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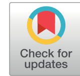
Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation



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ABSTRACT

Through the action of cortisol, stress can affect reproductive biology with behavioural and physiological alterations. Using mixed sex primary pituitary cultures from Atlantic cod (*Gadus morhua*), the present study aimed to investigate potential direct effects of basal and stress level cortisol on the pituitary in terms of cell viability and reproduction-related gene expression at different stages of sexual maturity. Stress level of cortisol stimulated cell viability in cells derived from sexually maturing and mature fish. In cells from spent fish, high cortisol levels did not affect cell viability in terms of metabolic activity, but did stimulate viability in terms of membrane integrity. Basal cortisol levels did not affect cell viability. Ethanol, used as solvent for cortisol, decreased cell viability at all maturity stages, but did generally not affect gene expression. Genes investigated were *fshb*, *lhb* and two Gnrh receptors expressed in cod gonadotropes (*gnrhr1b* and *gnrhr2a*). Cortisol had dual effects on *fshb* expression; stimulating expression in cells from mature fish at stress dose, while inhibiting expression in cells from spent fish at both doses. In contrast, cortisol had no direct effect on *lhb* expression. While *gnrhr2a* transcript levels largely increased following cortisol treatment, *gnrhr1b* expression decreased in cells from spent fish and was unaffected at other maturity stages. These findings demonstrate that cortisol can act directly and differentially at the pituitary level in Atlantic cod and that factors facilitating these actions are dose-dependently activated and vary with level of sexual maturity.

1. Introduction

Sexual maturation and reproduction in teleosts are regulated through the brain-pituitary-gonadal (BPG) axis. Hypothalamic neurons secrete gonadotropin-releasing hormone (Gnrh) onto gonadotrope cells in the pituitary, leading to the synthesis and release of follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), which in turn stimulate gonadal development, gametogenesis and steroid production. The axis itself is regulated through an array of environmental factors, such as photoperiod, temperature, population density and food availability, as well as internal modulators such as the levels of sex steroids, dopamine, kisspeptins and others (reviewed by Golan et al., 2014; Levavi-Sivan et al., 2010). In addition, stress has been shown to interfere with the BPG-axis and affect fish reproduction.

Stress can be defined as a state in which there is a perceived threat to an organism's homeostasis, real or imagined. The factor causing this threat is the stressor, and the physiological and behavioural responses attempting to re-establish homeostasis is the stress-response (Charmandari et al., 2005; McEwen, 2000; Wendelaar Bonga, 1997). In fish, the stress response is characterized by the activation of the brain-pituitary-interrenal (BPI) axis and of sympathetic fibres leading to release of catecholamines from chromaffin cells in the head kidney (Wendelaar Bonga, 1997). Activation of the BPI-axis enhances corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and glucocorticoid (GC) release (Flik et al., 2006). The main teleost GC is cortisol, produced by steroidogenic interrenal cells functionally equivalent to the mammalian adrenal cortex. In fish, as in mammals, high levels of cortisol have been demonstrated to act on all levels of the BPG-axis, affecting Gnrh and gonadotropin levels, gonadal development, gamete quality, vitellogenin production, sex steroid

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levels and sexual behaviour (reviewed in Goos and Consten, 2002; Leatherland et al., 2010; Milla et al., 2009; Schreck, 2010; Schreck et al., 2001). The results, however, are inconsistent between studies. For instance, in cortisol-implanted sexually maturing brown trout (*Salmo trutta*), pituitary gonadotropin levels decreased in both males and females, while in juvenile female eel (*Anguilla Anguilla*) pituitary Lh levels increased after cortisol exposure, both *in vivo* and *in vitro* (Carragher et al., 1989; Huang et al., 1999). Moreover, cortisol administration to vitellogenic ovarian follicles decreased 17β-estradiol and testosterone production in rainbow trout (*Oncorhynchus mykiss*) and Eurasian perch (*Perca fluviatilis*) (Mandiki et al., 2017; Reddy et al., 1999), but had no effect in goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*) or the New Zealand snapper (*Pagrus auratus*) (Pankhurst et al., 1995). The differential responses seem dependent on species-specific regulations, age, sex and maturational stage, as well as dose, manner and duration of the treatment.

In Atlantic cod (*Gadus morhua*), an important commercial saltwater species belonging to the relatively little studied order of Gadiformes fish, stress has been shown to induce irregular spawning intervals and lowered fertilization rates (Bogevik et al., 2012; Kjesbu, 1989; Kjesbu et al., 1990). Moreover, high cortisol levels altered reproductive behaviour and lead to increased number of abnormal larvae (Morgan et al., 1999), as well as differential expression of cytogenesis-linked genes inn eggs and embryo (Kleppe et al., 2013) in this species. However, stress or cortisol effects at the higher levels of the BPG-axis in Atlantic cod are not known. The objective of the present study was therefore to investigate potential direct effects of cortisol at the cod pituitary level, using primary cell cultures as the model system. After reaching puberty at 2–3 years of age in the wild, Atlantic cod spawn annually. Gonadal maturation commence during late autumn, with full maturation and multiple spawning occurring from January until May (Dahle et al., 2003; Kjesbu, 1989). The pituitary cultures were prepared from fish at different stages of sexual maturity (maturing, mature and spent), and exposed to cortisol in doses corresponding to basal and stress plasma levels. Impact on cell viability and gene expression of gonadotropin (*lhb* and *fshb*) and two cod gonadotrope Gnrh receptor isoforms (*gnrhr1b* and *gnrhr2a*) (von Krogh et al., 2017) were recorded.

2. Materials and methods

2.1. Animals

Atlantic cod (0.5–4.1 kg body weight) from the southern Norwegian coast were captured and kept for at least one week in the aquarium facilities at the University of Oslo before being sacrificed. While in captivity, they were fed shrimp daily. Aquaria were continuously perfused with seawater with a salinity of 28‰ and a temperature of 8–12 °C. The light cycle was adjusted to fit the natural night/day rhythm in Oslo (60°N). Both male and female cod were used in the study. A general permission to keep the animals in the facilities was given by the Norwegian animal research authority (S-2008/108215) and all animals were kept and handled in agreement with their requirements. 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).

2.2. Pituitary primary cell cultures

Dispersed pituitary primary cultures of mixed sexes were prepared using culture conditions optimized to the physiology of Atlantic cod (Hodne et al., 2012). Due to difficulties obtaining fish and the lack of external dimorphic sexual phenotypes in cod, in order to save fish, donors were not discriminated based on sex, but only on state of sexual maturity. Maturational status was determined based on visual inspection of the gonads (see von Krogh et al., 2017, supplementary data) and gonadosomatic index (GSI; [Gonadal weight / Total body weight] x 100). Two cultures were prepared from each stage (maturing, mature and spent) over a two-year period. The GSI (mean ± SD, M = males, F = females) for each stage was; maturing (M; 1.47 ± 1.78, F; 1.74 ± 0.67), mature (M; 3.60 ± 4.46, F; 4.71 ± 5.71) and spent (M; 0.11 ± 0.17, F; 0.62 ± 0.33). The sex ratios within cultures were; maturing stage (8F/7M and 7F/7M), mature stage (6F/2M, and 7F/2M) and spent stage (4F/5M and 3F/2M).

In short, dissected pituitaries were pooled ($n = 5–15$) and dissociated. Dispersed cells were seeded at a density of 1.5×10^5 cells/cm^2 in modified L-15 medium (Life Technologies, Carlsbad, CA, USA) and incubated at 12 °C in a humidified atmosphere of 0.5% CO_2 in air (pCO_2; 3.8 mmHg, which resulted in a medium pH of 7.85). After 24 h, culture media were replaced to remove damaged and detached cells.

2.3. Steroid exposure

At day 4 in culture, cells were exposed to cortisol. Stock solutions of cortisol (Sigma, St. Louis, MO, USA) were prepared by dissolving the steroid in 100% ethanol (EtOH; Kemetyl, Kolbotn, Norway). Before cell exposure, stocks were diluted to desired concentration in modified L-15 medium, with working solutions having a final EtOH concentration of 0.2% (34.25 mM). For each experiment, controls w/wo EtOH (solvent control/control blank) were included.

Two physiologically relevant doses of 10 ng/ml (2.76×10^{-8} M) and 100 ng/ml (2.76×10^{-7} M), corresponding roughly to Atlantic cod basal and stress plasma cortisol levels, respectively (King and Berlinsky, 2006; Staurnes et al., 1994), were applied. Six replicate wells in 24-well plates per treatment were prepared for gene expression analysis, whereas for viability tests, a minimum of six replicate wells in 96-well plates were used. Exposure lasted 72 h.

2.4. Viability testing

After seven days of culture (w/wo exposure), viability tests were performed using two non-toxic fluorescent indicator dyes, AlamarBlue (AB) and 5-carboxyfluorescein diacetate-actetoxymethyl ester (CFDA-AM) (both from Life Technologies). These assays indicate metabolic activity and plasma membrane integrity, respectively, and measure the conversion of a non-fluorescent dye into a fluorescent dye by enzymes present in intact and viable cells (Bopp and Lettieri, 2008). The test procedures were carried out as described by Hodne et al. (2012).

In short, cells were seeded, incubated and exposed to cortisol as described above. At day 7, 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 fluorescent products was measured simultaneously for both probes using a Bio-Tek FLX 800 fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Data was collected with the Gen5 (Gen5 Data Analysis Software, Bio-Tek Instruments Inc.) software.

As a positive control for cell toxicity, serving as intra/inter assay control, each plate included additional wells exposed to copper sulphate ($CuSO_4$) (0.156–2.5 mM) the last 24 h of culture ($n = 6$ per concentration). The effects from $CuSO_4$ exposure were comparable between individual plates and cultures, indicating the AB/CFDA-AM assays a stable system for cytotoxicity measurement in these cultures (see supporting information, Fig. S1).

2.5. Quantification of gene expression

2.5.1. RNA extraction and cDNA synthesis

Cells used for gene expression analysis were harvested at day 7. Total RNA was extracted in Trizol (Life Technologies) and resuspended in 10 µl RNase-free water (Ambion, Thermo Fisher Scientific, Waltham, MA, USA). DNase treated RNA (TURBO DNase-free (Ambion)) was quantified spectrophotometrically (NanoDrop, Thermo Fisher

Table 1
qPCR primers used in the present study.

Target	Reference		Primer sequence	Amplicon size (nt)	Efficiency
lhb	Hodne et al., 2010	Forward	5′-GTGGAGAAGAAGGGCTGTCC-3`	81	1.93
		Reverse	5′-GGACGGGTCCATGGTG-3`		
fshb	Hodne et al., 2010	Forward	5′-GAACCGAGTCCATCAACACC-3`	63	1.84
		Reverse	5′-GGTCCATCGGGTCCTCCT-3`		
gnrhr1b	von Krogh et al., 2017	Forward	5′-GCTACTCCCGAATCCTCCTC-3`	73	1.96
		Reverse	5′-CGCCTCAGGTATGACTCTCC-3`		
gnrhr2a	von Krogh et al., 2017	Forward	5′-TTCACCTTCTGCTGCCTCTT-3`	113	1.99
		Reverse	5′-TCCGTGGAGGAAAGATTGTC-3`		
bactin	Hodne et al., 2012	Forward	5′-TTCTACAACGAGCTGAGAGTGG-3	102	1.84
		Reverse	5′-CATGATCTGGGTCATCTTCTCC-3		
arp2	Hodne et al., 2012	Forward	5′-GGAGGTTAGAAGTAGCAAGGAGC-3′	107	1.94
		Reverse	5′-TGCTGACTCTCACGGAGTTG-3`		
ef1a	Hodne et al., 2010	Forward	5′-CCTTCAACGCCCAGGTCAT-3`	100	1.92
		Reverse	5′-AACTTGCAGGCGATGTGA-3`		
ubiquitin	Hodne et al., 2012	Forward	5′-TGTCAAAGCCAAGATTCAGG-3`	111	1.86
		Reverse	5′-TGGATGTTGTAATCCGAGAGG-3′		

Scientific), and the quality assessed by electrophoretic validation (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) of the RNA Integrity Number (RIN). Only RNA samples with RIN number above 8 were analysed 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.

2.5.2. Primers and reference genes

All qPCR primers (Table 1) were designed using Primer3 shareware (http://frodo.wi.mit.edu/primer3/input.htm). To test for possible hairpin loops and primer dimer formations, potential primers were further analysed using Vector NTI (Life Technologies). 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 reproductive function, *i.e.*, *lhb* (GenBank ID: DQ402374), *fshb* (GenBank ID: DQ402373), *gnrhr1b* (GenBank ID: GU332297) and *gnrhr2a* (GenBank ID: GU332298.1) was investigated. 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 quantification cycle value (Cq) geometric means and standard deviations (± Cq) of 27.06 (± 0.39), 21.96 (± 0.45), 22.19 (± 0.43) and 20.40 (± 0.39), respectively. In order to save sample, only one reference gene, *ef1a* (GenBank ID: DQ402371.1) was used for normalization of the qPCR data in the subsequent experiments.

2.5.3. qPCR analysis

qPCR analyses were carried out as previously described (Hodne et al., 2012; Weltzien et al., 2005), using the LightCycler 480 platform (Roche, Basel, Switzerland). 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). To account for plate-to-plate variation, a standard positive calibrator control in triplicate was also included on every plate. The positive calibrator was prepared by mixing cDNA from all individual samples analysed at the same time. The mixture was diluted and analysed in the same manner as the individual samples. 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 Cq. Directly following the PCR a melting curve analysis was performed 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):

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

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

2.6. Statistics

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, control samples were calculated in the same manner and included in the analysis. All data were tested for normality by the Shapiro-Wilk W test. In case of non-normality, log-transformed data gave a satisfactory fit to the normal distribution. To account for the non-independence of the data within each culture when comparing solvent control wells (w/0.2% EtOH) with control blank wells (wo/EtOH), a one-way analysis of covariance (ANCOVA) corrected for well replicates within each culture as a concomitant variable was run. The same model was used to compare solvent control cells with exposed cells within each maturational stage. Here, to elucidate differences between groups that showed a significant treatment effect, the Tukey–Kramer honestly significant difference (HSD) post-hoc test was conducted on the adjusted means. Significance level was set to 0.05.

3. Results

3.1. Effects of ethanol as a solvent

Control wells with and without 0.2% EtOH (solvent control and control blank, respectively) in the media were prepared for every primary cell culture. The AB and CFDA-AM viability assays performed on these cells revealed a statistically significant negative effect from EtOH on both the metabolic activity (Fig. 1A) and membrane integrity (Fig. 1B), respectively. Although consistent between maturational stages, the negative effects on cell viability appeared more pronounced in cells from sexually mature fish. No inhibition of gene expression was detected from the solvent, regardless of maturational state (Figs. 2 and 3). In contrast, the solvent increased gene expression of *fshb* and *lhb* in the spent stage (Fig. 2A and B). No solvent effects were detected on *gnrhr1b* and *gnrhr2a* expression (Fig. 3A and B). Note that data from

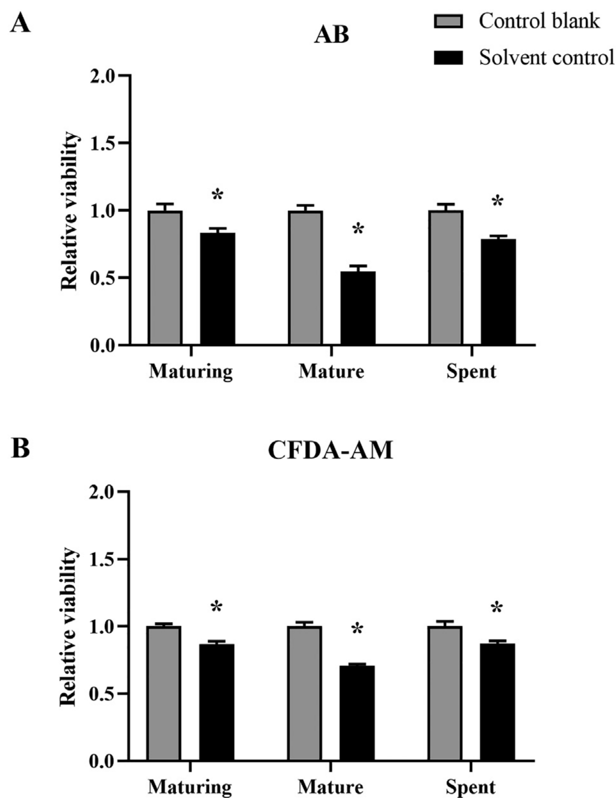

Fig. 1. Viability in terms of mitochondrial activity (AB assay; A) and membrane integrity (CFDA-AM assay; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 17–24). Statistical significance (*; $p < .05$) was assessed by ANCOVA.

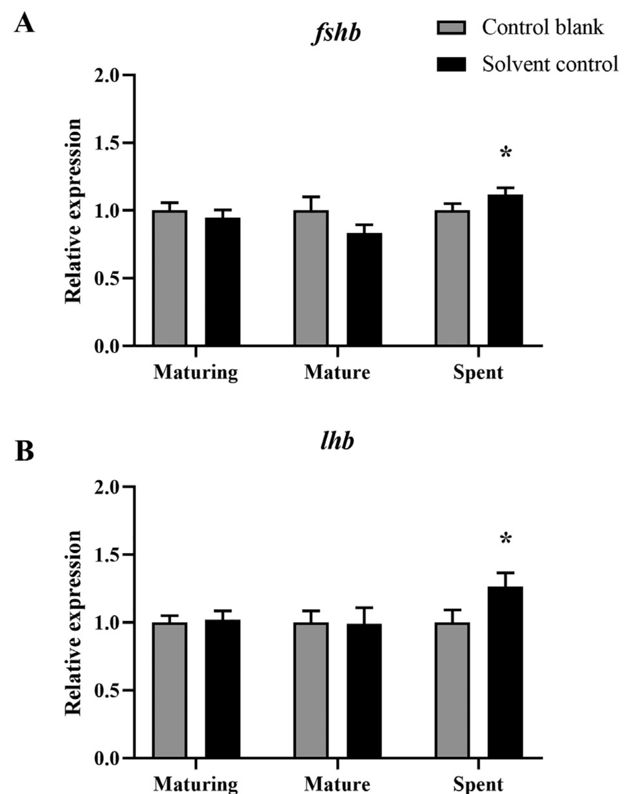

Fig. 2. Gene expression of gonadotropin subunits (*fshb*; A, *lhb*; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 12). Statistical significance (*; $p < .05$) was assessed by ANCOVA.

cortisol-exposed cells in the following sections are compared to effects seen in solvent control cells.

3.2. Cortisol exposure

3.2.1. Cell viability after cortisol exposure

Cell viability in terms of metabolic activity or membrane integrity (Fig. 4A and B, respectively) was unaffected by 72 h of basal cortisol level (10 ng/ml) exposure. On the other hand, stress levels of cortisol (100 ng/ml) stimulated both viability parameters in cells derived from maturing and mature fish. In spent fish, only the membrane integrity was significantly improved, although a similar tendency (p = .096) was seen also for the metabolic activity.

3.2.2. Gene expression after cortisol exposure

Depending on the target gene and time of year, cortisol treatment led to differential effects on gene expression, regarding both gonadotropin subunits (Fig. 5) and Gnrh receptors (Fig. 6).

Cortisol exposure had seasonally dependent effects on *fshb* expression (Fig. 5A). Basal cortisol levels had no effect on cells from mature and maturing fish, but decreased *fshb* transcription in cells from spent donors. In contrast, stress levels increased *fshb* expression at the mature stage, while decreasing expression in cells from maturing and spent fish, the decrease being more pronounced in the latter.

No significant changes in *lhb* transcript levels were seen following cortisol treatment (Fig. 5B), regardless of the maturational stage of the donor fish, although an increasing trend (p = .057) was observed in cells from mature fish exposed to stress level concentrations.

Similar to *fshb* expression, cortisol inhibited *gnrhr1b* expression in pituitary cells derived from spent fish (Fig. 6A). However, only basal levels of cortisol led to significantly reduced *gnrhr1b* transcript levels, although a similar trend was seen from stress level exposure (p = .095). In exposed cells derived from maturing and mature fish, *gnrhr1b* transcription levels followed those of solvent control. Regardless of season, a general increase in *gnrhr2a* expression was seen after stress level cortisol treatment (Fig. 6B), though statistically significant from solvent control only in cells derived from spent fish. Basal cortisol level did not affect *gnrhr2a* expression at any maturational stage.

4. Discussion

It is generally accepted that stress and high levels of cortisol can affect different aspects of reproduction, both in mammals and teleosts. The present study aimed to investigate direct effects of cortisol at the pituitary level in Atlantic cod and provides evidence that cortisol stimulates pituitary cell viability at stress-level concentrations and affects reproductive gene expression at both basal and stress levels in this species.

4.1. Methodical considerations

In Atlantic cod, peaking plasma cortisol levels during stress vary between studies and the severity of the stressor. Morgan et al. (1999) measured cortisol peaks of ~28 ng/ml in cod subjected to capture/confinement and ~127 ng/ml after otter trawl capture. Similar results to the latter value have been reported after exposure to a net stressor (~95 ng/ml; King and Berlinsky, 2006) and progressive hypoxia (~110 ng/ml; Herbert and Steffensen, 2005), while Perez-Casanova et al. (2008) measured ~450 ng/ml after acute temperature increase in juveniles. The stress dose of 100 ng/ml administered in this study must therefore be regarded to represent a mean of physiologically relevant

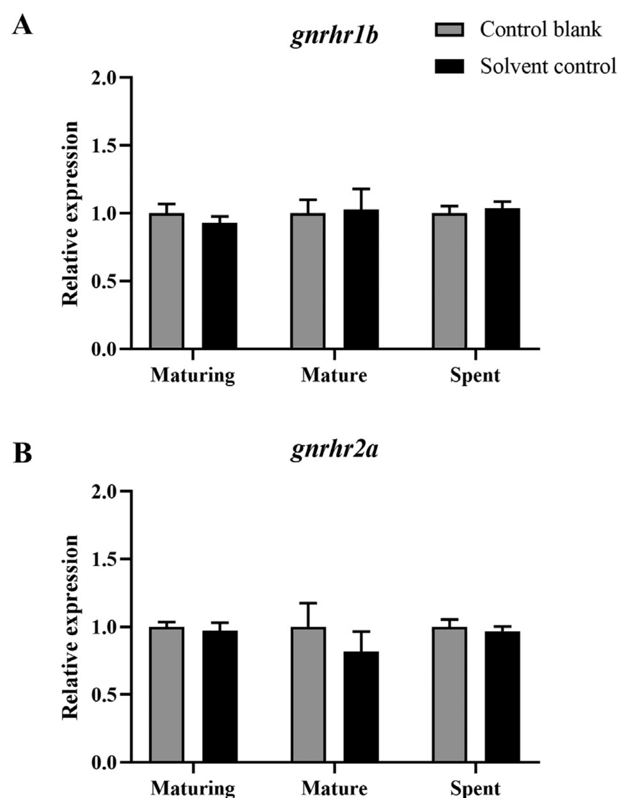

Fig. 3. Gene expression of two gonadotropin-releasing hormone receptors (*gnrhr1b*; A, *gnrhr2a*; B) in Atlantic cod pituitary cells after 7 days of primary culture, w/wo 0.2% EtOH (solvent control/control blank) added to the culture media for the last 72 h. Data is presented as mean fold change relative to mean control blank ± SEM (n = 12). Statistical significance (*; p < .05) was assessed by ANCOVA.

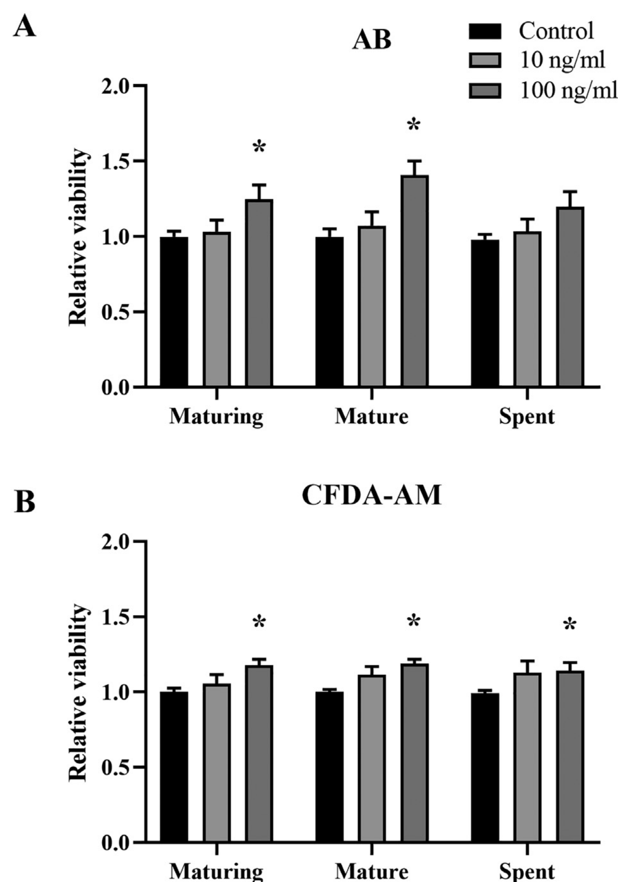

Fig. 4. Viability in terms of mitochondrial activity (AB assay; A) and membrane integrity (CFDA-AM assay; B) in Atlantic cod pituitary cells after 7 days of primary culture w/wo 72 h of cortisol exposure. Data is presented as mean fold change relative to mean solvent control ± SEM (*n* = 9–12). Statistical significance (*; p < .05) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

stress levels, and it cannot be excluded that the present results could have been different had the exposure dose resembled more extreme values. Glucocorticoids affect both sexes and do so in a dual manner, *i.e.* the effects can be both positive and negative, depending on the species in question, tissue investigated and maturational stage (Milla et al., 2009). However, there is reason to speculate that at least chronic stress could affect female reproduction harder than male reproduction as the nutritious investment is higher for eggs than sperm and because part of the allostatic load is reallocation of available metabolic resources (Leatherland et al., 2010). At the pituitary level, there are studies in rats showing differential effects between the sexes, both *in vivo* and *in vitro* (D' Agostino et al., 1990; Ringstrom et al., 1992; Suter and Schwartz, 1985a, 1985b). For instance, using perfused fragments of rat anterior pituitary, D'Agostino et al. (1990) found increased gonadotropin secretion by cortisol treatment in female donors, but not in males. In contrast, cortisol implantation of sexually maturing brown trout reduced pituitary gonadotropin levels, while maintaining plasma gonadotropin levels, in both sexes equally (Carragher et al., 1989). The present study pooled pituitary cells from both sexes in each culture prepared, and can therefore not contribute information regarding potential sex dependent mechanisms in Atlantic cod. The cultures prepared at the mature stage had seemingly a more pronounced decrease in viability following EtOH treatment than cultures from the other maturational stages. Since these cultures had a surplus of female donors, it is uncertain if the enhanced effect is a result of sex, *i.e.* that cells from females are more sensitive to EtOH, or of reproductive state, *i.e.* that during this period the cells are in such a state that they are more vulnerable.

The pituitary consists of many cell types, which may respond differentially to experimental treatment. Gene expression of Fsh and Lh subunits are probably limited to gonadotropes, whereas Gnrh receptors have been identified in several pituitary cell types, including corticotropes (Parhar et al., 2005). The cultures used in the present study were prepared from *in toto* pituitaries. Consequently, results presented here cannot be attributed to individual cell types within the pituitary. Furthermore, effects may be underestimated or undetected, as potential opposite effects from individual cells, either because of its type or because of the sex or history of its donor, would mask each other.

4.2. Cell viability

For relevant results in functional *in vitro* studies, it is crucial that the cells are physiologically healthy and stable while in culture. To ensure this, culture conditions should be adjusted to mimic the physiology of the animal in question. However, most fish *in vitro* studies are performed using mammalian protocols with only the temperature being adjusted to fish physiology. The present study used protocols optimized for Atlantic cod plasma osmolality, pCO2 and pH, as well as temperature (Hodne et al., 2012). These optimized conditions allow cultured Atlantic cod pituitary cells to maintain stable membrane potentials and steady Gnrh responses, along with the ability to fire action potentials for at least two weeks (Hodne et al., 2012). Moreover, cell metabolic activity and membrane integrity are significantly improved compared to traditional cell culture conditions.

Cortisol is a lipophilic steroid hormone and prior to cell media dilution, stock solutions are often prepared using a solvent, like EtOH. As many organic solvents, EtOH can be cytotoxic, and the working

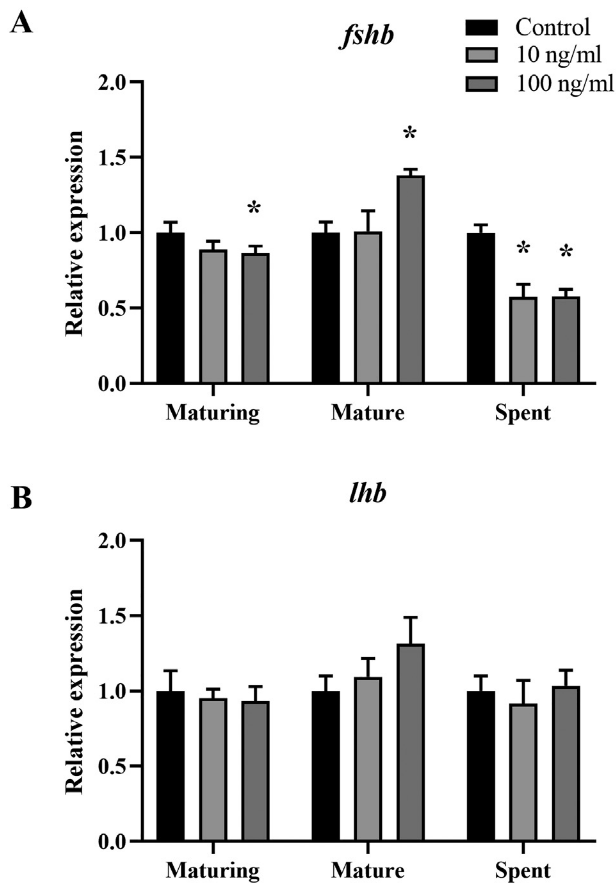

Fig. 5. Gene expression of gonadotropin subunits (*fshb*; A, *lhb*; B) in Atlantic cod pituitary cells after 72 h of cortisol exposure *in vitro*, presented as mean fold change relative to mean solvent control ± SEM (n = 6–12). Statistical significance (*; p < .05) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

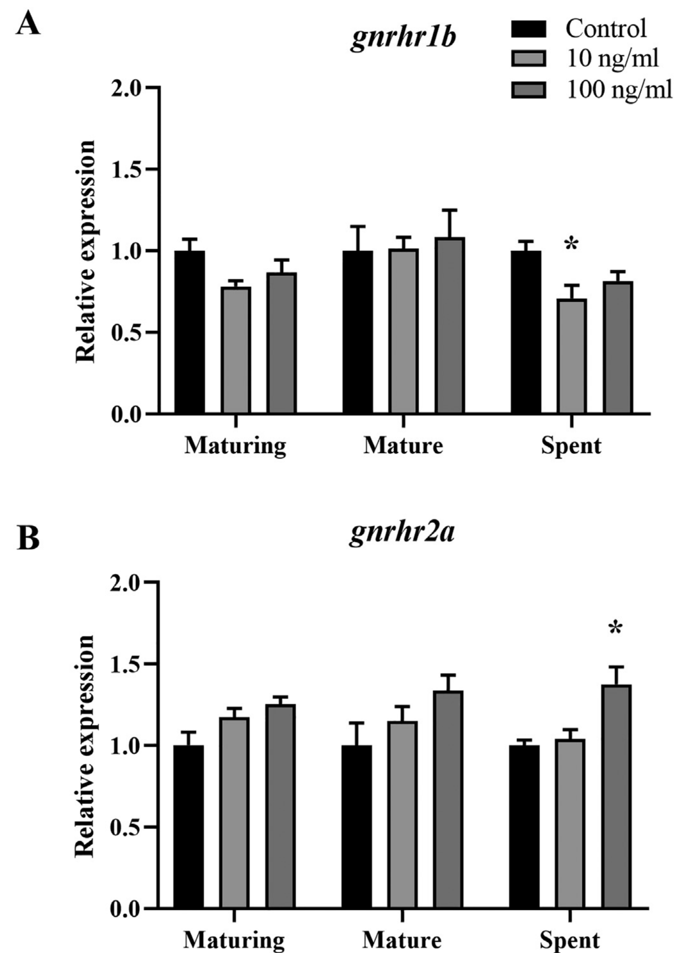

Fig. 6. Gene expression of two gonadotropin-releasing hormone receptors (*gnrhr1b*; A, *gnrhr2a*; B) in Atlantic cod pituitary cells after 72 h of cortisol exposure *in vitro*, presented as mean fold change relative to mean solvent control ± SEM (n = 6–12). Statistical significance (*; p < .05) between treated samples and control was assessed by ANCOVA followed by the Tukey HSD test.

concentration of 0.2% EtOH (34.2 mM) used in the present study caused negative effects on pituitary cell viability, both in terms of metabolic activity and membrane integrity, but did not negatively affect gene expression. Due to its small hydrophobic hydrocarbon chain, EtOH can physically insert into the lipid bilayer of membranes and alter their function and integrity (McKarns et al., 1997). Furthermore, EtOH is linked to mitochondrial dysfunction and increased levels of reactive oxygen metabolites (Baker and Kramer, 1999; Manzo-Avalos and Saavedra-Molina, 2010; McKarns et al., 1997), so the negative effects on cell viability seen here was not surprising. The addition of cortisol to the culture medium seemed to leave the cells more viable than solvent control cells. Positive effects on cell viability from exogenous steroid hormones have previously been observed using this pituitary cell model, where physiological concentrations of sex steroids increased viability at different maturity stages (von Krogh et al., 2017). The cortisol-induced increase in viability seen here was evident in cells at all reproductive stages, but in cells derived from spent fish, cortisol exposure had no significant effect on metabolic activity, indicating that the viability-stimulating mechanisms here are linked to factors that varies with sexual maturity. Furthermore, only stress-level dose of cortisol increased cell viability, which suggest that these factors are also dose-dependent in their activation. Cortisol exerts its action by binding to corticoid receptors (Cr), *i.e.* glucocorticoid receptors (Gr) or mineralocorticoid receptors (Mr), in the membrane or cytosol of its target cells. The level of available receptors, as well as the receptors' sensitivity to cortisol, regulate the strength of impact. Crs have been demonstrated in the pituitary and in gonadotropes of both mammals and teleosts (Breen et al., 2012; Kitahashi et al., 2007; Kononen et al., 1993; Pepels et al., 2004; Teitsma et al., 1999; Thackray et al., 2006), but are not yet investigated in cod, so their affinity for cortisol and potential pituitary regulation through the reproductive cycle in cod is presently not known. However, in the common carp (*Cyprinus carpio*), Stolte et al. (2008) demonstrated that one Mr. and two Gr isoforms were expressed in the pituitary and that all had differential sensitivity to cortisol, meaning that basal and elevated levels of cortisol could initiate differential cellular responses dependent on which receptor is activated. One such possible cellular response is mitosis, meaning that the increased viability observed presently could be a reflection of cell proliferation in cortisol treated wells. Though generally being considered inhibitory to cell proliferation, this is not always the case, and cortisol treatment did *e.g.* increase cell numbers at early stage embryogenesis in rainbow trout (Li et al., 2012). Still, as the viability scores of the cortisol treated cells in the current study never exceeded those of the control blank cells, with no EtOH added to the media, proliferation seems an unlikely explanation. It appears more probable then, that high-level cortisol somehow counteracts the cytotoxicity induced by EtOH. In addition to the well-known genomic effects, cortisol may act through non-genomic mediated mechanisms as well (Borski, 2000; Mommsen et al., 1999). One such proposed mechanism for steroids is intercalation into the phospholipid bilayer of the cell membrane, introducing structural and functional alterations (Falkenstein et al., 2000; Golden et al., 1998; Whiting et al., 2000). This might be a way for cortisol to reduce the

negative effects from EtOH on the membrane activity, but at the present stage, this remains a speculation and needs further elucidation. Nevertheless, for future studies it seems reasonable to recommend an even lower working concentration of EtOH.

4.3. Gene expression

In mammals, it has been suggested that some of the negative effects seen from stress and cortisol on reproduction is due to a reduction of Gnrh responsiveness in the pituitary (Breen and Karsch, 2004; Breen et al., 2012; Suter et al., 1988). This is mainly based on the observation that synthesis and release of LH, which normally increase following GnRH stimulation, decrease when coupled with glucocorticoids. For instance, rats receiving cortisol implantation and GnRH injection had lower plasma LH levels than rats receiving GnRH only (Suter et al., 1988). Similarly, restrained stress by itself did not affect mice *LHβ*-expression, but led to a significant reduction in *LHβ*-expression compared to unstressed mice when coupled with exogenous GnRH (Breen et al., 2012). The present study indicate that, similar to mammals, cortisol does not affect *lhb* synthesis directly in Atlantic cod. This is in contrast to data from juvenile eel, where *lhb* mRNA and Lh protein levels increased after cortisol exposure *in vitro*, demonstrating that in this species, cortisol effects on Lh are not necessarily Gnrh dependent (Huang et al., 1999). However, teleost data at the pituitary level are inconsistent and seem dependent on several factors, such as species and stage of sexual maturity. For instance, in sexually mature brown trout, acute stress was followed by high levels of both circulating cortisol and gonadotropin (Pickering et al., 1987), whereas neither confinement stress nor cortisol injections had any effect on plasma Lh in maturing rainbow trout (Pankhurst and Van Der Kraak, 2000). Furthermore, cortisol implantation decreased pituitary Lh levels in sexually maturing brown trout (Carragher et al., 1989), but increased pituitary Lh contents in juvenile eel (Huang et al., 1999). A study on male common carp fed cortisol-containing food during first sexual maturation reported unchanged pituitary Lh levels during pubertal development, but decreased Lh contents in the mature fish (Consten et al., 2001). The same study also investigated Lh release from dissected pituitaries, and found that basal Lh release was unaffected by cortisol treatment, whereas concurrent treatment with Gnrh and cortisol decreased Lh release compared to Gnrh alone in pituitaries from mature fish. Collectively, it seems clear that there are considerable differences between species regarding Lh synthesis and release, both in terms of direct and Gnrh-coupled effects. While no direct effects of cortisol on *lhb* mRNA was detected in the present study, except a stimulatory tendency in cells derived from mature donors, potential effect on Atlantic cod Lh regulation if cortisol is co-administered with Gnrh cannot be excluded and would be an natural next subject for future mechanistic studies. An additional cause to the observed differential effects between studies, especially regarding *in vitro* experiments, might be the solvent used to administer cortisol to the cells. Most studies described here has used EtOH as the solvent, with concentrations ranging from 0.01 to 1% (Summarized in Table S1, supplementary data). As very few studies include control blanks, it is hard to deduce the potential effects of EtOH on their experiments. To the best of our knowledge, no study has compared the effect of cortisol treatment with and without EtOH as a solvent. Future work investigating the potential interactions between EtOH and cortisol on pituitary cells might elucidate the observed differences between *in vitro* studies.

In addition to mechanisms downstream of the Gnrh receptor, such as reduced Lh synthesis or release, reduced Gnrh responsiveness might be caused by a down-regulation of available binding sites for the Gnrh ligand, *i.e.* the Gnrh receptors (Gnrhr). While *gnrhr1b* expression indeed was down-regulated in cells from spent cod, previous evidence points to Gnrhr2a as the main gonadotropin modulator in this species. This is based on the fact that *gnrhr2a* expression increases in concert with the gonadosomatic index and, while being co-expressed with *gnrhr1b* in both *lhb*- and *fshb*- expressing cells, *gnrhr2a* expression is more prominent than that of *gnrhr1b* and regulated by sex steroids (Hildahl et al., 2011; von Krogh et al., 2017). Here, basal cortisol levels had no effect on *grnhr2a* transcript levels, while stress levels generally increased *gnrhr2a* expression. This agrees with results from the mouse gonadotrope LβT2 cell line where dexamethasone, a synthetic glucocorticoid, increased *GnRHR* transcripts (Kotitschke et al., 2009; Turgeon et al., 1996). However, GnRHR numbers and GnRH affinity were unaffected by glucocorticoid treatment in rats *in vivo* (Suter et al., 1988). To our knowledge, this is the first teleost study to look at Gnrhr expression in the pituitary following cortisol treatment. Assuming translation of the mRNA into functional protein, the present result suggests that stress levels of cortisol enhances, rather than reduces, Gnrh responsiveness in terms of gonadotropin modulation in Atlantic cod.

As mentioned above, expression of *gnrhr2a* and *gnrhr1b* are evident in both gonadotropes in Atlantic cod (von Krogh et al., 2017). The distribution of these receptors in the other cell types of the cod pituitary is presently not known. However, Flanagan et al. (2007) showed that *Astatotilapia burtoni* somatotropes express *gnrh-r2-^{PEY}*, which is phylogenetically related to *Gadus morhua gnrhr1b*. It is therefore possible that Gnrhr1b is involved in growth hormone (Gh) regulation (Leatherland et al., 2010). Crs have been demonstrated in teleost Gh-cells (Kitahashi et al., 2007; Stolte et al., 2008), and both stress and high cortisol levels have been demonstrated to correlate with decreased somatic growth and plasma Gh levels (Farbridge and Leatherland, 1992; Small, 2004). Yet, whether *gnrhr1b* is expressed in cod somatotropes and Gnrhr1b involved in Gh regulation in this species, remains to be determined.

Unlike that of *lhb*, direct effects from cortisol on *fshb* expression was observed in the present study. However, the outcome seemed highly dependent on the stage of sexual maturation of the donor fish. In mature cod, cortisol at stress level enhanced *fshb* transcription. This is in concert with several studies on cortisol exposure performed on rats of both sexes, demonstrating both *in vivo* and *in vitro* that cortisol increases pituitary content of FSH and *FSHβ* mRNA, with and without exogenous GnRH (D' Agostino et al., 1990; Leal et al., 2003; Ringstrom et al., 1991; Suter and Schwartz, 1985b; Suter et al., 1988). Suter and Schwartz (1985a) suggested that the increase in FSH synthesis during a stressful situation might be a way for the animal to rapidly resume reproductive function after a period of stress has ended. However, in the present study, it cannot be ruled out that the increased *fshb* expression is a female cod phenomenon only, due to the skewed sex ratio in the cultures derived from mature fish. Studies from other teleosts indicate a negative effect on Fsh regulation from cortisol. In sexually maturing male common carps, *fshb* transcript levels decreased following cortisol treatment (Goos and Consten, 2002), whereas in immature cinnamon clownfish (*Amphiprion melanopus*), cortisol injection reduced plasma Fsh levels after both short- and long-term treatment (Choi et al., 2017). The present study found negative effects on *fshb* levels in cells derived from maturing and spent fish, the effect appearing more marked in the latter stage. During this spent stage, there is a quiescent period in Atlantic cod gonadal growth, with somatic growth and increasing energy reserves being prioritized (Pedersen and Jobling, 1989). The findings here suggest that in spent cod the recruitment of new gametes into proliferation is prohibited by cortisol through the down-regulation of *fshb*. As this occurred also at basal cortisol levels, it is possible that cortisol at this stage of the reproductive cycle is part of the homeostasis, making sure that the body does not prioritize gonadal recrudescence in times when offspring survival would be harder. The mechanisms at which similar concentrations of cortisol may act as an inhibitor of *fshb* during one stage and a stimulator during another is not established. It seems likely that either additional transcription factors interplay with the Crs at the binding sites of the *fshb* promotor causing activation or repression of the transcript, or that Cr receptors in Fsh cells are differentially regulated through sexual maturation, either in abundance or isoform expressed. The latter is indeed the case in tilapia, where single cell RT-PCR on Fsh cells revealed that while immature males expressed

both *gr1* and *gr2*, mature males expressed only *gr1*, but did so in significantly higher absolute number of *gr1* transcripts than the immature fish (Kitahashi et al., 2007). As the presence, let alone the regulation, of Crs is unknown in the cod pituitary, this would be an interesting topic for future studies.

5. Conclusions

The present study demonstrates that cortisol can exert direct and differential effects on pituitary cell viability and reproduction associated gene expression in an Atlantic cod primary culture system. Basal cortisol levels had no impact on cell viability and affected gene expression only in pituitaries from spent donor fish, while stress cortisol levels stimulated cell viability and affected gene expression at all maturational stages. The negative reproductive effects associated with high-level cortisol in cod is probably not a result of direct interaction with the *lhb* promotor, nor with a downregulation of *gnrhr* expression. However, cortisol can affect reproduction directly at the pituitary level through Fsh synthesis. As this effect was both positive and negative dependent on the reproductive stage of the donor fish, it is likely that some unmeasured elements, such as transcription factors, vary with sexual maturity in the cod gonadotropes and facilitate the action of cortisol.

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Conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.06.017.

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