Title: G protein-coupled estrogen receptor is not required for sex determination or ovary function in zebrafish

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ABSTRACT

Estrogens regulate vertebrate development and function through binding to nuclear estrogen receptors alpha and beta (ERα, ERβ) and the G protein-coupled estrogen receptor (GPER). Studies in mutant animal models demonstrated that ERα and ERβ are required for normal ovary development and function. However, the degree to which GPER signaling contributes to ovary development and function is less well understood. Previous studies using cultured fish oocytes found that estradiol inhibits oocyte maturation in a GPER-dependent manner, but whether GPER regulates oocyte maturation *in vivo* is not known. To test the hypothesis that GPER regulates oocyte maturation *in vivo*, we assayed ovary development and function in *gper* mutant zebrafish. We found that homozygous mutant *gper* embryos developed into male and female adults with normal sex ratios and fertility. Adult mutant fish exhibited normal secondary sex characteristics and fertility. Additionally, mutant ovaries were histologically normal. We observed no differences in the number of immature versus mature oocytes in mutant versus wild-type ovaries from both young and aged adults. Furthermore, expression of genes associated with sex determination and ovary function were normal in *gper* mutant ovaries compared to wild type. Our findings suggest that GPER is not required for sex determination, ovary development or fertility in zebrafish.

INTRODUCTION

Estrogens influence a wide range of physiological processes in reproductive and non-reproductive tissues in zebrafish [1-6]. Estrogens act by binding to nuclear estrogen receptors (ERα, ERβ), ligand dependent transcription factors that directly regulate gene expression [7-9]. Estrogens also activate the G protein-coupled estrogen receptor (GPER/GPR30) to elicit downstream signaling cascades [10-12]. In the zebrafish ovary, two ERβ paralogue genes (*esr2a* and *esr2b*) are essential in reproduction, folliculogenesis and maintenance of ovarian tissue, with mutations in both receptors resulting in female to male sex reversal [1]. However, less is understood regarding the role of GPER in sex determination, ovarian development and oogenesis in vertebrates.

Sexual determination in zebrafish is unique in that laboratory strains lack a sex chromosome and no sex-determining gene has been identified. Elevated temperatures and stress are associated with masculinization, but these results are conflicting [13-15]. It appears that multiple sex-related genes interact as a network to establish sex, a concept known as polygenic sex determination [16-18]. Several genes promote ovary differentiation and development, including aromatase (*cyp19a1a*) and forkhead box L2 (*foxl2a*, *foxl2b*) [17, 19-22]. Conversely, expression of anti-Mullerian hormone gene (*amh*) promotes testes differentiation and spermatogenesis [21, 23-25]. Additionally, exposure of juvenile zebrafish to exogenous estrogens can bias sex differentiation in favor of fully functioning and fertile females [23, 26]. Although levels of estrogens are

important for female sex determination and differentiation, the role of GPER in zebrafish sex determination and differentiation is unknown.

GPER was shown to regulate oocyte maturation *in vitro*. In carp (*Cyprinus carpio*) and zebrafish, maturation of cultured oocytes is regulated by estrogens and progestogens, where pharmacologic and RNA interference approaches demonstrated that estrogens inhibit maturation via GPER while progestogens promote maturation via membrane progesterone receptors [5, 27-30]. In ovaries cultured from golden hamsters (*Mesocricetus auratus*), estradiol promoted the formation of primordial follicles in a GPER-dependent manner [31].

While evidence from *in vitro* studies demonstrates that GPER regulates oocyte maturation and ovarian follicle development, evidence from *in vivo* studies suggests that GPER is not required for ovary development. *Gper* mutant mice showed no defects in fertility or gonad development [32, 33].

In carp and zebrafish ovaries, *gper* mRNA was detected in pre-vitellogenic, late-vitellogenic and post-vitellogenic oocytes but was not detected in ovarian follicle cells [27, 34]. Thus, *gper* expression in teleost ovaries is consistent with a cell-autonomous role in oocyte maturation. To better understand the role of GPER in the development and function of ovaries, we examined ovary histology, fertility and gene expression in adult wild-type and *gper* mutant zebrafish. Our findings suggest that GPER does not influence sex determination, gonad development or fertility in zebrafish.

METHODS

Animal care and husbandry

Zebrafish were housed at the University of Alabama at Birmingham (UAB) zebrafish facility in a recirculating water system (Aquaneering, Inc., San Diego CA) and kept at 28.5°C on a 14-h light, 10-h dark cycle. Zebrafish embryos were raised in E3B (60x E3B: 17.2 g NaCl, 0.75 g KCl, 2.9g $CaCl_2$-H_20, 2.39 g $MgSO_4$ dissolved in 1 L Milli-Q water; diluted to 1x in 9 L Milli-Q water plus 100 μl 0.02% methylene blue) and housed in an incubator at 28.5°C on a 14-h light, 10-h dark cycle, until 5 dpf. Wild-type zebrafish were AB strain [35]. *gper*^{uab102} mutant zebrafish, containing a 130 basepair deletion in the open reading frame, were used as described [2]. All procedures were approved by the UAB and Baylor College of Medicine Institutional Animal Care and Use Committees.

Genomic DNA isolation and genotyping analysis

Fin biopsies from wild type and *gper* mutant adults were digested in 100 μL lysis buffer (10 mM Tris pH 8.3, 50 nM KCl, 0.3% Tween 20) with 1 μL proteinase K (800 U/mL, New England Biolabs) per well in 96-well plates. Samples were incubated at 55°C for 8 hours to extract genomic DNA, followed by 10 minute incubation at 98°C to inactivate proteinase K. Samples were stored at -20°C. Genotyping was performed using polymerase chain reaction and gel electrophoresis as described [2].

Secondary sexual characteristics and sex ratios

Heterozygous *gper*$^{uab102/+}$ adults were bred to each other and offspring were raised to adulthood. At approximately 4-months of age progeny were genotyped and secondary sexual characteristics were examined visually. Zebrafish sex was determined by examining multiple secondary sexual characteristics including body shape, anal fin coloration, and presence or absence of a genital papilla (Figure 1, 2)[36]. Mendelian ratios were determined via genotyping. The total number of males and females for each genotype were counted and compared to heterozygous and wild-type animals from the same clutch (Table 1, Figure 1).

Histology

All histology experiments were performed on wild type or maternal zygotic homozygous *gper* mutant adult fish (MZ*gper*$^{uab102/102}$). *MZgper*$^{uab102/102}$ individuals were generated by crossing homozygous (*gper*$^{uab102/102}$) females to homozygous males. Whole-body 4-month and 10-month old adult *MZgper*$^{uab102/102}$ mutant and wild-type females (n = 4 females per genotype) were selected for histology based on secondary sexual characteristics and breeding trials. Fish were anesthetized in iced 0.2 mg/mL tricaine, decapitated with a razor blade, followed by a 2-day fixation in 4% formaldehyde. Specimens were either processed, embedded and sectioned as described [36] or shipped on ice to HistoWiz Inc. (New York, NY) where they were processed, embedded, sectioned (5 microns) and stained with hematoxylin and eosin (H & E) as follows: Processing of tissues into paraffin blocks was accomplished using an automated Peloris II tissue processor (Leica Biosystems, Buffalo Grove, IL) and embedded in Histoplast PE (Thermo Scientific). Tissues were dehydrated using the

following protocol: 50% ethanol (15 minutes, 45°C), 70% ethanol (15 minutes, 45°C) x 2, 90% ethanol (15 minutes, 45°C), 90% ethanol (15 minutes, 45°C), 90% ethanol (30 minutes, 45°C), 100% ethanol (45 minutes, 45°C), 100% xylene (45 minutes, 45°C), Parablock wax (30 minutes, 65°C) x 2 (Leica Biosystems) and Parablock wax (45 minutes, 65°C). Embedded specimens were cut into 5-micron sections and adhered to charged slides in a water bath. Slides were heated to 65°C for 10 minutes in an oven to melt paraffin and fully-adhere sections. Slides were rehydrated and stained using the a Tissue-Tek Prisma (Sakura) or by hand in glass containers using the following protocol: 100% xylene (5 minutes) x 2, 100% ethanol (2 minutes) x 2, 95% ethanol (1 minute), deionized (DI) H_2O (1 minute), hematoxylin (1 minute), DI H_2O (1 minute), defining solution (45 seconds) (Define MX-aq; Leica Biosystems), DI H_2O (1 minute), bluing agent (Fisher Scientific), DI H_2O (1 minute), 95% ethanol (15 seconds), eosin (30 seconds), 95% ethanol (2 minutes), 100% ethanol (3 minutes) x 2 and 100% xylene (5 minutes) x 2. Brightfield images of sections were obtained using a Zeiss Axio Observer.Z1 microscope with a Zeiss Axio MRc5 camera with 10x and 20x objectives (Figures 4). In addition, specimens processed at HistoWiz were imaged using a Leica Aperio AT2 slide scanner (Figure 5). A one-way ANOVA with Tukey's test for multiple comparisons was performed to determine significance ($p \leq 0.05$) in total number of oocyte per stage, all statistics were completed using GraphPad Prism software (version 7.0).

Breeding trials

MZgper$^{uab102/102}$ mutant females were bred once every two weeks with wild-type males (1 female: 1 male) and compared to wild-type crosses (1 female: 1 male) (n = 10 adult females per genotype). The number of individuals that spawned and the total number of oocytes released were counted after each breeding trial (Table 2, Figure 3). An unpaired t test was performed to determine significance, defined as $p \leq 0.05$, in number of oocytes released. Statistical analyses were performed using GraphPad Prism software (version 7.0).

RNA extraction and quantitative PCR

Total RNA was extracted from four dissected ovaries of 10-month old adult *MZgper*$^{uab102/102}$ and wild-type zebrafish according to manufacturer's guidelines for the TRIzol RNA isolation kit (Life Technologies, Carlbad, CA). Ovaries were homogenized individually in 500 μL TRIzol and DNase-treated with a TURBO DNA-free kit (Ambion) to remove genomic contamination. Quality of RNA was checked on a 1% agarose gel and quantity of RNA was determined using a Nanodrop ND-1000 ultraviolet-Vis spectrophotometer (Thermo Scientific). Expression of genes previously shown to be involved in sex differentiation were examined with quantitative reverse transcription PCR (qRT-PCR): *cyp19a1a*, *foxl2a, amh* and *rp113a* as a reference gene (Supplemental Table 1) [17, 37, 38]. Complementary DNA (cDNA) was synthesized via reverse transcription according to manufacturer's guidelines for the RETROscript First Strand Synthesis Kit (Fisher Scientific). Quantitative RT-PCR reactions were completed using SsoAdvanced Universal SYBR Green Supermix Kit (Bio-Rad Laboratories, Inc.) and run on a Bio-Rad CFX96 Touch Real-Time PCR Detection System using the following protocol: 98°C for 1 minute, followed by 34 cycles of 98°C for 10 seconds,

60°C or 61°C for 20 seconds, 72°C for 45 seconds and then 72°C for 2 minutes. Reactions were run in technical quadruplicates on 96-well plates and transcript quantification was measured using CFX Manager version 3.1 software (Bio-Rad Laboratories, Inc.). Cq expression values were averaged across technical replicates, primer efficiencies were measured and expression fold change values were calculated according to the ΔΔCt method [39]. An unpaired t test was performed on relative expression levels ($2^{-\Delta Ct}$) for individual biological replicates (n = 3-4 per gene per genotype) to determine significant differences in gene expression between mutants and wild type. For the graph in Figure 6, relative expression was normalized to wild type mean for each gene.

RESULTS

Male and female *gper*$^{uab102/102}$ mutants display normal sex ratios compared to wild-type and heterozygous siblings

To determine if GPER is involved in sex determination in zebrafish we crossed *gper*$^{uab102/+}$ heterozygous males and females and compared the number of male and female offspring across genotypes. Males were the dominant sex in all genotypes (+/+, *gper*$^{uab102/+}$, *gper*$^{uab102/102}$), which is consistent with previous studies demonstrating that approximately 70% of wild type fish are male in the UAB zebrafish research facility [40]. There was a 67% male bias in wild-type fish, a 68% male bias in heterozygous fish and a 75% male bias in homozygous *gper*$^{uab102/102}$ fish (Table 1, Figure 1). Therefore, we conclude that GPER does not regulate sex determination in zebrafish.

gper mutant males and females display normal secondary sexual characteristics compared to wild-type fish

Following our sex-ratio studies, we attempted to grossly assay fertility and ovary function by naturally breeding homozygous mutant females with homozygous mutant males. We found that homozygous mutant *gper* fish of both sexes are fertile. Therefore, we maintained *gper* mutants as homozygotes in our zebrafish colony by crossing homozygous adults to each other and raising maternal-zygotic homozygous embryos to adulthood (*MZgper*$^{uab102/102}$). To evaluate if *gper* is involved in the physical appearance

of males and females we examined secondary-sexual characteristics in *MZgper*$^{uab102/102}$ mutants compared to wild type. Wild-type males have a slender body shape, yellow anal fin and no genital papilla [36, 41]. Similarly, *MZgper*$^{uab102/102}$ mutant males displayed these same physical attributes (Figure 2A, B). In contrast, wild-type females display a rounded abdomen, a lighter colored anal fin and a large protruding genital papilla [36, 41]. Similarly, *MZgper*$^{uab10/102}$ mutant females displayed these same physical characteristics (Figure 2 C, D). Therefore, we conclude that *gper* does not influence the development of secondary sex characteristics.

gper mutant females spawn as frequently and release a similar number of oocytes on average as wild-type adult females

To examine if *gper* is involved in regulating fertility, we measured spawning frequency and the total number of oocytes released by *MZgper*$^{uab102/102}$ and wild-type females during three mating trials. On average, 63% of female wild-type and 56% of female *MZgper*$^{uab102/102}$ spawned during mating trials (10 fish per genotype per trial, n = 3 trials, Table 2, Figure 3). *MZgper*$^{uab102/102}$ females released an equivalent number of oocytes as did wild-type females. The average number of oocytes released by wild-type females was 131 ± 32 (SD), compared to 171 ± 35 released by *MZgper*$^{uab102/102}$ mutant females (Table 2, Figure 3, $p = 0.188$, unpaired t test). Therefore, we conclude that *gper* does not influence spawning or oocyte release.

gper mutant female ovaries are structurally similar to wild type at 4-months and 10-months of age

To evaluate if *gper* mutant ovaries show a defect in ovary development or oocyte maturation, we examined ovaries from 4-month old adult ovaries fish and counted the number of stage I, II and III oocytes present. *MZgper*$^{uab102/102}$ mutant ovaries were histologically similar to wild-type ovaries, with no significant difference in the number of stage I, II or III oocytes in mutants versus wild type (Figure 4). Ovary function declines with age [42], and it is possible that *gper* could influence the maintenance of normal ovary function, a phenotype that would not be apparent in young, 4-month old adults. To test whether *gper* influences ovary function in aged animals, we compared ovaries from mutant and wild type 10-month old zebrafish. We observed no difference in gross ovary organization and H & E staining between wild type and mutants. We also observed no significant differences in the number of stage I, II or III oocytes between 10-month old wild type and mutant zebrafish (Figure 5). Therefore, we conclude that *gper* does not regulate oocyte maturation or ovary function in laboratory zebrafish.

Genes associated with sex determination and gonad function are not differentially expressed between *gper* mutant and wild-type ovaries

It is possible that GPER influences sex determination and ovary function by regulating gene expression. For example, *androgen receptor* mutant zebrafish have grossly histologically normal ovaries in adulthood, but display abnormal expression of sexually dimorphic genes associated with gonad function [36]. To determine whether GPER is involved in regulation of sexual determination or ovary differentiation and function we compared levels of three genes associated with sexual differentiation and maintenance: *cyp19a1a* and *foxl2a*, which promote and maintain ovary differentiation

and function, and *amh*, which promotes testes differentiation [17, 24]. Expression levels of all three genes were not significantly different in *gper* mutants and wild-type adult zebrafish ovaries (Figure 6; *cyp19a1a* 2.01 ± 0.49 *MZgper* vs wild type, *foxl2a* 1.22 ± 1.53, *amh* 1.32 ± 1.50; $p>0.05$ in all cases, t test). Therefore, we conclude that GPER is not required for normal expression of genes associated with sex determination and maintenance.

DISCUSSION

Our results suggest that GPER is not necessary for ovarian development and function in zebrafish. *gper* mutant ovaries appeared normal and contained all stages of oocytes in similar quantities to wild-type fish. Fertility was not affected in *gper* mutant females, which released a similar number of oocytes during natural mating trials and bred at a similar frequency to wild-type females. We also found that GPER does not influence sex determination or secondary sexual characteristics. $gper^{uab102/102}$ mutant embryos developed into male and female adults in similar ratios as wild-type and heterozygous clutch mates. Adult $gper^{uab102/102}$ males and females displayed normal sexually dimorphic body shape and anal fin coloration. $gper^{uab102/102}$ females possessed a large protruding genital papilla, similar to wild-type females. Expression of three genes important for sex determination or ovary maintenance (*cyp19a1a*, *foxl2a*, *amh*) were expressed at similar levels in *gper* mutant and wild-type ovaries, suggesting that these genes are not regulated directly or indirectly by GPER.

Our results are consistent with results from *Gper* mutant mice [32, 33]. However, our results contradict previously published *in vitro* results. In several fish species

including zebrafish, estrogens were shown to inhibit meiotic maturation of cultured oocytes in a GPER-dependent manner [5, 27, 43, 44], yet we did not observe an oocyte maturation defect in *gper* mutant zebrafish. There are several explanations that could account for this discrepancy. One possibility is that assaying oocyte maturation *in vitro* does not reflect oocyte maturation *in vivo* with high fidelity. Studies examining the effects of estrogens on oocyte maturation *in vitro* use oocytes separated from follicle cells. In contrast, in the ovary, oocytes interact with follicle cells, extracellular matrix and circulating signaling molecules that could contribute to maintenance of meiotic arrest and make GPER unnecessary for maintenance of meiotic arrest in oocytes *in vivo*.

Another possibility is that cell signaling pathways compensate for lack of GPER signaling *in vivo*. Evidence suggests that, *in vitro*, GPER promotes meiotic arrest by increasing cGMP and cAMP levels [5, 45]. In the absence of GPER *in vivo*, perhaps another GPCR can compensate for GPER and drive an increase in cGMP and cAMP levels. Alternatively, there could exist novel or previously unappreciated estrogen receptors that contribute to estradiol-mediated inhibition of oocyte maturation in the absence of GPER.

A third possibility is that the *gper*^{uab102} mutation is a hypomorph or is rescued by genetic compensation. The *uab102* allele lacks 130 basepairs from the *gper* open reading frame. Even if this transcript is translated, the resulting protein would lack large portions of transmembrane domains 1 and 2. The resulting protein would not fold properly and would not be properly integrated into the cell membrane and is likely to be completely devoid of function. We cannot exclude the possibility that genetic compensation is occurring to rescue phenotypes in the *gper*^{uab102} deletion mutants [46].

In genetic compensation, upregulation of related genes occurs in response to a gene knockout. In the case of *gper*, there are no identified ohnologues or related genes in the zebrafish genome that are obvious candidates for genetic compensation. *gper*^{uab102} embryos were shown to have a heart rate phenotype [2]. Thus, if genetic compensation is occurring, it is compensating for an adult phenotype but not an embryonic phenotype.

Our results suggest that GPER is not required for normal sex differentiation, gonad development or gonad function in zebrafish. These findings are consistent with studies from *Gper* mutant mice, which also reported no gonad development phenotypes [32, 33]. While GPER has been demonstrated to regulate the development and function of non-gonadal tissues [2, 47-49], we conclude that, in contrast to nuclear estrogen receptors, GPER is dispensable for gonad formation and function.

Table 1. Mendelian and sex ratios in progeny derived from *gper*$^{uab102/+}$ crosses

A single population of offspring derived from harem breeding among male and female *uab105/+* heterozygotes. Total number of individual fish within each genotype is indicated, followed by percent of fish with indicated genotype. Numbers of male and females for each genotype are included in brackets, with sex based on presence of testis (♂) or ovary (♀).

	+/+	***uab102*/+**	***uab102/102***
Population 1	15 (27%) [10 ♂, 5♀]	28 (51%) [19 ♂, 9 ♀]	12 (22%) [9 ♂, 3 ♀]

Table 2. *MZgper*$^{uab102/102}$ spawn as frequently and release similar numbers of oocytes as wild-type females. 10-month old adult *MZgper*$^{uab102/102}$ or wild-type females were crossed 1:1 with wild-type males (n =10 individuals per genotype). The percent of pairs that spawned was determined by counting the number of females that released oocytes in each trial. For each trial of 10 crosses, the average number of oocytes released is shown (mean ± standard deviation).

Genotype of female paired with wild-type male	Trial 1	Trial 2	Trial 3
Wild type	60% spawned 126 oocytes ± 49	60% spawned 158 oocytes ± 55	70% spawned 113 oocytes ± 88
***MZgper*$^{uab102/102}$**	50% spawned 157 oocytes ± 65	50% spawned 212 oocytes ± 61	70% spawned 144 oocytes ± 50

Supplemental Table 1. List of primers used for qRT-PCR.

Primer Name	Primer sequences (5' to 3')	Citation
rp113a	Forward - TCTGGAGGACTGTAAGAGGTATGC	Tang *et al.* 2007
	Reverse - AGACGCACAATCTTGAGAGCAG	
cyp19a1a	Forward - AGATGTCGAGTTAAAGATCCTGCA	Jorgensen *et al.* 2008
	Reverse - CGACCGGGTGAAAACGTAGA	
foxl2a	Forward - CCCAGCATGGTGAACTCTTAC	Siegfried *et al.* 2008
	Reverse - CGTGATCCCAATATGAGCAGT	
amh	Forward - CACGAAGAGCAGGACAACAA	Siegfried *et al.* 2008
	Reverse - TGGAGCACACTCTGAACCAG	

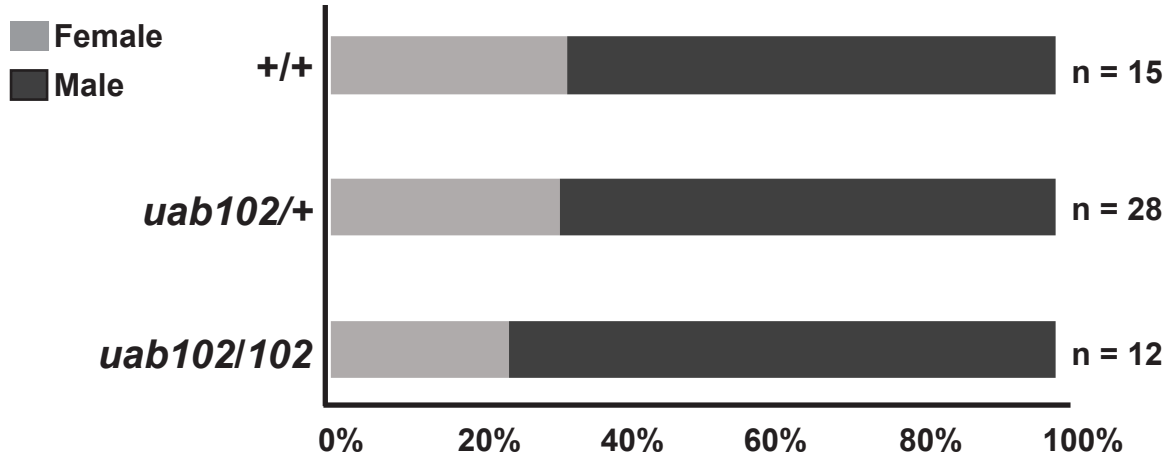

Figure 1. *gper*$^{uab102/102}$ mutant adults display normal sex ratios. Percentage of male and female progeny from crosses between *gper*$^{102/+}$ males and females. Offspring were observed to have 1:2:1 Mendelian ratios and sex ratios were similar across all three genotypes. Number of fish per genotype is included to the right of the graph.

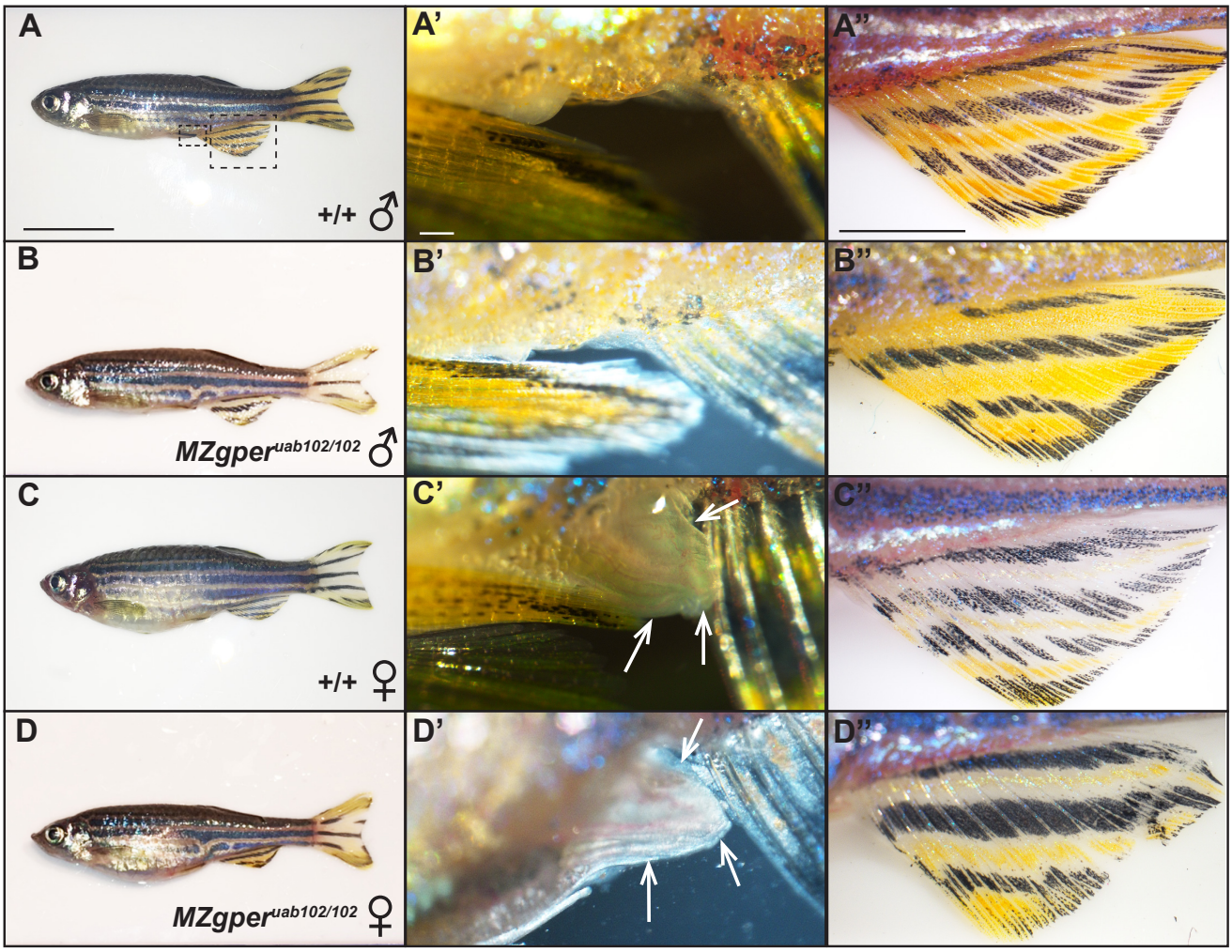

Figure 2. *MZgper*$^{uab102/102}$ mutant males and females display normal secondary sex characteristics. Male wild-type fish have a slender body shape (A), lack a genital papilla (A') and have a yellow anal fin (A''). Similarly, *MZgper*$^{uab102/102}$ mutants have a slender body shape (B), no genital papilla (B') and a yellow anal fin (B''). Wild-type females (C) and *MZgper*$^{uab102/102}$ females (D) have a rounded body shape with prominent protruding abdomen, large extended genital papilla (arrows in C', D') and whiter anal fin (C'', D''). (A', A'') High magnification images of boxed areas in A, which corresponds to all other similar images. Scale bars: 1 cm (A-D), 100 μm (A'-D'), 1000 μm (A''-D''). 10 females and 8 males per genotype were examined.

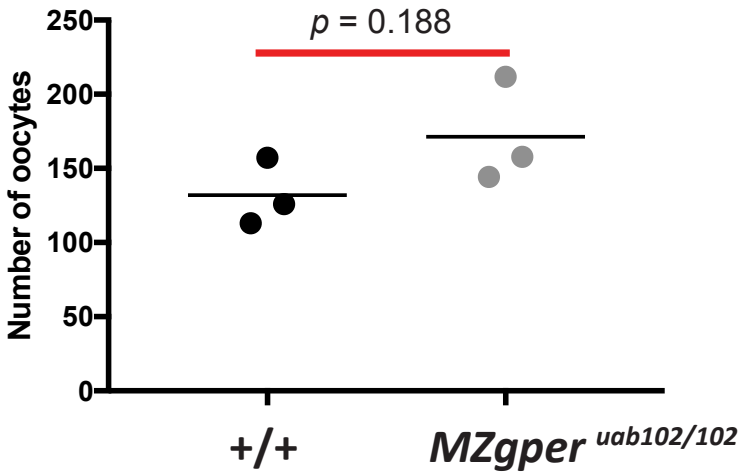

Figure 3. *MZgper*$^{uab102/102}$ mutants release on average a similar numbers of oocytes to wild-type fish during natural breeding trials. *MZgper* and wild-type 10-month old adult females were mated with wild-type males in 1:1 ratio (n = 10 fish per trial). Each dot represents the average number of oocytes released by spawning females in each mating trial (n =3 trials). *P* = 0.188, unpaired t-test.

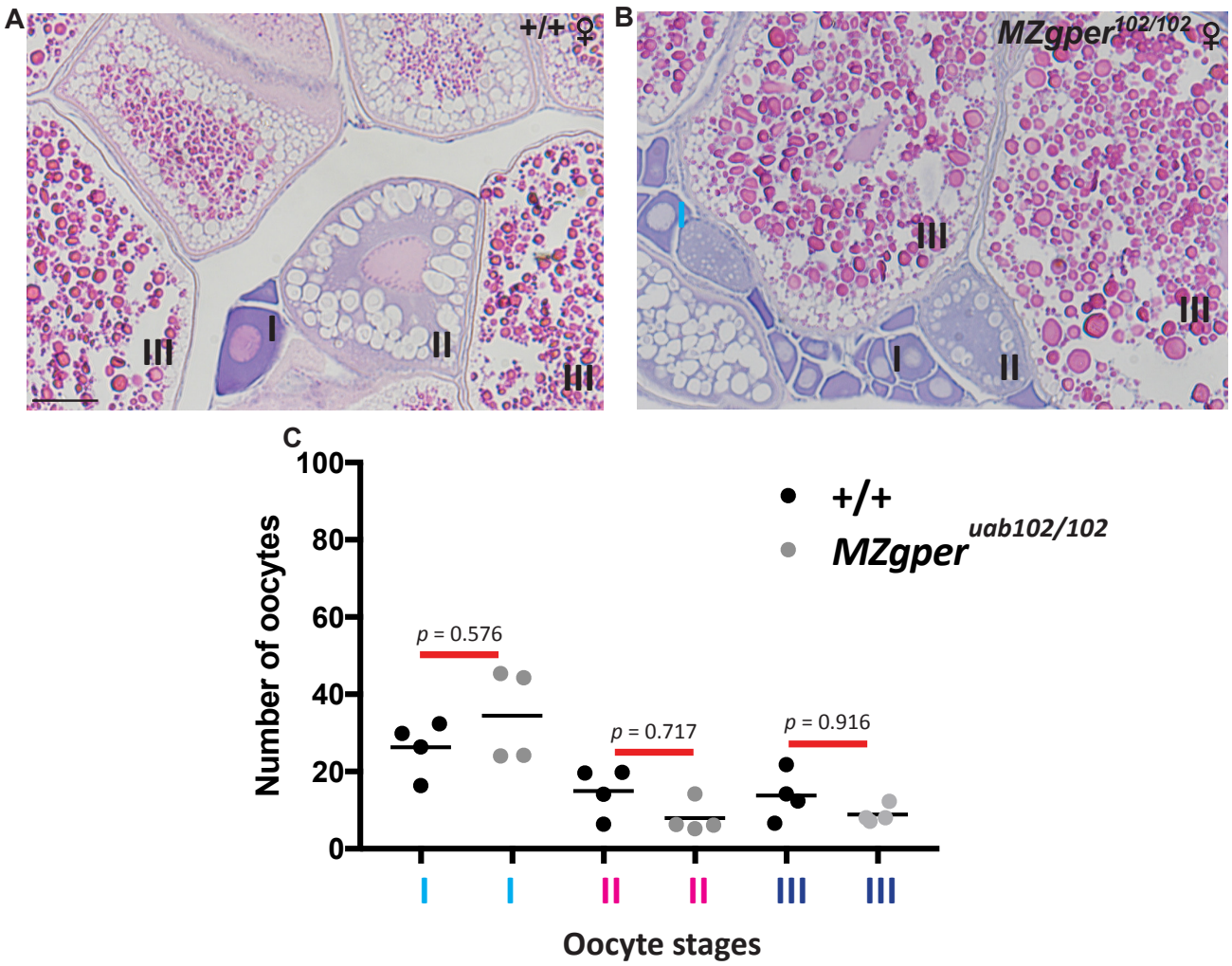

Figure 4. 4-month old *MZgper*$^{uab102/102}$ and wild-type ovaries contain similar quantities of all stages of oocytes. H & E staining of adult 4-month old wild-type (A) and *MZgper* (B) ovaries. Number of oocytes of each stage (I, II & III) are shown in (A, B) and quantified in (C) with *p*-values from one-way ANOVA with Tukey's test for multiple comparisons. Each dot represents average number of oocytes per histological section per fish (n = 4 fish per genotype, 15 sections per fish). Scale bar = 200 μm

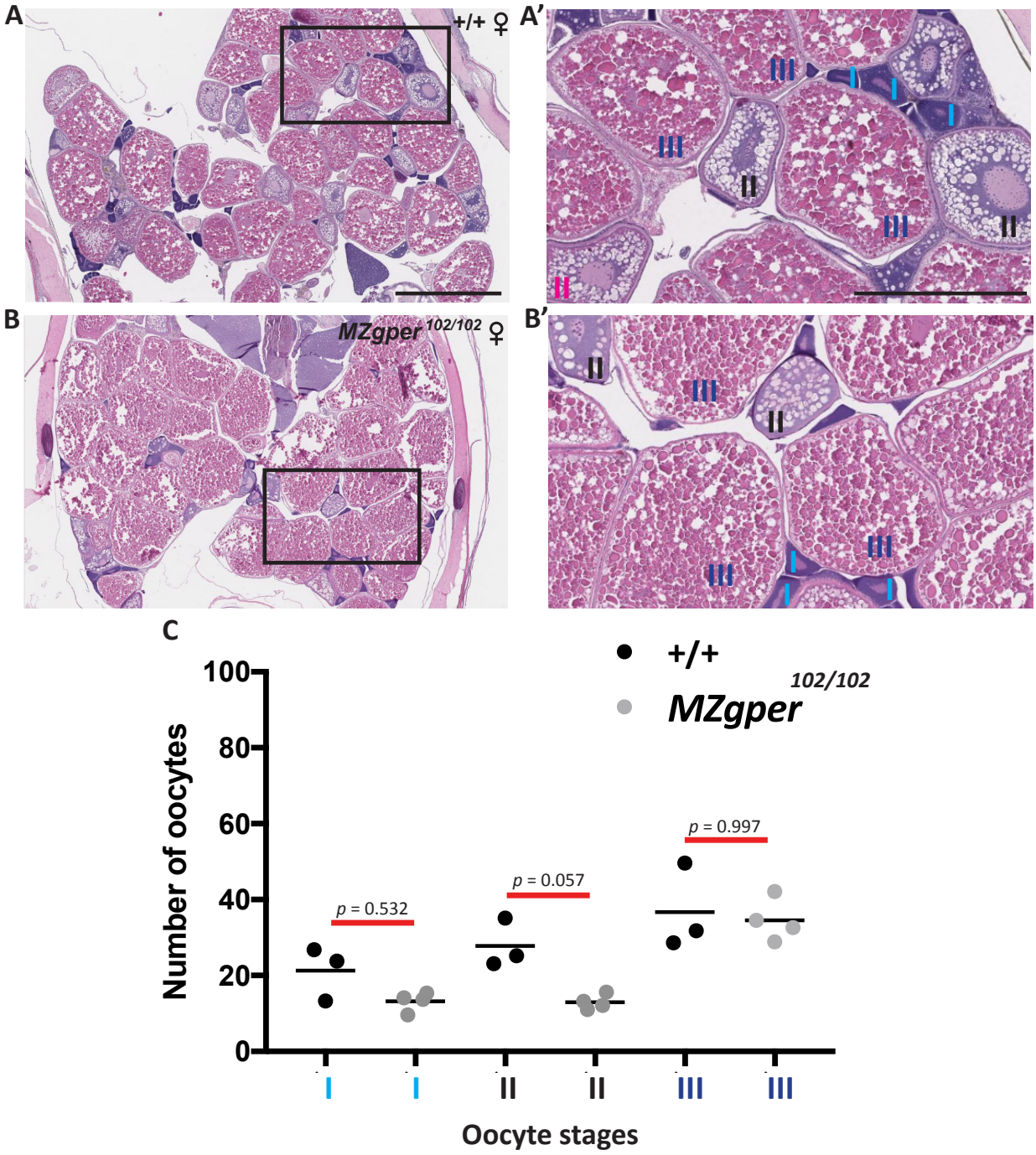

Figure 5. 10-month old *MZgper*$^{uab102/102}$ and wild-type ovaries contain similar quantities of all stages of oocytes. H & E staining of adult 10-month old wild type and *MZgper*$^{uab102/102}$ ovaries (A, B). Boxed areas are shown at higher magnification (A', B'). Number of oocytes according to stage (I, II & III) are labeled (A', B') and quantified (C) with p-values from one-way ANOVA with Tukey's test for multiple comparisons. Each dot represents average number of oocytes per histological section per fish (n = 3-4 fish per genotype, 15 sections per fish). Scale bars = 1 mm (A, B), 500 µm (A', B').

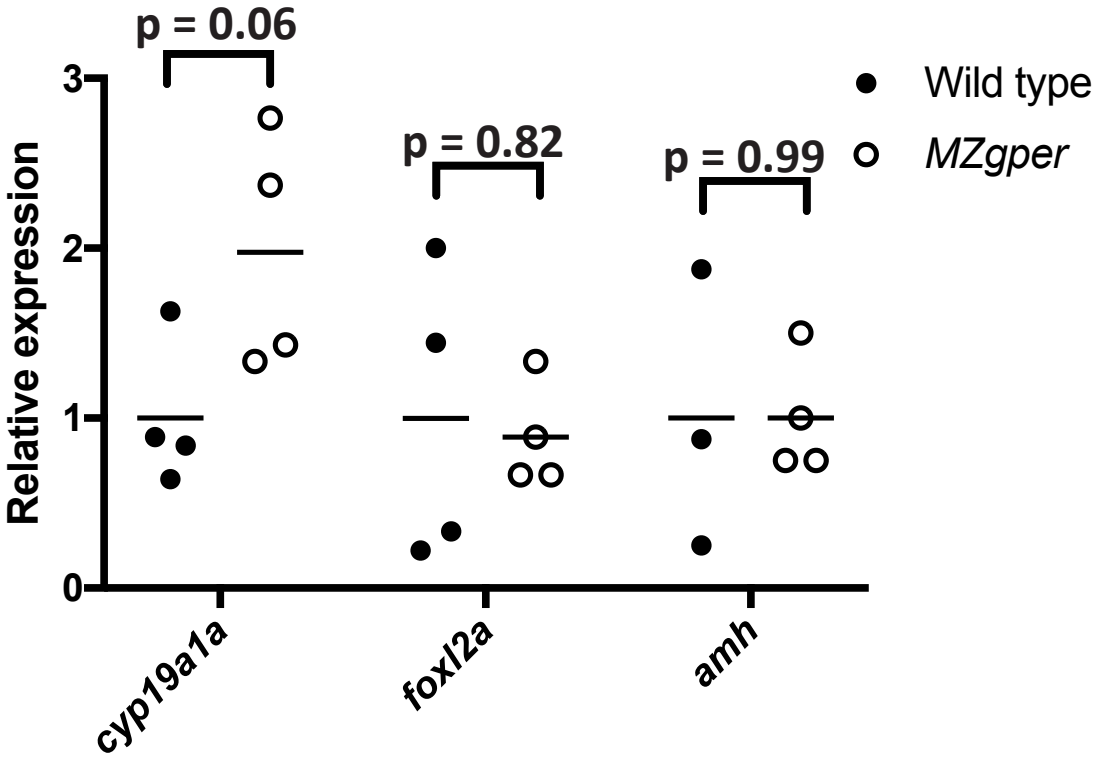

Figure 6. Quantitative RT-PCR of genes associated with sex determination and development in *MZgper*$^{uab102/102}$ mutant and wild-type ovaries. (A) qPCR analysis of *cyp19a1a*, *foxl2a* and *amh* mRNA in wild-type and *MZgper* mutant ovaries. Circles represent independent biological replicates, horizontal line is the mean, wild type mean set to 1 for each gene. Unpaired t-test showed no significant difference between gene expression in wild type and mutants.

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AUTHOR CONTRIBUTIONS

CMC and DAG conceived and designed the experiments, CMC performed the experiments, CMC and DAG analyzed/interpreted the data, SNR provided new tools/reagents, CMC and DAG wrote the manuscript. All authors approved the final manuscript.

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