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Effective “off-on” switch for fertility control in female zebrafish

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The implementation of a controllable sterility strategy is crucial for the commercialization of precise trait improvements in farmed fish using genome editing and sustainable development of fisheries. Our previous research has demonstrated that females deficient in pituitary gonadotropin *luteinizing hormone β -subunit (*lh β*)* or gonadal steroidogenesis gene *steroidogenic acute regulatory protein (*star*)* exhibit sterility due to impaired oocyte maturation and ovulation. Nevertheless, the effective restoration of fertility in *lh β* - or *star*-deficient females remains unsolved. This study has discovered that the administration of exogenous $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) at 100 and 300 $\mu\text{g/L}$ for 6 h (from 02:00 to 08:00 a.m.) effectively restores the fertility of *lh β* - or *star*-deficient females. Fertilized eggs from these mutant females can be raised without noticeable developmental defects for up to 3 weeks post-fertilization (wpf) compared to the wild-type (WT) control zebrafish. The increased expression levels of *adamts9* and *adam8b* in *lh β* - or *star*-deficient zebrafish females treated with DHP demonstrate a positive correlation with oocyte maturation and ovulation restoration. In contrast, exogenous DHP administration did not rescue the sterility phenotype observed in *progesterone receptor (*pgr*)*-deficient females. Building on our recent success in generating an all-female carp population through *cytochrome P450, family 17, subfamily A, polypeptide 1 (*cyp17a1*)*-depletion, our research presents a promising and effective strategy for an “off-on” switch for managing fertility in genome-edited cyprinids. The strategy would offer practical guidance and theoretical justification for developing “controllable fertility” in all-female fish, which would support the sustainable development of fisheries by promoting the use of novel biotechnologies in aquaculture in an eco-friendly manner.

KEYWORDS

gonadotropin, luteinizing hormone, DHP, controllable sterility, eco-friendly manner

Introduction

Advances in genome editing technologies, such as transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats associated with Cas9 (CRISPR/Cas9), provide effective and precise tools for enhancing fish traits for aquaculture purposes. For example, improved growth and feed conversion efficiency have been observed in gibel carp (*Carassius gibelio*) and all-female common carp (*Cyprinus carpio*) populations by depleting the *phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (pik3r1)*, and *cytochrome P450, family 17, subfamily A, polypeptide 1 (cyp17a1)* loci, respectively (Huang et al., 2021; Zhai et al., 2022a). However, potential spread of edited alleles into wild-type (WT) stocks limits use of genome-edited fish in aquaculture. To circumvent this, we have dedicated ourselves to developing a strategy by creating an all-female fish population (Zhai et al., 2022a) and its sterilization (Gratacap et al., 2019; Okoli et al., 2022). In this study, we establish a “off-on” switch for fertility control of female fish to breed and maintain desirable traits of sex-controlled breeding. Building on our recent success with the all-female carp population generated via the *cyp17a1*-depletion strategy, a practicable “off-on” technique for sterility in female cyprinids will be exhilarating particularly.

In vertebrates, pituitary gonadotropins are critical for ovarian development throughout the reproductive cycle, a multifaceted biological process (McGee and Hsueh, 2000). The pituitary gonadotropin, Lh β , a member of the glycoprotein hormone family, binds to its receptor Lhcgr on the granulosa or theca cells to regulate of oocyte growth, development and maturation (Gharib et al., 1990). Mutation of *LH* or *Lhcgr* in both humans and mice can result in ovarian hypogonadism and infertility due to defective gonadal steroidogenesis (Lei et al., 2001; Zhang et al., 2001; Ma et al., 2004; Huhtaniemi and Themmen, 2005; Huhtaniemi, 2006; Li and Ge, 2020). Ovarian development in zebrafish can be categorized into five stages: primary oocyte growth (stage I), accumulation of cortical alveoli (stage II), vitellogenesis (stage III), and maturation (stages IV and V). Stage IV includes stage IVa (onset of oocyte maturation and 3 h before lights on), and stage IVb (oocytes mature but before ovulation, 1 h before lights on) (Liu et al., 2018). Stimulation from Luteinizing hormone-releasing hormone (LHRH) prompts Lh secretion from the pituitary gland, resuming the meiotic cell cycle and inducing germinal vesicle breakdown (GVBD), an indicator of oocyte maturation. In stage V, the completely developed eggs are released and prepared for spawning (Nagahama et al., 1995; Nagahama and Yamashita, 2008).

In zebrafish, the crucial regulatory roles of Lh in ovarian steroidogenesis have been documented. Briefly, LH activates the intracellular signaling pathway (cAMP/PKA/CREB), the production of maturation-induced hormone and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP), and oocyte maturation by binding to Lhcgr, which is located on the theca cells of the ovary (Ascoli et al., 2002; Nagahama and Yamashita, 2008; Levavi-Sivan et al., 2010). In theca cells, cholesterol undergoes conversion to 17 α -hydroxy-progesterone and testosterone. These hormones are then respectively converted to DHP and estradiol in the granulosa cells (Clelland and Peng, 2009). Lh binds to Lhcgr to increase *insulin-like*

growth factor 3 (igf3) expression as well, which subsequently binds to the *insulin-like growth factor 1 receptor (igf1r)* and initiates oocyte maturation and ovulation (Li et al., 2018). Lh can also initiate follicle activation and promote follicle growth via the Fsh receptor (Fshr), which compensates for the absence of Fsh in folliculogenesis (Zhang et al., 2015). Steroidogenic acute regulatory protein (Star), an enzyme responsible for transporting cholesterol into the inner mitochondrial membrane for DHP production, has been identified as a downstream target of Lh for oocyte maturation in zebrafish (Shang et al., 2019) and some other teleost fishes (Nagahama and Yamashita, 2008). However, the specific functions and mechanisms of progestin signaling in initiating oocyte maturation and ovulation in zebrafish requires further elucidation.

Recently, we reported that the maturation-arrested oocyte phenotypes could be partially rescued by the administration of DHP precursors, pregnenolone or progesterone, including DHP itself, in *lh β* - or *star*-deficient females (Shang et al., 2019). Progestin signals can be mediated through membrane progestin receptor α (mPR α) and nuclear progestin receptor (Pgr). Arrested oocyte maturation and mature oocytes trapped within the follicular cells were observed in *mPRs*-deficient and *npr*-deficient females, respectively (Zhu et al., 2015; Wu et al., 2020). These results indicate that the progestin signaling may be disrupted in *lh β* - or *star*-deficient females due to impaired steroidogenesis in mutant fish. However, the exact pathological mechanisms responsible for impaired oocyte maturation and ovulation in *lh β* - or *star*-deficient females remain elusive, hindering the development of effective restoration strategies for their fecundity.

A ovulation-impaired phenotype was also observed in the *pgr*-deficient females (Liu et al., 2018). In preovulatory follicular cells (stage IVb) of female zebrafish, the expression levels of four metalloproteinases, including *ADAM metalloproteinase with thrombospondin type 1 motif, 9 (adamts9)*, *ADAM metalloproteinase domain 8b (adam8b)*, *ADAM metalloproteinase with thrombospondin type 1 motif, 1 (adamts1)* and *matrix metalloproteinase 9 (mmp9)*, increased dramatically in WT females, but reduced in *pgr*-/- zebrafish (Liu et al., 2018). *In vitro* experiments conducted on pre-ovulatory follicles have demonstrated that DHP upregulates *adamts9* expression in a dose-, time-, and Pgr-indispensable manner (Liu et al., 2018). *adamts9* expression in pre-ovulatory follicular cells is also regulated by human chorionic gonadotropin (hCG, a LH analog), which has been demonstrated to be Lhcgr-dependent, but not Pgr-dependent (Liu et al., 2020). These findings suggest that the regulation of *adamts* expression in pre-ovulatory follicular cells may involve multiple contributing factors.

In this study, we examined the effects on oocyte maturation/ovulation caused by deficiencies in *lh β* or *star* in female zebrafish. The strategy for restoring fecundity in these mutants has been continuously refined through synchronized assessments of the gonadal anatomy, fertility capacity, and the efficacy of each chosen steroid compound, along with its dosage and duration (Chen et al., 2013; Zhu et al., 2015; Lau et al., 2016; Tang et al., 2016; Lu et al., 2017; Yin et al., 2017; Crowder et al., 2018; Tang et al., 2018; Yu et al., 2018; Zhai et al., 2018; Li et al., 2020; Shu et al.,

2020). Our results indicate that treatment of exogenous DHP effectively restores the fecundity of *lhβ*- or *star*-deficient female zebrafish. The main findings of this study using genome editing models provide a valuable basis for developing an “off-on” switch to control female fertility in teleosts.

Materials and methods

Animals

Zebrafish were maintained under standard conditions at 28.5°C, in a circulated water system with a 14 h light and 10 h dark cycle as previously described (Westerfield, 2000). The *lhβ*, *star* and *pgr* heterozygous males and females were inbred to generate population that contained *lhβ*, *star* and *pgr* homozygotes, respectively (Tang et al., 2016; Shang et al., 2019). Mutant *lhβ* has an 8 bp deletion in the second exon, *star* has a 1 bp deletion in the second exon, and *pgr* has a deletion of the flanked genomic fragment (13.40 kb) between the first and the sixth exon.

Natural mating

Natural mating was conducted as previously described (Shu et al., 2020). Briefly, WT, *lhβ*- or *star*-deficient females were kept with WT males in a breeding tank with an insert and a divider in the middle. The divider was removed at 08:00 the following morning and the ratio of spawning, fertilization, and survival were recorded in every tank.

Administration with DHP

DHP (CAS No. 1662-06-2, P712080, Toronto Research Chemicals, Canada) was dissolved in Dimethylsulfoxide (DMSO), and the stock solutions of 10 g/L was sub-packed and stored in -80 °C. The *lhβ*- and *star*-deficient females were subjected to immersion treatments with 100 and 300 μg/L DHP from 02:00 to 08:00, respectively.

hCG intraperitoneal injection and administration with DHP

The hCG (hor-250, PROSPEC, Israel) was dissolved in water at 25 IU/μL as stock solution and the working solution was diluted to 1/5 with normal saline. Intraperitoneal hCG injection was conducted as described in a previous study, with minor modifications (Kinkel et al., 2010). After anesthetization, *lhβ*- or *star*-deficient females were quickly placed on a piece of wet gauze and carefully injected with 10 μL of the working solution into the pelvic midline fins at 00:00 using a microsyringe. *lhβ*- and *star*-deficient females were subsequently administrated with the immersion treatments with 100 and 300 μg/L DHP from 02:00 to 08:00, respectively.

Collection of follicular cells from stage IV follicles

The ovaries were dissected, and follicular cells of preovulatory follicles at stage IV were collected approximately 1 h prior to ovulation with lights on. Follicular cells, collected from 100

follicles per fish were separated from stage IV follicles using precision tweezers, according to a previous study with minor modifications (Liu et al., 2018).

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the follicular cells of *lhβ*- or *star*-deficient females with Trizol reagent (15596026, Ambion, TX, USA). 500 ng of the RNA template was used for reverse transcription and cDNA synthesis using the EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (AE311-03, Transgen, China). The qPCR was conducted using PerfectStart™ Green qPCR SuperMix (AQ601-02, Transgen, China) according to a previous study (Shi et al., 2022). qPCR was conducted using a Bio-Rad Real-Time System (Bio-Rad Systems, USA). All the mRNA levels were calculated as the fold change relative to *eukaryotic translation elongation factor 1 alpha 1 (ef1a)*. The primers of *adamts9*, *adam8b*, *adamts1*, and *mmp9* used for qPCR are listed in Table 1.

Histological analysis

Hematoxylin and eosin staining was performed as previously described (Zhai et al., 2022b). Briefly, fish were euthanized with MS-222 and the ovary was isolated for fixation in Bouin's solution. Fixed samples were dehydrated, infiltrated and embedded in paraffin for sectioning on a Leica microtome (RM2235, Leica Biosystems, German). Paraffin sections were stained with hematoxylin and eosin and examined microscopically using a Nikon Eclipse Ni-U microscope (Nikon, Tokyo, Japan). The scale bar is displayed in each image.

Statistical analysis

Detailed information regarding the number of zebrafish used per experiment is provided for each experiment and the corresponding figure. All analyses were conducted with the GraphPad Prism 6.0 software program and the differences were evaluated using the student's t-test. The results were expressed as the mean ± SD. For all statistical comparisons, a P value 0.05 was used to indicate a statistically significant difference.

Results

WT females could be induced to spawn after DHP administration

For the regular WT zebrafish natural mating group, one male and one female were placed overnight in a tank separated by a transparent divider. The following morning, the divider was removed for natural fish mating (Figure 1A). Ovulation of mating females was induced by overnight housing with the mating males (Figures 1B–I, Table 2). If a female was not primed by an overnight housing with a male before the mating (no priming group) (Figure 1J), this naïve single female would not naturally engage in ovulation and mating behavior with a male (Figures 1K–R, Table 2).

TABLE 1 Primers used in this study for qPCR.

Gene	Primer direction ^a and sequence (5'-3')	Product size (bp) ^b	References
<i>adams9</i>	F: TCACCCAACCCCGATTTCG	224	(Liu et al., 2018)
	R: CAAGAGCGCTGTTCATGGG		
<i>adam8b</i>	F: CCTGGCATCCACAATTGCAC	254	(Liu et al., 2018)
	R: CATTACCACAGACAGGCCCA		
<i>adams1</i>	F: ACACCGTGCACTCCAGATTTC	247	(Liu et al., 2018)
	R: GGCTACGGCCTCCAAAAGAT		
<i>mmp9</i>	F: TCTGCCTTTGAGGACCACCT	260	(Liu et al., 2018)
	R: CCGAAAGCTGCATCAGTGAA		
<i>ef1a</i>	F: AAGATCGGCTACAACCCCTGC	107	(Shang et al., 2019)
	R: TTCCATCCCTTGAACCAGCC		

^aF, Forward; R, Reverse.
^bbase pair.

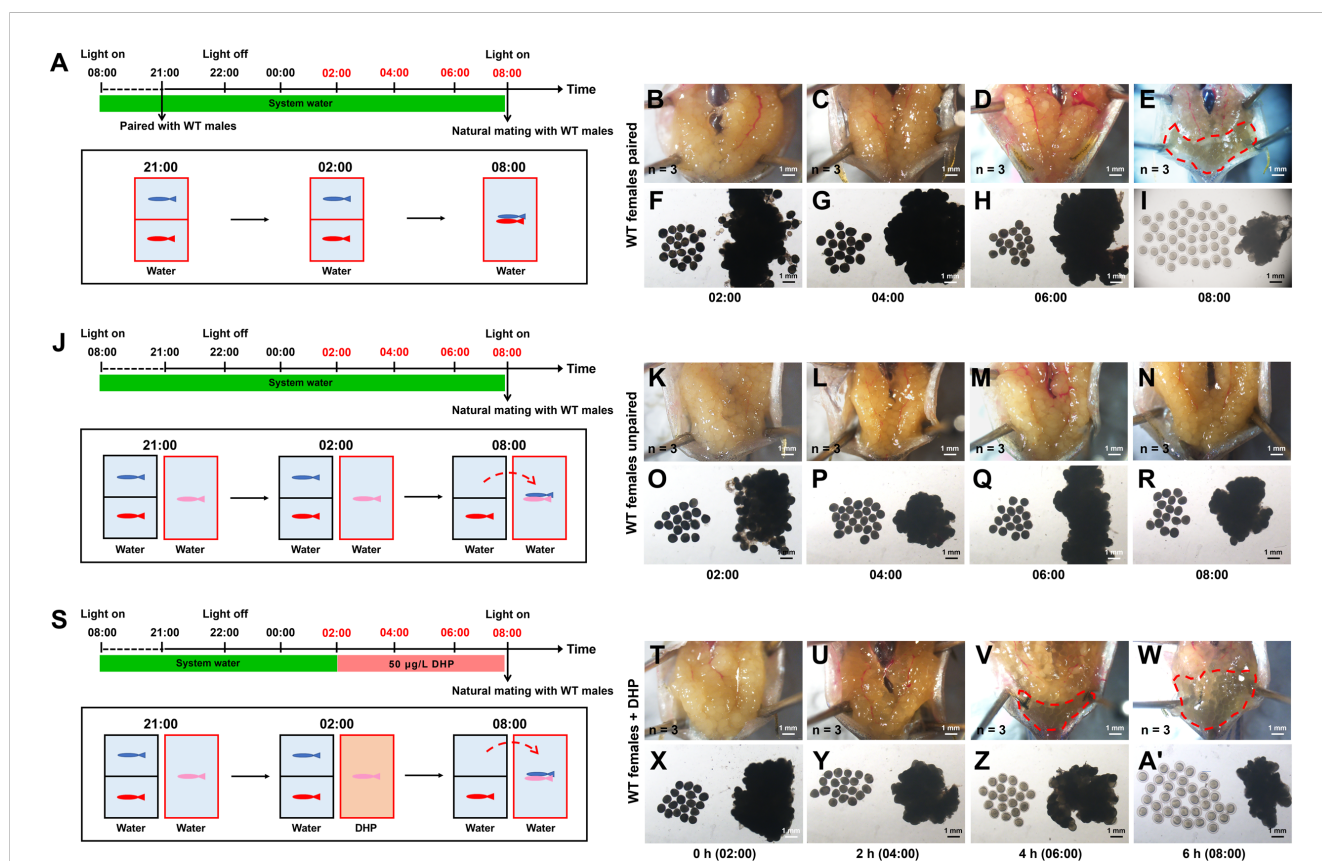


FIGURE 1

WT females could be induced to spawn after DHP administration. (A) Schematic illustration of the spawning test for paired WT females (depicted in red). One WT female (depicted in red) and one WT male (depicted in blue) are housed overnight in the tank, separated by a transparent divider. At 08:00 (light on), the divider is removed, then the spawning between the paired WT female and WT male succeeds. (B–I) The anatomical examination and microphotographs of follicles are sampled from paired WT females at 02:00, 04:00, 06:00, and 08:00 (n = 3 at each timepoint). (J) Schematic illustration of the spawning test for unpaired WT females (depicted in pink). WT females (depicted in red) are housed overnight in the tank with WT male (depicted in blue), then natural mating of the unpaired WT females (pink) with the paired males (blue) failed. (K–R) The anatomical examination and microphotographs of follicles sampled from unpaired WT females at 02:00, 04:00, 06:00, and 08:00 (n = 3 at each timepoint). (S) Schematic illustration of the spawning test for unpaired WT females (depicted in pink) treated with DHP (tank depicted in brown). WT females are exposed to 50 µg/L DHP from 2:00 to 8:00, then natural mating with males (depicted in blue) which are from another tank paired with WT females (depicted in red) overnight succeeds. (T–A') The anatomical examination and microphotographs of follicles sampled from WT females treated with 50 µg/L DHP for 0 h (02:00), 2 h (04:00), 4 h (06:00), and 6 h (08:00) (n = 3 at each timepoint). The samples at the timepoints depicted in red are harvested for oocyte analysis. (B, F, K, O, T, X) 0 h (C, G, L, P, U, Y) 2 h (D, H, M, Q, V, Z) 4 h (E, I, N, R, W, A') 6 h.

TABLE 2 Comparison of the fecundities of wild-type zebrafish female induced by overnight housing and DHP treatment.

Group	Ratio of spawning (%)	Number of spawning eggs	Ratio of fertilization (%)	Ratio of membrane break (%)	Ratio of survival at 3 wpf (%)	Fecundity index*	Relative fecundity ratio** (%)
WT + overnight housing of the mating partner	92.50 ± 8.29 ^a	257.83 ± 100.94 ^a	91.15 ± 6.09 ^a	96.94 ± 2.24 ^a	85.00 ± 7.83 ^a	179.13	100.00
WT + DHP treatment	88.87 ± 7.87 ^a	243.11 ± 55.60 ^a	60.63 ± 9.39 ^b	88.10 ± 7.56 ^b	76.71 ± 8.50 ^a	88.54	49.43

N = 6–10 for each group.

*Fecundity index = Ratio of spawning (%) × Number of spawning eggs × Ratio of fertilization (%) × Ratio of membrane break (%) × Ratio of survival at 3 wpf (%).

**Relative fecundity ratio = Fecundity index of the treated mutant group / Fecundity index of wild-type control fish group.

Different letters in the table represent significant differences compared with the control group (P < 0.05).

However, if the WT female was treated with 50 µg/L DHP for 6 h from 02:00 to 08:00, the effective ovulation could be seen in DHP treated female (Figures 1S–A'). When a pair of DHP-treated females and males was set up for mating after 6 h DHP treatment, the DHP treated WT females could naturally mate with the WT males and spawn (Table 2). Although the fertilization ratio of the offspring from DHP administrated WT females and WT males was significantly lower than that of the natural mating group, the ratios of membrane breakage and survival at 3 wpf were unaffected (Table 2). These results indicate that DHP treatment can effectively replace overnight housing with male fish to induce the ovulation and spawning in female fish. The viabilities of the naturally fertilized eggs from primed by overnight housing and DHP treatment were equivalent.

Anovulation defects in *lhβ*- or *star*-deficient females rescued with the DHP administration

Previously, the phenotypes of oocyte maturation and ovulation were observed in *lhβ*- or *star*-deficient females. We found that the follicles in the *lhβ* mutants did not enter stage V as it normally occurred in the WT control females between 06:30 and 07:30 when mated with WT males (Shang et al., 2019). We first examined the restorative effects of DHP on ovulation in these females. The *lhβ*- and *star*-deficient females were exposed to 100 and 300 µg/L DHP, respectively, from 02:00 to 08:00 (Figure 2A). At 02:00, the follicles from the dissected ovaries of *lhβ*-deficient females were non-transparent (Figures 2B, F); however, after 2 h DHP administration (at 04:00), the follicles became semi-transparent (Figures 2C, G), marking the beginning of oocyte maturation. More apparently, the anatomical examination of *lhβ*-deficient females exposed to 100 µg/L DHP for 4 h (at 06:00) was sufficient to induce maturation of oocytes, as evidenced by the microphotographs of follicles, which had become transparent embedded in the dissected ovaries and few eggs with egg membrane formed (characterized as stage V) (Figures 2D, H). At 08:00, when the *lhβ*-deficient females were exposed to DHP for 6 h, more eggs with membrane formed, suggesting that the follicles reached to stage V, and released into the vicinity of the genital pore in *lhβ*-deficient females (Figures 2E, I). The successful induction of follicles at different time points after DHP administration was also

supported by the observed oocyte maturation and ovulation in *star*-deficient females (Figures 2J–Q). These results suggest that the arrested oocyte maturation and ovulation in *lhβ*- or *star*-deficient females *in vivo* could be effectively rescued by DHP administration.

Infertility of *lhβ*- or *star*-deficient females was rescued with DHP administration

Following the sketches depicted in Figure 2A, the rescue of the infertility phenotypes in *lhβ*- or *star*-deficient females administrated diverse combinations of the hormones, including DHP and hCG respectively, were evaluated with natural mating with WT males (Table 3). For comparison of the rescue efficiency, the “fecundity index” was adopted for representing the capacity of each experimental mating group to produce healthy offspring. The “relative fecundity” indicates the rescue efficiency of each treatment, represented by the fecundity index of the treated mutant group/fecundity index of WT control group (Table 3). After a 6 h treatment (from 02:00 to 08:00), the *lhβ*-deficient females were placed in a new breeding tank with an insert at the bottom and a divider in the middle. WT males, which had been kept with WT females in another breeding tank contained system water that night, were transferred into the tank with the DHP-treated *lhβ*-deficient females. Natural mating was observed between WT males and DHP-treated *lhβ*-deficient females. Compared with the hCG treatment, the administration of DHP effectively rescued the defects of oocyte maturation and ovulation of the *lhβ*-deficient females, as the spawned and fertilized eggs with the genotype of *lhβ*+/- were observed (76.00 ± 23.32% *lhβ*-deficient females spawned, 25.90 ± 12.28% alive eggs were fertilized, 68.11 ± 31.45% fertilized eggs broke the egg membrane, and 59.80 ± 39.56% fertilized eggs survived to juvenile stage) (Table 3).

Noteworthy, the administration of 100 µg/L DHP for 6 h, which was sufficient to rescue the defects of oocyte maturation and ovulation in the *lhβ*-deficient females, did not work well in the *star*-deficient females, as no spawned eggs were observed, even with artificial squeeze (Data not shown). The *star*-deficient females were subsequently exposed to 300 µg/L DHP from 02:00 to 08:00. Strikingly, 6 h DHP administration at 300 µg/L effectively rescued the defects of oocyte maturation and

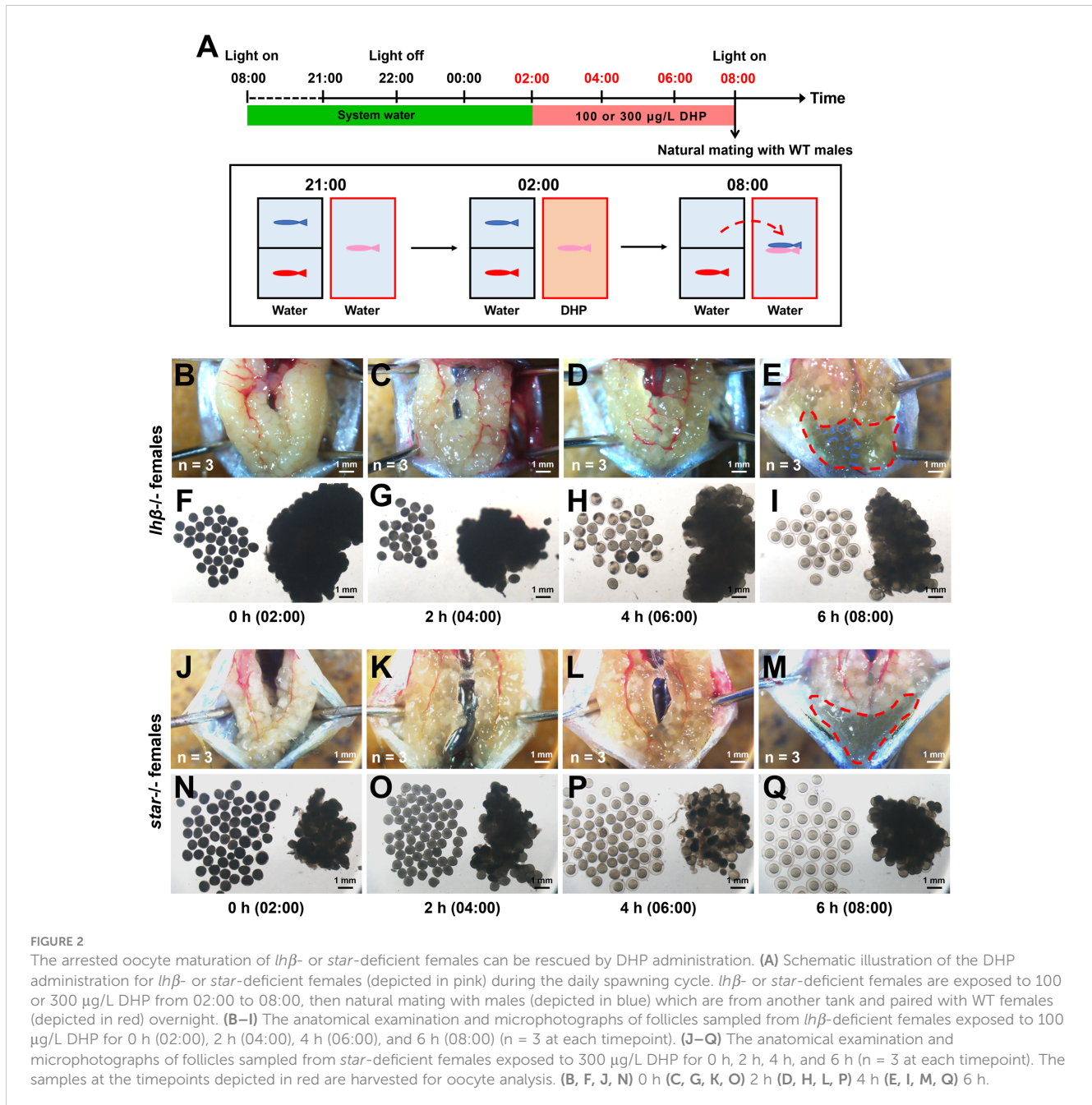


FIGURE 2

The arrested oocyte maturation of *lhβ*- or *star*-deficient females can be rescued by DHP administration. (A) Schematic illustration of the DHP administration for *lhβ*- or *star*-deficient females (depicted in pink) during the daily spawning cycle. *lhβ*- or *star*-deficient females are exposed to 100 or 300 µg/L DHP from 02:00 to 08:00, then natural mating with males (depicted in blue) which are from another tank and paired with WT females (depicted in red) overnight. (B–I) The anatomical examination and microphotographs of follicles sampled from *lhβ*-deficient females exposed to 100 µg/L DHP for 0 h (02:00), 2 h (04:00), 4 h (06:00), and 6 h (08:00) (n = 3 at each timepoint). (J–Q) The anatomical examination and microphotographs of follicles sampled from *star*-deficient females exposed to 300 µg/L DHP for 0 h, 2 h, 4 h, and 6 h (n = 3 at each timepoint). The samples at the timepoints depicted in red are harvested for oocyte analysis. (B, F, J, N) 0 h (C, G, K, O) 2 h (D, H, L, P) 4 h (E, I, M, Q) 6 h.

ovulation in the *star*-deficient females (Table 3), resulting in the observation of the spawned and fertilized eggs with the genotype of *star*^{+/-} (51.90 ± 18.84% *star*-deficient females spawned, 72.87 ± 9.92% alive eggs were fertilized, 47.81 ± 26.25% fertilized eggs broke the egg membrane, and 66.04 ± 32.40% larvae of them survived to juvenile stage) (Table 3).

The developmental outcomes of the fish from the control females, the *lhβ*- or *star*-deficient females and WT males were also evaluated. The fertilized eggs at the early stage during embryonic development (12 h post-fertilization, hpf), larval stage (72 hpf and 5 days post-fertilization, dpf), and juvenile stage (3 weeks post-

fertilization, wpf) from the control females and from the *lhβ*- or *star*-deficient females and WT males were comparable as evaluated by the gross appearance (Supplementary Figures 1A–L, Table 3). At 1-month post-fertilization (mpf), the body weight, body length and full length of the fish from control females were comparable with rescued fish from the *lhβ*- or *star*-deficient females mated with WT males (Supplementary Figures 1M–O). These results suggest that DHP at 100 or 300 µg/L from 02:00 to 08:00 is sufficient to rescue ovarian maturation and ovulation defects of *lhβ*- or *star*-deficient females as evaluated with natural mating with WT males, while the overall efficiency of *star*-

TABLE 3 Efficiencies of the fecundity rescue of *lhβ*- and *star*-deficient females with diverse steroids treatments.

Group	Ratio of spawning (%)	Number of spawning eggs	Ratio of fertilization (%)	Ratio of membrane break (%)	Ratio of survival at 3 wpf (%)	Fecundity index*	Relative fecundity ratio** (%)
WT Control	91.48 ± 9.02 ^a	266.11 ± 83.96 ^a	80.54 ± 22.35 ^a	94.19 ± 6.62 ^a	93.56 ± 8.97 ^a	172.79	100.00
<i>lhβ</i> ^{-/-}	0	0	0	0	0	0	0
<i>lhβ</i> ^{-/-} + hCG	0	0	0	0	0	0	0
<i>lhβ</i> ^{-/-} + DHP	76.00 ± 23.32 ^a	193.14 ± 81.39 ^a	25.90 ± 12.28 ^b	68.11 ± 31.45 ^b	59.80 ± 39.56 ^b	15.50	8.97
<i>lhβ</i> ^{-/-} + hCG + DHP	84.00 ± 14.97 ^a	288.50 ± 136.70 ^a	24.57 ± 12.95 ^b	28.60 ± 21.78 ^c	35.68 ± 21.62 ^b	6.08	3.52
<i>star</i> ^{-/-}	0	0	0	0	0	0	0
<i>star</i> ^{-/-} + hCG	0	0	0	0	0	0	0
<i>star</i> ^{-/-} + DHP	51.90 ± 18.84 ^b	251.75 ± 52.91 ^a	72.87 ± 9.92 ^b	47.81 ± 26.25 ^b	66.04 ± 32.40 ^b	30.06	17.40
<i>star</i> ^{-/-} + hCG + DHP	66.67 ± 9.43 ^b	224.80 ± 91.63 ^a	40.02 ± 19.05 ^c	72.36 ± 25.63 ^b	71.60 ± 22.32 ^b	31.07	17.98

N = 6–10 for each group.

*Fecundity index = Ratio of spawning (%) × Number of spawning eggs × Ratio of fertilization (%) × Ratio of membrane break (%) × Ratio of survival at 3 wpf (%).

**Relative fecundity ratio = Fecundity index of the treated mutant group / Fecundity index of WT control fish group.

Different letters in the table represent significant differences compared with the control group (P < 0.05).

deficient females administrated with 300 μg/L DHP for 6 h is better than those of DHP-treated *lhβ*-deficient females.

DHP administration upregulated *adam8b* and *adamts9* expression in pre-ovulatory follicle cells of WT females mimicked the effect of natural mating with WT males

It is known that the metalloproteinases, including *adamts9* and *adam8b*, are required for zebrafish ovulation, and their expression significantly increased at 1 h prior to ovulation (Liu et al., 2018; Liu et al., 2020). Both metalloproteinases, *adamts9* and *adam8b*, but not *adamts1* and *mmp9* (Figures 3A–D, column 2 vs. 1), were significantly upregulated in the pre-ovulatory follicle cells of WT females paired with WT males 1 h before light on compared to those of unpaired WT females. Intriguingly, in the follicular cells of WT females exposed to DHP for 2 h (from 05:00 to 07:00) exhibited upregulated expression of *adamts9* and *adam8b* compared to the unpaired WT females, mimicking the effect of natural mating with WT males (Figure 3D, column 3 vs. 2).

DHP administration restores *adam8b* and *adamts9* expression in pre-ovulatory follicle cells of *lhβ*- or *star*-deficient females

Both metalloproteinases, *adamts9* and *adam8b* (Figures 4, 5A–E, column 2 vs. 1), but not *adamts1* and *mmp9* (Figures 4, 5F, G, column 2 vs. 1), were significantly upregulated in the pre-ovulatory follicle cells of control females paired with WT males 1 h before light on. Contrarily, the increased expression of *adamts9* and

adam8b in follicular cells was not observed in *lhβ*- or *star*-deficient females paired with WT males 1 h before light on (Figures 4, 5D, E, column 4 vs. 3). We hypothesized that this may be one of the major reasons for arrested oocyte maturation and ovulation. Intriguingly, the follicular cells of *lhβ*- or *star*-deficient females exposed to DHP for 2 h (from 05:00 to 07:00) exhibited upregulated *adamts9* and *adam8b* expression, compared to the untreated fish (Figures 4, 5D, E, column 5 vs. 3). These results suggest that the upregulated *adamts9* and *adam8b* expression may be induced by DHP to restore the final maturation of oocyte and ovulation in *lhβ*- or *star*-deficient females.

Pgr signaling is indispensable for DHP-induced ovulation in zebrafish

Genomic progesterin signaling and ovulation require the participation of Pgr, and *pgr* depletion is known to cause ovulation failure but does not affect oocyte maturation in zebrafish (Zhu et al., 2015). Our observations in the *pgr*-deficient female zebrafish replicated the manifestations of impaired ovulation reported in previous studies (Zhu et al., 2015). In control females paired with WT males, normal mature stage V oocytes that were released into the vicinity of the genital pores were observed at 08:00 (Figure 6A). However, anovulation with mature stage V oocytes trapped within the ovary was observed in *pgr*-deficient females (Figure 6B). The results of histological analysis demonstrated that the outer layer cells of the mature follicles failed to breakdown in the *pgr*-deficient fish (Figure 6C, D). Moreover, we found that the anovulation defects observed in *pgr*-deficient female zebrafish could not be rescued by DHP administration at a range of concentrations (Figure 6E–H). These results suggest that Pgr signaling is indispensable for DHP-mediated ovulation in zebrafish.

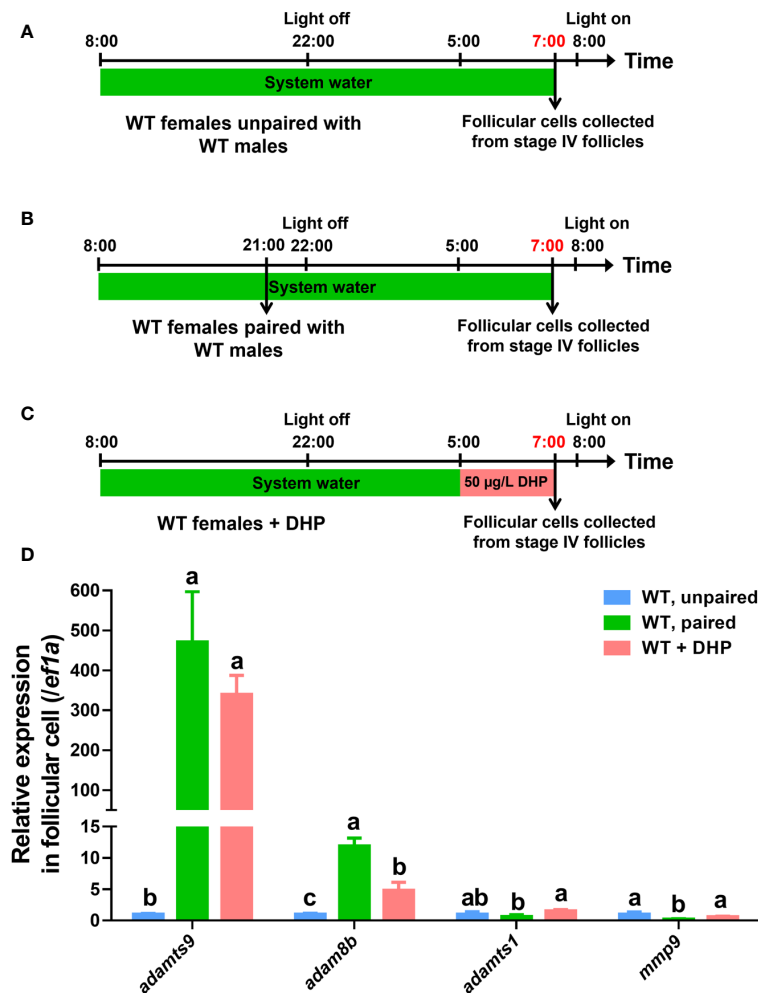


FIGURE 3

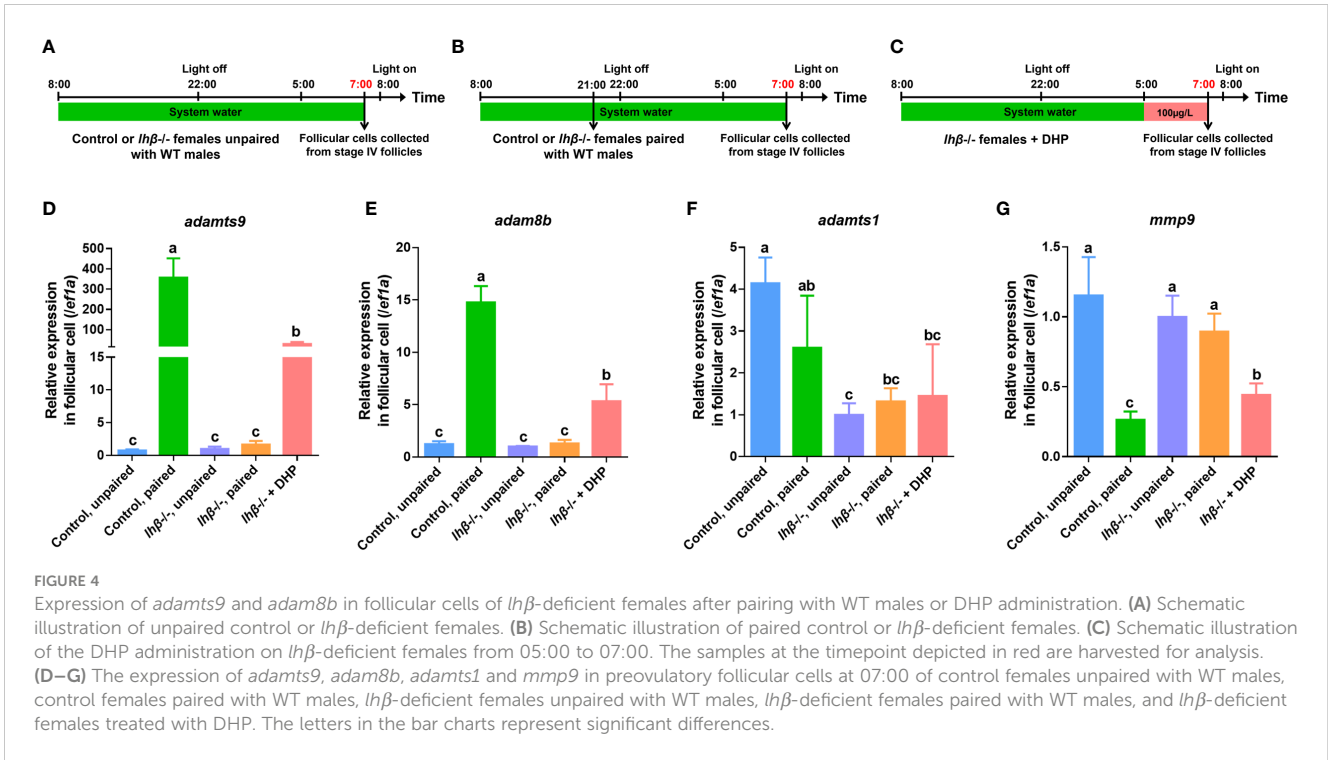
Expression of *adamts9* and *adam8b* is upregulated after DHP administration. (A) Schematic illustration of unpaired WT females. (B) Schematic illustration of paired WT females. (C) Schematic illustration of the DHP administration on WT females from 05:00 to 07:00. The samples at the timepoint depicted in red are harvested for analysis. (D) The expression of *adamts9*, *adam8b*, *adamts1*, and *mmp9* in pre-ovulatory follicular cells at 07:00 of paired WT females or WT females exposed to DHP are comparatively evaluated with that of control females unpaired with WT males. The letters in the bar charts represent significant differences.

Discussion

Although several studies have been conducted in zebrafish, the precise physiological processes and mechanisms involved in oocyte maturation and ovulation remain unclear. This study investigated the replacement of overnight mating partner housing with DHP treatment for oocyte maturation and ovulation induction in WT zebrafish. Based on these trails, DHP was selected to rescue sterility phenotype in *lhβ*- or *star*-deficient females. After multiple trials to determine the appropriate compound, dosage, and duration, it has been discovered that DHP and DHP + hCG administration can achieve consistently restore fertility in *lhβ*- or *star*-deficient females. The increased levels of *adamts9* and *adam8b* present in follicular cells of *lhβ*- or *star*-deficient females, as well as WT females exposed to overnight housing pairing or DHP treatment, demonstrated a positive relationship with the induction of oocyte maturation and ovulation induction. Since DHP administration failed to rescue the ovulation failure observed in *pgr*-deficient female zebrafish despite

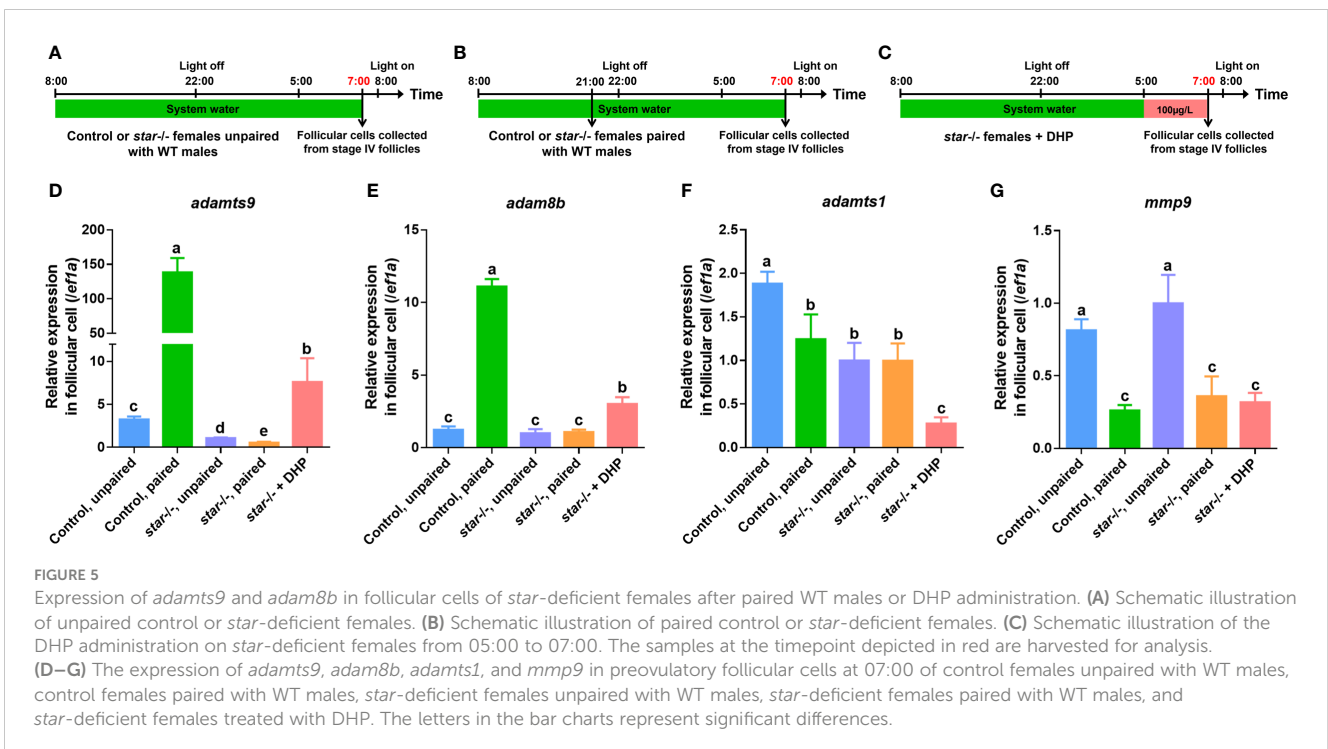
the presence of mature stage V oocytes (Zhu et al., 2015), we concluded that *Pgr* is imperative for DHP-mediated ovulation.

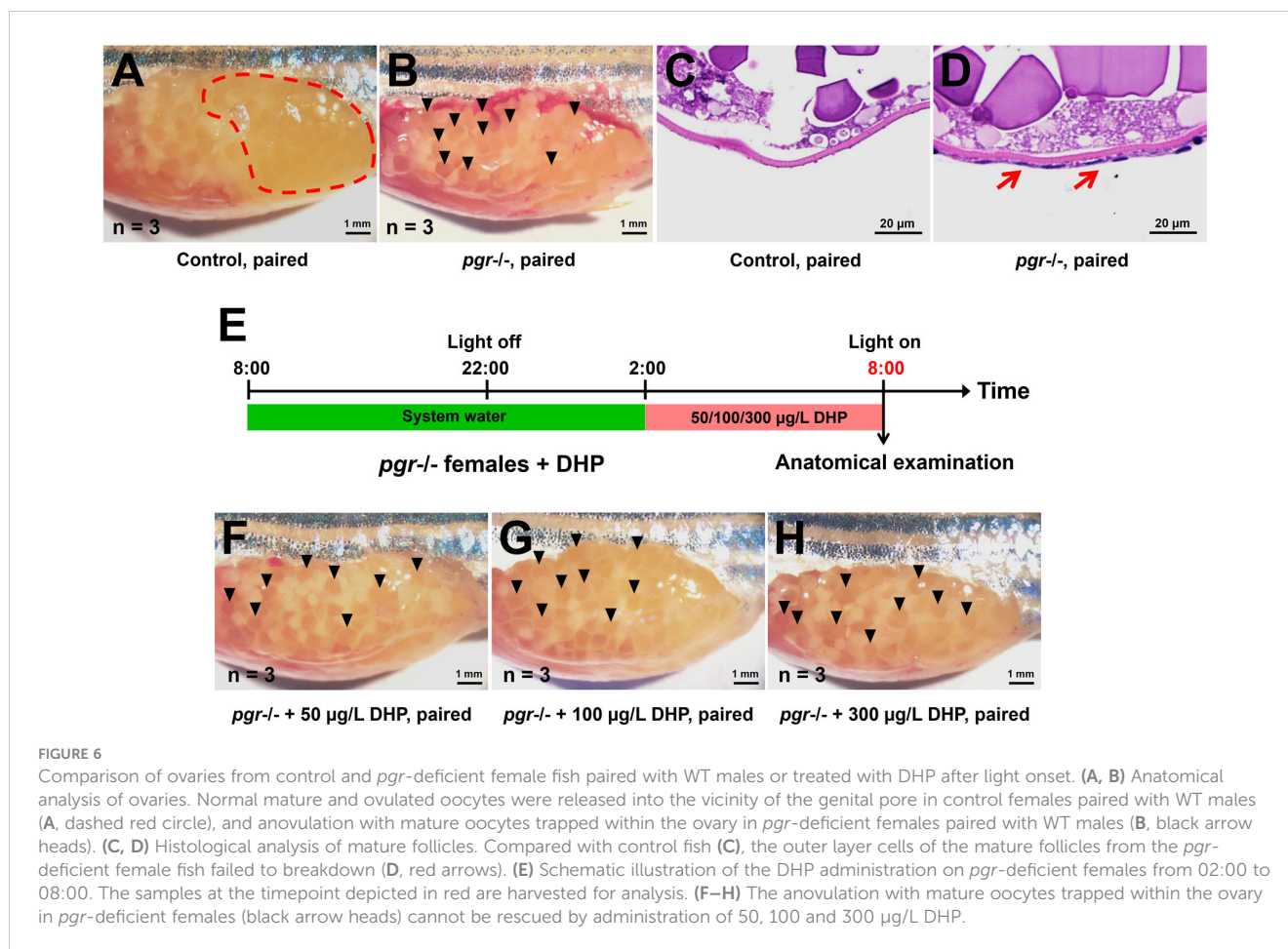
The process of oocyte maturation and ovulation lasts for a few hours in fish within the spawning cycle. In the preovulatory follicles of WT female zebrafish, the relative expression of *lhβ* began to increase from 03:30, and reached the peak at 05:30, the beginning of oocyte maturation (follicles started to become semi-transparent) (Shang et al., 2019). Similarly, the transcript and protein levels of *Pgr* begin to increase at 05:00 (prior to maturation) and peak level at 06:00–07:00 (in the middle of maturation) in female zebrafish (Liu et al., 2018). DHP is the major maturation-inducing hormone in zebrafish (Nagahama and Yamashita, 2008; Tokumoto et al., 2011; El Mohajer et al., 2022). Previously, we found that *Lhβ* regulates DHP synthesis in zebrafish by targeting *star*, the first step of gonad steroidogenesis from cholesterol (Shang et al., 2019). This is supported by the observations in *lhβ*-, or *star*-, or *mprs*-deficient females, wherein the oocyte maturation was impaired (Wu et al., 2020). These findings suggest that *Lhβ*/*Star*/*Progesterone* signaling is



closely related to oocyte maturation. However, most of the conclusions that DHP promotes oocytes maturation were conducted on stage IV follicles *in vitro*. Here, the assessment of DHP in oocyte maturation and ovulation was firstly examined *in vivo* from 02:00 to 08:00 in *lhβ*- or *star*-deficient females with DHP administration at 100 and 300 μg/L, respectively. Oocytes become to be transparent following GVBD due to fusion of yolk proteins,

allowing more lights to pass through. At 04:00, after 2 h DHP treatment, the follicles became semi-transparent, indicating the beginning of oocyte maturation. At 06:00, after 4 h DHP treatment, the follicles became transparent, and very few oocytes formed egg membranes, marking the completion of oocyte maturation (stage V) started. At 08:00, after 6 h DHP treatment, the transparent follicles were not trapped in the vicinity of the





ejaculatory pore and were ready to spawn, forming egg membranes followed by dissection. These results provided direct *in vivo* evidence supporting that oocyte maturation and ovulation occurred effectively in *lh β* - or *star*-deficient females after DHP administration.

The *lh β* - or *star*-deficient females reared in control medium (1‰ DMSO system water) exhibited no egg-laying when naturally mated with WT males. Nevertheless, DHP treatment from 02:00 to 08:00 effectively rescued the defects in oocyte maturation and ovulation of *lh β* - or *star*-deficient females when naturally mated with WT males, i.e. the restored spawned and fertilized eggs (Figure 2, Table 3). Noteworthy, the spawning ratio of the *lh β* - or *star*-deficient females and the fertilization ratio of the offspring were decreased compared to the WT control group (Table 3). One possibility is that the spawned eggs may not be in their most appropriate status for fertilization with the above treatment method. Therefore, further efforts to optimize the process, including concentration of the chemical reagent, mode of the administration, or other factors, are needed to improve the quality of the eggs from the mutant females. This hypothesis could be partially supported by the observations that the ratio of membrane break and survival at 3 wpf of the offspring from the DHP + hCG treated *star*-deficient females mated with WT males were higher than with the females with DHP treatment only

(Table 3). Intriguingly, the survived fish (*lh β* ^{+/-} or *star*^{+/-}) displayed normal growth performance (body weight, body length and full length) compared with the control fish (*lh β* ^{+/+} or *star*^{+/+}) as observed at 1 mpf (Supplementary Figure 1).

Metalloproteinases are important for zebrafish ovulation. The relative expression of metalloproteinases, *adamts9*, *adam8b*, *mmp9*, and *adamts1*, was upregulated in the follicular cells of pre-ovulatory follicles at stage IV, approximately 1 h prior to ovulation and light on (Liu et al., 2018). Among the four metalloproteinases, *adamts9* and *adam8b*, were significantly upregulated in follicular cells of control females paired with WT males, and WT females, *lh β* - or *star*-deficient females exposed to DHP for 2 h (from 05:00 to 07:00), but not in *lh β* - or *star*-deficient females paired with WT males. Thereby, it is reasonable to speculate that dysregulated expressions of *adamts9* and *adam8b* in follicular cells of preovulatory follicles at stage IV of *lh β* - or *star*-deficient females might be due to insufficient DHP synthesis. Thus the insufficient expressions of *adamts9* and *adam8b* may be the cause for the impaired oocyte maturation and ovulation, which is in agreement with a previous report on the reduced expression of *adam8b* and *adamts9* in the follicular cells of preovulatory follicles of *pgr*^{-/-} females (Liu et al., 2018). We discovered that a concentration of 50 $\mu\text{g/L}$ can induce oocyte maturation, ovulation, and spawning in WT females (Figure 1, Table 2). Upregulated *adamts9* and *adam8b* expression in the

follicular cells of the pre-ovulatory follicles of control females exposed to DHP from 05:00 to 07:00 mimicked the effect of natural mating with WT males (Figure 3D).

Anovulation, wherein mature oocytes were retained in the ovary, was observed in *pgr*-deficient females when mated with WT males. However, DHP administration was ineffective in restoring ovulation in *pgr*-deficient females when paired with WT males, indicating that the critical role of Pgr in ovulation. This is further demonstrated by the distinct phenotypes of *pgr*- and *lhβ*- or *star*-deficient females, which display impaired ovulation and oocyte maturation, respectively. Therefore, it is reasonable to hypothesize that Star plays a role in the downstream LH signaling pathway, leading to the direct synthesis of DHP to enhance oocyte maturation and ovulation, with ovulation being dependent on Pgr.

Previously, it has been reported that the intraperitoneally injection of hCG, DHP precursors (pregnenolone or progesterone), and DHP itself, partially rescued the maturation-arrested oocyte phenotypes in *lhβ*-deficient females (Shang et al., 2019; Chu et al., 2014). Among the chemicals, hCG administered via intraperitoneal injection successfully induced the maturation of a small number of follicles and ovulation in *lhβ*-deficient females. However, the report lacked clarity regarding the restorative effects of natural spawning and fertilization (Chu et al., 2014). Herein, we also evaluated the combined effect of hCG injection and DHP administration for 6 h (hCG injection at 00:00) and found no spawning and fertilized eggs from the hCG treated *lhβ*-deficient females mated with WT males. However, compared with the *star*-deficient females exposed to 300 μg/L DHP only, the improvement of the ratio of spawning, membrane broken and survival at 3 wpf were observed in *star*-deficient females with hCG injection and 300 μg/L DHP administration for 6 h ($66.67 \pm 9.43\%$, $72.36 \pm 25.63\%$, and $71.60 \pm 22.32\%$, respectively). The overall relative fecundity ratio, a novel concept evaluated in this study was that *star*-/- zebrafish treated with DHP and hCG exhibited a ratio at 17.98%. It is noteworthy that there was a limited improvement in these parameters in *lhβ*-deficient females after hCG injection and administration of 100 μg/L DHP for 6 h (Table 3). These observations suggest that although hCG is a LH analog, its function in regulating oocyte maturation and ovulation may differ in various animal species.

In channel catfish, sterilization was achieved through genetic editing of LH using a modified zinc finger nuclease technology with electroporation. This accomplishment not only enhances the comprehension of the roles of LH in farmed fish, but also represents progress towards fertility control in females of transgenic fish (Qin et al., 2016). CRISPR/Cas9 techniques have been suggested to improve economic traits. However, the application of sterilization technology can be utilized to establish fertility control strategies for genetically modified farm fish. Fertility-controlled fish, which are unable to breed naturally, offer natural biosecurity benefits by preventing escapee from mating with the WT stock and protecting intellectual property rights (Okoli et al., 2022). To the best of our knowledge, this study represents the first model with the manipulation of sex steroid-related genes, which opens up prospects for its application in the aquaculture industry, particularly in the genetic engineering of farmed fish with

the aim of improving economic traits. Notably, our recent research involving zebrafish and common carp revealed that all homozygous *cyp17a1*-deficient fish gonads developed into testes with proper spermatogenesis; however, they lacked the typical male sexual characteristics and mating behaviors (Zhai et al., 2018; Zhai et al., 2022a). All offspring resulted from the artificial fertilization of the neomale common carp (*cyp17a1*-/-;XX genotype) sperm with the eggs from WT females (*cyp17a1*+/-;XX genotype) developed into all-female common carp. The genotype (*cyp17a1*+/-;XX genotype), all-ovarian differentiation ($n > 500$) and significant growth advantage have been confirmed (Zhai et al., 2022a). The fertility of this genome-edited all-female population of common carp presents a potential eco-risk, thus hindering its application in aquaculture. This strategy offers the possibility of addressing the challenging situation. However, the “controllable fertility” strategy proposed in this study offers a solution by enabling the production and sterilization of the all-female population (*cyp17a1*+/-;*lhβ* (or *star*)-/-;XX). This can be achieved by crossing the *cyp17a1*-/-;*lhβ* (or *star*)-/-;XX males (neomales) with *cyp17a1*+/-;*lhβ* (or *star*)-/-;XX females, who are administered DHP. Fortunately, no obvious defect in fertility of *lhβ*-/- or *star*-/- male zebrafish was observed, suggesting that mutation of *lhβ* or *star* did not affect fertility in male fish (Shang et al., 2019). Based on the achieved relative fecundity ratio of *star*-/- zebrafish treated with the DHP and hCG procedure, a ratio of 17.98% was obtained (Table 3). This ratio suggests that more than 10,000 healthy fingerlings can be produced from each *star*-/- common carp with its egg-carrying capacity, providing an effective rescue strategy for farmed fish. However, further improvements will be required to enhance rescue efficiency. Overall, the genome-edited population of all-female carp rendered infertile via this procedure could impede the ecological risks by preventing the interbreeding of genome-edited escapees with WT carp stocks, as they are unable to reproduce naturally.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Guiding Principles for the Care and Use of Laboratory Animals, Institute of Hydrobiology, Chinese Academy of Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

SS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. YZ: Data curation, Formal analysis,

Investigation, Methodology, Writing – review & editing. JH: Investigation, Validation, Visualization, Writing – review & editing. QL: Investigation, Resources, Validation, Writing – review & editing, Methodology. XJ: Resources, Supervision, Visualization, Writing – review & editing. JH: Resources, Software, Supervision, Writing – review & editing. GZ: Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Writing – original draft. ZY: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2024.1381305/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

The gross appearance of offspring at diverse stages from control females, DHP-administrated *lh β* - or *star*-deficient females naturally mated with WT males. (A–C) Representative image of the gross appearance of embryos at 12 hpf. (D–F) Representative image of the gross appearance of larvae at 72 hpf. (G–I) Representative image of the gross appearance of larvae at 5 dpf. (J–L) Representative image of the gross appearance of fish at 21 dpf. (A, D, G, J) Offspring from control females naturally mated with WT males. (B, E, H, K) Offspring from *lh β* -deficient females exposed to 100 μ g/L DHP for 6 h and naturally mated with WT males. (C, F, I, L) Offspring from *star*-deficient females exposed to 300 μ g/L DHP for 6 h and naturally mated with WT males. (M) The bar chart represents body weight of the fish at 1 mpf. (N) The bar chart represents body length of the fish at 1 mpf. (O) The bar chart represents full-length of fish at 1 mpf. The letter a in the bar charts indicates no significant difference.

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