Progestin increases the expression of gonadotropins in pituitaries of male zebrafish

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Abstract

Our previous study showed that the *in vivo* positive effects of 17α,20β-dihydroxy-4-pregnen-3-one (DHP), the major progestin in zebrafish, on early spermatogenesis was much stronger than the *ex vivo* ones, which may suggest an effect of DHP on the expression of gonadotropins. In our present study, we first observed that *fshb* and *lhb* mRNA levels in the pituitary of male adult zebrafish were greatly inhibited by 3 weeks exposure to 10 nM estradiol (E_2). However, an additional 24 h 100 nM DHP exposure not only reversed the E_2-induced inhibition, but also significantly increased the expression of *fshb* and *lhb* mRNA. These stimulatory effects were also observed in male adult fish without E_2 pretreatment, and a time course experiment showed that it took 24 h for *fshb* and 12 h for *lhb* to respond significantly. Because these stimulatory activities were partially antagonized by a nuclear progesterone receptor (Pgr) antagonist mifepristone, we generated a Pgr-knockout ($pgr^{-/-}$) model using the TALEN technique. With and without DHP *in vivo* treatment, *fshb* and *lhb* mRNA levels of $pgr^{-/-}$ were significantly lower than those of $pgr^{+/+}$. Furthermore, *ex vivo* treatment of pituitary fragments of $pgr^{-/-}$ with DHP stimulated *lhb*, but not *fshb* mRNA expression. Results from double-colored fluorescent *in situ* hybridization showed that *pgr* mRNA was expressed only in *fshb*-expressing cells. Taken together, our results indicated that DHP participated in the regulation of neuroendocrine control of reproduction in male zebrafish, and exerted a Pgr-mediated direct stimulatory effect on *fshb* mRNA at pituitary level.

Key Words
- 17α,20β-dihydroxy-4-pregnen-3-one
- gonadotropin
- nuclear progesterone receptor
- zebrafish
- pituitary

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Introduction

Gonadal steroid hormones play key roles in regulating gametogenesis, but they also exert both positive and negative feedback effects at the hypothalamic and pituitary levels. In tetrapod vertebrates, progesterone (P4) is the dominant ovarian progestin, which regulates reproductive behavior (Blaustein 1986) and exerts feedback effects on

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both pituitary gonadotropin secretion (Turgeon & Waring 1990, 2000, Waring & Turgeon 1992) and hypothalamic gonadotropin-releasing hormone (GNRH) release (O'byrne *et al*. 1991, Skinner *et al*. 1998). 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β,21-trihydroxy-pregn-4-en-3-one (20β-S) are the most potent and biologically relevant progestins in teleosts (Scott *et al*. 2010). In male fish, progestins induce spermiation (Ueda *et al*. 1985), increase seminal fluid production (Baynes & Scott 1985), and stimulate spermatozoa motility (Miura *et al*. 1992, Tubbs & Thomas 2008). Studies in Japanese eel (*Anguilla japonica*) demonstrated that DHP induces the entry of male germ cells into meiosis (Miura *et al*. 2006). Our recent studies in zebrafish showed that DHP treatment increased proliferation and differentiation of early spermatogonial generation (Chen *et al*. 2013). Interestingly, the *in vivo* effect of DHP on spermatogonia was more prominent than that *ex vivo*. One likely mechanism is that the effects of DHP *in vivo* may involve both direct action at the testicular level and indirect modulation of pituitary gonadotropin release. Alternatively, *in vitro* incubation could also lead to dysregulation of many paracrine and autocrine signaling around testes that are important for DHP signaling.

In mammalian models, some studies reported no effect of progesterone on luteinizing hormone (LH) (Kerrigan *et al*. 1993, Park *et al*. 1996), while others using immortalized gonadotrope-derived Lβ T2 cells (L beta T2 gonadotrope cell line, Turgeon *et al*. 1996) demonstrated that progesterone suppressed LH β subunit gene expression, in contrast to its stimulatory effect on the expression of follicle-stimulating hormone (FSH) β subunit (Thackray *et al*. 2006, 2009). In teleosts, much progress has been made in understanding the regulation of the GNRH-Fsh/Lh system by androgens and 17β-estradiol (Zohar *et al*. 2010), but the potential roles of progestins in the neuroendocrine regulation of reproduction have received little attention. In female tilapia, DHP has been implicated in the regulation of Fsh and Lh release (Levavi-Sivan *et al*. 2006), but these DHP-mediated effects are considered to play a minor role in comparison to the dominant actions of androgen and 17β-estradiol (Van der Kraak 2009). Lack of information hinders an overall understanding of steroid hormones in regulating reproductive processes in teleosts.

The effects of progestins are mainly mediated through an intracellular nuclear progestin receptor (Pgr) that belongs to the nuclear receptor family (Conneely *et al*. 1986). In addition, multiple membrane progestin receptors (mPRs), which have no structural similarity to Pgr, also can mediate the nonclassical action of progestin (Zhu *et al*. 2003, Hanna & Zhu 2011, Tan & Thomas 2015). In zebrafish, the Pgr is expressed robustly in the preoptic region of the hypothalamus (Hanna *et al*. 2010), and membrane progestin receptors (mPRs) in scattered cells in the pituitary (Hanna & Zhu 2009), suggesting potential roles of progestins in the brain–pituitary complex and the involvement of multiple progestin receptors and signaling pathways.

Recently, we have clearly shown that Pgr knockout ($pgr^{-/-}$) causes completely anovulation and infertility in female zebrafish, but male knockout fish is still fertile (Zhu *et al*. 2015). In current study, we focused on the effects and molecular mechanisms of progestin (DHP) signaling in male zebrafish. We demonstrate that DHP can enhance the expression of gonadotropins (*fshb* and *lhb* transcripts) in the pituitaries of male adult zebrafish, independently of estradiol (E_2) inhibition. We also provide clear evidence from studies of Pgr antagonist, knockout and *in situ* to indicate that effects of progestin (DHP), especially, on *fshb* are mediated directly at least in part by Pgr that is expressed in Fsh-expressing cells in the pituitary of male zebrafish.

Materials and methods

Zebrafish husbandry

The experimental fish were Tübingen strain, which were housed in the zebrafish (*Danio rerio*) facility (ESSEN, China) and maintained in recirculating freshwater (pH 7.2–7.6) at 28°C with a 14L:10D photoperiod (lights on at 08:00). The fish were fed three times per day with commercial tropical fish food (Otohime B2, Reed Mariculture, Campbell, CA, USA), using standard conditions for this species (Westerfield 2000). All the fish used were mature adult males (age 4–6 months, body length 2.4–2.8 cm, body weight 275–440 mg). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University.

Generating and characterizing $pgr^{-/-}$ zebrafish using TALENs

Instead of using an unit assembly protocol and a modified FokI (Huang *et al*. 2011, Zhu *et al*. 2015), we used a Golden Gate TALEN assembly protocol and wild type (WT) FokI (Cermak *et al*. 2011) to generate TALEN expression vectors targeting one locus as per the previous target (Zhu *et al*. 2015) located in the first exon (Fig. 1A) of the

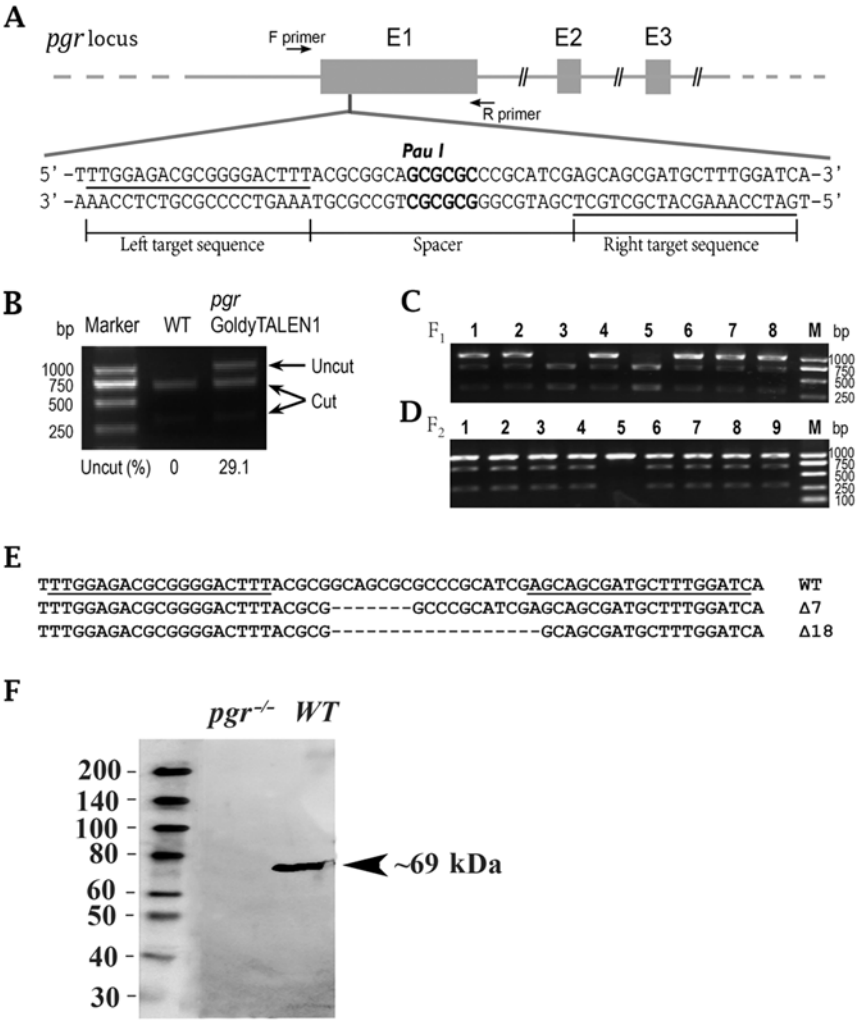

Figure 1
Genomic structure of the rzebrafish *pgr* gene, TALEN targets, and a representative mutation induced by TALENs. (A) TALENs were designed to target the first exon of the *pgr* gene. The left and right binding sites of TALENs are underlined, and a restriction enzyme cleavage site with PauI used for mutation analysis is highlighted in bold. Forward and reverse primers for PCR amplification of the genomic fragment including the target region are also indicated by the arrow lines. (B) Mutation detection in injected F0 founder embryos using PauI restriction enzyme digestion (mutation efficiency 29.1%). (C) Genotype examination of F1 adult zebrafish using restriction enzyme digestion and tailfin clipping. Fish were individually numbered and are indicated on the top of the figure. No. 3 and 5 were wild-type fish, No. 1, 2, 4, 6, 7, and 8 were heterozygote fish. (D) Genotype examination of F2 adult zebrafish. Individual fish were numbered and are indicated on the top of the figure. No. 1, 2, 3, 4, 6, 7, 8, and 9 were heterozygote fish; No. 5 was a homozygote fish. (E) DNA sequences of wild type or Pgr mutant. Nucleotide deletion is indicated by dashed lines. The numbers at the right side show the number of deleted base pairs. (F) Western blot of the Pgr of zebrafish testes. Sample types are indicated on the top of the figure. F, forward; R, reverse; No., number; WT, wild type; $pgr^{-/-}$, Pgr knockout.

zebrafish *pgr* gene (Ensembl No. ENSDARG00000035966). Thereafter, assembled *pgr* GoldyTALEN expression vectors were linearized using SacI (Thermo Scientific), and transcribed into mRNAs using the Mmessage mMACHINE T3 Transcription Kit (Life Technologies).

Approximately 1 nL containing ~75 pg of each TALEN mRNA was injected into the yolk of each embryo at the one-cell stage. For examining the mutation rate of the injected embryos, a pool of genomic DNA was extracted from 30 injected embryos or the same number of WT embryos. With genomic DNA as template, a DNA fragment containing the TALEN target site was amplified using PCR (Fig. 1A and Table 1), followed by restriction endonuclease (PauI; Thermo Scientific) digestion, and checked with agarose gel electrophoresis. Area densities of cut and uncut bands on the gel were measured using the Gel-Pro Analyzer Program (Media Cybernetics, Rockville, MD, USA) (Fig. 1B), mutation efficiency being calculated as (%) = uncut/(cut + uncut) × 100%.

To obtain homozygous mutant zebrafish, adult (>90 dpf) F0 founder fish were outcrossed with WT fish. From each cross, a pool of genomic DNA was extracted from 30 randomly selected F1 embryos, and the status of the TALEN target site was analyzed via PCR amplification, and restriction enzyme digestion as described above. Based on the mutation efficiency, the remaining F1 embryos from F0 founder fish with high rates of germline transmission were raised to adulthood and were genotyped individually using tail fin-clip screening assay in order to obtain heterozygous F1. Based on the DNA sequence of the TALEN target sites, heterozygous F1 fish with the same frame shift mutation were intercrossed to produce F2 offspring (Fig. 1C). Homozygous Pgr-knockout ($pgr^{-/-}$), heterozygotes ($pgr^{+/-}$) and homozygous wild type ($pgr^{+/+}$) in F2 fish were identified via PCR amplification followed by restriction enzyme digestion analysis as described above (Fig. 1D).

In order to examine the *pgr* mRNA sequence in $pgr^{-/-}$ male fish, cDNA was synthesized using a Revert Aid First

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Table 1 Primers used in present study.

Primer name	Primer sequence (5′–3′)	Expected size (bp)	GenBank accession number	Purpose
pgr-TALEN Forward	AGGAATACGTCGCACACTTT	965	Ensembl No. ENSDARG00000035966	Positive gene Knockout fish screening
pgr-TALEN Reverse	CGCCATTTTAATTCGACCTC			
pgr-Forward	GGGTCTCGCTGCGTAATTTT	841	Ensembl No. ENSDARG00000035966	RT-PCR
pgr-Reverse	GCCTGGTAGCACTTTCGAAG			
ef1a-Forward	GGCTGACTGTGCTGTGCTGATTG	409	BC064291	Real-time PCR
ef1a-Reverse	CTTGTCGGTGGGACGGCTAGG			
fshb-Forward	CAGATGAGGATGCGTGTGC	281	AY424303	Real-time PCR
fshb-Reverse	ACCCCTGCAGGACAGCC			
lhb-Forward	GCAGAGACACTTACAACAGCC	145	AY424304/AY424305	Real-time PCR
lhb-Reverse	AAAACCAAGCTCTGAGCAGCC			

Strand cDNA Synthesis Kit (Thermo Scientific) from the total RNA extracted from pooled pituitary samples using RNAzol reagent (MRC, Cincinnati, OH, USA). The specific PCR product was obtained using the Pgr-primers (Table 1), followed by cloning and Sanger sequencing.

In order to examine if Pgr protein was absent in $pgr^{-/-}$ male fish, testicular samples of WT and $pgr^{-/-}$ fish were collected for western blot assay as described previously (Hanna *et al*. 2010). Total protein samples were extracted by immediately placing freshly excised testis into 1×SDS buffer, denatured by boiling for 5 min, and then cooled on ice. Equal amounts (60 µg) of protein samples were loaded and separated using a 12% SDS–PAGE gel and were transferred onto a PVDF membrane. The membrane was blocked in TBS containing 0.1% (v:v) Tween 20 and 1% (w:v) bovine serum albumin for 1 h at room temperature (RT), before the membrane was incubated with Pgr antibody (Hanna *et al*. 2010) for 24 h at 4°C. After five washes with TBST, the membrane was incubated for 1 h at RT with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, v:v). A Bio-Rad ECL kit was used to detect signals on the PVDF membrane. Protein size was determined by comparing blotted protein size to a biotinylated protein ladder (Cell Signaling Technology, #7727s) following the manufacturer's directions.

Expression of *fshb* and *lhb* mRNA during the diurnal cycle

To examine the expression of *fshb* and *lhb* mRNA levels in the pituitary during the diurnal cycle, mature WT male fish (>90 dpf) from the same batch were randomly chosen. Pituitary samples were collected every 4 h starting at 10:00 until 06:00. Since maximum levels were found at 06:00, expression levels of WT and $pgr^{-/-}$ fish were compared at 06:00. Three pituitaries were pooled as one sample for these mRNA quantification studies.

In vivo exposure to sex steroids

Using a zebrafish model of estrogen-induced androgen insufficiency (De Waal *et al*. 2009), adult (>90 dpf) males were kept for 3 weeks in water containing 10 nM estradiol (E_2) (Sigma-Aldrich). Fish were then exposed again to 10 nM E_2 either with or without 100 nM DHP (Sigma-Aldrich) for another 24 h. From the same batch of male fish, animals without E_2 pretreatment were exposed to 100 nM DHP for 24 h. Male fish were killed in ice water; pituitaries were collected (three pituitaries were pooled as one sample), frozen quickly in dry ice, and stored at −80°C for RNA extraction.

For other *in vivo* exposure experiments, adult (>90 dpf) male fish were randomly divided into the control and DHP treatment groups. To determine an appropriate exposure concentration, fish were exposed to 10 or 100 nM DHP for 24 h (starting at 13:00), while the control group was exposed to the vehicle (ethyl alcohol at a concentration of 0.0001%, v:v). For time course experiments, treatment groups were exposed to 100 nM DHP at 13:00, and pituitary samples were collected as described above at 16:00, 19:00, 01:00, and 13:00 to determine appropriate length of exposure time.

To study if the effects of DHP on *fshb* and *lhb* transcript levels were Pgr-dependent, male fish were exposed to DHP (100 nM) and Pgr-specific antagonist (RU486, 0.1 or 1 µM) for 24 h. An additional control group was exposed only to 1 µM RU486 for 24 h. In addition, $pgr^{-/-}$ male fish were exposed to the vehicle (as control, 0.0001% ethyl alcohol, v:v) or DHP (100 nM) for a period of 24 h. We also quantified the basal *fshb* and *lhb* mRNA levels in $pgr^{+/+}$ fish.

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Pituitary *ex vivo* exposure to sex steroids

Pituitaries of mature male fish were removed, washed three times with Hank's balanced salt solution (without magnesium and calcium) in a 24-well plate (NEST, Rahway, NJ, USA), and then incubated individually in basal culture medium consisting of 15 mg/mL Leibovitz's L-15 medium (Invitrogen), supplemented with 10 mM Hepes (Merck), 0.5% w/v BSA (MULT Sciences, China), 200 U/mL penicillin, and 200 mg/L streptomycin (Invitrogen); pH was adjusted to 7.4 with NaOH. Pituitary samples were incubated for 24 h in basal culture medium containing either 100 nM DHP, or 0.0001% (v:v) ethyl alcohol as control. Incubation was carried out in a humidified air atmosphere at 28°C for 24 h, and three pituitaries were pooled to obtain one sample for gene expression analysis.

Gene expression analysis

Total RNA was extracted from pituitary samples using RNAzol reagent (MRC, Cincinnati, OH, USA). Three pituitaries were pooled as one sample to obtain sufficient RNA for cDNA synthesis. The same amount of total RNA (1.0 μg) was used for the synthesis of the first-strand cDNAs using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions.

The relative expression levels of *fshb*, *lhb,* and the house-keeping-gene *ef1a* were determined using real-time quantitative PCR (qPCR) with gene-specific primers (Table 1), which had been examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (2×10^2–2×10^7 copies/2 μL). qPCR was performed in a 20 μL reaction mixture on the 7500 FAST real-time PCR detection system (Applied Biosystems) using default settings. Copies of *ef1α*, which showed no significant difference among different stages, were used as internal control. The relative mRNA levels of the target genes were determined using the comparative C_t method (Schmittgen & Livak 2008).

Double-colored fluorescent *in situ* hybridization

The colocalization of *pgr* with either *fshb* or *lhb* mRNA in zebrafish pituitary was investigated using double-colored fluorescent *in situ* hybridization (FISH), as described previously (Chen & Ge 2012), except that cryosections were used. In brief, pituitaries of adult male zebrafish were dissected and fixed in 4% w/v paraformaldehyde in PBS at 4°C overnight, followed by immersion in 25% w/v sucrose in PBS at 4°C until sinking, and then embedded in optimal cutting compound (Tissue-Tek, Sakura, USA) by freezing in liquid nitrogen. Embedded pituitaries were processed for serial frozen sectioning at 10 μm thickness, rehydrated and digested with proteinase K (5 μg/mL; Roche Applied Science) at 37°C for 5 min, followed by hybridization with fluorescein-labeled and DIG-labeled RNA probes at 55°C overnight. The probes for the detection of *fshb*, *lhb* and *pgr* mRNA were generated as described previously (Chen *et al*. 2010, Chen & Ge 2012). After hybridization, the cryosections were washed with 2×saline-sodium citrate (SSC; 0.06 M NaCl and 0.006 M sodium citrate) for 30 min at RT, 30% v/v formamide deionized in 2×SSC for 15 min at 65°C, 0.2×SSC for 15 min at 65°C, and 0.2×SSC for 15 min at RT. After washing, a TSA Plus Cyanine3/Fluorescein (TSA-Cy3) System (PerkinElmer) was used to detect the hybridization signal. The first signal was detected using HRP-conjugated anti-fluorescein antibody (Roche Applied Science) with TSA Fluorescein following the manufacturer's instructions. In order to detect the second signal, cryosections were incubated in 1% H_2O_2 for 60 min to deactivate the HRP from the first staining. HRP-conjugated anti-DIG antibody (Roche Applied Science) were added to the sections, followed by detection with the TSA-Cy3 system. After mounting with the medium for fluorescence reagent (Vector Laboratories, Burlingame, CA, USA), the slides were observed and the images recorded using a Zeiss LSM 780 NLO two photon laser scanning system (Zeiss).

Statistical analysis

All data are presented as mean±S.E.M. of the mean. Depending on the experimental setup, data were analyzed using either Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test to assess statistical differences between two or more groups. The analyses were performed using GraphPad Prism 4 software package (GraphPad Software).

Results

Generating the *pgr*$^{-/-}$ zebrafish model and knockout phenotype

Mutant lines targeting two different loci with three different frame shift mutations caused by small deletions and/or insertions of nucleotides in the first exon of *pgr* genomic sequences have been generated and characterized using a unit assembly protocol and modified FokI (for details, Zhu *et al*. 2015). In current study, we targeted same locus as one

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of the previous targets (Fig. 1A) using a different TALEN assembly protocol and wild type FokI (Cermak *et al*. 2011), and successfully generated a new knockout line with a different frame shift (7-bp deletion) and a premature stop codon (Fig. 1B, C, D and E). Pgr protein was undetectable in the testes of *pgr*$^{-/-}$ fish (Fig. 1F). We found exact same anovulation and infertility in newly generated knockout female zebrafish, which validated our previous finding (Zhu *et al*. 2015). Similarly, we found that Pgr knockout had no obvious effect in male fertility. So this new Pgr line was chosen for the following experiments.

E_2 inhibited but DHP stimulated the expression of *fshb* and *lhb*, independently from E_2 inhibition

Both *fshb* and *lhb* transcripts in the pituitaries of male zebrafish decreased significantly to 2 (*fshb*) or 20% (*lhb*) of the control after 3 weeks of E_2 (10 nM) exposure (Fig. 2). By contrast, expression of the *fshb* and *lhb* transcripts in the pituitaries of male zebrafish increased significantly compared with the control when male fish were exposed to DHP both with or without E_2 (Fig. 2).

Dose and time-dependent stimulatory effects of progestin on the expression of *fshb* and *lhb*

Because pituitary *fshb* and *lhb* transcript levels increased to similar levels in response to DHP irrespective of an E_2 pretreatment, the subsequent DHP exposure experiments were carried out without E_2 pretreatment.

Both *fshb* and *lhb* transcripts in the pituitaries of male fish increased significantly (fivefold for *fshb*, sevenfold for *lhb*) when fish were exposed to 100 nM DHP water for 24 h; while 10 nM DHP exposure had no significant effects (Fig. 3A and B). The time course experiment indicated that significant increases of *fshb* and *lhb* transcript levels in response to DHP (100 nM) required at least 24 h and 12 h exposure, respectively (Fig. 3C and D).

RU486 inhibited DHP-induced expression of *fshb* and *lhb* in the pituitary

Effects of a Pgr-specific antagonist (RU486) were examined in order to determine if DHP-induced *fshb* and *lhb* expression was mediated through Pgr. RU486 alone, up to a concentration of 1 μM, did not alter *fshb* and *lhb* expression levels (Fig. 4), but RU486 significantly inhibited the stimulatory effects of DHP on the expression of *fshb* and *lhb*, in a dose-dependent manner (Fig. 4).

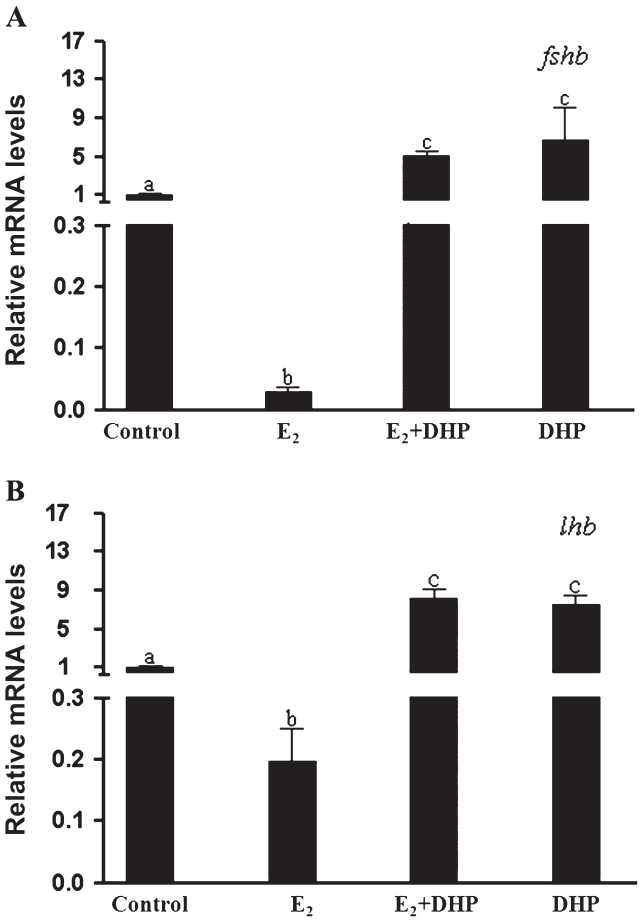

Figure 2
Effects of E_2 and DHP administration on *fshb* (A) and *lhb* (B) mRNA levels in the pituitaries of wild-type male zebrafish. Zebrafish were maintained in the presence of E_2 (10 nM) alone for 3 weeks (E_2) or in the additional presence of 100 nM DHP during the last 24 h (E_2+DHP) of the total 3-week-long exposure to E_2 *in vivo*. The same batch of male zebrafish without E_2 pretreatment were treated with DHP (100 nM) for 24 h *in vivo*. The levels of *fshb* and *lhb* mRNA were determined using qPCR and normalized to the internal control gene (*ef1a*). Data are expressed as the mean ± S.E.M. (n=6) relative to the respective transcript levels of the control value. Mean values marked with different letters are significantly different from each other (P < 0.05).

Daily expression levels of *fshb* and *lhb* transcripts in the pituitary of *pgr*$^{-/-}$ male fish

Expression of *fshb* and *lhb* mRNA showed distinct diurnal changes in WT male fish. Both transcripts of *fshb* and *lhb* were low in the late morning (10:00) after spawning and in the afternoon (14:00). The levels increased in the evening (18:00) and reached peak levels in early morning (06:00), 2 h before the lights came on and the start of spawning activity (Fig. 5A and B). The highest transcript level of *fshb* was ~7.5-fold higher than the lowest one, while that of *lhb* was ~4.0-fold higher than the lowest one.

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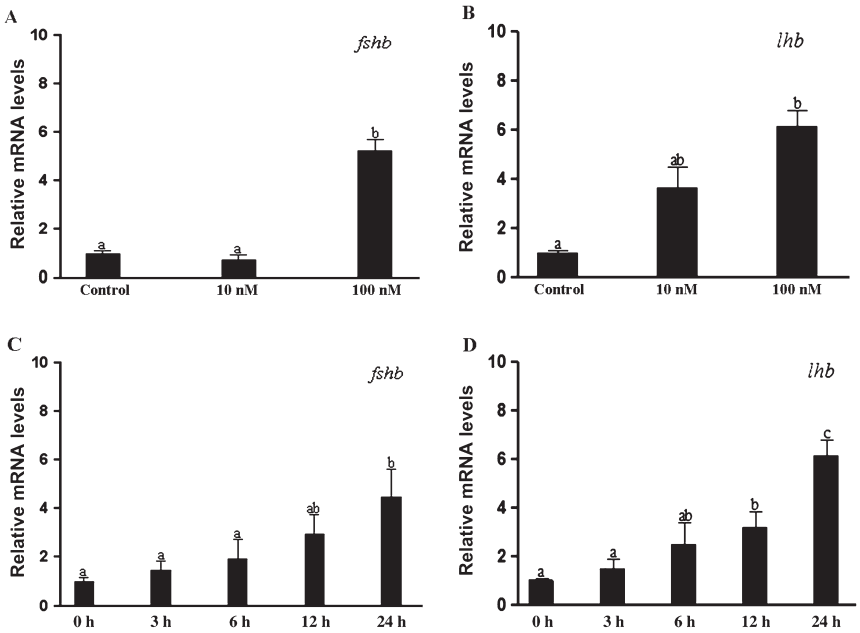

Figure 3
Dose- (A and B) and time (C and D)-dependent stimulation of progestin on the expression of *fshb* and *lhb* transcripts in the pituitaries of wild-type male zebrafish *in vivo*. Male zebrafish were treated with DHP (10 or 100 nM) for 24 h (13:00–13:00 next day) *in vivo* (panels A and B). Thereafter, based on the dose-response experiment, an optimal dose level (100 nM) was selected in the time course experiment (panels C and D). Transcript levels of *fshb* or *lhb* at time zero without progestin exposure are defined as one, and data (mean ± S.E.M., $n=6$) are expressed as fold changes to the zero value. Mean values marked with different letters are significantly different from each other ($P<0.05$).

Furthermore, we observed that there was no difference in *fshb* or *lhb* transcript levels between control WT male (i.e., not treated with TALENs) and $pgr^{+/+}$ male pituitaries collected at 06:00 (Fig. 5C and D). This excluded potential TALEN induced off-target effects on the site that might affect the reproductive system. In addition, the levels of *fshb* and *lhb* mRNA of $pgr^{-/-}$ male fish were significantly lower than those of WT and $pgr^{+/+}$ collected at 06:00 (Fig. 5C and D).

Effects of DHP on the expression of *fshb* and *lhb* in $pgr^{-/-}$ male zebrafish

Surprisingly, we found that DHP still significantly increased the mRNA levels of *fshb* and *lhb* in $pgr^{-/-}$ fish, but the magnitudes of the increases were significantly lower than those observed in WT fish (Fig. 6A and B).

To examine whether DHP acts directly at the pituitary level to increase *fshb* and *lhb* mRNA levels, an *ex vivo* pituitary culture system was used. Both *fshb* and *lhb* transcripts increased significantly (~2-fold for *fshb*, ~2.5-fold for *lhb*) in the cultured pituitary tissue fragments of both WT and $pgr^{+/+}$ males exposed to 100 nM DHP for 24 h (Fig. 7). Furthermore, compared with the $pgr^{-/-}$ control, there was a significant increase (~twofold) of *lhb* transcript levels in pituitary fragments of $pgr^{-/-}$ fish following exposure to 100 nM DHP for 24 h (Fig. 7B), while *fshb* transcript levels no longer responded to DHP in pituitaries of $pgr^{-/-}$ males (Fig. 7A).

pgr mRNA were expressed in *fshb*-expressing cells but not in *lhb*-expressing cells

Because the above results suggested that at least some part of DHP effects on gonadotropin hormone (GTH) expression were likely mediated by *pgr*, we further investigated if *pgr* mRNA was expressed in gonadotropin cells for a direct action of DHP. Using double-colored FISH, which could detect the expression of two genes in the same section as reported previously (Chen & Ge 2012), we observed that the *fshb*-expressing cells scattered individually in the pituitary, whereas the *lhb*-expressing cells normally formed aggregates (Fig. 8A), which is consistent with a recent report (Golan *et al.* 2016). Because the expression level of *pgr* mRNA was much lower than those of *fshb* and *lhb* mRNA in the pituitary, the FISH signals of *pgr* mRNA were observed only in a few cells scattered individually (Fig. 8B, C, D, E and F). The FISH signal of *pgr* transcript was mostly observed in *fshb*-expressing cells (Fig. 8C, D, E and F), but none in *lhb*-expressing cells (Fig. 8B).

Discussion

Gonadal steroids exert negative or positive feedbacks on gonadotropin synthesis and secretion in various vertebrates. In the present study, we investigated the potential roles of DHP in regulating gonadotropin transcription in adult male zebrafish using morphological, physiological, and molecular approaches. The results

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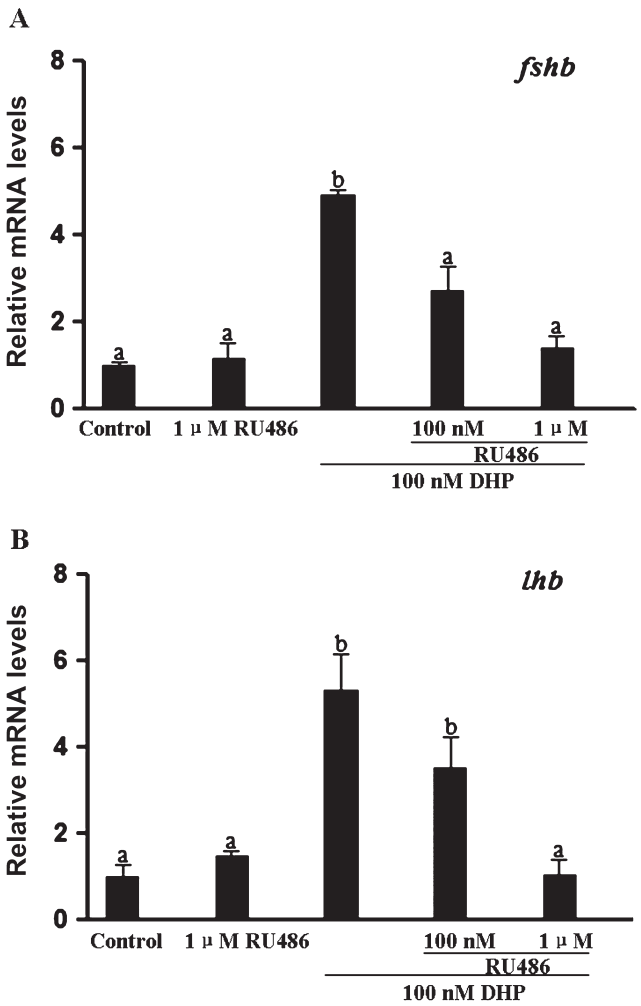

Figure 4
Effects of RU486 on DHP-stimulated *fshb* and *lhb* transcript expression levels of the wild-type (WT) male zebrafish. Data are expressed as the mean ± S.E.M. ($n = 6$) relative to the respective transcript levels of the WT control (A and B). Mean values marked with different letters are significantly different from each other ($P < 0.05$).

showed that DHP was able to stimulate the transcription levels of both *fshb* and *lhb*. Importantly, using *pgr*$^{-/-}$ fish, we demonstrated that the stimulatory effect of DHP on *fshb* mRNA was mediated by the Pgr which was expressed in *fshb*-expressing pituitary cells.

We first applied the long-term E_2 treatment model to examine the effects of DHP on gonadotropin subunit expression *in vivo*. Our results showed that DHP significantly increased gonadotropin subunit mRNA expression in the presence and absence of E_2. This was different from that in mammalian models, in which many studies have shown that progesterone can suppress *LH* mRNA levels in the presence of estrogen (Abbot *et al*. 1988, Simard *et al*. 1988, Corbani *et al*. 1990). Therefore, it seemed that there was no interaction between DHP and E_2 in regulating GTH subunit expression in male zebrafish.

Extensive studies in goldfish have demonstrated that waterborne DHP is a pheromone which can increase LH, steroid, and seminal fluid production (Stacey & Sorensen 1986, Dulka *et al*. 1987, Kobayashi *et al*. 2002). Because of the exposure via water in the present study, it is possible that DHP exerted its stimulatory effects as a pheromone. While the zebrafish ovary can produce steroid glucuronides, including 17,20β-P-glucuronide, which are attractive to males, 17,20β-P-glucuronide has never been tested as a sex pheromone in male zebrafish. However, 17,20β-P-sulfate is the only steroid that males appear to be able to smell (Belanger *et al*. 2010). Sex pheromones usually induce a rapid endocrine response at low concentrations (pM and low nM). For example, exposure of goldfish to 0.5 nM DHP increases serum LH levels after only 15 min (Dulka *et al*. 1987). However, in the present study, the significant response of *fshb* and *lhb* to DHP exposure required 24 h and 12 h, respectively, reaching maximum levels after 24 h exposure, and DHP induced the upregulation of *fshb* mRNA expression only at a high concentration (100 nM). Moreover, using the primary pituitary *ex vivo* culture system, we observed a stimulatory effect of DHP on GTH expression. Taken together, it was most likely that DHP induced GTH upregulation, especially *fshb* mRNA expression, through an endocrine pathway.

Several studies in fish report that plasma LH levels are very low during the early spermatogenesis stage and become detectable when germ cells entered meiosis; however, the increase is not prominent until the spawning season (Schulz *et al*. 2010). Similarly, the highest levels of circulating progestins are observed during the entire spermiation process, especially during the spawning season (Schulz *et al*. 2010). One physiological mechanism to explain this coincidence is that LH induced the production of DHP (Schulz *et al*. 2010). Our previous study in zebrafish also demonstrates that recombinant zebrafish Lh induces DHP production *ex vivo* (Chen *et al*. 2010). Data from the present study showed that DHP had a positive feedback on *lhb* mRNA expression, which was in agreement with previous studies in female tilapia (Levavi-Sivan *et al*. 2006). These results might indicate that LH is the main factor regulating the production of the maturation-inducing hormone DHP.

An increase of *fshb* mRNA levels during spermiation is recorded in several teleost species, which may be due to environmental factors (i.e., temperature and photoperiod) (Schulz *et al*. 2010). In the present study, we report

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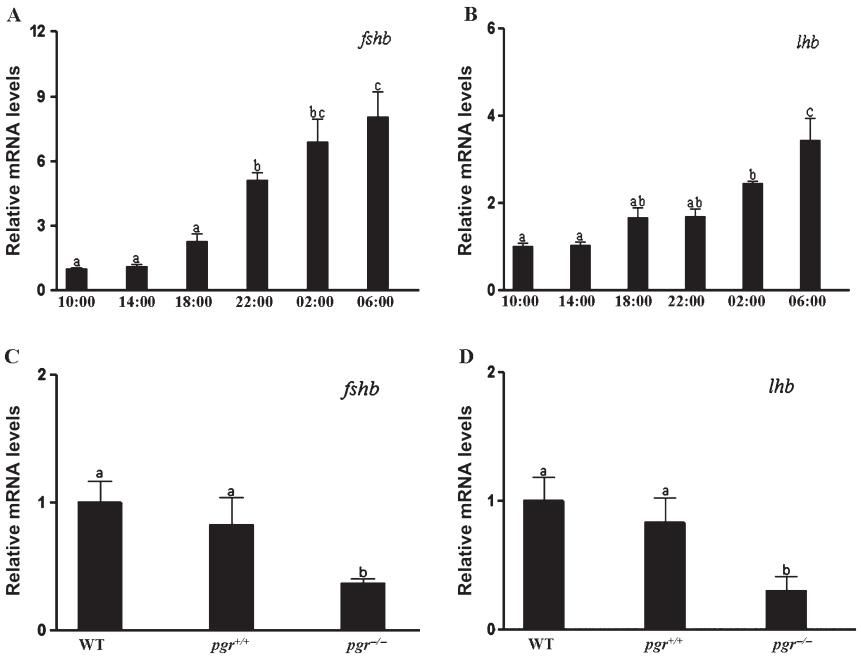

Figure 5
Diurnal changes of *fshb* (A) and *lhb* (B) transcripts in the pituitaries of the wild-type (WT) male zebrafish, and the levels of *fshb* (C) and *lhb* (D) in the pituitaries of the WT, $pgr^{+/+}$, and $pgr^{-/-}$ male zebrafish collected at 06:00. Data are expressed as the mean ± S.E.M. ($n=6$) relative to the respective transcript levels at 10:00 (A and B) or WT (C and D). Mean values marked with different letters are significantly different from each other ($P < 0.05$).

that DHP increased *fshb* mRNA levels in male zebrafish. It is worth noting that this stimulatory effect required a high concentration of DHP (100 nM), which is supposed to happen during spawning. Therefore, we examined the diurnal changes of *fshb* expression levels, and the results indicated that both *fshb* and *lhb* transcripts reached peak levels at 06:00, 2 h before spawning. Interestingly, a previous study in female zebrafish also reports an obvious elevation of *fshb* expression at 01:00 and 04:00 before final oocyte maturation at 07:00 (So *et al.* 2005). Taken together, these results suggested that FSH may be involved in final gamete maturation, which is different from the current view that FSH in fish is involved in early gametogenesis, that is, promoting the early stage of spermatogenesis in the testis, and stimulating follicle growth or vitellogenesis in the ovary. As a continuous daily breeder with an asynchronous spermatogenic cyst, it is most likely that zebrafish initiate the early stages of spermatogenesis at some point of time every day. Results from our present study supported the suggestion from our previous study that DHP-induced early spermatogenesis is mainly mediated by triggering the release of gonadotropins (Chen *et al.* 2013). Recent studies in zebrafish indicate that FSH promotion of proliferation and differentiation of spermatogonia does not require androgen, but can also be mediated by suppressing an inhibitor of spermatogenesis (Skaar *et al.* 2011), or by inducing a stimulator of spermatogenesis (Igf3) (Nóbrega *et al.* 2015). Moreover, the testes in $fshr^{-/-}$ males showed significant retardation in growth with delayed spermatogenesis and gonad formation, though male fertility did not seem to be affected (Zhang *et al.* 2015*a*). In contrast to significant effects and infertility due to *lh*-knockout in female fish, male fertility was also apparently not affected in zebrafish (Chu *et al.* 2014, Zhang *et al.* 2015*b*). However, our present study clearly indicates expressions of *fshb* and *lhb* mRNA are regulated by feedback signaling of progestin in zebrafish. Taken together, FSH, LH, and progestin are working in concert to appropriately regulate gonadal growth, puberty onset, daily recruitment, and maturation of germ cells in male zebrafish. Clearly, additional studies are required to understand effects, regulation, and conserved functions of LH, FSH, progestin, and their receptors during reproduction and evolution.

Three classes of progestin receptors (Pgr, mPR, and progesterone receptor membrane component (Pgrmc)) are reported in vertebrates (Thomas 2008, Zhu *et al.* 2008). Previous reports and the present results showed that both Pgr and mPR are expressed in the zebrafish hypothalamus and pituitary (Hanna & Zhu 2009, Chen *et al.* 2010, Hanna *et al.* 2010), so that DHP may induce GTH expression via Pgr or/and mPRs. The results from present study showed that the Pgr antagonist RU486 partially blocked the stimulatory effects of DHP. Furthermore, using a pituitary *ex vivo* culture system, we did not observe any stimulatory effect of DHP on *fshb* expression in the $pgr^{-/-}$ model. Moreover, results from double-colored FISH showed that most of the FISH signals revealed by the *pgr* probe were observed in *fshb*-expressing cells, but not

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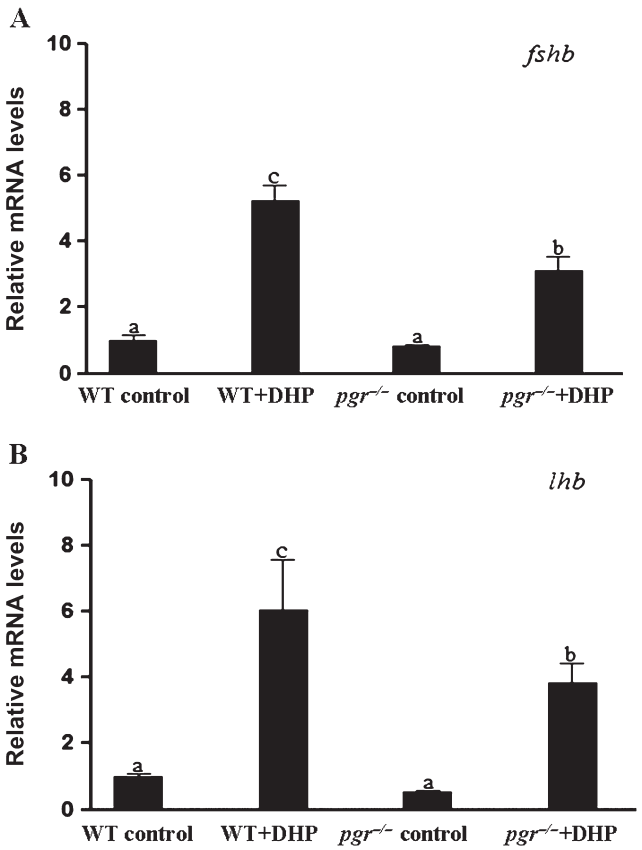

Figure 6
Stimulatory effects of DHP in the expression of *fshb* (A) and *lhb* (B) transcripts in the pituitaries were significantly blocked in *pgr*$^{-/-}$ male zebrafish. Wild type (WT + DHP group) and *pgr*$^{-/-}$ (*pgr*$^{-/-}$ + DHP group) male zebrafish were treated with (100 nM) DHP for 24 h (13:00–13:00 next day). Wild-type control (WT control) and *pgr*$^{-/-}$ control groups were treated with the vehicle (0.0001%) for 24 h (13:00–13:00 next day). The levels of *fshb* and *lhb* mRNA were determined using qPCR and normalized to the internal control gene (*ef1a*). Transcript levels of *fshb* and *lhb* obtained at the WT control are defined as one, and data (mean ± S.E.M., $n=6$) are expressed as fold changes to the WT control. Mean values marked with different letters are significantly different from each other ($P < 0.05$).

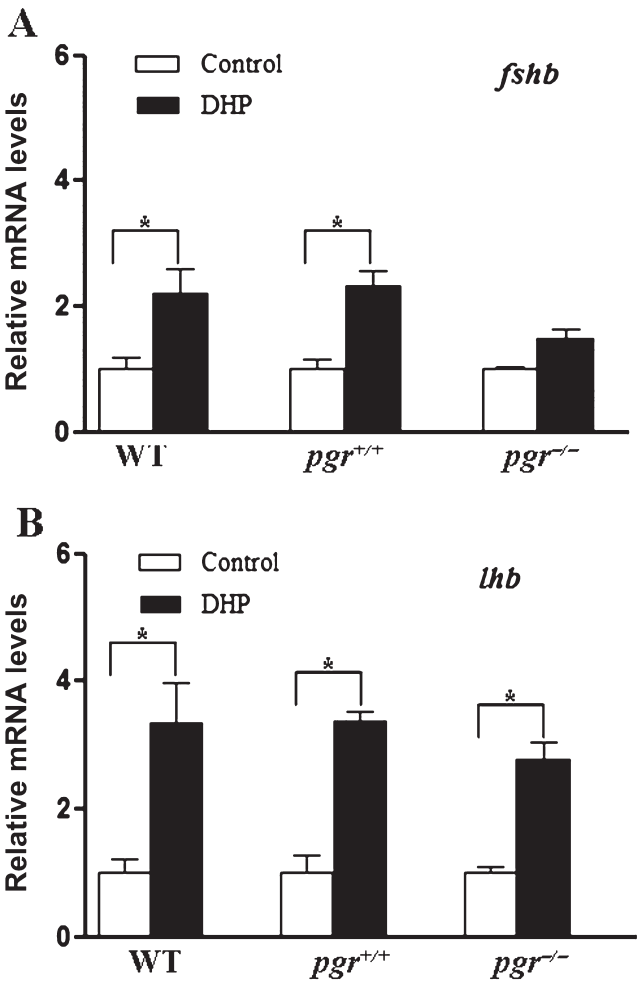

Figure 7
Ex vivo effects of DHP administration on *fshb* (A) and *lhb* (B) mRNA levels. Pituitary fragments of wild type (WT), *pgr*$^{+/+}$, and *pgr*$^{-/-}$ male zebrafish were treated with 100 nM DHP for 24 h (13:00–13:00 next day). WT, *pgr*$^{+/+}$, and *pgr*$^{-/-}$ controls were treated with the vehicle (0.0001%) for 24 h (13:00–13:00 next day). The levels of *fshb* and *lhb* mRNA were determined using qPCR and normalized to the internal control gene (*ef1a*). Transcript levels of *fshb* or *lhb* obtained at the WT control are defined as one, and data are expressed as the mean ± S.E.M. ($n=6$) relative to the respective transcript levels of the WT control value. Bars marked with * are significantly different from their respective controls ($P<0.05$).

in *lhb*-expressing cells. Our results clearly indicated that DHP exerted a direct stimulatory effect on *fshb* mRNA expression which was mediated by Pgr at the pituitary level. In mammals, it has been demonstrated that the full suppressive effect of progesterone on *Lhb* gene expression requires the unique amino-terminal region of the Pgr, but this suppression does not require direct binding of Pgr to the Lhb promoter although it is recruited to the endogenous promoter in live cells (Thackray *et al*. 2009). By contrast, progesterone directly activates *Fshb* through binding of Pgr in the proximal FSH promoter (Thackray *et al*. 2006). The molecular mechanism of progestins on *fshb* gene expression is conserved in vertebrates during evolution. Further study is necessary to show whether other progestin receptors are involved in mediating the stimulatory effect of DHP on *lhb* gene expression in fish.

Interestingly, DHP still caused upregulation of GTH expression *in vivo* in *pgr*$^{-/-}$ male fish, suggesting that non-nuclear receptor mediated pathways in other brain regions, most likely in the hypothalamus, might be involved in the stimulatory effects of DHP on GTH. However, the relevant information is less documented in teleosts. In mammals, the molecular mechanisms of progesterone on GNRH were also unambiguous. Classic Pgr (Skinner *et al*. 1998),

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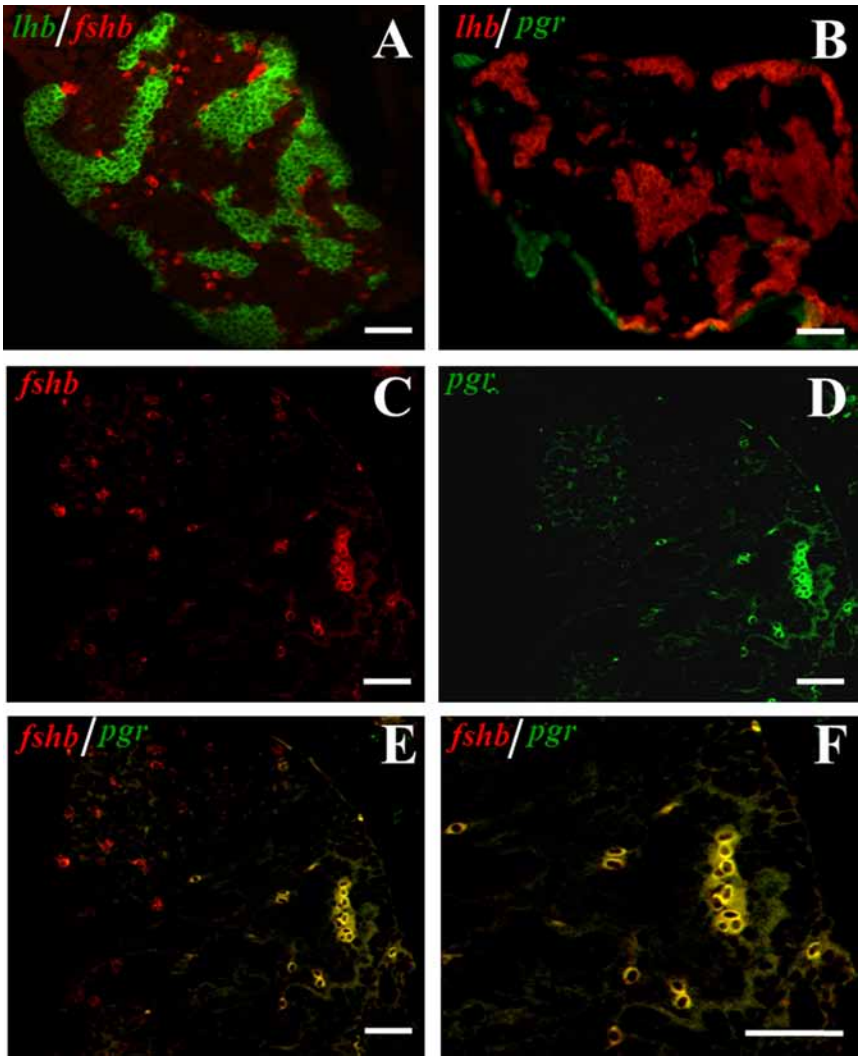

Figure 8
pgr mRNAs were expressed in *fshb*-expressing cells but not in *lhb*-expressing cells. (A) Cryosection stained for *fshb* (red) and *lhb* (green); (B) Cryosection stained for *lhb* (red) and *pgr* (green); (C and D) Cryosection stained for *fshb* (red) and *pgr* (green); and (E and F) Cryosection stained for *fshb* (red) and *pgr* (green); scale bars = 50 μM. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-16-0073.

PAQR (Sleiter *et al*. 2009) and PGRMC1 (Bashour & Wray 2012) are suggested as the key players in mediating progesterone action on GNRH. Further research is needed to address the involvement of other progestin receptors in mediating this effect.

In teleost fish, estrogenic compounds can inhibit androgen synthesis and prevent spermatogenesis (Van der Ven *et al*. 2003, 2007, Pawlowski *et al*. 2004, Van den Belt *et al*. 2004), but the mechanisms remain unclear. A study in zebrafish suggests that *in vivo* exposure to E_2 causes a state of androgen insufficiency, involving feedback mechanisms on the hypothalamus–pituitary system (De Waal *et al*. 2009). In the present study, we observed *in vivo* a clear inhibitory effect of E_2 on *fshb* and *lhb* mRNA levels, similar to what is described in the closely related goldfish (Kobayashi *et al*. 2000, Huggard-Nelson *et al*. 2002). However, direct estrogenic stimulation of pituitary cells elevates *fshb* and *lhb* transcript levels in zebrafish (Lin & Ge 2009). Therefore, we concluded that in zebrafish, long-term exposure to E_2 inhibited stimulatory or strengthened inhibitory signaling toward the pituitary gonadotropin cells, and these effects overruled the direct, stimulatory effects of E_2 on gonadotropin subunit expression.

In summary, the present study indicated that DHP played an important role in the regulation of gonadotropin production in the male zebrafish pituitary. It is likely that the effect of DHP on gonadotropin gene expression may have resulted from integration of multiple effectors acting on the hypothalamus, as well as the pituitary. In addition, Pgr was the main but not the only receptor type for mediating this effect. However, at the pituitary level, DHP exerted a Pgr-mediated direct stimulatory effect on *fshb* mRNA. Despite this, $pgr^{-/-}$ male fish are fertile (Zhu *et al*. 2015), which is similar to a mice model (Schneider *et al*. 2005), and the biological

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significance of this stimulatory effect of DHP on gonadotropin gene expression remains to be clarified.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C L W was involved in entire study. D T L performed TALEN synthesizing, mutation screen. W T C performed double-colored fluorescent *in situ* hybridization. W G, Y Z, and W S H supervised the project and prepared manuscript drafting. S X C conceived and supervised the project, analyzed results, and wrote the paper.

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