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Endogenous regulation of 11-deoxycorticosterone (DOC) and corticosteroid receptors (CRs) during rainbow trout early development and the effects of corticosteroids on hatching.

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Abstract :

Clear evidence for a physiological role of the mineralocorticoid-like hormone 11-deoxycorticosterone (DOC) and the mineralocorticoid receptor (MR) in fish is still lacking. Efforts to demonstrate an osmoregulatory role for this hormone has so far not been conclusive, while a few scattered studies have indicated a role for DOC in development and reproduction. In this study, we investigate the onset of *de novo* DOC synthesis in parallel with endogenous corticosteroid receptor mRNA production from fertilization to the swim-up stage in rainbow trout. Whole egg DOC content decreased from fertilization until hatching followed by an increase to pre-fertilization levels just after hatching. Onset of *de novo* transcription of corticosteroid receptor mRNA's was observed shortly after the midblastula transition; initially glucocorticoid receptor 2 (GR2) followed by MR and then GR1. Non-invasive introduction of DOC or cortisol at fertilization resulted in altered corticosteroid receptor regulation and accelerated hatching date, suggesting a regulatory role in trout ontogenesis of both hormones through MR signaling pathway. The results presented in this study suggest a possible physiological role of the DOC-MR signaling pathway during fish ontogenesis, at fertilization and just after hatching.

Highlights

► Large amount of DOC deposited in rainbow trout eggs. ► Onset of *de novo* DOC synthesis in the trout egg just after hatching. ► Onset of *de novo* CRs transcription (GR2<MR<GR1) shortly after midblastula transition. ► Cortisol and DOC treatments accelerate hatching date. ► Cortisol and DOC treatments affect regulation of GR1, GR2 and MR.

Keywords : 11-deoxycorticosterone Cortisol Mineralocorticoid receptor Glucocorticoid receptor Fish Development

1. Introduction

Corticosteroids are a group of cholesterol-derived hormones regulating metabolism, ion homeostasis, and the stress response during the life-span of animals. From their function in tetrapods, corticosteroids are divided in two subgroups; glucocorticoids (cortisol and corticosterone) which govern regulation of growth, metabolism, immune function, behaviour and stress (Mommsen et al. 1999; Young et al. 2004) and mineralocorticoids (aldosterone), which regulates not only hydromineral balance, but also cardiovascular remodelling, fat storage, energy balance and behaviour (reviewed in Viengchareun et al., 2007). This clear division of tasks does not seem to apply to teleost fish, where the absence of aldosterone (Balment and Henderson 1987; Jiang et al. 1998) seemingly leaves cortisol with both the glucocorticoid and the mineralocorticoid functions described in tetrapods. It is well-established that cortisol, besides regulation of classical glucocorticoid tasks, also governs osmoregulatory functions in fish (reviewed by McCormick, 2001). However, the recent identification of a mineralocorticoid receptor (MR) and its potential physiological ligand in fish, 11-deoxycorticosterone (DOC) might reveal a more complicated corticosteroid signaling axis than previously expected (Prunet et al., 2006). Thus, the MR signaling pathway has been suggested to be involved in brain-dependent behavioural and visual responses and also the stress responses (Sakamoto et al., 2016; Kiilerich et al., 2017) but largely not in osmoregulatory functions (McCormick et al., 2008; Takahashi and Sakamoto, 2013; Sakamoto et al., 2016) although conflicting literature exists (Kiilerich et al., 2011a,b,c).

Besides regulation of the important physiological processes described above, cortisol also participates in regulation of major developmental changes. This was initially shown in metamorphosis of larval stages in flounder (de Jesus et al. 1990; de Jesus et al. 1991) and pre-adaptive development of saltwater tolerance in juvenile salmon (Mommsen et al. 1999). More recently, the role of cortisol has been highlighted in the metamorphosis of eels (Rousseau et al.

2008) and skin ionocyte ontogenesis in zebrafish embryos (Cruz et al., 2013; Trayer et al., 2013). Furthermore, Morpholino knock-down of the glucocorticoid receptor (GR) in zebrafish, demonstrate decisive roles of the cortisol-GR axis during embryonic development. The dramatic developmental defects of GR knock-down induced severe malformations of neural, vascular, and visceral organs (Pikulkaew et al., 2011; Nesan et al., 2012). Moreover, increased concentration of cortisol in newly-fertilized eggs caused disruption in cardiogenesis (see review by Nesan and Vijayan, 2012). Taken together, these findings support a major role of cortisol during the developmental program of fish through the GR signaling pathway. In salmonids, artificial elevation of cortisol levels in eggs, either pre- or post-fertilization, has long-lasting consequences on stress responsiveness, behaviour, metabolic physiology, morphology and survival of fry (Eriksen et al., 2007, Auperin and Geslin, 2008, Sloman, 2010; Colson et al. 2015), which may occur through early unknown developmental effects although little is known regarding the signaling pathways.

DOC and MR are also involved in many different physiological processes. For instance, DOC and MR have been associated with spermiation and oocyte maturation in several fish species (reviewed by Milla et al. 2009) and precocious hatching in medaka (Cloud, 1981), t hus linking the mineralocorticoid axis in fish to reproduction and development. However, contrarily to cortisol action, attempts to demonstrate an involvement of MR signaling pathway in ionocyte ontogenesis at the level of skin have been unsuccessful, thus supporting a lack of osmoregulatory role (Cruz et al., 2013; Trayer et al., 2013). Despite these first reports, we are far from having a clear idea of the physiological and developmental roles of the DOC/MR signaling axis.

To examine the contribution of the two corticosteroid (DOC and cortisol) signaling axes in the regulation of embryonic development in rainbow trout, we initially describe the endogenous content of DOC and compare this with the regulation of corticosteroid receptor (CR) transcript levels from fertilization to the fry-stage. Furthermore, we examine the effect of an early increase in DOC and

cortisol levels on gene regulation and hatching. We use the natural uptake of water just after fertilization to transiently boost the cortisol and DOC levels in rainbow trout eggs in a non-invasive manner. Consequences of these hormonal treatments were analysed at the level of endogenous corticosteroid receptor mRNA regulation and by observation of various developmental parameters such as hatching date, egg and fry mortality and the number of malformations.

2. Materials and Methods

2.1 Eggs and fertilization

Eggs and sperm were gently stripped from sexually mature female and male rainbow trout (*Oncorhyncus mykiss*) under anaesthesia (0.05% 2-phenoxyethanol) either at the INRA-LPGP fish facility or at the INRA/SEDI fish farm (Sizun, France) and subsequently transported on ice to INRA-LPGP facility. For each experiment a pool of eggs and sperm were obtained from several female and male rainbow trout and fertilized at 10 ºC for 30 min-1 hour in ActiFish® (IMV, L'Aigle, France) with or without hormones (*i.e.* control, DOC or cortisol, see below for a descriptive protocol). Fertilized embryos were subsequently divided into an appropriate number of incubators (10cm×10cm) supplied with 10 ºC flow-through re-cycled facility water until the end of the experiment. Sampled eggs were quickly frozen and stored at -80 ºC while fry were euthanized with an overdose of 2-phenoxyethanol before sampling and stored at -80 ºC.

2.2 Total embryo DOC content and corticosteroid mRNA during development:

From a total of ~ 5000 fertilized eggs distributed in 4 incubators, 8 pools of 10 eggs (or fry) were randomly sampled for RNA extraction, at each time-point. Ten pools of 20 eggs (or 15 fry)were also sampled at each time-points for DOC extraction. Fry were euthanized with an overdose of 2-phenoxyethanol before sampling to avoid stress effects on the measured parameters. Sampling time-points were as follows: 1 and 17 hour post fertilization (hpf) and 1, 2, 3, 6, 10, 14, 17 (eyed stage), 20, 27, 34 (hatching), 44, 48 and 55 (swim-up stage) day post fertilization (dpf).

2.3 Effect of cortisol and DOC on hatching and CR mRNA regulation during development:

4000 eggs from 3 different females were collected in a glass beaker before adding a mix of sperm from 2 different males. After 5 minutes incubation 500 ml incubation medium (100 ml Actifish® +

400 ml water) containing 55 µM cortisol or DOC (final concentration) or ethanol vehicle (control, final concentration 0.4% EtOH) was added and the fertilized embryos left to take up water and hormone for 30 minutes. This dose of corticosteroids was chosen from a pilot experiment where this was the lowest dose which accelerated hatching. Levels of DOC and cortisol were measured in the eggs between treatment and hatching, ranging respectively from 1000 to 136 ng/g body weight and from 440 to 342 ng/g body weight (data not shown). These hormonal treatments led to a~1000 and ~50-100 fold increase in DOC and cortisol levels, respectively, compared to non-treated eggs. The differences in cortisol and DOC levels in eggs after a similar treatment may be related to lower polarity of DOC of which uptake by eggs should be greater when compared to cortisol. Finally, this dose is lower than the dose (100 µM) used for treating zebrafish embryos by bathing them with synthetic glucocorticoids (Wilson et al., 2013, 2016) and less than that shown to have morphological effects in zebrafish (Hillegass et al., 2008). Eggs from each treatment group were washed several times in facility water before distributing each group in 4 separate incubators for each treatment supplied with flow-through recycled facility water. At 1 hpf, 1, 2, 3, 6, 10, 14, 17, 20, 27, 34, 44, 48 and 55 dpf the number of dead embryos was recorded and removed from the incubators. At 1 hpf, 1, 2, 3, 6, 10, 14, 17, 20, and 27 dpf 6 pools of 10 eggs were sampled for total RNA extraction. A detailed survey of the number of hatched embryos was conducted every day from day 27 to day 37 after fertilization. At the swim-up stage (55 dpf) fry were euthanized with an overdose of 2-phenoxyethanol before counting and visual determination of malformations. Malformations were categorized in groups representing yolk sac resorption defects (YSD), crooked body, small size, cyclopia, prognathia, tail absence, siamese, albino or miscellaneous bodily malformations according to Bonnet et al. (2007) and expressed as a percentage of fry alive at day 55.

2.5 Analyses:

2.5.1 Hormone extraction and DOC measurement

For DOC extraction 20 eggs or 15 larvae were homogenized in 750 µl water using a T18 ULTRA-TURRAX homogenizer (IKA-WERKE, Staufen, Germany) followed by 3 freeze–thaw cycles. Recovery of steroids from the eggs was assessed by addition of 4000 cpm ^{3}H-DOC (American Radiolabelled chemicals, St Louis, MO, USA*)* to the homogenate. Samples were kept at -80 °C until all the samples were ready for extraction. For extraction of steroids samples were kept for 2 hours at room temperature to ensure full thawing of the samples before adding 2 ml ethylacetate/cyclohexane (50/50, vol/vol). The supernatant was removed after freezing at -20 ºC for at least 1 hour followed by another round of ethylacetate/cyclohexane extraction. After ethylacetate/cyclohexane evaporation, extracted steroids were re-dissolved in 60 µl ethanol. 50µl of the steroid-ethanol solution were injected in a HPLC column (Agilent, Massy, France) and passed through a mobile phase containing acetonitrile/water (80/20, v/v) acidified with 0.01% sulphuric acid. The collected organic chromatographic elution fractions were evaporated to dryness and re-dissolved in 250 µl assay buffer (0.01 M NaH_2PO_4, 0.01 M Na_2HPO_4, 0.9% NaCl, and 0.1% gelatine, pH 7.25). The recovery of steroids was assessed by measurement of ^{3}H-DOC in 40 µl of re-dissolved chromatographic fraction with 100 µl ethanol in 2.5 ml scintillation liquid on a Tri-Carb 2100 TR Liquid Scintillation Analyzer (Packard or Perkin-Elmer) resulting in an average recovery of steroids at 24.8% ± 60 SD. The low recovery rate was most likely due to a significant but unavoidable formation of an insoluble vitellogenin-ethylacetate/cyclohexane solid phase containing some of the steroids. Thus, the recovery rate for ^{3}H-DOC in each individual sample was used to calculate the endogenous DOC content in the same sample. Measurement of DOC was done in duplicates using a RIA assay as previously described (Kiilerich et al. 2011c).

2.5.2 Cortisol measurement

Extraction and measurement of cortisol were carried out as described by Auperin and Geslin (2008). Recovery of cortisol was assessed with ^{3}H-cortisol using a similar protocol as for DOC and was estimated to 69% ± 12 SD. As this parameter was quite stable between samples, we did not adjust each individual sample for cortisol recovery rate.

2.5.3 RNA extraction and qPCR

The 10 eggs collected at each time-point were homogenized in 4 ml TRIzol (Invitrogen, Carlsbad, CA, USA) and RNA extracted using a high-salt precipitation step according to manufacturers' recommendations. Concentration and purity of the RNA was assessed using A260/A280 and A260/A320 measurements on a Nanodrop device (Thermo Scientific, Wilmington, DE, USA). RNA quality was assessed on selected samples using a BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). 1.5 µg RNA were subjected to DNase treatment (0.5 units RQ1 DNase (Promega) per sample incubated at 37 ºC for 30 min) before it was used for cDNA synthesis with 1 µg random primers (Promega, Madison, WI, USA), 0.5 mM dNTPs (Promega), 200 units MMLV reverse transcriptase (Promega) in the presence of 25 units RNasin (Promega) in a total volume of 25 µl. After denaturation of the RNA at 70 ºC for 5 min, reverse transcription (RT) conditions were 30 ºC for 10 min, 37 ºC for 1 hour and 95 ºC for 10 min. RT reactions were randomly distributed in two 96 well plates and diluted 3 times in water to a total volume of 75 µl after completion of the cDNA synthesis. For qPCR 1 µl diluted cDNA were added to 10 µl Fast SYBR® Green Master mix (Applied Biosystems, Carlsbad, CA, USA), 250 nM forward and 250 nM reverse primer in a total volume of 20 µl. Cycling conditions were 95 ºC for 20 seconds followed by 40 cycles of 95 ºC for 3 seconds and 60 ºC for 30 seconds on a Step-One Plus real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA). Melting curve analysis was routinely carried out for each qPCR run with 0.3 ºC intervals from 65 to 90 ºC.

2.5.4 Normalization, amplification efficiency and absolute quantification

18S rRNA was used as normalization gene using 1 µl of 1/1000 diluted cDNA with identical QPCR conditions as described above. Amplification efficiency was determined on each plate for each primer-set. 2 µl undiluted cDNA from each sample were pooled and diluted 2, 4, 8, 16 and 32 times in duplicates. For 18S rRNA a dilution series of 500, 1000, 1500 and 2000 was used. The Pfaffl equation was used to calculate the relative copy number of the target and normalization genes and normalized values were obtained by dividing the relative levels of the target gene with the relative levels of the normalization gene.

An absolute quantification of GR1, GR2 and MR primers were previously described in Kiilerich et al. (2011c) allowing a direct comparison of mRNA levels among these three primer-sets.

2.5.5 Primers

To ensure specific detection of GR1, GR2 and MR primers were designed against the A/B-domain and the QPCR product were subjected to gel electrophoresis to verify amplification of only one PCR product. Primer sequences (5´-3´) are as follows: GR1 forward: CCATCGTCAAGCGGGAAGAG, GR1 reverse: GGAACTCCACGCTAAGGGATTTATTC (GenBank Acc. No. Z54210), GR2 forward: CTCCGCTTTCTCCAGCAGCTA, GR2 reverse: GTGAGCCACCCCGTAGTGACAG (GenBank Acc. No. AY495372), MR forward: GAAACAGATGATCCGCGTGGT, MR reverse: TGGATCAGGGTGATTTGGTCCT (GenBank Acc. No. AF209873), 11β-hydroxysteroid dehydrogenase 2 forward: AAGGGACGCATCGTCACAATCT, 11β-hydroxysteroid dehydrogenase 2 reverse: AACAGGTTGAGAGCTGCCTTGG (GenBank Acc. No. AB104415), StAR forward: ACATGGAGCAGATGGGAGAC, StAR reverse: GCACAGCGAACACTAACGAA, (GenBank Acc. No. NM_001124202), 3β-HSD forward: AGAAGCCTATCCTGCCCATC, 3β-HSD reverse:

CATGTCCCTCTGAGCCCTCCG, (GenBank Acc. No. S72665), CYP21 forward: CGTGTCTCTATGGGGCTCTC, CYP21 reverse: GGCTTCCTCTGATGTTTTGC, (GenBank Acc. No. EU246942), 18S rRNA forward: CGGAGGTTCGAAGACGATCA, 18S rRNA reverse: TCGCTAGTTGGCATCGTTTATG (GenBank Acc. No. AF309412). All primers were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.5.6 Statistical analysis

Whole-egg DOC content and QPCR data on natural variations in CR's and corticosteroid synthesis enzymes were analyzed with a one-way ANOVA followed by multiple t-tests with Bonferroni correction. Effects on hatching date was analysed using a loglogistic model (similarly to Geffroy and Simon, 2013):

$$y = 0 + [(1 - 0) / [1 + \exp(b(\log(x) - \log(e))]],$$

where b is the slope of the curve around the point f inflexion (e); e is the time required to reduce hatching by 50% (HT50; i.e. point of inflexion); x is the time and y is the response (hatching). The HT50 determined by the equation allowed comparing curves while considering all replicates within each treatment group, at each time. Data were then compared using a Wilcoxon test, and p values were adjusted using the Holm method.

The regulation of corticosteroid receptors after hormone treatment were analyzed with two-way ANOVAs (with treatment and time as factors) followed by Bonferroni corrected pairwise comparisons at each time-point. Differences were considered significant when $p<0.05$. Statistical analyses were carried out using Prism 4.03 (GraphPad Software Inc., La Jolla, CA, USA). Hatching time modelling was done using the add-on package adrc (Ritz and Streibig, 2005) of the R software (R Development Core Team 2009).

3. Results

3.1 Whole embryo corticosteroid synthesizing capacity during early development

Endogenous production of DOC from its cholesterol precursor is dependent on transcription, translation and activity of a subset of proteins involved in the steroid synthesis, specifically steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 21-hydroxylase (CYP21). qPCR analyses of these three enzymes show that the capacity to synthesize DOC is present before hatching, at least at the transcript level (fig. 1). While there is a steady increase from fertilization to hatching, interrupted only by a sharp peak at 17 dpf (emergence of eyed eggs) of 3β-HSD and CYP21 (fig. 1B, C) mRNA levels, the regulation of StAR appears different (fig. 1A). Maternal StAR transcripts are maintained at the fertilization level until the onset of endogenous transcriptional activity at the midblastula transition (MBT, 2dpf), whereafter the mRNA level significantly decrease until it becomes undetectable by qPCR at 10 dpf. Endogenous StAR mRNA transcription was initiated just after the eyed stage and increased onwards to the swim-up stage.

3.2 Corticosteroid receptors and 11β-HSD2 mRNA levels during embryonic development

At 1 hpf, GR2 is the highest expressed CR compared to GR1 and MR, while the latter are expressed at similar levels (absolute expression levels in fig. 2A, B and C: 8.0±1.28 (GR1), 29.6±4.75 (GR2) and 7.1±0.94 (MR) $p<0.0001$). GR2 is the first CR transcript to be endogenously transcribed, just after the midblastula transition (MBT) around 2 days post fertilization (dpf, fig. 2C), although the difference is significant only at 17 dpf. A maximum of a four-fold induction compared to 1 hpf is reached at 17 dpf at the eyed stage, after which the GR2 level decreases and stabilizes at a lower level throughout the rest of the time-course (figure 2C). MR is the second CR to be *de novo* transcribed in the eggs: There is an initial pronounced increase in egg MR content between 3 dpf

and 10 dpf after which the MR level steadily increases towards a 13.5-fold expression level at 48 dpf as compared to 1 hpf – one week before total yolk-sac resorption (fig. 2A). Finally, GR1 transcription is initiated after 10 dpf, and becomes significantly different from the basal level (roughly between 1 hpf and 10 dpf) at the eyed egg stage at 17 dpf (fig. 2B). After a minute decrease, GR1 levels increase again from hatching to 48 dpf, reaching a maximum of an 8-fold induction (fig. 2B). An increase in the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme mRNA occurs just before hatching, between 27 and 34 dpf, and steadily increases towards 55 dpf at the swim-up stage (fig. 2D).

3.3 Whole embryo DOC content from fertilization to hatching stage

High levels of DOC were present in the eggs just after fertilization (131 pg/egg, fig. 3). From fertilization until hatching a slow and constant decrease in whole embryo DOC content, reflecting usage and/or breakdown of maternal DOC, was observed. After reaching the lowest level at day 34 (45 pg/egg just immediately before hatching), a significant increase and a peak in DOC content (up to 271 pg/egg just after hatching) is followed by a stabilization of the DOC level (at 173 pg/egg) towards the swim-up stage (fig. 3).

3.4 Early incubation with cortisol and DOC affect gene expression and hatching date of rainbow trout eggs

Treatment of eggs with DOC or cortisol at fertilization affected the transcript levels of all three corticosteroid receptors. Interestingly, the effect was not observed immediately after treatment, but first from day 14 to 27 post fertilization (figure 4) for both treatments. DOC treatment resulted in an up-regulation of MR and GR1 levels at 20 and 27 dpf (figure 4A, B), but a down-regulation of GR2 levels at 14 dpf followed by up-regulation at 17, 20 and 27 dpf (figure 4C). Cortisol caused up-

regulation of MR, GR1 and GR2 levels at 27 dpf and a down-regulation of MR at 14 dpf. The effect of DOC was only significantly different from that of cortisol at 20 dpf for all three CR transcripts.

The early boost of egg hormone content did not affect wet weight of the eggs and fry, the mortality during the sampling time-course (not shown) nor the number of malformed fry at the end of the experiment at 55 dpf (fig. 5A). Fry malformations were visually assessed and categorized into 9 groups, where yolk sac resorption defects (YSD), torsions of the body, and prognathia were the most frequent. Miscellaneous bodily malformations were grouped together with other relatively rare malformations such as small size, cyclopia, tail absence, siamese and albino.

Both DOC and cortisol treatment at fertilization significantly accelerated hatching (HT50 DOC: 31.47 vs Control: 32.16, p-value = 0.02; HT50 Cortisol: 31.87 vs Control: 32.16, p-value = 0.03 fig. 5B). Furthermore, DOC treatment also tended to result in accelerated hatching when compared to cortisol treatment (p-value = 0.06, fig. 5B).

4. Discussion

The aim of this study was to describe and investigate the roles of the corticosteroid signaling system in rainbow trout embryonic development in order to clarify the role of the mineralocorticoid-like axis, DOC and MR. A detailed time-course study of the corticosteroid signaling elements is presented during rainbow trout early development from fertilization to the swim-up stage on the level of mRNA transcription of MR, GR1, GR2 and 11β-HSD2, along with the protein involved in DOC synthesis, i.e. StAR, 3β-HSD and CYP21 and onset of *de novo* synthesis of DOC. Overall, these data support the involvement of cortisol and GR signaling axis in trout early development and suggest that the MR/DOC system may also play a role during embryonic development, although the functional role of this system still remain to be clarified. The effect of exogenous corticosteroids (cortisol or DOC) on regulation of the corticosteroid receptors (GR1, GR2 and MR) and timing of hatching bring some new elements of response regarding the role of both cortisol and DOC.

4.1 Role of DOC in rainbow trout early development

Here we show, for the first time in fish, the course of whole egg DOC content from fertilization to the swim-up stage. First of all, a large amount of maternal produced DOC is deposited in the eggs before ovulation, which is then gradually broken down during the early parts of embryogenesis to reach as low a level as ~4.5 pg/individual just before hatching. Interestingly, a ~60-fold increase in whole egg DOC levels – reaching fertilization levels – is seen just after hatching and sustained until the swim-up stage. Based on the regulation of DOC synthesis enzymes, StAR, 3β-HSD and 21-hydroxylase *de novo* DOC synthesis is possible from day 20 post fertilization, however DOC levels first increase between day 34 to 44 – coincident with hatching. Since these enzymes are also involved in cortisol synthesis pathway, the ability of this enzymatic system to be involved in

cortisol production in trout eggs has also to be considered. Actually, Auperin and Geslin (2008) have observed a decrease in whole body cortisol level from fecundation until eyed stage but followed by an increase in cortisol levels in trout eggs at 27 dpf indicating capability of eggs to synthetize this corticosteroid at least 6 days before hatching. Such picture observed in rainbow trout embryo-larvae stages appeared significantly different from what has been reported in zebrafish where both cortisol levels and steroidogenic enzyme gene expressions increased after hatching (Alsop and Vijayan, 2008). Our data on DOC levels during embryogenesis suggest that DOC may be important not only during early development in trout embryos but also after hatching in relation with the organization of aquatic life.

4.2 Regulation of the corticosteroid receptors

The detailed time-course conducted here show differential regulation of the three corticosteroid receptors, MR, GR1 and GR2, in parallel with 11β-HSD2 during trout embryonic development. Based on the chronology of which the CR´s are *de novo* transcribed, GR2 (at 3 dpf) and MR (at 6 dpf) are suggested to be important transcription factors in the very early parts of embryogenesis just after mid-blastula transition, whereas this is probably not the case for GR1 (17 dpf). These early changes in GR2 and MR expression also correlates with high DOC and cortisol levels as previously discussed. Additionally, the higher GR2 mRNA level compared to GR1 and MR suggest that GR2 is the most important glucocorticoid receptor during the developmental time-course presented here, especially at the beginning of *de novo* transcription at the MBT and emergence of eyed eggs. However, while GR2 exhibits the highest expression level at fertilization, a greater fold increase of MR during the days following mid-blastula transition suggests some importance of signaling through this receptor as well. Altogether, these data support the role of cortisol and GR signaling in developmental programming and suggest that the MR signaling pathway may also be involved in

the regulation of these biological processes in rainbow trout. These conclusions based on measurements of mRNA abundance should be confirmed by measurement of protein levels.

The increase in corticosteroid receptor (GR and MR) levels during embryonic development has also been investigated in other fish species. An overall up-regulation of MR from fertilization until hatching has been observed in zebrafish (Alsop and Vijayan, 2008; Pikulkaew et al. 2010) but not in medaka (Trayer et al., 2013). Concerning GR expression in fish embryos, various contradictory patterns have been described in the literature: In zebrafish where a single GR has been identified (Schaaf et al. 2008), a decrease from fertilization until hatching was followed by an increase in GR mRNA levels until the feeding stage (Alsop and Vijayan 2008; Pikulkaew et al. 2010). In medaka where 2 GR forms have been described (Kim et al., 2011), a similar decrease of GR1 and GR2 transcript levels within 36 hours after fertilization has been observed by Trayer et al. (2013). This is somewhat discrepant with what has been reported in the present study on trout. On the other hand, the up-regulation of GR1 in trout eggs around the eyed stage does not coincide with the up-regulation of GR1 after hatching in sea bass (Pavlidis et al. 2011), and is directly contradictory to a decrease described in tilapia (Tagawa et al. 1997). Concerning GR2, the up-regulation of mRNA levels at the midblastula transition (MBT) was not observed in sea bass, where there was no significant regulation of GR2 mRNA from the embryo to the juvenile stage (Pavlidis et al. 2011). Variations between these studies and the present study most likely reside in species specific developmental differences and/or differences in sampling time-points.

4.3 Physiological role of the corticosteroid signaling systems

Presence of DOC and its mineralocorticoid receptor mRNA at significant levels in trout eggs suggests that the mineralocorticoid signaling system is active during embryogenesis and may be involved in regulation of developmental processes in rainbow trout. Moreover, our data also

support the perception that glucocorticoid signaling (cortisol/GR1, cortisol/GR2) is involved in such regulation, in agreement with previous studies conducted in zebrafish (Pikulkaew et al., 2011; Nesan and Vijayan, 2012). With whole egg DOC levels ranging between 0.2-6.5 nM (volume of an average trout egg is 62.77 ± 14.49 mm^3 (Bonislawska et al. 2001)) and an MR EC_{50} value of 0.1 nM (Sturm et al. 2005), activation of MR by DOC is theoretically possible at all stages of development, assuming that the hormone is equally distributed in the egg with no compartmentalization.

However, based on the respective affinities of cortisol and DOC for MR (Sturm et al., 2005), one could anticipate that DOC may be competing with cortisol for MR binding as both hormones are present in the egg at the same time. DOC activation of MR is possible when cortisol levels are less than 10-fold compared to DOC, since MR has a 10-fold lower EC_{50} value for DOC than cortisol (Sturm et al. 2005) – at least *in vitro*. When comparing cortisol (Auperin and Geslin, 2008) and DOC (present study) concentrations in trout eggs, we find ~3-4 times less DOC than cortisol between fertilization and day 20 whereas this ratio increase from day 27 until hatching on day 34. Finally, after hatching this ratio decreases to 3-6 fold difference because of a large increase in DOC levels on day 44 and later. These data may suggest that DOC could be important during the first phases of embryogenesis and in the late phases before hatching. Interestingly, another important factor also to be considered is *de novo* transcription of the MR-protective enzyme 11β-HSD2, which is responsible for cortisol degradation and most likely ensure DOC signaling through MR in the presence of cortisol. Our data on 11β-HSD2 expression from the present study (fig.2) suggest that this enzyme activity may only be present just after hatching and later, coinciding with increase in cortisol levels (Auperin and Geslin, 2008) but also with a large increase in DOC levels (present study). This supports a possible role of the DOC-MR axis in the regulation of development after hatching. Such hypothesis has already been suggested by Alsop and Vijayan (2008) for regulation of zebrafish embryogenesis. Overall, these results suggest a possible involvement of DOC-MR

pathway in the regulation of early embryogenesis and later during the larval development phase. Regarding glucocorticoid receptors the relatively low concentrations of cortisol in the egg (ranging from 10-25 nM (Auperin and Geslin, 2008)) favor cortisol signaling through GR2 compared to GR1 (EC_{50} value: 0.7 and 46 nM, respectively (Bury et al. 2003)). This is consistent with the very early up-regulation of GR2 after the MBT and a significantly higher mRNA level of this receptor compared to GR1. However, it is worth noting that the increase in GR1 mRNA from hatching at 34 dpf to 48 dpf (one week before complete yolk sac resorption) is coincident with emergence of a functional HPI-mediated stress-response in rainbow trout (Auperin and Geslin, 2008), suggesting GR1 may somehow partake in establishment and maturation of a stress-responsive feedback mechanism in the developing larvae.

4.4 Effect of increased cortisol and DOC on hatching

The possible role of DOC and cortisol on hatching was investigated by transiently boosting the *in ovo* corticosteroid levels at fertilization Despite the very large increase in cortisol (~7.5 fold) or DOC (from ~150 to ~20 fold) after treatment at fertilization which stay high until hatching, we did not see any morphological defects or increases in mortality after treatment. This is in agreement with previous studies where rainbow trout eggs transiently exposed to cortisol around fertilization did not induce detectable developmental defects (Auperin and Geslin, 2008; Sloman, 2010; Li et al. 2010). Higher doses of synthetic corticosteroid (dexamethasone) have also been used in zebrafish without inducing gross morphological defects (Wilson et al., 2013, 2016). However, the method used to treat fish eggs is important as other studies have shown significant morphological developmental defects after exposure to either pharmacological high cortisol levels (Li et al. 2010) or chronic cortisol treatment (Hillegass et al. 2008) probably reflecting abnormal activation/inactivation of the cortisol-GR signaling system.

Our data not only confirm the accelerating effect of cortisol treatment on hatching but show that DOC has similar effect. As we did not observe similar levels of DOC and cortisol in eggs following the same treatment, it is difficult to extrapolate if one corticosteroid is more efficient or not compared to the other. This interesting observation lead us to suggest that the MR signaling system is probably involved in this effect of both corticosteroid hormones as DOC cannot activate GR receptors (Bury et al., 2003). However, as low levels of DOC have been observed at hatching (see figure 3), we suggest that cortisol is probably the corticosteroid which binds and activates MR at that period. The acceleration of hatching in trout eggs after DOC treatment observed in the present study is in agreement with one previous study in medaka, where DOC treatment during egg development resulted in a decrease of hatching time regardless of the time of DOC exposure (0, 4, 8 dpf, Cloud, 1981). This led the author to suggest that DOC probably induced precocious release of hatching enzyme from the medaka larvae around hatching. Similarly, cortisol treatment was shown to accelerate hatching time in damselfish, leading to the suggestion that cortisol affected embryonic development rhythms and thereby inducing changes in the timing of hatching (Gagliano and McCormick, 2009). In zebrafish and medaka, augmenting endogenous corticosteroid levels by treating eggs with dexamethasone or cortisol advanced time of hatching, an effect which was shown to involve GR signaling pathway (Wilson et al., 2013, 2016; Nesan and Vijayan, 2013; Trayer et al., 2013). Therefore, hatching appears to be modulated in several fish species by corticosteroid hormones (cortisol and/or DOC) which underlies their physiological relevance during embryonic development.

4.5 Effect of increased cortisol and DOC on CR mRNA regulation

Treatment of trout eggs with corticosteroid hormones at fertilization did not immediately affect corticosteroid receptor transcript levels (GR1, GR2 and MR) - effects of cortisol and DOC were firstly seen 2-3 weeks after exposure for all three transcripts. The more pronounced effect of DOC

compared to cortisol on CR expressions may be related to higher DOC levels in eggs after treatment as previously discussed. Similarly to what has been discussed in the previous paragraph, we suggest that these effects on CR expression may be mediated by MR signaling pathway. This also suggests a possible connection between the high hormone levels of maternal origin at fertilization which lasts until hatching, altered regulation of corticosteroid receptors 3 weeks after mid-blastula transition, and acceleration of hatching date. Deciphering these complex regulations would require further studies which have not been developed in the present study.

5. Conclusion

This study is the first to describe presence of significant levels of DOC and the onset of *de novo* DOC synthesis in fish eggs. High DOC levels at fertilization and also just after hatching associated with significant 11β-HSD2 expression, together with significant MR transcript levels after the mid-blastula transition and onwards presented here, suggest a possible physiological role for the DOC-MR signaling pathway during fish ontogenesis. However, we cannot exclude the possibility that cortisol may be also involved in the activity of the MR signaling pathway - especially after the eyed stage. Both DOC and cortisol possess the potential of regulating hatching of trout eggs which underlines the physiological relevance of the MR signaling pathway in the regulation of embryonic development. Whether these effects of DOC and cortisol on hatching are related to a global acceleration of the developmental program in trout embryos or/and the regulation of hatching enzyme synthesis or activity (see review by Yamagami, 1996) would deserve further studies. Additionally, we also observe substantial regulation of GR1 and GR2 transcription during development from fertilization to hatching and effects of cortisol on regulation of CRs, although less potent compared DOC-MR. Thus, the results presented in this study, lead us to suggest that the DOC-MR signaling pathway is a functional and relevant member of the corticosteroid signaling

system in fish aside the more established cortisol-GR axis. In conclusion, both DOC and cortisol possess the potential of regulating hatching of trout eggs which underlines the physiological relevance of the corticosteroids in embryonic development. Whether these effects of DOC and cortisol on hatching are related to a global acceleration of the developmental program in trout embryos or/and the regulation of hatching enzyme synthesis or activity (see review by Yamagami, 1996) would deserve further studies.

6. Declaration of interests

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

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Figure legends

Figure 1: Whole egg mRNA transcript levels of proteins involved in DOC synthesis : (A) StAR (steroidogenic acute regulatory protein), (B) 3β-HSD (3β-hydroxysteroid dehydrogenase) and (C) CYP21 (21-hydroxylase). Overall effect of time was detected by one-way ANOVA for all transcripts ($p<0.0001$). Values with no letters in common are significantly different as determined by multiple t-tests with Bonferroni correction (mean±SEM, n=8).

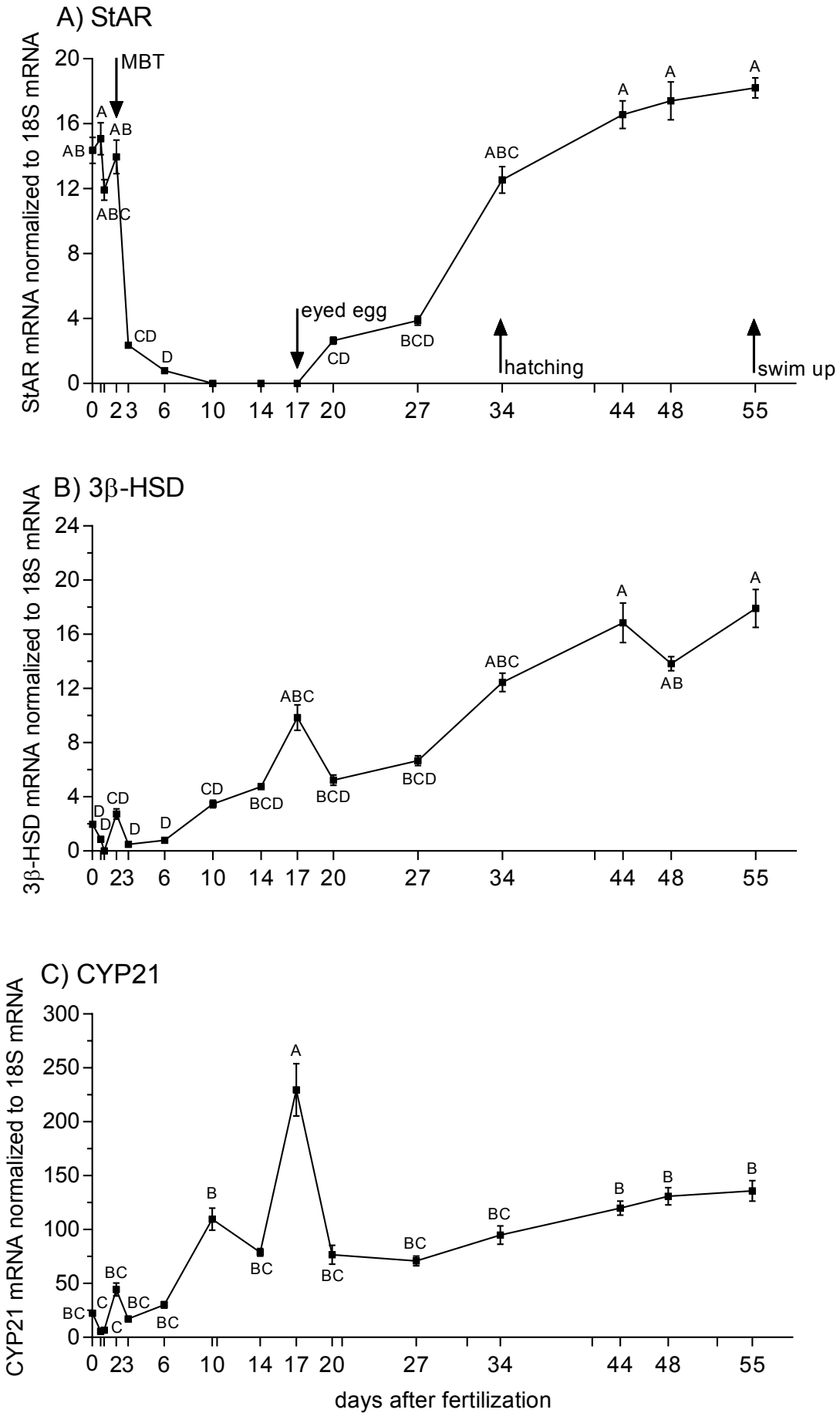
Figure 2: Whole egg mRNA transcript levels of (A) MR, (B) GR1, (C) GR2 and (D) 11β-HSD2 from fertilization to the swim-up stage. Overall effect of time was detected by one-way ANOVA for MR, GR1, 11β-HSD2 ($p<0.0001$) and GR2 ($p<0.0005$). Values with no letters in common are significantly different as determined by multiple t-tests with Bonferroni correction (mean±SEM, n=8).

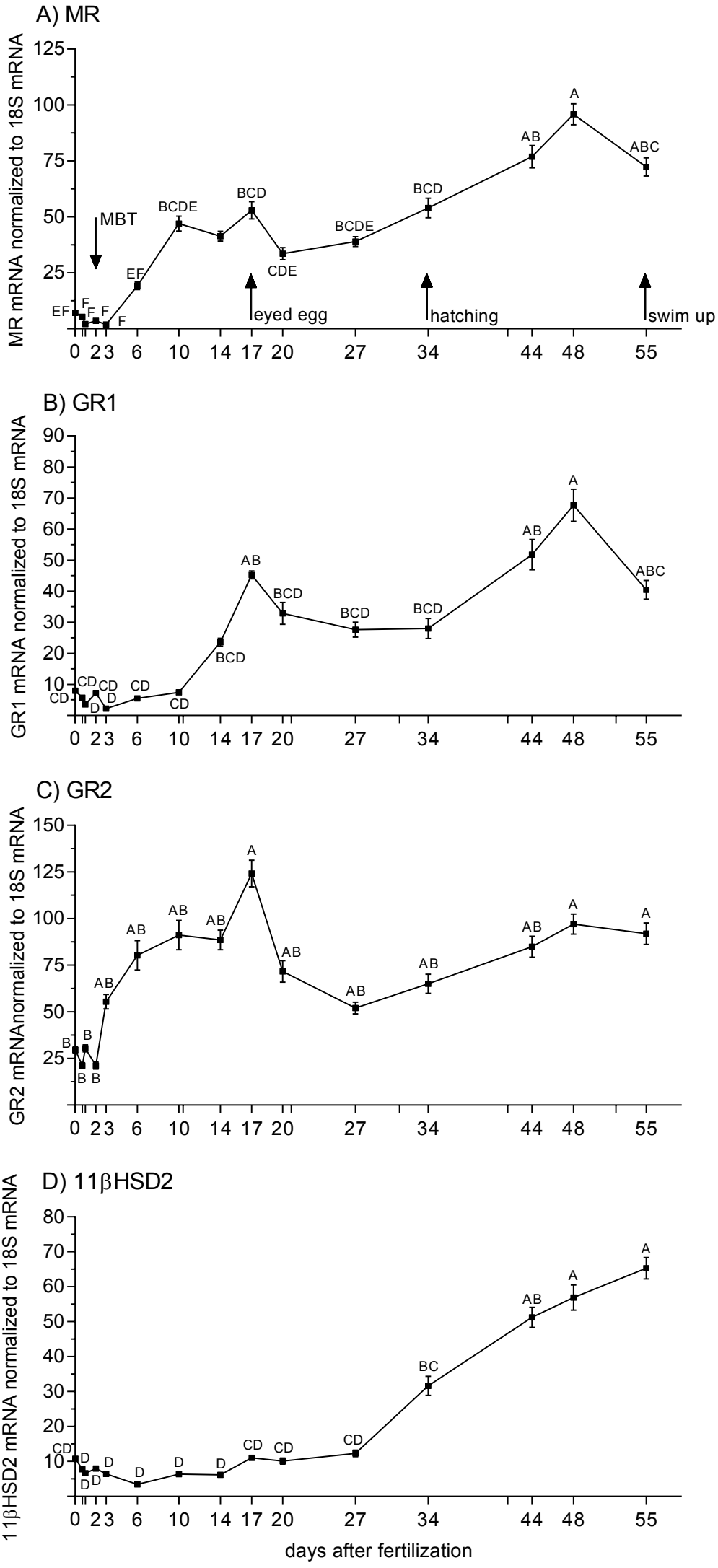
Figure 3: Whole egg DOC concentration during embryogenesis from fertilization to the swim-up stage (mean±SEM, n=10). There is an overall effect on time ($p<0.0001$) as detected by one-way ANOVA and values with no letters in common are significantly different as determined by multiple t-tests with Bonferroni correction. MBT – midblastula transition.

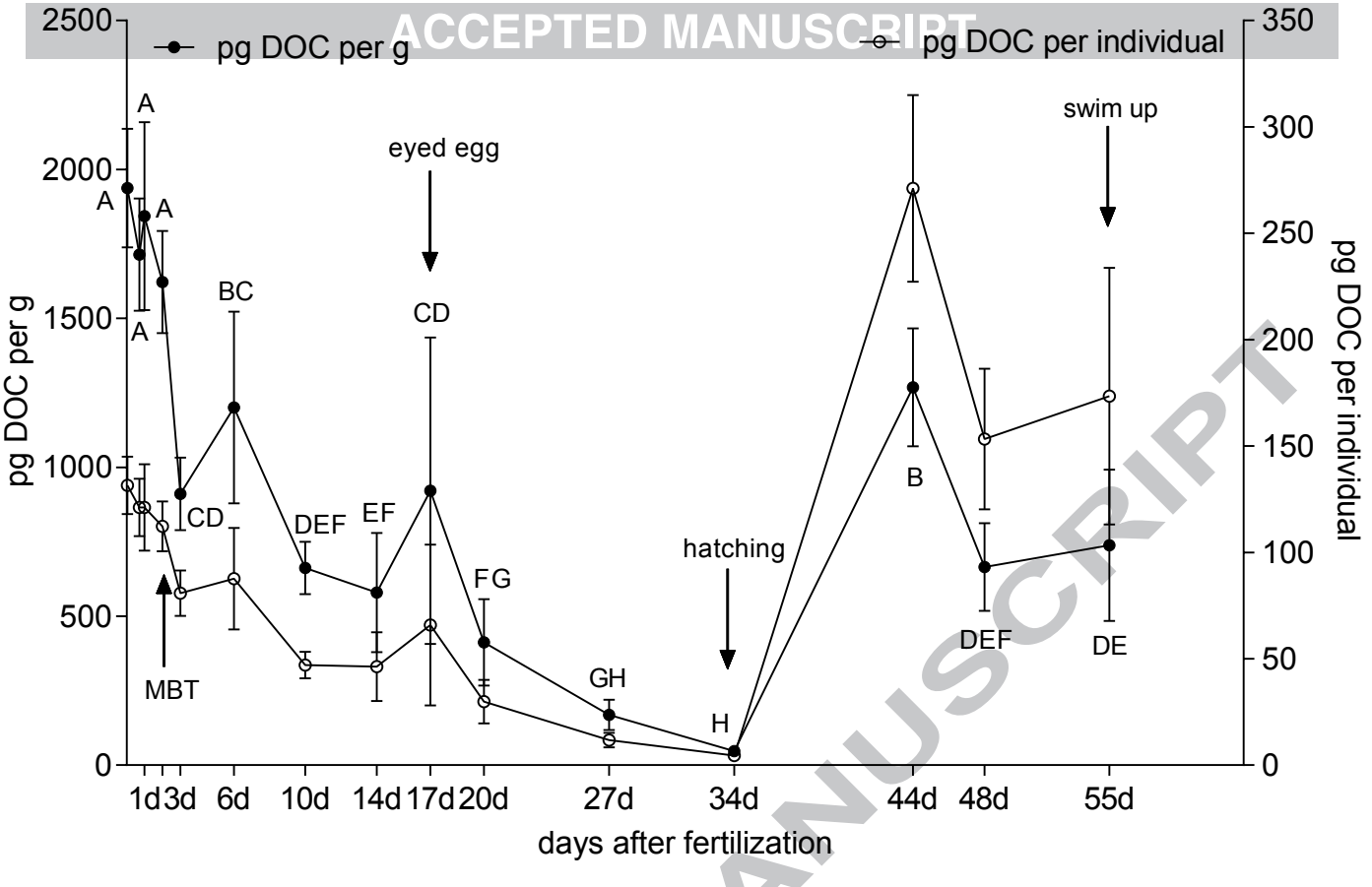
Figure 4: Regulation of (A) MR, (B) GR1 and (C) GR2 transcript levels from fertilization to day 27 after *in ovo* DOC or cortisol treatment. There is no overall significant effect of DOC or cortisol treatment on MR transcript levels as determined by two-way ANOVA but there was a significant interaction between treatment and time which led us to carry out Bonferroni corrected pairwise comparisons at each time point. Overall significant effect of DOC and cortisol on GR1 transcript levels ($p<0.0005$ and $p<0.05$, respectively) was detected along with an overall effect of DOC on

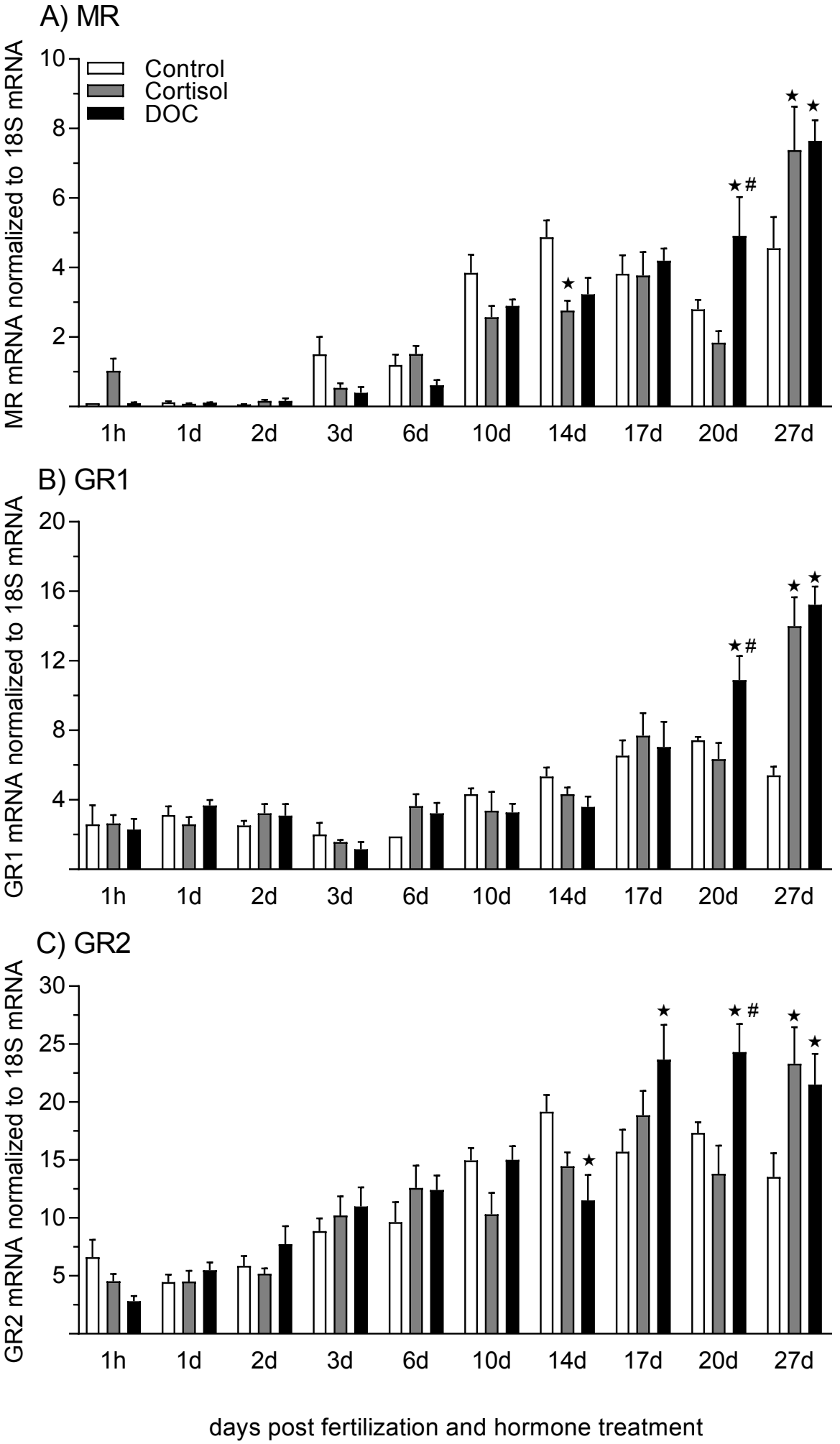
GR2 transcript levels ($p<0.05$) and significant difference between DOC and cortisol treatment for GR2 ($p<0.05$) (mean±SEM, n=8). A star denotes significant difference from control and # denotes significant difference between cortisol and DOC groups at different time points as determined by Bonferroni corrected pairwise comparisons.

Figure 5: Number of malformations at the swim-up stage and survey of hatching date after hormone treatment at fertilization. (A) The percentage of malformed fry at the swim-up stage (55 dpf) in DOC and cortisol treated eggs. YSD – yolk sac resorption defects. There was no effect of either treatment compared to control as determined by two-way ANOVA ($p>0.05$) (mean±SEM, n=4). (B) Effect of DOC and cortisol on hatching date is depicted as percentage of the total number of hatched embryos. There is an overall effect of DOC ($p<0.0001$) and cortisol ($p<0.0001$) compared to control and an overall effect of DOC compared to cortisol ($p<0.0001$) as determined by two-way ANOVA. A star denotes significant difference from control and # denotes significant difference between cortisol and DOC groups as determined by multiple t-tests with Bonferroni correction (mean±SEM, n=4).

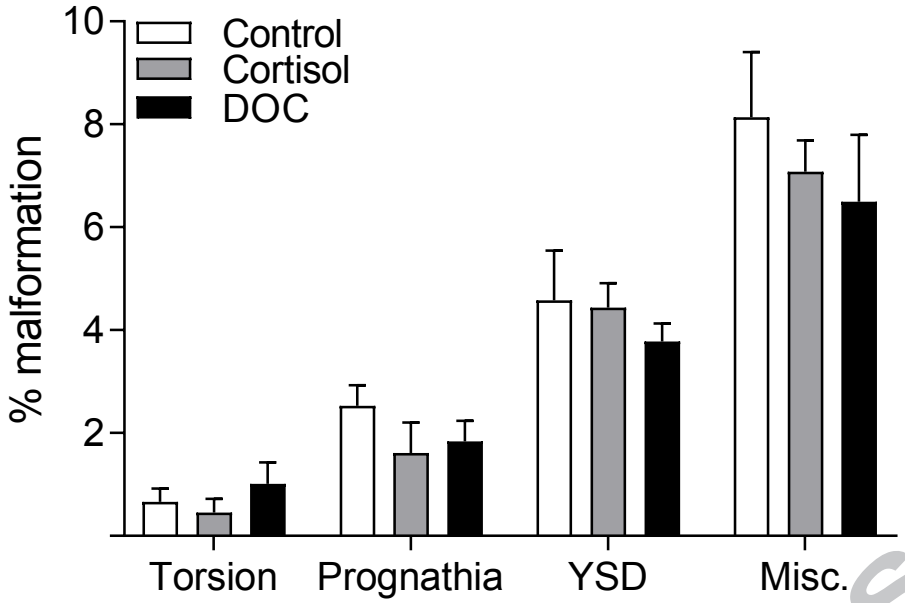

A) StAR
StAR mRNA normalized to 18S mRNA
MBT
eyed egg
hatching
swim up
B) 3β-HSD
3β-HSD mRNA normalized to 18S mRNA
C) CYP21
CYP21 mRNA normalized to 18S mRNA
days after fertilization


A) MR
MR mRNA normalized to 18S mRNA
MBT
eyed egg
hatching
swim up
B) GR1
GR1 mRNA normalized to 18S mRNA
C) GR2
GR2 mRNAnormalized to 18S mRNA
D) 11βHSD2
11βHSD2 mRNA normalized to 18S mRNA
days after fertilization




A) MR
Control
Cortisol
DOC
MR mRNA normalized to 18S mRNA
10
8
6
4
2
1h
1d
2d
3d
6d
10d
14d
17d
20d
27d
B) GR1
GR1 mRNA normalized to 18S mRNA
20
16
12
8
4
C) GR2
GR2 mRNA normalized to 18S mRNA
30
25
20
15
10
5
days post fertilization and hormone treatment

A)



B)