Sex steroids differentially regulate *fshb*, *lhb* and *gnrhr* expression in Atlantic cod (*Gadus morhua*) pituitary

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Abstract

Depending on the stage of gonad maturation, as well as other factors, gonadal steroids can exert either a positive or negative feedback at the brain and pituitary level. While this has been demonstrated in many teleost species, little is known about the nature of steroid feedback in Gadiform fish. Using an optimized *in vitro* model system of the Atlantic cod pituitary, the present study investigated the potential effects of two physiologically relevant doses of estradiol, testosterone (TS) or dihydrotestosterone (DHTS) on cell viability and gene expression of gonadotropin subunits (*fshb/lhb*) and two suggested reproduction-relevant gonadotropin-releasing hormone receptors (*gnrhr1b/gnrhr2a*) during three stages of sexual maturity. In general, all steroids stimulated cell viability in terms of metabolic activity and membrane integrity. Furthermore, all steroids affected *fshb* expression, with the effect depending on both the specific steroid, dose and maturity status. Conversely, only DHTS exposure affected *lhb* levels, and this occurred only during the spawning season. Using single-cell qPCR, co-transcription of *gnrhr1b* and *gnrhr2a* was confirmed to both *fshb*- and *lhb*-expressing gonadotropes, with *gnrhr2a* being the most prominently expressed isoform. While steroid exposure had no effect on *gnrhr1b* expression, all steroids affected *gnrhr2a* transcript levels in at least one maturity stage. These and previous results from our group point to *Gnrhr2a* as the main modulator of gonadotropin regulation in cod and that regulation of its gene expression level might function as a direct mechanism for steroid feedback at the pituitary level.

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Introduction

Sexual maturation in fish, as in other vertebrates, is controlled by the physiological connection between the brain–pituitary–gonad (BPG) axis compartments; gonadotropin-releasing hormone (Gnrh) secreted from hypothalamic neurons onto the pituitary stimulates the synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which in turn stimulate gonadal steroidogenesis and gametogenesis. Although the exact mechanisms are not known, activation of the Gnrh system seems to be the key event in the onset of teleost puberty (Weltzien *et al.* 2004). Gnrh exerts its action via specific Gnrh receptors (Gnrhr) located in target cell membranes. Several isoforms of Gnrhr exist in teleosts, and these may be differentially expressed during maturation and spawning. In Atlantic cod (*Gadus morhua*), four Gnrhr gene orthologues (*gnrhr1b*, *gnrhr2a*, *gnrhr2b* and *gnrhr2c*) have been identified (Hildahl *et al.* 2011), all of which were expressed in the brain, and three of

which (*gnrhr1b*, *gnrhr2a* and *gnrhr2c*) were expressed in the pituitary. The expression of *gnrhr2c* was low and not seasonally regulated, while *gnrhr1b* expression showed increasing trends in expression during the mature and spent stages. *gnrhr2a* expression appeared to be closely correlated to gonadal maturation, indicating this forms the most likely mediator of the gonadotrope Gnrh response. Unlike mammals, teleost fish have two types of gonadotropes, individually producing either Fsh or Lh. Gnrh has been shown to stimulate both *fshb*- and *lhb*-expressing cells in cod (Hodne *et al.* 2013), but the Gnrh receptor type expressed in individual gonadotropes is yet to be determined.

The activation of the BPG axis and pubertal development is under the control of both external and internal factors, as well as feedback mechanisms within the axis itself (Dufour *et al.* 2010, Levavi-Sivan *et al.* 2010, Taranger *et al.* 2010, Zohar *et al.* 2010) and ensures that reproduction occurs at a time of optimal survival of the offspring. The synthesis and/or release of gonadotropins

due to sex steroid feedback has been confirmed in several teleost species by measuring protein or transcript levels after steroid supplementation/replacement *in vivo* or after steroid exposure *in vitro* (Billard 1978, Kobayashi *et al.* 1989, Larsen & Swanson 1997, Borg *et al.* 1998, Holland *et al.* 1998, Khan *et al.* 1999, Cavaco *et al.* 2001, Huggard-Nelson *et al.* 2002, Aroua *et al.* 2007). The nature of the feedback can differ depending on maturational stage, hormone concentration, species, sex and other factors (Saligaut *et al.* 1999, Okuzawa 2002, Yamaguchi *et al.* 2006, and see Levavi-Sivan *et al.* 2010 and references therein).

Atlantic cod is a marine multibatch spawner with group-synchronous gonadal development, and spawns multiple times from February until May (Brander 1994). Reports describing the gonadotropic interplay in multibatch spawners are scarce compared to single batch spawners such as salmonids and eel, and little is known on the role of steroid feedback at this level in Atlantic cod or in Gadiformes in general. In contrast to single or total spawners, in which gametes develop synchronously, both multibatch and daily spawners display gametes at different stages of development at any one time. These two contrasting spawning strategies probably display differences in the gonadotropin regulation of gametogenesis, including different responses to circulating steroids at the higher levels of the BPG axis.

The objective of the present study was to investigate direct effects of sex steroid exposure on pituitary gonadotropin and *Gnrhr* gene expression during different stages of sexual maturity in Atlantic cod, using dispersed pituitary primary cultures as the model system. To acquire a greater understanding of the regulatory elements in gonadotropes, a secondary objective was to identify the expression profiles of *gnrhr* isoforms in individual *fshb*- and *lhb*-producing pituitary cells.

Materials and methods

Animals

Wild Atlantic cod (0.51–4.10 kg body weight) of both sexes were caught by trawling on the south coast of Norway. Following capture, fish were immediately transported to the University of Oslo and kept in recirculating aquaria supplied with 28‰ seawater. Fish were kept under ambient conditions of temperature (8–12°C) and photoperiod (60°N), and fed daily with frozen shrimp. All animals were kept and handled in agreement with the provisions enforced by the Norwegian Animal Research Authority. A specific approval for this study was not needed, as the animals themselves were not experimentally treated (Norwegian legislation for use of animals in research, Chapter II, §6). All procedures were approved by the Ethical Committee for Animal Experiments at the University of Oslo.

Dispersed pituitary cell cultures

Dispersed primary cultures of mixed sexes were prepared from pituitaries according to Hodne and coworkers (2012), with culture conditions optimized for Atlantic cod parameters in respect to osmolality, temperature, pCO₂ and pH. The optimized conditions allowed the pituitary cultures to be physiologically stable and viable for at least two weeks (see Supplementary data and Hodne *et al.* 2012, see section on Supplementary Data given at the end of this article). The pituitaries were sampled throughout the year to reflect different reproductive stages of cod, from maturation to the spent stage. Maturational status was determined based on macroscopic inspection of the gonads (see Supplementary data) and gonadosomatic index (GSI; (gonad weight/total body weight) × 100). Six cultures were included in the experiment, two from each of the following maturity stages; maturing, mature and spent.

In short, dissected pituitaries were pooled ($n=5-15$), washed with modified phosphate-buffered saline (PBS; Life Technologies) and chopped into approximately 1 mm³ pieces, before being treated with trypsin (type II-S, Sigma; 1 mg/mL PBS). The trypsin digestion was ended by replacing the solution with PBS containing trypsin inhibitor (type I-S, Sigma; 1 mg/mL) supplemented with approximately 1 µg/mL DNaseI (Sigma). Next, the tissue fragments were mechanically dissociated in ice-cold PBS, before the solution was filtered and centrifuged. Subsequently, the pellet was resuspended in modified L-15 and the dispersed cells seeded at a density of 1.5×10^5 cells/cm² in 24/96-well plates (Corning) precoated with poly-L-lysine (0.1 mg/mL, Sigma). The cells were incubated at 12°C in a humidified atmosphere of 0.5% CO₂ in air (pCO₂; 3.8 mmHg, which resulted in a medium pH of 7.85). After 24 h, medium was replaced to remove damaged and detached cells.

Steroid exposure

Stock solutions of 17β-estradiol (E2), testosterone (TS) and the non-aromatizable androgen dihydrotestosterone (DHTS) (all from Sigma) were prepared by dissolving the steroids in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway). Stock solutions were stored at –20°C for no longer than 3 months and diluted in modified L-15 medium before cell exposure. To prevent possible reactions between steroids and plastic, all solutions were prepared in sterile glass tubes. The working solutions for exposure had an EtOH concentration of 0.2%. For each experiment, two controls, either with or without EtOH were included. At day 4 of culture, the cells were exposed to either a low or high physiologically relevant dose of steroid (E2; 2.5 and 25 ng/mL ($\sim 9.2 \times 10^{-9}$ and 9.2×10^{-8} M), TS and DHTS; 2 and 20 ng/mL ($\sim 6.9 \times 10^{-9}$ and 6.9×10^{-8} M)) (Dahle *et al.* 2003, Norberg *et al.* 2004, Almeida *et al.* 2009) and incubated for an additional 72 h. Solvent control cells were exposed to 0.2% EtOH in the same manner. For gene expression analysis, six replicate wells in 24-well plates per treatment were prepared, whereas for viability tests, six replicate wells in 96-well plates were used.

Viability testing

A classical approach to measure cell viability is using the vital stain Trypan blue. However, in cod pituitary cultures, a proportion of the cells appear naturally blue under the microscope, making this approach suboptimal (see [Supplementary data](#)). So to gain information about the condition of the cells after 7 days in culture (w/wo exposure), viability tests were performed using two non-toxic fluorescent indicator dyes, AlamarBlue (AB) and 5-carboxyfluorescein diacetate-acetoxymethyl ester (CFDA-AM) (both from Life Technologies). Both assays measure the conversion of a non-fluorescent dye into a fluorescent dye by enzymes present in intact and viable cells and indicate metabolic activity and plasma membrane integrity respectively ([Bopp & Lettieri 2008](#)). Test procedures were carried out as described in [Hodne and coworkers \(2012\)](#). In short, cells were seeded, incubated and exposed to steroids as described previously. As a positive control for cell toxicity, an additional set of wells were prepared and incubated in modified L-15 medium. At day 6, the medium of these cells was replaced with L-15 supplemented with 2.5, 0.625 or 0.156 mM CuSO₄ ($n=6$). At the highest concentration, Cu²⁺ is lethal to the cells. The CuSO₄ wells were included on every plate used in the viability assays and served as both intra- and inter-assay-positive controls.

At day 7, the culture medium in all wells was replaced with 100 µL Tris buffer (50 mM, pH 7.5) containing both 5 % AB and 4 µM CFDA-AM (from 4 mM stock in DMSO). After 30 min of incubation, the concentration of the fluorescent products was measured simultaneously for both probes with a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) and the software Gen5 was used for data collection (Gen5 Data Analysis Software, Bio-Tek Instruments Inc.).

Quantification of gene expression

RNA extraction and cDNA synthesis

Cells used for gene expression analysis were harvested at day 7 of culture. A cell lysate was produced by removing

the culture medium, washing with ice-cold PBS, adding 1 mL TRIzol (Life Technologies) and stirring the well content with a pipette. The lysate was transferred to an Eppendorf tube and instantly frozen in liquid N₂ and stored at −80°C. Total RNA was extracted in TRIzol following standard procedures, with the exception of adding 1.5 µL GlycoBlue (Ambion, Life Technologies) to the isopropanol step for visualization of the RNA pellet. The RNA was resuspended in 10 µL RNase-free water (Ambion). DNase-treated RNA (TURBO DNase-free (Ambion)) was quantified spectrophotometrically (NanoDrop, Thermo Scientific, Wilmington, DE, USA), and the quality was assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies) of the RNA integrity number (RIN). Only RNA samples with RIN number above 8 were analyzed further. First-strand cDNA synthesis was performed on 500 ng total RNA using random hexamer primers and Super Script III (Life Technologies), according to standard procedures, and stored at −20°C until qPCR.

Primers and reference genes

The qPCR primers were designed using Primer3 shareware (<http://frodo.wi.mit.edu/primer3/input.htm>). Potential primers were further analyzed using Vector NTI (Life Technologies) to test for possible hairpin loops and primer dimer formations ([Table 1](#) for sequence details). In each pair, one primer was targeted to an exon–exon border to avoid amplification of genomic DNA. The expression of four different genes specifically related to pituitary function was investigated, i.e., *fshb* (GenBank ID: DQ402373), *lhb* (GenBank ID: DQ402374), *gnrhr1b* (GenBank ID: GU332297) and *gnrhr2a* (GenBank ID: GU332298.1). To allow accurate normalization of the qPCR, the stability of four reference genes, *arp2*, *bactin*, *ubiquitin* and *ef1a* was tested using Bestkeeper Software ([Pfaffl et al. 2004](#)), giving Cq geometric means and standard deviations ($\pm$ Cq) of 27.06 ($\pm$ 0.39), 21.96 ($\pm$ 0.45), 22.19 ($\pm$ 0.43) and 20.40 ($\pm$ 0.39) respectively. For subsequent experiments, *ef1a* was used for normalization of the qPCR data.

Table 1 qPCR primers used in the present study.

Target	Reference	Primer sequence	Amplicon size (nt)	Efficiency
<i>lhb</i>	Hodne et al. (2010)	Forward: 5'-GTGGAGAAGAAGGGCTGTCC-3' Reverse: 5'-GGACGGGTCCATGGTG-3'	81	1.93
<i>fshb</i>	Hodne et al. (2010)	Forward: 5'-GAACCGAGTCCATCAACACC-3' Reverse: 5'-GGTCCATCGGGTCCTCCT-3'	63	1.84
<i>gnrhr1b</i>	This study	Forward: 5'-GCTACTCCCGAATCCTCCTC-3' Reverse: 5'-CGCCTCAGGTATGACTCTCC-3'	73	1.96
<i>gnrhr2a</i>	This study	Forward: 5'-TTCACCTTCTGCTGCCTCTT-3' Reverse: 5'-TCCGTGGAGGAAAGATTGTC-3'	113	1.99
<i>bactin</i>	Hodne et al. (2012)	Forward: 5'-TTCTACAACGAGCTGAGAGTGG-3' Reverse: 5'-CATGATCTGGGTATCTTCTCC-3'	102	1.84
<i>arp2</i>	Hodne et al. (2012)	Forward: 5'-GGAGGTTAGTAGCAAGGAGC-3' Reverse: 5'-TGCTGACTCTCACGGAGTTG-3'	107	1.94
<i>ef1a</i>	Hodne et al. (2010)	Forward: 5'-CCTTCAACGCCAGGTCAT-3' Reverse: 5'-AACTTGCAGGCGATGTGA-3'	100	1.92
<i>ubiquitin</i>	Hodne et al. (2012)	Forward: 5'-TGTCAAAGCCAAGATTCAGG-3' Reverse: 5'-TGGATGTTGTAATCCGAGAGG-3'	111	1.86

qPCR analysis

qPCR analyses were carried out using the LightCycler 480 platform (Roche), as described previously (Weltzien *et al.* 2005, Hodne *et al.* 2012). All samples were run in duplicate, and in every round, three non-template negative control (NTC) reactions were conducted for each primer pair by substituting the cDNA template with nuclease-free water (Ambion). In addition, to account for plate-to-plate variation, a standard positive calibrator control in triplicate was included on every plate. Each PCR reaction (10 µL) mixture contained 5 µL of SYBR Green I master mix (Roche), 1 µL (5 µM) of forward primer, 1 µL (5 µM) of reverse primer and 3 µL of diluted (1:10) cDNA. The qPCR reactions were carried out using an initial step for 10 min at 95°C to activate the *Taq* polymerase, followed by 42 cycles consisting of 10 s at 95°C (denaturation), 10 s at 60°C (annealing) and elongation at 72°C for 6 s. The fluorescence was measured after each elongation and used for determining the quantification cycle values (Cq). A melting curve analysis was performed directly following the PCR by continuously reading the fluorescence while slowly heating the reaction mixture from 65°C to 98°C. qPCR efficiencies (E) were determined based on cDNA dilution curves, which, together with the Cq values, were used to calculate the relative expression (Pfaffl 2001, Roche 2001):

Relative expression $n =$

$$E_{\text{target}}^{\Delta Cq(\text{calibrator-sample})} * E_{\text{reference}}^{\Delta Cq(\text{sample-calibrator})}$$

The qPCR assay specificity was confirmed by agarose gel electrophoresis and sequencing.

Single-cell qPCR

In order to analyze *gnrhr* expression patterns in individual gonadotropes, qPCR was performed on transcripts harvested from single cells, as described by Hodne and coworkers (2010). To secure single-cell transcripts, the patch-clamp technique in whole cell configuration was used. During these experiments, cells were maintained in artificial extracellular solution (EC) comprising (mM): 150 NaCl, 5 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 1.8 glucose and 10 HEPES/NaOH (pH 7.85) at 12°C, 320 mosmol. Patch pipettes were made from borosilicate glass with filament and had a resistance between 2 and 5 MΩ. Before fire polishing, the patch pipettes were silanized (Sigmacote, Sigma) to prevent extracellular contamination from attaching to the glass surface. The patch pipettes were filled with ribonuclease-free intracellular solution (mM): 120 CH₃SO₃K, 20 KCl, 10 HEPES/NaOH, 20 sucrose (pH 7.2), 290 mosmol. Following whole cell configuration, the cytosol was harvested into the pipette by applying gentle suction through tubing connected directly to the pipette holder. The cell content was transferred to 0.5 mL RNase-free tubes immediately after harvesting for RNA linear pre-amplification using MessageBOOSTER cDNA Synthesis Kit (Epicenter Biotechnologies, Madison, WI, USA). Transferred cytosol from individual cells was eluted into 3 µL of Quick Extract solution from the MessageBOOSTER cDNA Synthesis Kit together with Oligo(dT) primer containing a T7 promoter and SuperScript III (Life Technologies). Reverse transcriptase was used to synthesize first-strand cDNA from

poly(A) RNA. After the second-strand cDNA synthesis, a high-yield *in vitro* transcription reaction was used to amplify the poly(A) RNA (mRNA). All single-cell qPCR was performed on cells from sexually mature cod, sampled during the spawning season.

Statistical analysis

Statistical analysis was performed using the JMP Pro12 software (SAS Institute Inc, Cary, NC, USA). Fold-changes of exposed samples relative to their respective solvent control mean were calculated and used in the subsequent analysis for both gene expression and viability data. To maintain control variance in the data set, each control sample was calculated in the same manner and included in the analysis. All data were tested for normality using the Shapiro–Wilk *W* test. In case of non-normality, log-transformed data gave a satisfactory fit to the normal distribution. Potential differences between control cells w/wo EtOH were tested by a one-way ANOVA. Differences in gene expression or viability between treatments were investigated with the Tukey–Kramer HSD test. Significance level was set to 0.05.

Results

Solvent control

For every primary cell culture, control wells with and without 0.2% EtOH (solvent) in the media were prepared. Viability assays performed on these cells revealed a significant negative effect from EtOH on both metabolic (mitochondrial) activity and membrane integrity (data not shown). However, qPCR analysis did not detect any influence on gene expression from the solvent (data not shown). Note that data from steroid exposed cells in the following sections are compared to effects seen in solvent control cells.

Cod pituitary cell viability after 7 days of primary culture

In general, 72 h of sex steroid exposure increased both metabolic activity (AB assay) and membrane integrity (CFDA-AM assay) (Figs 1, 2 and 3), with strong correlation between effects from each assay. No negative effects from the steroids were detected.

E2 exposure significantly increased viability compared to solvent control during all three reproductive stages (Fig. 1), with the exception of cells from maturing fish exposed to low (2.5 ng/mL) dose, which showed no effect from the treatment.

Androgen treatment significantly increased metabolic activity in cells from maturing and mature fish. Interestingly, in cells from mature fish the low dose (2 ng/mL) of DHTS gave a significantly stronger effect on metabolic activity than the high dose (Fig. 3). TS exposure stimulated membrane integrity in maturing and mature stages (Fig. 2), whereas effects from DHTS

Figure 1 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of estradiol exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold-change relative to solvent control $\pm$ S.E.M. ($n=8-12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P<0.05$).

was seen only during the former with this parameter. No effect on viability was detected from androgen treatment on cells from spent fish.

Figure 2 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of TS exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold-change relative to solvent control $\pm$ S.E.M. ($n=8-12$). Asterisk indicates statistical difference from control ($P<0.05$).

Figure 3 Viability in Atlantic cod pituitary cells after 7 days of primary culture and 72 h of DHTS exposure, in terms of mitochondrial activity (AB) and membrane integrity (CFDA-AM). Data are presented as mean fold change relative to solvent control $\pm$ S.E.M. ($n=8-12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P<0.05$).

Cod pituitary gene expression after 7 days of primary culture

Gene expression in individual gonadotropes

In order to identify which GnRh receptors (Gnrhr) are expressed in cod gonadotropes, cytosol from individual non-exposed *fshb*- or *lhb*-expressing cells from mature fish was analyzed by single-cell qPCR. Ten *fshb*-expressing and 10 *lhb*-expressing cells were assessed, and 7 of each cell type expressed *gnrhr2a*. In the remaining cells, no *gnrhr* expression was detected. Of the 7 *gnrhr2a*-positive cells, three also expressed *gnrhr1b*, both in *fshb*- and *lhb*-expressing cells (Fig. 4), meaning both isoforms are expressed in individual gonadotropes. However, *gnrhr1b* expression was low and close to the detection limit of the qPCR assay (Cq values from 35 to 38). Thus, *gnrhr2a* seems to be the predominant receptor in both gonadotrope cell types, at least during spawning season (Cq values from 32 to 34).

Gene expression in steroid-exposed cells

Following 72 h of steroid exposure, gene expression of gonadotropin β -subunits and two GnRh's in the pituitary cells was determined by qPCR (Figs 5, 6 and 7). In general, steroid treatment had differential effects on gene expression, dependent on the type of steroid, target gene and time of year. Common for all three steroids was that

Figure 4 Transcripts of *gnrhr1b* and *gnrhr2a* in *fshb*- and *lhb*-expressing cells in Atlantic cod. Melting curve analysis of qPCR products from a single-cell was performed directly after the qPCR and plotted as the negative change in fluorescence per time as a function of temperature. Each qPCR product is reflected in a specific melting peak in the melting curve analysis. Expression of both *gnrhr1b* and *gnrhr2a* was detected in (A) *fshb*-expressing cells and (B) *lhb*-expressing cells.

gnrhr2a transcript levels showed the strongest treatment response, while, regardless of season, no response on *gnrhr1b* expression was observed.

In more detail, a significant increase in *gnrhr2a* levels was seen after high-dose E2 (25 ng/mL) exposure in cells from mature fish, while both E2 doses increased *gnrhr2a* expression during the spent stage (Fig. 5). Gonadotropin transcript levels were mainly unaffected by E2, with the exception of a significant increase in *fshb* expression following high-dose treatment in mature fish. A similar trend was observed with the expression of *lhb* ($P=0.08$).

The effects from TS exposure on *gnrhr2a* expression were seasonally dependent (Fig. 6). During maturation, no effects were seen, whereas the high dose (20 ng/mL) stimulated expression during the mature stage. Interestingly, in cells from spent fish, the effect of the low TS dose (2 ng/mL) on *gnrhr2a* expression was significantly different from both control and the high TS dose. A similar pattern was seen with *fshb*, where

the low dose stimulated expression during maturation, while the high dose had no effect. During the mature stage, there were no observed effects on *fshb* expression, while transcript levels decreased following high-dose TS treatment in cells from spent fish. TS exposure had no statistically significant effects on *lhb* expression, although a decreasing trend was observed during the spent stage ($P=0.13$).

While E2 and TS stimulated *gnrhr2a* expression during mature and spent stages, DHTS affected *gnrhr2a* only in cells from maturing fish (Fig. 7), with the high-dose (20 ng/mL) exposure increasing expression. For gonadotropin gene expression, an effect from DHTS treatment was seen in cells from maturing fish. While the low DHTS dose (2 ng/mL) resulted in increased expression levels of both *fshb* and *lhb*, the high DHTS dose only significantly increased *lhb* expression levels, although *fshb* expression levels were also increased, but not significantly ($P=0.067$).

Discussion

Gonadotropes are important regulators of reproduction in vertebrates and are themselves subject to regulation from both hypothalamic input and systemic feedback. Using primary pituitary cell cultures as model system, this study describes the effects of sex steroid (E2, TS and DHTS) exposure (72 h) on cell viability and gene expression of gonadotropin β -subunits (*fshb* and *lhb*) and two GnRh receptors (*gnrhr1b* and *gnrhr2a*) during three stages of sexual maturity in Atlantic cod. In addition, it demonstrates that both *fshb*- and *lhb*-producing gonadotropes can co-express two GnRh receptor genes, *gnrhr1b* and *gnrhr2a*.

Cell viability

Very few studies have investigated pituitary cell viability after steroid exposure in fish, which is somewhat surprising as in most *in vitro* studies steroids are generally solubilized using solvents such as ethanol (EtOH) or dimethyl sulfoxide (DMSO), chemicals known to elicit deleterious effects on the health and properties of cultured cells (Baker & Kramer 1999, Santos *et al.* 2002). In this study, steroids were dissolved in EtOH to a final exposure concentration of 0.2%. This concentration did not affect gene expression of any gene analyzed, but did negatively affect the viability of the cells as compared to those exposed to EtOH-free media, both in terms of metabolic activity and membrane integrity. Consequently, all steroid exposed cells were compared to that of the solvent control. The negative effects on cell viability seen from EtOH were generally reversed with the addition of steroids. One proposed mechanism for the non-genomic effects of steroids is intercalation into the phospholipid bilayer,

Figure 5 Gene expression in Atlantic cod pituitary cells after 72 h of estradiol exposure *in vitro*, presented as mean fold-change relative to solvent control $\pm$ S.E.M. ($n=6-12$). Asterisks indicate statistical significant difference from control ($P < 0.05$).

Figure 6 Gene expression in Atlantic cod pituitary cells after 72 h of TS exposure *in vitro*, presented as mean fold-change relative to solvent control $\pm$ S.E.M. ($n=6-12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P < 0.05$).

Figure 7 Gene expression in Atlantic cod pituitary cells after 72 h of DHTS exposure *in vitro*, presented as mean fold change relative to solvent control $\pm$ S.E.M. ($n=6-12$). Asterisk indicates statistical difference from control, while dagger indicates statistical difference between doses ($P<0.05$).

introducing structural and functional alterations of the cell membrane (Golden *et al.* 1998, Falkenstein *et al.* 2000, Whiting *et al.* 2000). In the present study, using physiological sex steroid concentrations, no negative effects on membrane integrity in cod pituitary cells was observed. This was not surprising, as membrane perturbations such as fluidity changes and leakage occur mainly at high, non-physiological, concentrations (Clarke *et al.* 1990, Shivaji & Jagannadham 1992). However, some differential effects were seen. While all steroids generally stimulated membrane integrity, E2 did so regardless of maturational state. While TS had no effect on pituitary cells from spent fish, DHTS had no effect on cells from both spent and mature fish. A similar pattern was evident from the metabolic activity assay. E2 stimulated cell metabolism at all three reproductive stages, while the androgens stopped being stimulatory in cells from the spent stage. This strongly implies that for androgens, there is a seasonal effect in terms of both membrane integrity and cellular metabolism. In spent fish, the endocrine activity of gonadotropes is low, with *fshb* and *lhb* synthesis being at its lowest level in the reproductive cycle (Mittelholzer *et al.* 2009). Although the present study was performed on all pituitary cell types and not just gonadotropes, it is tempting to speculate that there might be an association between gonadotropic endocrine activity and the androgen interplay with the plasma membrane and cellular metabolism in spent fish.

Gonadotropin expression

With the exception of the goldfish (*Carassius auratus*), that has been thoroughly studied (Kobayashi *et al.* 1989, 2000, Huggard *et al.* 1996, Sohn *et al.* 1998, 2001, Huggard-Nelson *et al.* 2002), most studies on teleost steroid feedback have been on single or total spawners. Tables 2, 3, 4 and 5 summarize studies on steroid effects on gonadotropin β -subunit mRNA synthesis in multibatch and asynchronous spawners (Tables 2, 3, 4 and 5: Hermaphrodite species are omitted; Tables 4 and 5: Studies using gonadectomized fish are omitted due to possible pain/stress interaction with reproduction). Common for the *in vitro* studies is that steroid exposure leads to no or stimulating effects on gonadotropin transcript levels, indicating that positive feedback can occur through mechanisms directly at the pituitary level. Inhibiting effects from steroids on gonadotropin transcripts have rarely been observed *in vitro*, but in the male African catfish (*Clarias gariepinus*) 24-h sex steroid (11-ketotestosterone (11-KTS), TS or E2) exposure inhibited *lhb* expression (Rebers *et al.* 2000). In the present study, TS inhibited *fshb* expression in spent cod. There are therefore likely, at least in some species, mechanisms that facilitate negative feedback directly at the pituitary level also.

Due to high levels of aromatase, the enzyme complex that converts TS to E2, in the brain and pituitary of teleosts

Table 2 *In vitro* studies of sex steroid effects on gonadotropin subunit gene expression in immature multibatch spawning fish^a.

Species	DHTS ¹ /11-KTS ²			E2			TS			Sex	Dose (s)	Time	References
	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>				
Goldfish (<i>Carassius auratus</i>)	NM	NM	NM	NM	NM	NM	NM	NM	↑	M + F	2–5000 ng/mL	15 h	Huggard <i>et al.</i> (1996)
Goldfish	NM	NM	NM	2 ng/mL: (↑) 20 ng/mL: — 100–200 ng/mL: ↑	↑	NM	NM	NM	NM	M + F	2–200 ng/mL	15–16 h	Huggard-Nelson <i>et al.</i> (2002)
Goldfish	NM	NM	NM	NM	NM	NM	—	1/100 nM: (↑) 10 nM: ↑	NM	M + F	1–100 nM	168 h	Sohn <i>et al.</i> (2001)
Nile tilapia (<i>Oreochromis niloticus</i>)	NM	NM	NM	—	↑	NM	NM	NM	NM	F	10 nM–150 μM	48 h	Levavi-Sivan <i>et al.</i> (2006)
Tilapia hybrid (<i>O. niloticus</i> x <i>O. aureus</i>)	NM	NM	NM	NM	NM	NM	12/24/72 h: — 36/48 h: ↑	—	—	M	10 nM	12–72 h	Melamed <i>et al.</i> (1997)

^aImmature fish are defined here as both those that have not yet reached puberty and those that have spawned previously, but are now in early gonadal recrudescence. Hermaphrodite species are omitted.

F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; —, No effect.

Table 3 *In vitro* studies of sex steroid effects on gonadotropin subunit gene expression in mature multibatch spawning fish^a.

Species	DHTS ¹ /11-KTS ²			E2			TS			Sex	Dose (s)	Time	References
	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>	<i>lhb</i>				
Goldfish (<i>Carassius auratus</i>)	NM	NM	NM	NM	NM	NM	NM	2–20 ng/mL: — 2000 ng/mL: ↑	—	M + F	2–2000 ng/mL	15 h	Huggard <i>et al.</i> (1996)
Goldfish	NM	NM	NM	NM	NM	NM	10 nM: — 100 nM: (↑) 1 nM: —	—	—	M + F	10–100 nM	48 h	Sohn <i>et al.</i> (2001)
Zebrafish (<i>Danio rerio</i>)	NM	NM	NM	↑	↑	↑	10–100 nM: ↑	10–100 nM: ↑	1 nM: —	M + F	1–100 nM	72 h	Lin and Ge (2009)
Marine medaka (<i>Oryzias melastigma</i>)	NM	NM	NM	↑	↑	↑	NM	NM	NM	M + F	1 μM	24 h	Tse <i>et al.</i> (2013)
Tilapia hybrid (<i>O. niloticus</i> x <i>O. aureus</i>)	NM	NM	NM	NM	NM	NM	0.01–0.1 nM: (↑) 1–100 nM: —	(↑)	(↑)	M	0.1–100 nM	48 h	Melamed <i>et al.</i> (1997)
African catfish (<i>Clarias gariepinus</i>)	NM	0.1–2 ng/mL: — ² 10 ng/mL: ↑ ² 50 ng/mL: (↑) ²	NM	NM	0.001–0.02 ng/mL: — 0.1–10 ng/mL: ↑	NM	NM	NM	NM	M	0.001–50 ng/mL	48 h	Rebers <i>et al.</i> (2000)
African catfish	NM	24 h: NM ^{1/2} 48 h: — ^{1/(↑)2}	NM	NM	24 h: ↓ 48 h: (↑)	NM	NM	24 h: ↓ 48 h: ↑	—	M	50 ng/mL	24/48 h	Rebers <i>et al.</i> (2000)

^aHermaphrodite species are omitted.

F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; —, No effect; ↓, significant decrease; ¹, DHTS; ², 11-KTS.

Table 4 *In vivo* studies of sex steroid effects on gonadotropin subunit gene expression in immature multibatch spawning fish^a.

Species	DHTS ¹ /11-KTS ²			E2		TS		Sex	Dose (s)	Time	References
	<i>fshb</i>	<i>lhb</i>		<i>fshb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>				
Goldfish (<i>Carassius auratus</i>)	↓ ²	↓ ²		↓	↑	↓	(↑)	M	200 µg/fish	14 days	Sohn <i>et al.</i> (1998)
Goldfish	↓ ²	↓ ²		↓	↑	↓	↑	F	200 µg/fish	14 days	Sohn <i>et al.</i> (1998)
Goldfish	NM	NM		NM	NM	NM	12–48 h: (↓) 72–96 h: ↑	M+F	20 µg/fish	12–96 h	Huggard <i>et al.</i> (1996)
Goldfish	NM	NM		NM	NM	NM	0.2–2 µg: ↑ 20 µg: —	M+F	0.2–20 µg/fish	24 h	Huggard <i>et al.</i> (1996)
Goldfish	NM	NM		0.02 µg: — 0.2–2 µg: ↑	↑	NM	NM	M+F	0.02–2 µg/fish	24 h	Huggard-Nelson <i>et al.</i> (2002)
Goldfish	5 µg ² : ↓ 50 µg ² : ↓ ↓ ¹	5 µg ² : — 50 µg ² : — (↑) ¹		5 µg: ↑ 50 µg: ↓ ↓	5 µg: ↑ 50 µg: ↓ (↑)	5 µg: ↓ 50 µg: ↓ ↓	5 µg: ↑ 50 µg: ↓ ↑	M+F	5/50 µg/g pellet 2% BW pellet/d 20 mg/kg BW	14 days	Kobayashi <i>et al.</i> (2000)
European sea bass (<i>Dicentrarchus labrax</i>)								M+F	20 mg/kg BW	12 days	Mateos <i>et al.</i> (2002)
Atlantic croaker (<i>Micropogonias undulatus</i>)	NM	NM		1 µg/g: ↓	1 µg/g: —	NM	NM	M+F	1/5 µg/g BW	120 h	Banerjee and Khan (2008)
Nile tilapia (<i>Oreochromis niloticus</i>)	NM	NM		5 µg/g: ↓ 250 µg/kg: ↓ 500 µg/kg: ↓	5 µg/g: ↑ 250 µg/kg: (↓) 500 µg/kg: ↓	NM	NM	F	250/500 µg/kg BW	6 days	Levavi-Sivan <i>et al.</i> (2006)
Red sea bream (<i>Pagrus major</i>)	— ²	↑ ²		—	—	—	—	M	0.68 mg steroid/kg BW	30 days	Yamaguchi <i>et al.</i> (2006)
African Catfish (<i>Clarias gariepinus</i>)	NM	—/↓ ²		NM	↑	NM	↑	M	30 µg/g BW	14 days	Cavaco <i>et al.</i> (2001)

^aImmature fish are defined here as both those that have not yet reached puberty and those that have spawned previously, but are now in early gonadal recrudescence. Gonadectomized fish and/or hermaphrodite species are omitted.
 BW, body weight; F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; —, No effect; (↓), nonsignificant decrease; ↓, significant decrease;
¹, DHTS; ², 11-KTS.

(Dietel *et al.* 2010), effects from TS exposure might occur through both androgenic and estrogenic mechanisms. The level of brain aromatase gene expression and enzyme activity is itself modulated by steroids and has been shown to fluctuate with the reproductive stage and season in several species (Andersson *et al.* 1988, Pasmanik & Callard 1988a, Gonzalez & Piferrer 2003, Dietel *et al.* 2010). Hence, the amount of TS aromatization might vary and many studies on potential sex steroid feedback therefore include a non-aromatizable androgen to distinguish between actions mediated through estrogen receptors (Er) and androgen receptors (Ar). Despite 11-KTS being non-aromatizable and generally the most important teleost androgen, DHTS was chosen as the non-aromatizable androgen in this study, in part because this androgen has a high binding affinity for the Ar (Sperry & Thomas 1999, Jørgensen *et al.* 2007), and partly because it is often used in studies to demonstrate androgen effects (Martyniuk *et al.* 2013). Although the presence, let alone the biological activity, of DHTS has not been demonstrated in most teleosts, its synthesis and plasma levels has been reported in both male and female fathead minnow (*Pimephales promelas*) (Margiotta-Casaluci *et al.* 2013). In another species, the urohaze-goby (*Glossogobius olivaceus*), DHTS was the main product of *in vitro* steroidogenesis in testes tissue (Asahina *et al.* 1985). In addition, enzymatic activity of 5-α reductase, the enzyme that converts TS to DHTS, has been demonstrated in different tissues, including the brain and pituitary, in several teleost species (Pasmanik & Callard 1988a, Latz & Reinboth 1993, Margiotta-Casaluci *et al.* 2013), so it is likely that DHTS plays a biological role not yet described in teleosts. In the present study, DHTS stimulated *fshb* expression in cells from mature cod, which, to our knowledge, is the first time effects on *fshb* expression from a non-aromatizable androgen have been demonstrated *in vitro* in a multibatch spawner. Furthermore, DHTS, but not T, affected *lhb* expression. This result is in contrast to a study performed on mature male African catfish, where a relatively high dose (50 ng/mL) of DHTS had no effect on *lhb* expression *in vitro*, whereas a similar dose of TS did, presumably following aromatization to E2 (Rebers *et al.* 2000). In Atlantic cod, high E2 dose (20 ng/mL) had a stimulatory trend on *lhb* transcription in mature fish, but this result was not statistically significant. While TS had no effect on *lhb* levels, all steroids affected *fshb* expression at some point during the reproductive cycle, indicating that both estrogenic and androgenic mechanisms are involved in its regulation at the pituitary level in the cod. In a recent study on Atlantic cod, Nagasawa and coworkers (2014) demonstrated a seasonal variation in gonadal Er and Ar gene expression levels. To our knowledge, there is no available data regarding Er/Ar seasonal variation at the pituitary level in cod, although an earlier study reported changes in pituitary Er transcript levels over the reproductive cycle in the largemouth

Table 5 *In vivo* studies of sex steroid effects on gonadotropin subunit gene expression in mature multibatch spawning fish^a.

Species	DHTS ¹ /11-KTS ²		E2		TS		Sex	Dose (s)	Time	References
	<i>fshb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>	<i>fshb</i>	<i>lhb</i>				
Goldfish (<i>Carassius auratus</i>)	— ²	(↑) ²	—	↑	—	(↑)	M	200 µg/fish	14 days	Sohn <i>et al.</i> (1998)
Goldfish	(↑) ²	— ²	(↓)	—	—	—	F	200 µg/fish	14 days	Sohn <i>et al.</i> (1998)
Goldfish	NM	NM	NM	NM	NM	↑	M+F	0.02–20 µg/fish	24 h	Huggard <i>et al.</i> (1996)
African Catfish (<i>Clarias gariepinus</i>)	NM	↑ ²	NM	NM	NM	NM	M	10, 20 and 100 µg/kg BW	5 days	Rebers <i>et al.</i> (1997)

^aGonadectomized fish and/or hermaphrodite species are omitted.

BW, body weight; F, female; M, male; NM, not measured; ↑, significant increase; (↑), nonsignificant increase; —, No effect; (↓), nonsignificant decrease; ¹, DHTS; ², 11-KTS.

bass (*Micropterus salmoides*) (Martyniuk *et al.* 2009). In the current study, the steroids had differential effects on gonadotropin subunits expression at the different reproductive stages. Fluctuations in Er/Ar levels could change tissue sensitivity or responsiveness to estrogens/androgens and might serve as a possible explanation to this observation. Furthermore, as mixed sex cultures were used in these experiments, no information about potential sex-dependent regulation can be provided. It is possible that results from one sex could mask results from the other. Potential male vs female differences regarding both gonadotropin steroid sensitivity and Er/Ar levels through the reproductive cycle would be interesting and relevant topics for future studies in Atlantic cod.

Gonadotropin-releasing hormone receptor expression

In cell cultures prepared from intact pituitaries, all pituitary cell types, not just the gonadotropes, are present. Previously, Gnhr gene expression or protein has been demonstrated in gonadotropes, somatotropes (ST), lactotropes, thyrotropes (TT), melanotropes, corticotropes (CT) and somatolactotropes (SLT) (Illing *et al.* 1999, Parhar *et al.* 2002, 2005, Flanagan *et al.* 2007). In Nile tilapia (*Oreochromis niloticus*), Parhar and coworkers (2005) conducted a study examining receptor expression in individual pituitary cells. They showed that mRNA of receptors phylogenetically close to *gnrhr1b* was expressed in all cells types except TT, SLT (mature fish) and CT (immature fish). Similarly, mRNA of receptors closely related to *gnrhr2a* was detected in all cell types except immature fish TT and CT cells. The same study also showed that individual cells could concurrently express several isoforms of Gnhr and that both *fshb* and *lhb*-producing gonadotropes expressed both *gnrhr1b* and *gnrhr2a*. In the present study, it was demonstrated that this also is true in Atlantic cod, with co-expression of both receptors detected in 30% of the gonadotropes investigated. While this points toward a role for both receptors in gonadotropin regulation, in both *fshb*- and *lhb*-expressing cells, *gnrhr2a* was clearly the predominant receptor. In a study on Gnhr gene expression over the reproductive cycle in female Atlantic cod, Hildahl and coworkers (2011) showed that, in whole pituitary samples, only *gnrhr2a* isoform expression

levels followed the same profile as the GSI, and was significantly upregulated during periods of reproductive activity. Even though the observed expression pattern could not be confined to only gonadotropes, these results indicate that *gnrhr2a* is the main receptor mediating gonadotropin expression in cod.

In teleosts, as well as mammals, steroid treatment has been shown to modify pituitary responsiveness to Gnhr and regulate Gnhr mRNA abundance (Quinones-Jenab *et al.* 1996, Cowley *et al.* 1998, Yen *et al.* 2002, Levavi-Sivan *et al.* 2006, Lin *et al.* 2010). In the current study, *gnrhr1b* showed no, whereas *gnrhr2a* showed strong, sensitivity to steroid exposure. All sex steroids tested were able to stimulate the expression of *gnrhr2a*, which would, assuming its translation into functional protein, increases the sensitivity of gonadotropes to Gnhr stimulation and acts as a direct route for a positive feedback at the pituitary level. However, the mechanisms through which steroids affect *gnrhr2a* expression is not clear, as the stimulation occurred at different times of the year dependent on the steroid. During maturation, *gnrhr2a* transcripts were upregulated by DHTS exposure, while at later stages, DHTS did not affect its expression. Conversely, no effect was seen from E2 or TS during maturation, but both hormones stimulated *gnrhr2a* transcription in cells from mature and spent fish. In general, a close correlation exists between circulating steroid levels and GSI in Atlantic cod, with low plasma levels during early gonadal recrudescence, increasing during maturation, peaking at spawning, before declining rapidly in spent fish (Dahle *et al.* 2003). The steroid sensitivity of *gnrhr2a* does thus not correlate with circulating steroid levels. As with the gonadotropin regulation, seasonal and differential regulation or location of Ar/Er and their respective isoforms might be one explanation to this (Pasmanik & Callard 1988b, Harbott *et al.* 2007).

In the pituitary primary cultures used in the present study, control levels of *gnrhr1b* expression (data not shown) were quite stable over the reproductive cycle except for a small decrease during spawning season, and no effect on its transcription was seen following steroid exposure. In individual cells from mature fish, single-cell qPCR demonstrated that while *gnrhr1b* was co-expressed with *gnrhr2a* in the gonadotropes, the transcript levels

were low and sometimes undetectable. Taken together, these results indicate that, in Atlantic cod, *Gnrhr1b* is not a part of the sex steroid feedback mechanism at the pituitary level and its gene expression is more abundant in other pituitary cell types than gonadotropes. In cod, as in many other teleosts, somatic growth rate is low during times of gonadal growth and spawning (Hansen *et al.* 2001). The growth rate is mediated mainly through the somatotrophic axis (Dai *et al.* 2015), where growth hormone (Gh) production may be stimulated by *Gnrh* (Marchant *et al.* 1989). Since *gnrhr1b* transcript levels decreased during spawning season in control cells and have been demonstrated in STs in other species (Flanagan *et al.* 2007), a tempting hypothesis for future studies is that this receptor is involved in Gh regulation.

Conclusions

In the present study, it was demonstrated that sex steroids (E2, TS and DHTS) stimulate cell viability and have the ability to directly affect transcript levels of gonadotropins and two *Gnrhr* orthologues in the Atlantic cod pituitary. Cell viability was not correlated to gene expression, as steroids induced overall stimulating effects on metabolic activity and membrane integrity, while transcript levels had more seasonally dependent patterns. Co-transcription of *gnrhr1b* and *gnrhr2a* was confirmed to both *fshb*- and *lhb*-expressing gonadotropes using single-cell qPCR, with *gnrhr2a* clearly being most prominently expressed. While steroid exposure had no effect on *gnrhr1b* expression, all steroids stimulated *gnrhr2a* levels at some stage in the reproductive cycle. These results point to *Gnrhr2a* being the main modulator of gonadotropin regulation in Atlantic cod, and that regulation of its gene expression level might function as a direct mechanism for positive steroid feedback at the pituitary level. The expression of *fshb* showed greater steroid sensitivity than that of *lhb* and was affected by all steroids at some reproductive stage, while only DHTS was able to affect *lhb* expression. The mechanisms behind the differential steroid sensitivity is not clear, but might be due to different hormonal regulatory elements in the promoter region of the genes, the level/existence of Er/Ar in cell types that express the individual genes or that these levels vary with season.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0208>.

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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