E., LIN, H., YEH, S.-D., ALTUWAIJRI, S.,
618 ZHOU, X., XING, L., BOYCE, B. F., HUNG, M.-C., ZHANG, S., GAN, L. & CHANG, C. 2002.
619 Generation and characterization of androgen receptor knockout (ARKO) mice: An in
620 vivo model for the study of androgen functions in selective tissues. *Proceedings of the*
621 *National Academy of Sciences,* 99**,** 13498.
622 YIN, Y., TANG, H., LIU, Y., CHEN, Y., LI, G., LIU, X. & LIN, H. 2017. Targeted Disruption of Aromatase
623 Reveals Dual Functions of cyp19a1a During Sex Differentiation in Zebrafish. *Endocrinology,*
624 158**,** 3030-3041.
625 YONG, L., THET, Z. & ZHU, Y. 2017. Genetic editing of the androgen receptor contributes to impaired
626 male courtship behavior in zebrafish. *J Exp Biol,* 220**,** 3017-3021.
627 YU, G., ZHANG, D., LIU, W., WANG, J., LIU, X., ZHOU, C., GUI, J. & XIAO, W. 2018. Zebrafish androgen
628 receptor is required for spermatogenesis and maintenance of ovarian function. *Oncotarget,*
629 9**,** 24320-24334.
630 ZHAI, G., SHU, T., XIA, Y., LU, Y., SHANG, G., JIN, X., HE, J., NIE, P. & YIN, Z. 2018. Characterization of
631 sexual trait development in cyp17a1-deficient zebrafish. *Endocrinology***,** en.2018-00551-
632 en.2018-00551.

633 ZHANG, Q., YE, D., WANG, H., WANG, Y. & SUN, Y. 2020. Zebrafish cyp11c1 knockout reveals the
634 roles of 11-ketotestosterone and cortisol in sexual development and reproduction.
635 *Endocrinology*.
636 ZIV, L., MUTO, A., SCHOONHEIM, P. J., MEIJSING, S. H., STRASSER, D., INGRAHAM, H. A., SCHAAF, M.
637 J., YAMAMOTO, K. R. & BAIER, H. 2013. An affective disorder in zebrafish with mutation of
638 the glucocorticoid receptor. *Mol Psychiatry,* 18**,** 681-91.

639

640

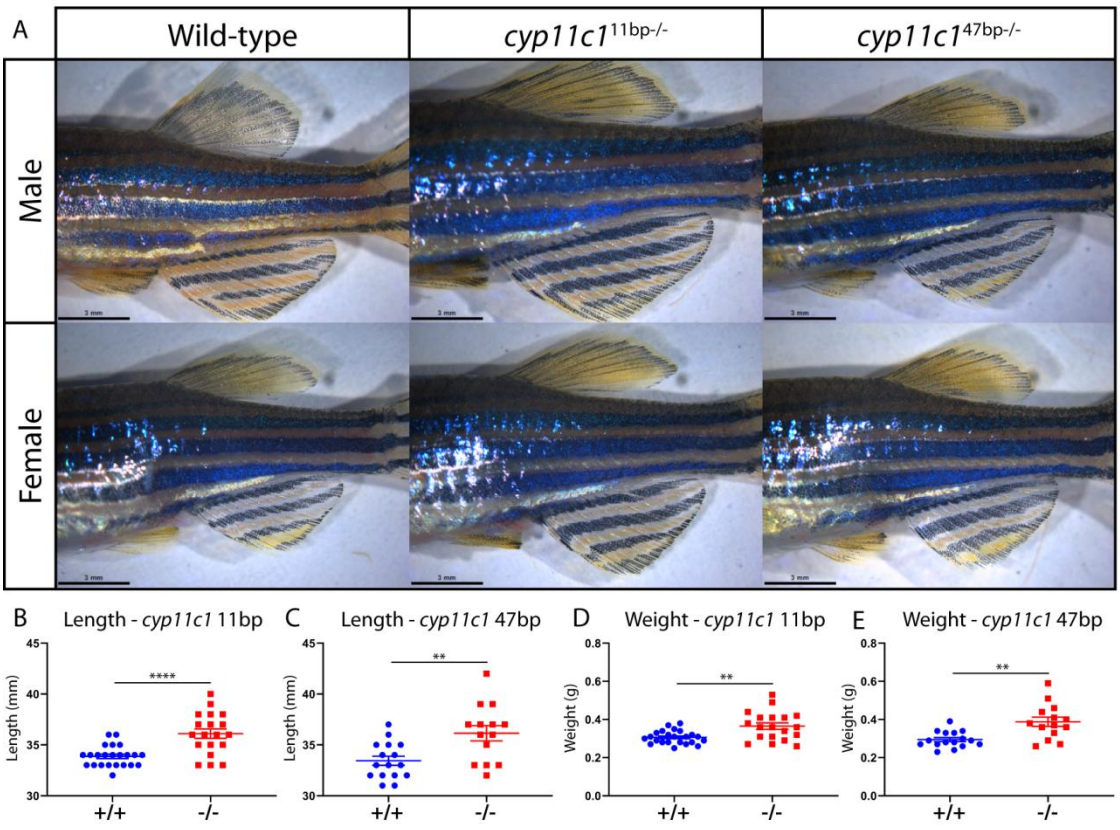
641 **Figures and Tables**



642

643 **Figure 1. The roles of Cyp11c1 (11β-hydroxylase) in androgen and glucocorticoid biosynthesis in**
644 **zebrafish.** Cyp11c1 is required for the final stage of glucocorticoid biosynthesis: the conversion of
645 11-deoxycortisol to cortisol. 11-ketotestosterone, the principal zebrafish androgen, may be
646 produced via two pathways beginning with androstenedione. The major pathway (solid arrows)
647 involves 11β-hydroxylation of androstenedione by Cyp11c1 to produce 11β-
648 hydroxyandrostenedione, whereas the minor pathway (dashed arrows) requires 11β-hydroxylation
649 of testosterone by Cyp11c1 to produce 11β-hydroxytestosterone.

650



651

652 **Figure 2. Feminisation of secondary sex characteristics and increased length and weight in**
653 ***cyp11c1*$^{-/-}$ mutant male zebrafish. A:** *Cyp11c1*$^{-/-}$ mutant male zebrafish exhibited fin pigmentation
654 patterns more commonly seen in female zebrafish. Wild-type male zebrafish exhibited pale dorsal
655 fins and strongly orange striped anal fins. *Cyp11c1*$^{-/-}$ mutant male zebrafish exhibited reduced
656 orange pigmentation in the anal fin and green-yellow pigmentation in the dorsal fin, like that seen in
657 wild-type females. **B-E:** Length and weight were significantly increased in homozygous mutants from
658 both *cyp11c1* mutant alleles (**B, D:** 11bp: wild-type n=23 mutant n=19, length p=<0.0001 weight
659 p=0.0016. **C, E:** 47bp: wild-type n=16 mutant n=14, length p=0.0034 weight p=0.0010). Statistical
660 analysis was by unpaired *t*-tests, **** p<0.0001, ** p<0.01.

661

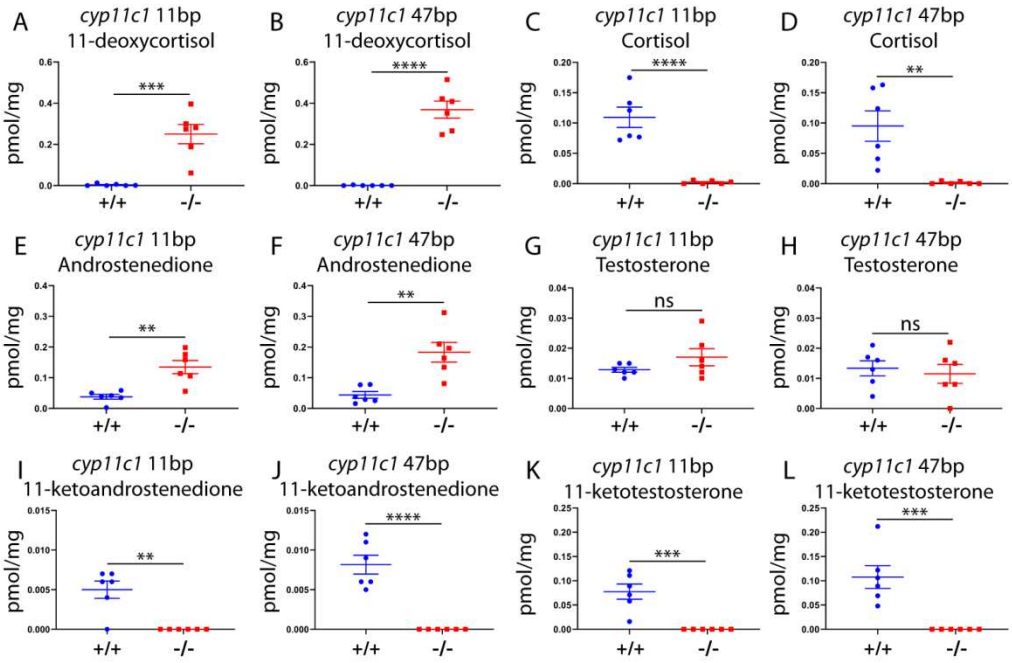
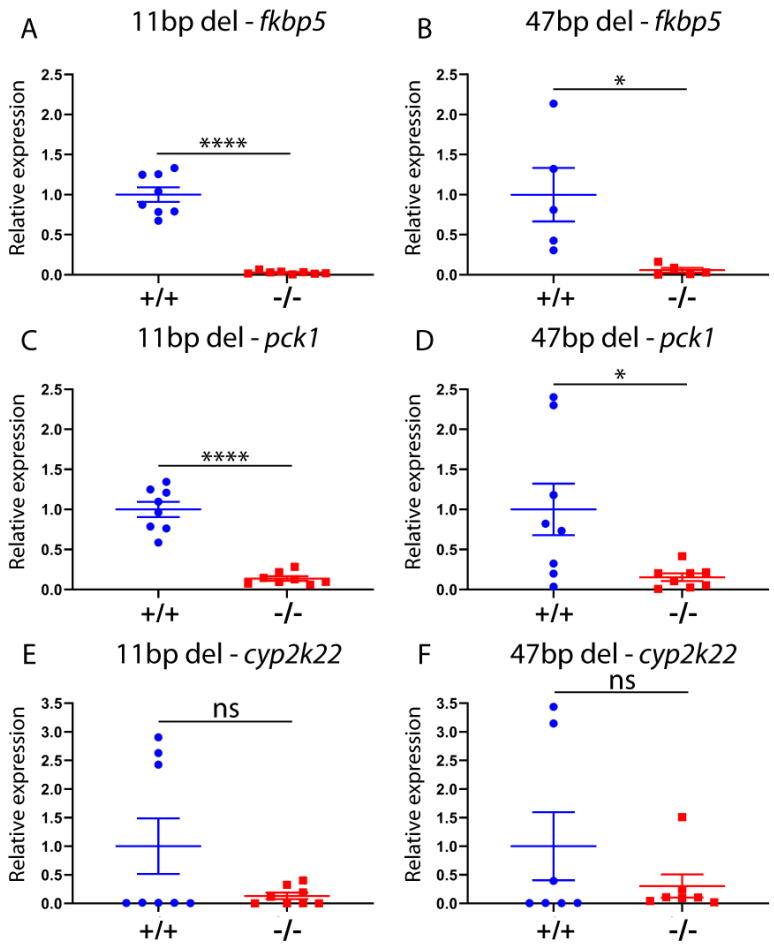
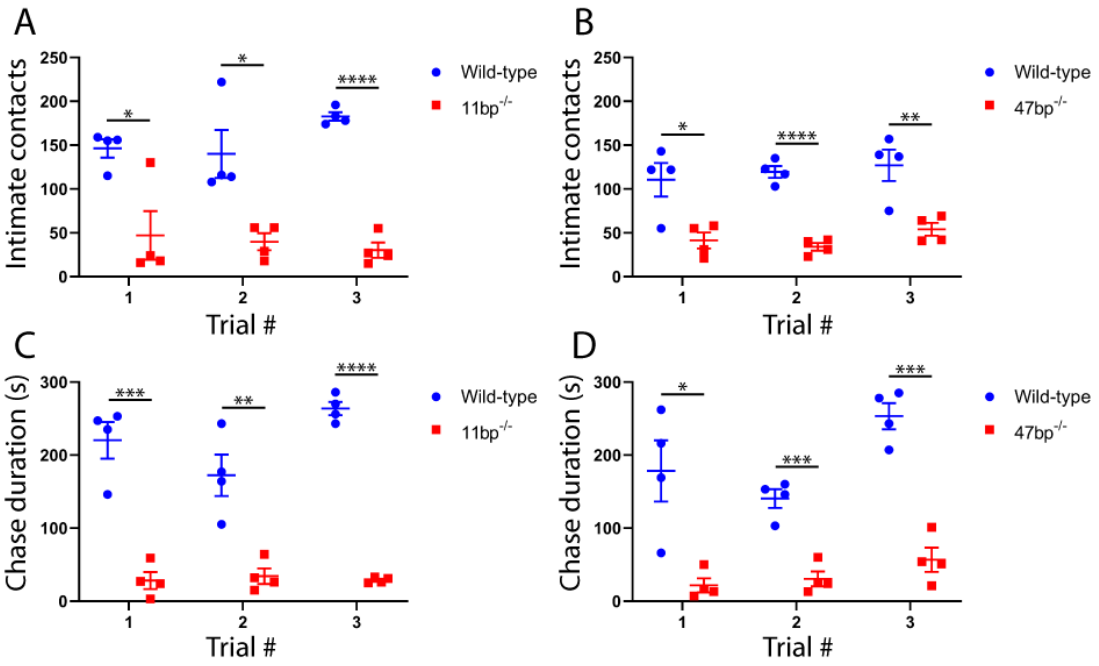


Figure 3. Steroid profile of adult male *cyp11c1*$^{-/-}$ mutant zebrafish is consistent with 11β-hydroxylase deficiency. Steroid hormone concentrations were quantified in whole adult zebrafish (n=6) by LC-MS/MS. Concentrations of cortisol (**C+D**) (11bp p<0.0001, 47bp p=0.0039) were profoundly reduced 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 mutation of *cyp11c1.* Results analysed by unpaired *t*-tests, ** p<0.01, *** p<0.001, **** p<0.0001.

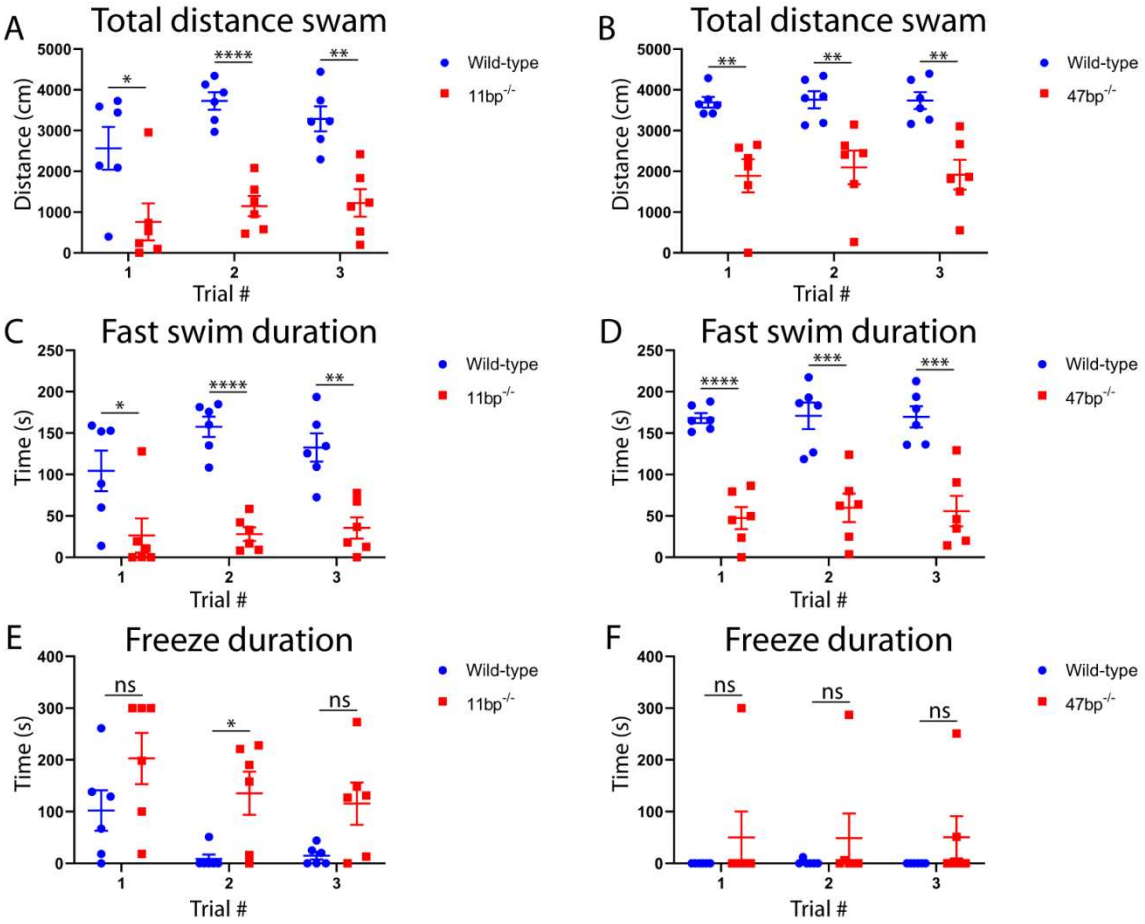


675

676 **Figure 4. Decreased expression of steroid responsive genes in the livers of Cyp11c1-deficient male**
677 **zebrafish.** Expression of steroid responsive genes in *cyp11c1*$^{-/-}$ mutant male zebrafish and wild-type
678 siblings was quantified by qPCR with *ef1a* as the reference gene. Expression of robustly
679 glucocorticoid responsive genes *fkbp5* (11bp wild-type n=8, mutant n=8, p<0.0001; 47bp wild-type
680 n=5, mutant n=5, p=0.0230) and *pck1* (11bp wild-type n=8, mutant n=8, p<0.0001, 47bp wild-type
681 n=8, mutant n=8, p=0.0212) was significantly reduced in the livers of *cyp11c1*$^{-/-}$ mutant male
682 zebrafish. Quantification of the expression of *cyp2k22* (11bp wild-type n=8, mutant n=8, p=0.2345,
683 47bp wild-type n=7, mutant n=7, p=0.6200, Mann-Whitney tests), a proposed androgen responsive
684 gene, revealed an apparent reduction in expression in *cyp11c1*$^{-/-}$ mutant male zebrafish, however
685 this did not achieve statistical significance. Data analysed by unpaired *t*-tests unless otherwise
686 stated, * p<0.05, **** p<0.0001.

687 

688 **Figure 5. Stereotypical breeding behaviours are reduced in pairings with Cyp11c1-deficient male**
689 **zebrafish.** *Cyp11c1*$^{-/-}$ mutant (n=4) and wild-type sibling male (n=4) zebrafish were paired with wild-
690 type females and behaviour was analysed during the first five minutes of breeding. This procedure
691 was repeated on three separate occasions. Intimate contacts (**A+B**) (11bp: 1 p=0.0270, 2 p=0.0270, 3
692 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,
693 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
694 trials and in both 11bp and 47bp deletion alleles. Results analysed using multiple *t*-tests with the
695 Holm-Sidak method, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



696

697 **Figure 6. Cyp11c1-deficient male zebrafish exhibit decreased locomotor activity.** In order to assess
698 locomotion, *cyp11c1*$^{-/-}$ mutant male zebrafish and wild-type siblings (n=6) were exposed to open
699 field tests. Tests were repeated on three occasions, and total distance swam, fast swimming
700 duration and freezing duration were recorded. Total distance swam (**A+B**) (11bp: 1 p=0.0265, 2
701 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**)
702 (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
703 consistently reduced in both *cyp11c1*$^{-/-}$ mutant alleles across all trials. Freeze duration (**E+F**) (11bp: 1
704 p=0.1418, 2 p=0.0402, 3 p=0.0708; 47bp: 1 p=0.5740, 2 p= p=0.5740, 3 p= p=0.5740) was
705 consistently increased in *cyp11c1*$^{-/-}$ mutant male zebrafish, however this was only significant in one
706 trial (**E**). Results were analysed using multiple *t*-tests with the Holm-Sidak method, * p<0.05, **
707 p<0.01, *** p<0.001, **** p<0.0001.

708

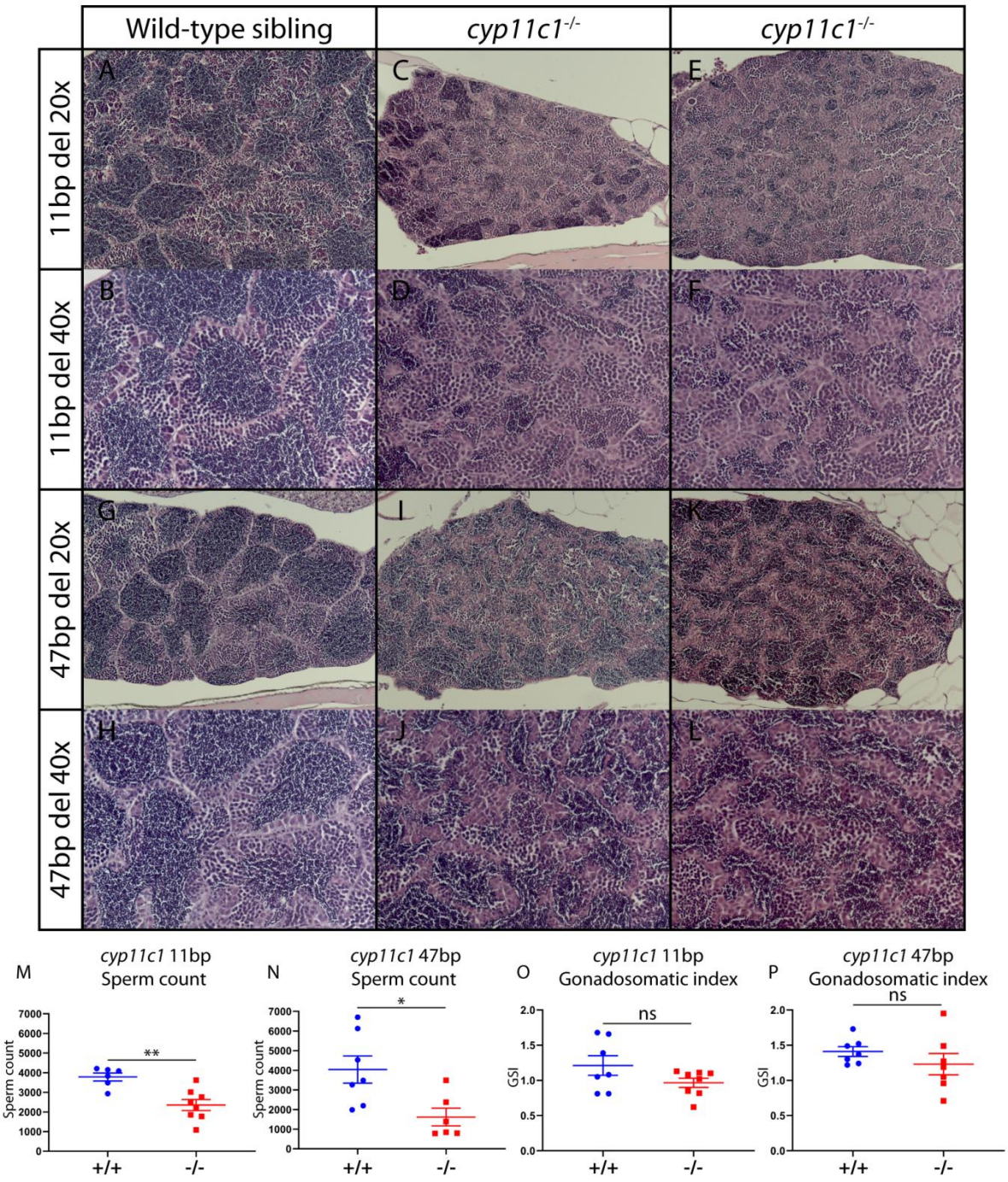
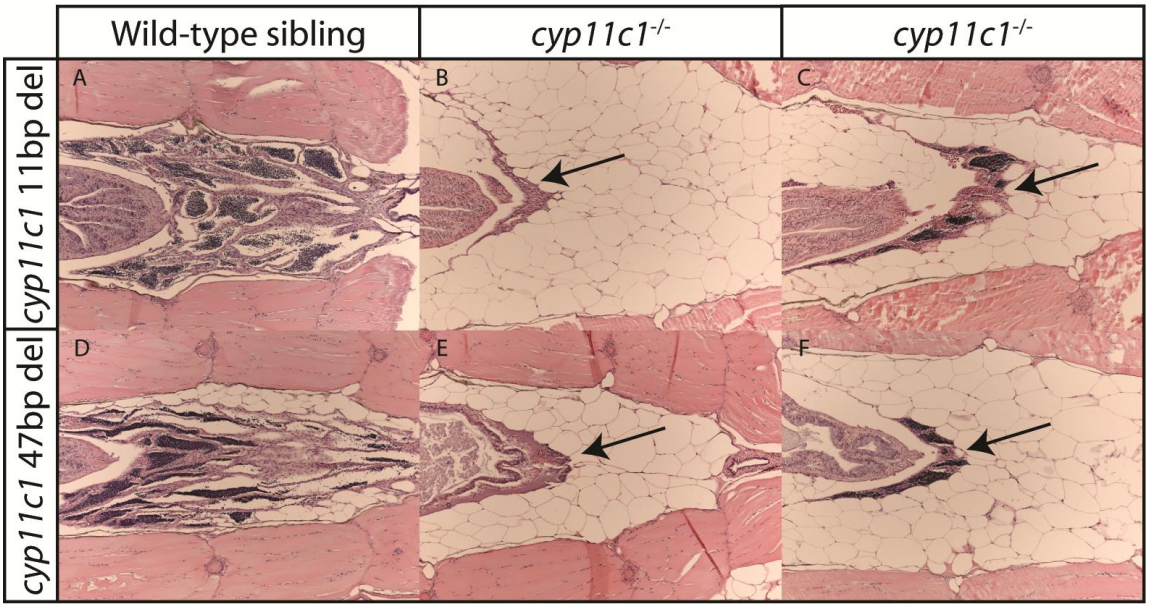


Figure 7. Mutation of *cyp11c1* results in disorganised seminiferous tubules and reduced numbers of spermatozoa. The morphology and composition of *cyp11c1*$^{-/-}$ mutant (n=5 per allele) and wild-type sibling (n=3 per allele) testes was assessed by H&E staining. The testes of wild-type sibling fish (**A,B,G,H**) contained defined seminiferous tubules whereas *cyp11c1*$^{-/-}$ testes (**C-F, I-L**) contained poorly defined seminiferous tubules and comparatively fewer mature sperm. *Cyp11c1* mutant zebrafish exhibited significantly reduced sperm counts compared to wild-type siblings (**M+N**) (11bp

716 wild-type n=6, mutant n=8, p=0.0023; 47bp wild-type n=7, mutant n=6, p=0.0167). No change in GSI
717 was observed (**O+P**) (11bp wild-type n=7, mutant n=8, p=0.1201; wild-type n=7, 47bp mutant n=7,
718 p=0.3046). Data analysed by unpaired *t*-tests, * $p<0.05$, ** $p<0.01$.

719

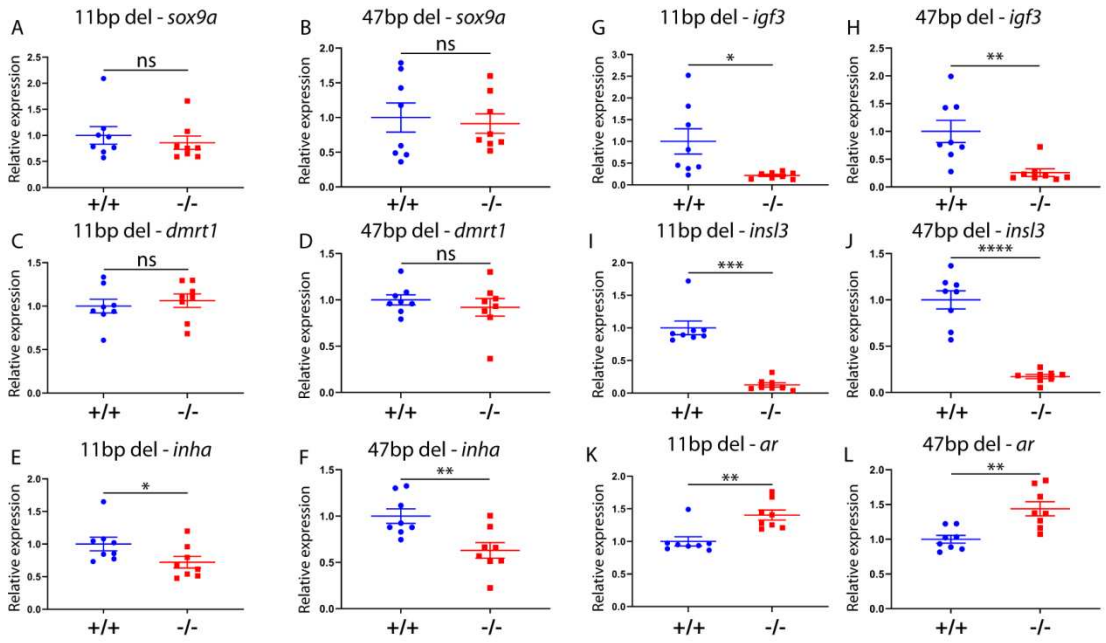
720



721

722 **Figure 8. The spermatic ducts of *cyp11c1*$^{-/-}$ mutant zebrafish are hypoplastic. A+D:** Spermatic ducts
723 of wild-type sibling zebrafish appeared as extensive networks of spermatozoa containing tubules
724 situated between the intestine and renal collecting duct. **B+C+E+F:** Spermatic ducts of *cyp11c1*$^{-/-}$
725 mutant zebrafish were comparatively smaller compared to those of wild-type siblings. *Cyp11c1*$^{-/-}$
726 spermatic ducts (arrows) appeared immediately posterior to the intestine and were severely
727 underdeveloped, though some contained mature sperm. Images captured at 10x magnification.

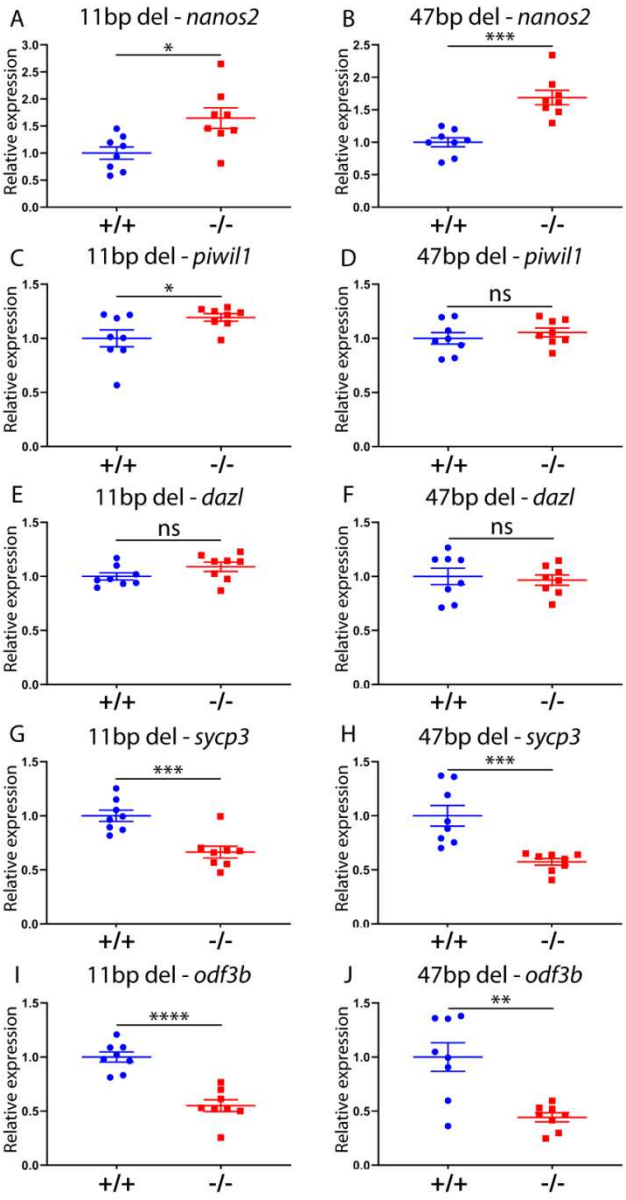
728



729

730 **Figure 9. Expression of pro-male and spermatogenic genes in the testes of *cyp11c1*$^{-/-}$ zebrafish.** The
731 expression of pro-male transcription factors *sox9a* (**A+B**) (11bp n=8, p=0.4418, Mann-Whitney test;
732 47bp n=8, p=0.7337) and *dmrt1* (**C+D**) (11bp n=8, p=0.5802; 47bp n=8, p=0.4756) was unchanged in
733 Cyp11c1-deficient zebrafish. Expression of the hypothalamus-pituitary-gonadal axis regulator inhibin
734 alpha *(inha)* was significantly reduced in *cyp11c1* mutant zebrafish (**E+F**) (11bp n=8, p=0.0499, Mann-
735 Whitney test; 47bp n=8, p=0.0062). Expression of the spermatogenic factors *igf3* (**G+H**) (11bp n=8,
736 p=0.0183; 47bp n=8, p=0.0011, Mann-Whitney test) and *insl3* (**I+J**) (11bp n=8, p=0.0002, Mann-
737 Whitney test; 47bp n=8, p<0.0001) was profoundly reduced in the testes of *cyp11c1*$^{-/-}$ mutant
738 zebrafish. Expression of the androgen receptor *(ar)* was significantly upregulated in *cyp11c1*$^{-/-}$
739 mutant zebrafish testes (**K+L**) (11bp n=8, p=0.0047, Mann-Whitney test; 47bp n=8, p=0.0020). Data
740 analysed by unpaired *t*-tests unless otherwise specified, * p<0.05, ** p<0.01, *** p<0.001, ****
741 p<0.0001.

742

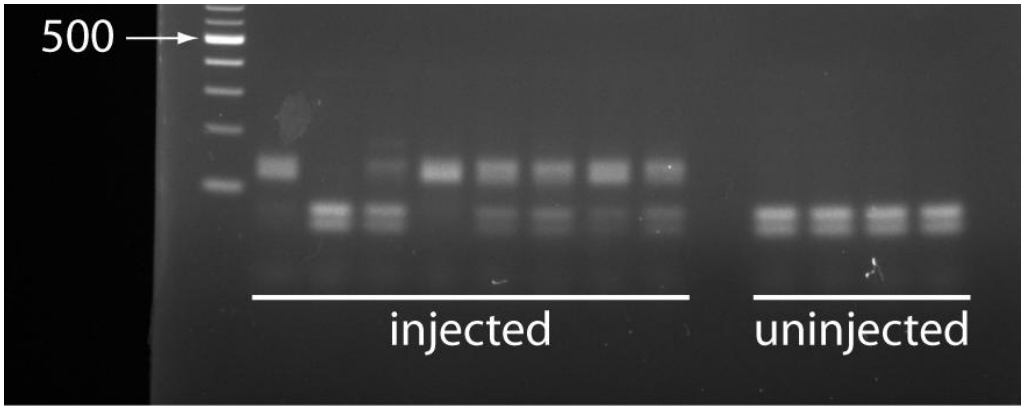


743

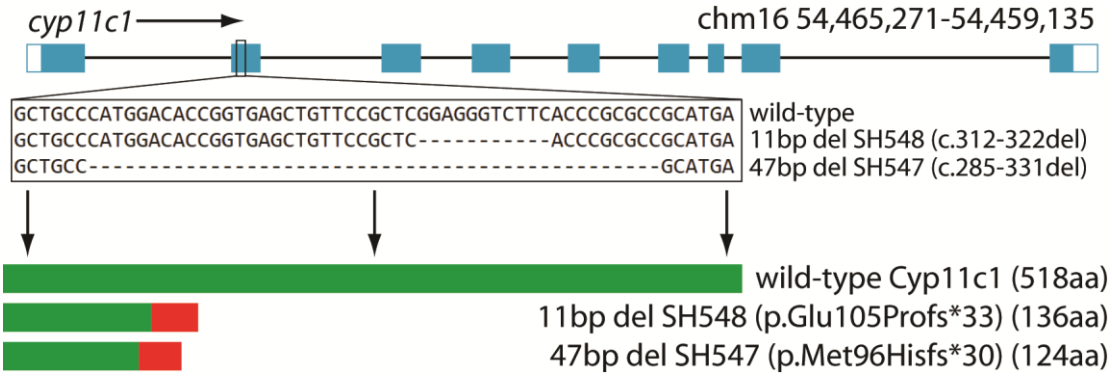
744 **Figure 10. Expression of spermatogenic marker genes in the testes of *cyp11c1*$^{-/-}$ zebrafish.** qPCR
745 was employed to measure the expression of marker genes for several spermatogenic stages. The
746 expression of *nanos2*, a marker of type A undifferentiated spermatogonia, was increased in *cyp11c1*$^{-}$
747 $^{/-}$ zebrafish (11bp n=8, p=0.0112; 47bp n=8, p=0.0001). The expression of *piwil1*, a marker of both
748 undifferentiated and differentiated type A spermatogonia was significantly increased in zebrafish
749 homozygous for the 11bp deletion allele (n=8, p=0.0405), but was not changed in those carrying the
750 47bp deletion allele (n=8, p=0.4385). Expression of the type B spermatogonia marker *dazl* was not
751 affected by mutation of *cyp11c1* (11bp n=8, p=0.1213; 47bp n=8, p=0.7009). Expression of the

752 spermatocyte and spermatid markers *sycp3* and *odf3b* was significantly reduced in the testes of
753 *cyp11c1*$^{-/-}$ zebrafish. (*sycp3*: 11bp n=8, p=0.0006; 47bp n=8, p=0.0008; *odf3b*: 11bp n=8, p<0.0001;
754 47bp n=8, p=0.0013). Data analysed by unpaired *t*-tests, * p<0.05, ** p<0.01, *** p<0.001, ****
755 p<0.0001.

756



Supplementary figure 1. *Cyp11c1* CRISPR design and screening assay. A two-part CRISPR system was used to generate mutations in *cyp11c1*. The Cas9 cut site overlapped a BslI restriction site. 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, mutated alleles were identified by incomplete digestion.

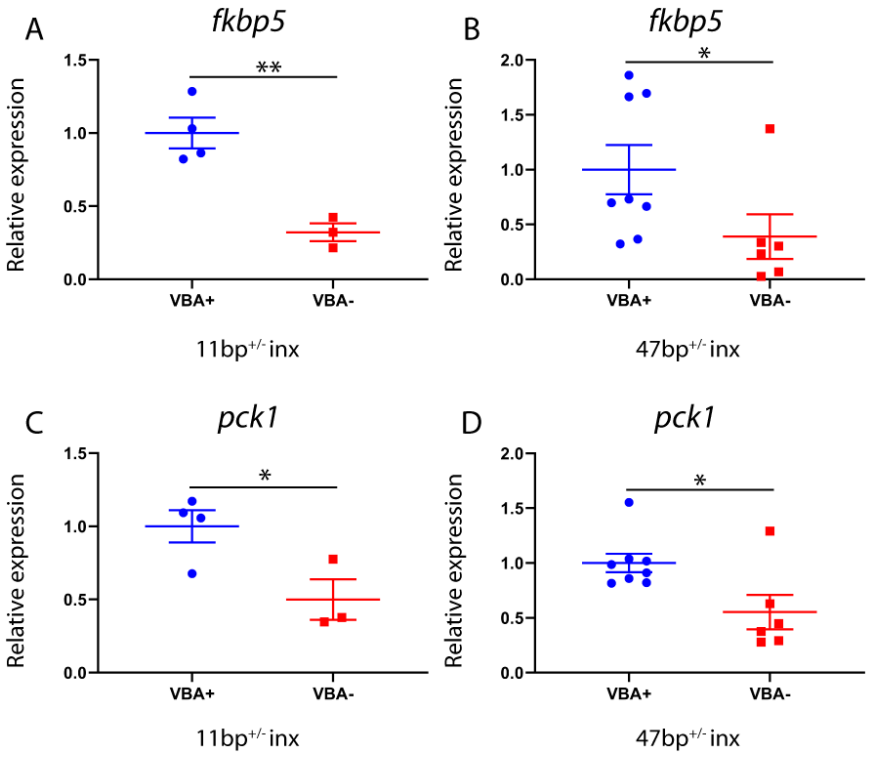


765

766 **Supplementary figure 2. Mutations introduced into *cyp11c1* by CRISPR/Cas9.** We used a
767 CRISPR/Cas9 strategy to introduce mutations into *cyp11c1*, resulting in production of several stable
768 *cyp11c1* mutant zebrafish lines. *Cyp11c1* is located on chromosome 16 and our CRISPR target site
769 was in exon 2. Two mutant alleles were utilised in this study: an 11bp deletion allele (SH548) (c.312-
770 322del) and a 47bp deletion allele (SH547) (c.285-331del). Wild-type Cyp11c1 is 518 amino acids in
771 length, our 11bp and 47bp deletion alleles are predicted to produce protein products truncated to
772 136 amino acids (p.Glu105Profs*33) and 124 amino acids (p.Met96Hisfs*30) in length respectively.
773 Protein products are depicted as green bars, the red portion of the mutant proteins comes after the
774 mutation site and does not align to the wild-type protein product.

775

776



777

778 **Supplementary figure 3. Decreased expression of glucocorticoid responsive genes in VBA- larvae.**
779 VBA- (mainly *cyp11c1*$^{-/-}$) and VBA+ (wild-type/heterozygous) larvae were identified from the progeny
780 of an incross of *cyp11c1*$^{+/-}$ adult zebrafish. VBA typed larvae were pooled, RNA was extracted, and
781 the expression of glucocorticoid responsive genes *fkbp5* and *pck1* was determined by qPCR.
782 Expression of both *fkbp5* (A+B) (11bp: VBA+ n=4, VBA- n=3, p=0.0039; 47bp: VBA+ n=8, VBA- n=6,
783 p=0.0200) and *pck1* (C+D) (11bp: VBA+ n=4, VBA- n=3, p=0.0349; 47bp: VBA+ n=8, VBA- n=6,
784 p=0.0293) was significantly decreased in VBA- samples from both alleles indicating decreased
785 glucocorticoid production in VBA- larvae. Results analysed using unpaired *t*-tests, * indicates p<0.05,
786 ** indicates p<0.01.

787

Table 1. Infertility in *cyp11c1*$^{-/-}$ mutant male zebrafish

Allele	Genotype	Total number of crosses	Number of 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 1. *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 2. 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 2. Testes were dissected from male zebrafish and homogenised in a 50x mass:volume dilution of HBSS. Sperm solutions were used to fertilise eggs collected from wild-type females.

801 **Table 3. Proportion of fish producing sperm samples during gamete expression experiments**

Genotype	Number 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

802 *2/6 samples from fish which produced sperm contained negligible sperm numbers.

803

804

805 **Supplementary table 1. Primer sequences for qPCR experiments**

Gene	Forward	Reverse	Citation
ar	AGATGGGCGAATGGATGGAT	AGAACACTTTGCAGCTTCCG	(Oakes et al., 2019)
cyp2k22	CGCTGTCAAACCTACGAGAC	GGGGCAGTTTTGTTTCAAATGG	(Oakes et al., 2019)
dazl	ACTGGGACCTGCAATCATGA	AATACAGGTGATGGTGGGGC	(Oakes et al., 2019)
dmrt1	GGCCACAAACGCTTCTGTAA	ATGCCCATCTCCTCCTCTTG	(Oakes et al., 2019)
ef1a	GTGGCTGGAGACAGCAAGA	AGAGATCTGACCAGGGTGGTT	(Oakes et al., 2019)
fkbp5	TTCCACACTCGTGTTCGAGA	ACGATCCCACCATCTTCTGT	(Griffin et al., 2016)
igf3	GTAGACCAGTGTTGTGTGCG	ATTCCTCATCTCGCTGCAGA	(Oakes et al., 2019)
inha	CAGAGCTGTGCACCATGTAG	CCAGGTCCAGCATCAGAAGA	(Oakes et al., 2019)
insl3	TCGCATCGTGTGGGAGTTT	TGCACAACGAGGTCTCTATCCA	(Safian et al., 2016)
nanos2	AAACGGAGAGACTGCGCAGAT	CGTCCGTCCCTTGCCTTT	(Safian et al., 2016)
odf3b	GATGCCTGGAGACATGACCAA	CAAAGGAGAAGCTGGGAGCTT	(Assis et al., 2016)
pck1	TGACGTCCTGGAAGAACCA	GCGTACAGAAGCGGGAGTT	(Griffin et al., 2016)
piwil1	ATACCGCTGCTGGAAAAAGG	GCAAGACACACTTGGAGAACC	(Safian et al., 2016)
sox9a	CGGAGCTCAAAACTGTG	CGGGGTGATCTTTCTTGTGC	(Oakes et al., 2019)
sycp3	AGAAGCTGACCCAAGATCATTCC	AGCTTCAGTTGCTGGCGAAA	(Assis et al., 2016)

806

807

Supplementary table 2. VBA sorting of larvae produced from a *cyp11c1*$^{+/-}$ incross

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%)

Supplementary table 2. The progeny of an incross of adult *cyp11c1* heterozygotes carrying either the 11bp or 47bp deletion allele were subjected to sorting by visual background adaptation (VBA) at 4-5dpf and subsequently genotyped. Fish identified as having an intact VBA response (VBA+) were exclusively wild-type or heterozygous for *cyp11c1* mutations. 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. Fishers exact test: 11bp $p<0.0001$, 47bp $p=0.0003$.