Glucocorticoid receptor activation following elevated oocyte cortisol content is associated with zygote activation, early embryo cell division, and IGF system gene responses in rainbow trout

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Abstract

Increased *in ovo* cortisol content of rainbow trout oocytes from ∼3·5 to ∼5·0 ng.oocyte^{-1} before fertilization enhances the growth of embryos and juveniles and changes the long-term expression pattern of IGF-related genes. This study used embryos reared from oocytes enriched with cortisol and the glucocorticoid receptor (GR) antagonist, RU486, to determine whether the growth-promoting actions of cortisol involve GR protein activation and modulation of *gr* expression. Whole-mount *in situ* immunohisto-fluorescence studies of zygotes showed that enhanced oocyte cortisol increased the immunofluorescent GR signal and activated the relocation of GR from a general distribution throughout the cytoplasm to an accumulation in the peri-nuclear cytoplasm. *In ovo* cortisol treatment increased the number of embryonic cells within 48-h post-fertilization, and RU486 partially suppressed this cortisol stimulation of cell duplication. In addition, there was complex interplay between the expression of *gr* and *igf* system-related genes spatiotemporally in the different treatment groups, suggesting a role for GR in the regulation of the expression of development. Taken together, these findings indicate an essential role for GR in the regulation of epigenomic events in very early embryos that promoted the long-term growth effects of the embryos and juvenile fish. Moreover, the pretreatment of the oocyte with RU486 had a significant suppressive effect on the maternal mRNA transcript number of *gr* and *igf* system-related genes in oocytes and very early stage embryos, suggesting an action of antagonist on the stability of the maternal transcriptome.

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Introduction

Cortisol of maternal origin is present in the oocytes and embryos of several fish species (reviewed by Leatherland *et al.* (2010)), and maternal plasma cortisol levels during the period of oocyte vitellogenesis determine oocyte cortisol content (Eriksen *et al.* 2006, 2007, Veillette *et al.* 2007, Leatherland *et al.* 2010). In salmonid fish, increased oocyte cortisol content brings about changes in the embryonic epigenome, resulting in changes in the phenotype of the progeny (Li *et al.* 2010, 2011, Eriksen *et al.* 2011). In addition to its potency as a regulator of intermediary metabolism in fish (Leatherland 2010, Vijayan *et al.* 2010), cortisol is involved in several aspects of normal gametogenesis and embryogenesis in fish. These include oocyte maturation and hydration (Milla *et al.* 2006, 2009, Leatherland *et al.* 2010, 2012), hatching (Barry *et al.* 1995, Sampath-Kumar *et al.* 1995), sex differentiation (Hayashi *et al.* 2010, Yamaguchi *et al.* 2010), embryo growth (Eriksen *et al.* 2006, 2007, Li *et al.* 2010), and modulation of the embryonic immune response (Li *et al.* 2011). Thus, elevated cortisol levels in the oocytes, and therefore in early embryonic cells, have the potential to interfere profoundly with many aspects of normal embryogenesis (reviewed by Leatherland *et al.* (2010, 2012)).

In mammals, maternal stressor-related increases in cortisol delivery to the embryo have been associated with increases in embryo mortality, smaller progeny, depressed immune function, reduced adrenal and gonadal mass, behavioral and morphological alteration, increased activation of the sympathetic nervous system, various neurological disorders, and impaired stress responses (reviewed by Godfrey *et al.* (2010), Matthews & Phillips (2010) and Hochberg *et al.* (2011)). Far less is known about the actions of cortisol and the effects of maternal cortisol levels on the early development of fish embryos; this study attempts to enhance current knowledge pertaining to the effects of maternal cortisol on early developmental events, particularly actions on the epigenome that determine the juvenile or adult physiological phenotype.

In recent studies, we found that an increase in oocyte cortisol content in rainbow trout (*Oncorhynchus mykiss*) oocytes from ∼3·5 ng.oocyte^{-1} (untreated) to ∼5·0 ng.oocyte^{-1} (cortisol treated) before fertilization resulted in an increase

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in the growth of embryos and adults reared from these oocytes (Li *et al.* 2010). This one-time exposure could not be explained on the basis of increased feeding efficiency or changes in GH secretion, but the growth responses were associated with changes in the patterns of the expression genes encoding insulin-like growth factors (IGFs), GHs, and their receptors. In particular, in the embryos reared from cortisol-enriched oocytes, *igf1* expression was significantly enhanced from the zygote to 21-days post-fertilization (dpf) embryos and *igf2* was enhanced in the 21-dpf embryos (Li *et al.* 2010). The mechanistic link between the one-time exposure of oocytes to elevated cortisol levels to the long-lasting growth promoting action has yet to be established.

This study was undertaken to determine whether these very early embryonic responses to cortisol were associated with the interaction of the hormone with glucocorticoid receptors (GRs; Veleiro *et al.* 2010). GRs, together with *gr* mRNA transcripts, are ubiquitous in the cytosol of post-embryonic cells (reviewed by Leatherland *et al.* (2010), Schreck (2010) and Vijayan *et al.* (2010)). Before ligand activation, the GRs are associated with chaperone molecule complexes (reviewed by Leatherland *et al.* (2010)). Ligand-activated GR dissociates from the chaperone complex and translocates toward the nucleus in association with skeletal microfibrils in the cytosol (Heitzer *et al.* 2007, Fitzsimons *et al.* 2008) the activated GR then enters the nucleus via the nuclear pores. Within the nucleus, the GR interacts with DNA in the promoter region of many genes, acting as a transcription factor. In this study, we examine whether changes in *in ovo* cortisol content in the presence or absence of the steroid receptor antagonist, RU486 (mifepristone), affected GR activation and translocation in the zygote. The study also examined the effects of *in ovo* cortisol (in the presence or absence of RU486) on the rate of early cell division of the embryo and the expression of *gr* and *igf* genes during the period of increased embryo genome activation (Li *et al.* 2007, 2010).

Materials and Methods

Experimental design, collection of gametes, and fertilization process

The University of Guelph Animal Care Committee approved the experimental protocols used in this study. All experiments were conducted at the Alma Aquaculture Research Station (Alma, ON, Canada).

The collection and processing of oocytes for *in ovo* treatments were as described for previous studies (Raine *et al.* 2004, Aluru *et al.* 2010, Li *et al.* 2010, 2011). Milt and naturally ovulated oocytes were collected from five male and five female 3+ year-old rainbow trout (*O. mykiss*), respectively, during the peak ovulatory period (mid-October). For 6 weeks before this, the female fish were examined weekly to determine whether ovulation had occurred, and if so, to harvest the oocytes from the peritoneal cavity of each fish by lateral palpation of the abdomen. Thus, for the mid-October collection, the maximum duration of the retention of the oocytes in the peritoneal cavity was 7 days, which for salmonid fish does not result in biochemical changes that lead to over-ripening and loss of fertility of the oocytes (Lahnsteiner 2000, Bahrekazemi *et al.* 2009).

The oocytes and accompanying ovarian fluid of five fish collected on the same day were pooled and gently mixed together to ensure homogeneity, before randomly allocating equal volumes of oocytes to the treatment groups for a 3-h prefertilization treatment as described below. Following the incubation, an aliquot of the milt pool was added to each group and the oocytes and milt were gently mixed together. A small volume of water was then added and mixed with the oocytes/ovarian fluid to activate the sperm; the excess milt was then rinsed from the mix by introducing a slow stream of water into each container. The eggs were then left undisturbed to allow their zona pellucida to begin the water hardening process. Following water hardening, the fertilized oocytes of each treatment group were randomly assigned among custom-designed Plexiglas wells in Heath incubator trays for rearing of the embryos (three replicates per treatment group).

This protocol was adopted because it introduces some genetic variation in the stock of oocytes before their allocation to different treatments, and it has been found to produce repeatable responses both within and between sampling seasons in studies of factors that affect embryo gene expression (Li *et al.* 2010). For purposes of consistency, this paper describes the results obtained from a single reproductive season (Fall of 2008); however, independent measures of fertilization and hatching rates were observed in two reproductive seasons (Fall of 2008, 2009), and cell counts involving confocal imaging were observed in three reproductive seasons (Fall of 2008, 2009, Spring of 2009). In all cases, the findings from the three reproductive seasons exhibited a similar among-treatment pattern of responses (Li 2011).

Cortisol and RU486 treatments of ovulated oocytes

A pool of ∼10 000 oocytes were divided into four treatment groups of approximately equal size for prefertilization incubation. The oocytes were immersed either in ovarian fluid alone or in ovarian fluid containing cortisol (hydrocortisone; Sigma) with or without RU486 (mifepristone; Sigma) to form the four treatment groups: control (Con), cortisol treatment (Ct), RU486 treatment (RU), and cortisol+RU486 treatment (Ct+RU)). The final concentrations of cortisol and RU486 were 100 and 1000 ng/ml ovarian fluid respectively. The cortisol- and RU486-enriched ovarian fluid was prepared by mixing 0·1% by volume of an ethanol stock solution of the compounds to the ovarian fluid; an equal volume of ethanol alone was added to the ovarian fluid of the control group. Previous studies (Li *et al.* 2010) have shown that incubating rainbow trout oocytes in ovarian

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fluid containing 100 ng/ml cortisol increases the oocyte cortisol content from ∼3·5 ng.oocyte^{-1} in the untreated group to ∼5·0 ng.oocyte^{-1}. The oocyte concentrations of the steroid RU486 could not be measured, but it was assumed that the partitioning of RU486 between the ovarian fluid and the ooplasm was similar to that for cortisol. The concentration of RU486 in the ovarian fluid was in a range that has been shown to have antagonistic effects on GR function in several species of fish (Vijayan *et al*. 1994, Aluru *et al*. 2004, Scott *et al*. 2005, Veillette *et al*. 2007, Vizzini *et al*. 2007, Alderman *et al*. 2012).

The incubations were carried out in the dark for 3 h and the oocytes were then fertilized, water hardened, and aliquots were placed in Heath incubator trays for the rearing of the embryos, as described earlier. Throughout the embryo rearing period, the Heath incubators were supplied with constantly running aerated well water at 8·5 °C. The embryos were reared in the trays until just before the 'swim up' stage (51 dpf) when they were transferred to 0·7 m^3 grow-up tanks for transition from late-stage embryos to exogenously feeding early-stage embryos.

The fertilization rate was calculated at the eyed-stage embryos (26 dpf) and expressed as a percentage of the total number of prefertilization oocytes. The hatching rate was calculated as the number of hatched live embryos (38 dpf) as a percentage of the total number of prefertilization eggs. The mortality rates were calculated by counting the surviving embryos at 51 dpf (swim up stage) and 62 dpf (just before first feeding); the values are expressed as the percentage of the total number of hatched live embryos.

Whole-mount in situ *immunostaining of GR protein in zygotes*

Zygotes collected at 2-h post-fertilization (hpf) were fixed in 4% paraformaldehyde in PBS (pH 7·5) for 4–6 h, washed several times in PBS, and maintained in PBS at 4 °C for whole-mount *in situ* immunohistochemical staining, which was carried out within 14 days of fixation.

The zona pellucida of each 2-hpf zygotes was removed before the whole-mount *in situ* GR immunofluorescence staining, and the denuded zygotes were placed in 72-well microplates (Nalge Nunc, Rochester, NY, USA). The protocol used was that described previously by Madan *et al*. (2007). Briefly, the denuded zygotes were incubated at room temperature for 1 h in 5% donkey serum in PBS containing 0·01% Triton X-100 to eliminate nonspecific antibody binding and then washed twice in 1% donkey serum in PBS containing 0·005% Triton X-100 for 30 min in each at 37 °C. The primary GR antibody used was anti-trout GR IgG serum raised in rabbits (Sathiyaa & Vijayan 2003). This antibody has been proven to be an effective GR anti-serum in rainbow trout (Vijayan *et al*. 2003, Aluru *et al*. 2004, Gravel & Vijayan 2006). The antibody was used at a 1:500 dilution in PBS. The denuded zygotes were incubated overnight at 4 °C; negative controls were incubated in PBS alone. The samples were then washed three times for 30 min at 37 °C in PBS, followed by incubation in a secondary antibody (donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (FITC; Invitrogen)) for 1 h at 37 °C. The denuded zygotes were then washed twice in PBS in darkness at 37 °C and treated with 4,6-diamidino-2-phenylindole (DAPI; 1:2000) for 20–30 min, followed by three 20- to 30-min washes in PBS. Finally, the denuded zygotes were mounted on slides in Vectashield mounting medium (Fluorguard) for fluorescence staining (Vector Laboratories, Inc., CA, USA); from this point, the zygotes were protected from light. Fluorescence images were taken using a Zeiss LSM 410 confocal microscope (Olympus Canada, Markham, ON, Canada; 10–40× objective magnification at 405 nm wavelength (for DAPI) and 488 nm wavelength (for FITC)). The images were then captured and stored as TIFF files; Z-stack images by Z-scan were saved as Multiview TIFF files and analyzed by the Zeiss LSM Software package.

Cell counts in early embryos

Paraformaldehyde-fixed embryos from 18 to 48 hpf were placed in 72-well microplates, washed several times in PBS, and incubated in DAPI (0·5 μg/ml); the incubation was carried out at room temperature, in the dark, for 20–30 min depending on the size of the embryo. The embryos were then washed several times in PBS to remove background staining and mounted on slides. Images of the DAPI-stained embryos were taken under a u.v. microscope, and the number of embryonic cells or nucleoli was counted depending on the stage of the embryos. For embryos older than 36 hpf, it was not possible to identify individual cells because of the small cell size and large number of cells; however, the nucleoli were visible, and therefore, counts of nucleoli were used as a relative indicator of the number of cells. Image-Pro Software (Media Cybernetics, Inc., Silver Spring, MD, USA) was used to count the nucleoli from chosen areas of the embryo images (at least five counts per image, five to ten embryos for each stage in total); manual counts were made to verify the software count numbers.

Total RNA content, relative gene expression ratios, and the correlation between the expression of genes encoding for GR and IGF system components

Zygotes or whole embryos were taken immediately after fertilization (t0) and again at 1, 2, 7, and 13 dpf. All samples were fixed in RNAlater (Ambion, TX, USA) for the extraction of total RNA. Five to ten RNAlater-fixed oocytes or embryos were pooled for each sample (depending on size), and the total RNA extracted as described previously (Li *et al*. 2007). Qiagen RNeasy Mini kits (Qiagen Science) were used to isolate embryo total RNA, and the RNA extracts were treated with DNase I to remove any genomic DNA following the manufacturer's instructions. The extracted RNA was quantified by NanoDrop (Thermo Scientific,

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Table 1 Effects of *in ovo* cortisol and RU486 treatments on the fertilization rates of oocytes and the hatching and mortality rates of embryos between hatching and swim up (51 dpf) and hatching and first feeding (64 dpf)

	Treatment groups			
	Con	Ct	RU	Ct+RU
Fertilization (%)	89·8	94·0	92·7	95·0
Hatching (%)	74·1	77·6	75·3	73·9
Mortality: hatching to swim up (%)	4·5	2·1	2·0	2·7
Mortality: hatching to first feeding (%)	7·8	6·7	10·3	11·3

Washington, DC, USA), and the total amount of RNA per embryo was determined.

A 1 μg sample of the DNase I-treated total RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. A 20 μl sample of the cDNA synthesis reaction was diluted 1:5 by volume with DNase-free water to prepare the working solution of the cDNAs in the real-time PCR gene quantification. Four replicates of cDNA from each treatment were produced in this study. Cytoplasmic actin gene (GenBank no: CB 494180) was used as the housekeeping gene; this gene had been found to be a suitable housekeeping gene by the Bestkeeper Software program for embryo developmental work (Pfaffl *et al*. 2004, Li 2011). A relative expression method for developmental embryos was used to quantify the expression of *gr1*, *gr2*, *igf1*, *igf2*, *igf-rIa*, and *igf-rIb* genes as described previously (Li & Leatherland 2008), in which each gene was quantified against its standard and then normalized to the housekeeping gene. In order to examine the correlation between gene expression, additional replicates of total RNA and cDNA were made from each of the four treatment groups of the 7-dpf embryos.

Statistical analysis

Data pertaining to the treatment effects on early embryo cell numbers, total RNA content of early embryos, and relative gene expression were analyzed by one-way ANOVA to examine the differences during embryo development in each treatment and within each development stage among the treatments. Where tests for normality or equal variance failed, Kruskal–Wallis's one-way ANOVA on ranks was used, followed by Dunn's pair-wise multiple comparison method. Where F values indicated statistical significance ($P<0{\cdot}05$), the Holm–Sidak multiple comparison or Student–Newman paired comparison method was performed. Data pertaining to rates of fertilization, hatching, and mortality were analyzed by χ^2 analysis. The relationship of the relative gene expression between *grs* and *igfs* in the 7-dpf stage of embryos was tested using Pearson's correlation analysis. For all statistical tests, a probability level of $P<0{\cdot}05$ was considered to be significant.

Results

Oocyte rates of oocyte fertilization, embryo hatching, and mortality

As shown in Table 1, there were no significant differences in the percent fertilization of oocytes, the rates of hatching of the embryos, the morbidity rates from hatching to 51 dpf, or the hatching rates to 64 dpf among the four treatment groups.

Zygote activation, early cell division, and GR distribution in early embryonic cells

Following fertilization, the cytoplasm of the zygote migrated rapidly to the animal pole and formed a thin layer overlying the yolk. In fresh tissue, it appeared that partially digested droplets of yolk surrounded and underlay the zygote, whereas in the 4% paraformaldehyde-fixed tissues, the early cells overlaid a cone-like region of digested yolk (Fig. 1).

In the whole-mount *in situ* GR immunofluorescent preparations of 2-hpf zygotes of the Con and RU treatment groups, the immunofluorescence signal was dispersed throughout the cytosol, with no indication of perinuclear aggregation of immunofluorescence (Fig. 2e, f1, f2, k and l). Conversely, in the 2-hpf zygotes that were reared from oocytes of the two cortisol treatment groups (Ct and

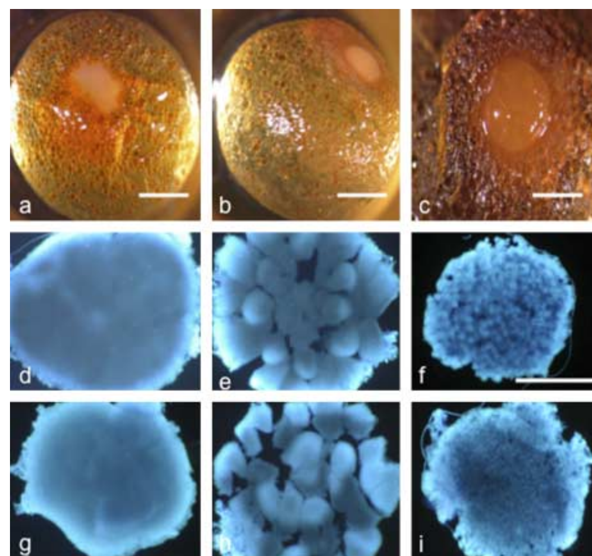

Figure 1 Whole-mount light microscopic images of rainbow trout early embryos: (a, b, and c) show eggs fixed in 4% paraformaldehyde at 2 hpf (zygote; a), 12 hpf (four-cell embryo; b), and 18 hpf (eight-cell embryo; c). The bar represents 100 μm. (d and e) show embryos reared from oocytes that were incubated in ovarian fluid alone, before fertilization (control): (d, e, and f) show 18-, 24-, and 48-hpf embryos respectively. (g, h, and i) show embryos reared from oocytes that were incubated in cortisol-enriched ovarian fluid for 3 h before fertilization (cortisol treatment): (g, h, and i) show 18-, 24-, and 48-hpf embryos respectively. The bar in (f) represents 50 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0030.

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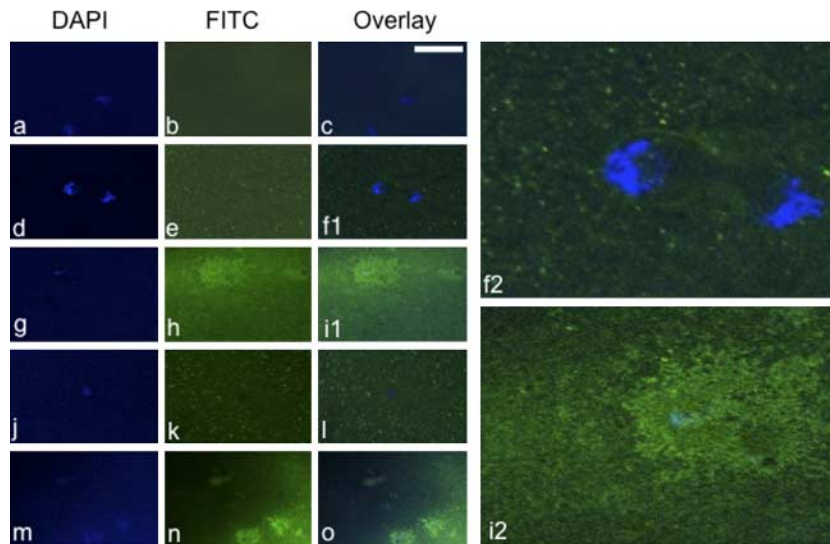

Figure 2 Whole-mount *in situ* GR immunohistofluorescence confocal microscopic images taken by confocal microscopy, showing the effects of incubation of oocytes in ovarian fluid alone (control; d, e, f1 and f2), or ovarian fluid enriched with cortisol alone (cortisol treatment; g, h, i1 and i2), RU486 alone (RU486 treatment; j, k, and l), or cortisol and RU486 together (cortisol+RU486 treatment; m, n and o) on the distribution of GR distribution in the zygote. (a, b, and c) are negative controls (no primary GR antibody in the control group). For each row, the left-hand column is DAPI stained, the middle column is stained with the fluorescence dye Alexa Fluor 488-labeled (FITC) donkey anti-rabbit IgG conjugated with/without rabbit anti-trout GR antibody (primary antibody), and the right-hand column is the overlain images. (f2 and i2) are enlarged images of figures f1 (control) and i1 (cortisol treatment) respectively. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-12-0030.

Ct+RU), in addition to a marked increase in the immunofluorescent signal relative to the non-cortisol treatment groups, there was a distinct aggregation of the GR in the cytosol surrounding the nucleus (Fig. 2h, i1, i2, n and o).

The effects of cortisol and/or RU486 treatment of oocytes on early embryo cell proliferation

There were four cells in the 12-hpf and eight cells in the 18-hpf embryos (Fig. 1b and c), indicative of a 6-h cell cycle for rainbow trout embryonic cells maintained at an ambient temperature of 8·5 °C. There were no significant differences in the number of cells in the embryos of the four treatment groups at 18, 24, and 36 hpf (Fig. 1f and i; Table 2). Because of the reduction in the size of the embryo cells as they proliferated, it was not possible to determine cell number after the 24-hpf stage; however, DAPI-stained nucleoli were visible in each nucleus, and counts of nucleoli were used as an index of differences in cell proliferation between treatment groups at that stage (Table 2). In the 48-hpf embryos, the Ct treatment group had significantly ($P<0{\cdot}05$) more nucleoli compared with the controls (Table 2). In addition, whereas the Ct+RU treatment group had significantly ($P<0{\cdot}05$) more nucleoli than the control group (Con), there were no significant differences between the RU and the Ct+RU treatment groups (Table 2).

Table 2 Effects of *in ovo* cortisol and RU486 treatments on the number of cells or nucleoli in 24–48 hpf embryos

	Treatment groups			
	Con	Ct	RU	Ct+RU
24 hpf (cells)	17·4±0·6	20·4±0·7	18·1±1·6	19·8±2·0
36 hpf (nucleolus)	193±7	218±16	202±29	197±23
48 hpf (nucleolus)	733±55*[,a]	1473±133*[,b]	912±178*[,a,c]	1084±134*[,c]

Data are shown as mean±S.E.M. ($n=4$–5); means with similar lower case superscript letters within a row are not significantly different from one another at that embryo stage; asterisks indicate significant ($P<0{\cdot}01$) differences from 36-hpf embryos within the same treatment group.

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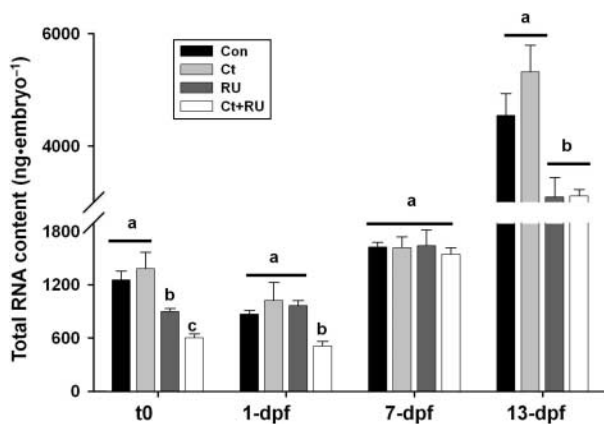

Figure 3 The effects of incubation of oocytes in ovarian fluid enriched with cortisol with and without RU486 on zygote and early total embryo RNA content (ng.embryo^{-1}); t0 are freshly fertilized zygotes; 1, 7, and 13 dpf represent the age of embryos. Data are shown as mean±S.E.M. (n=4). Bars with similar subscript letters are not significantly different from one another within the same age group. See the text for differences between age groups.

The effects of cortisol and/or RU486 treatment of oocytes on the total RNA content of early embryos

In the eggs sampled immediately after fertilization (t0), the total RNA contents of the eggs in the Con and Ct treatment groups were similar (Fig. 3), but in the values of the two RU486 treatment groups (RU and Ct+RU) were significantly lower ($P<0·01$) than those of the two non-RU486 treatment groups (Con and Ct; Fig. 3); moreover, the total RNA content of the Ct+RU treatment group was significantly lower ($P<0·05$) than that of the RU treatment group (Fig. 3).

By 7 dpf, total embryo RNA content had increased to levels that were significantly ($P<0·05$) higher compared with the 1-dpf embryos, but there were no significant differences among the treatment groups. The total embryo RNA contents at 13 dpf for all treatment groups were significantly higher ($P<0·01$) compared with newly fertilized eggs (t0) and 1- and 7-dpf embryos (Fig. 3); in addition, the total RNA content of the two RU486 treatment groups (RU and Ct+RU) was significantly lower ($P<0·05$) than that of the Con and the Ct treatment groups (Fig. 3).

The effects of cortisol and/or RU486 treatment of oocytes on the relative expression of gr1 and gr2 in embryos

In the t0 samples, the relative expression of *gr1* (ranging from ∼0·4 to ∼1·2%) tended to be higher than that of *gr2* (ranging from ∼0·09 to ∼0·35%; Fig. 4). However, as development proceeded, there was a decrease in the relative expression of *gr1* from t0 to 13 dpf, whereas the relative expression of *gr2* increased between 1 and 13 dpf (Fig. 4).

In the t0 sample, the relative expression of both *gr1* and *gr2* was significantly lower ($P<0·01$) in the two RU486 treatment groups compared with the control and cortisol treatment groups (Fig. 4). Moreover, the expression level of *gr2* in the Ct+RU treatment group was significantly lower ($P<0·05$) than that of the RU treatment group (Fig. 4). Conversely, the relative expression of *gr1* in the Ct treatment group was significantly higher ($P<0·05$) than that of the Con group (Fig. 4).

By 1 dpf, the expression levels of both *gr1* and *gr2* in the Con group had fallen significantly ($P<0·05$) relative to the t0 samples (Fig. 4). For *gr1*, there were no significant differences among treatments except for the Ct+RU treatment group, which was significantly lower ($P<0·05$ or $P<0·01$) than the other three treatment groups (Fig. 4). For *gr2*, the relative expression in the two cortisol-treated groups was significantly lower ($P<0·01$) than that in the two other groups (Fig. 4).

In both the 7- and 13-dpf embryos, there were no significant differences in the relative expression of *gr1* among the four treatment groups (Fig. 4). Conversely, for *gr2*, the among-treatment pattern that was seen in the 1-dpf embryos was again evident in the 7-dpf embryos, but in a muted form, as the expression levels in all four treatment groups were increasing; the only significant differences ($P<0·05$) were between the Con and the two cortisol treatment groups (Ct and Ct+RU; Fig. 4). In the 13-dpf embryos, the pattern of relative expression of *gr2* among the four treatment groups was similar to that seen in the t0 samples, but the mean values were approximately tenfold higher in the 13-dpf embryos; the expression levels of the two groups reared from RU486-treated oocytes were significantly ($P<0·01$) lower than those of the two non-RU486-treated groups (Fig. 4).

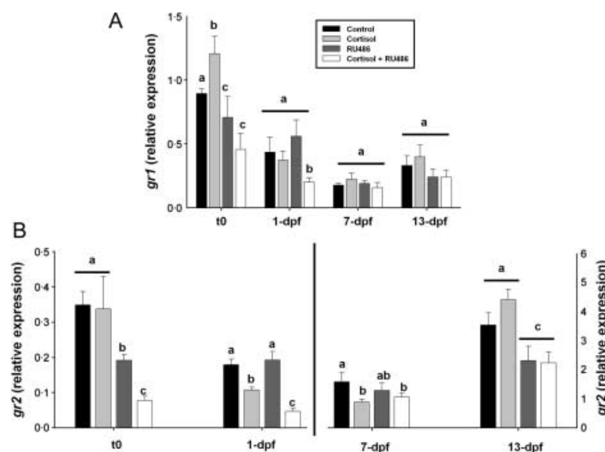

Figure 4 The effects of incubation of oocytes in ovarian fluid enriched with cortisol with and without RU486 on the relative expression of *gr1* (A) and *gr2* (B) by zygotes and early embryo. See the legend to Fig. 3. Data are shown as mean±S.E.M. (n=4). Bars with similar subscript letters are not significantly different from one another. See the text for differences between age groups.

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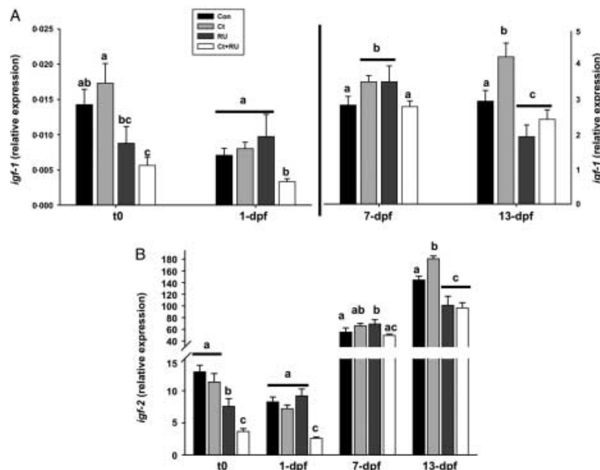

Figure 5 The effects of incubation of oocytes in ovarian fluid enriched with cortisol with and without RU486 on the relative expression of *igf1* (A) and *igf2* (B) by zygotes and early embryo. See the legend to Fig. 3. Data are shown as mean ± S.E.M. ($n=4$). Bars with similar subscript letters are not significantly different from one another. See the text for differences between age groups.

The effects of cortisol and/or RU486 treatment of oocytes on the relative expression of IGF system genes in embryos

Relative levels of *igf1* and *igf2* mRNA transcripts The relative abundance of *igf2* mRNA was ∼20 times more than that of *igf1* mRNA at all developmental stages (Fig. 5A and B). For both genes, the relative abundance of mRNA was significantly elevated ($P<0{\cdot}05$) in the 7- and 13-dpf embryos relative to the t0 eggs and 1-dpf embryos (Fig. 5A and B).

The relative expression of *igf1* in the t0 sample was similar in the Con and Ct treatment groups, and although the values for the two RU486 treatment groups tended to be lower than that for the non-RU486 treatment groups, only that of the Ct+RU treatment group was significantly lower ($P<0{\cdot}01$; Fig. 5A). An expression pattern similar to that of *igf1* was found for *igf2* in the t0 sample, except that the two non-RU486 treatment groups were significantly higher ($P<0{\cdot}01$) than both of the RU486 treatment groups (Fig. 5B).

The among-treatment pattern of the relative expression of *igf1* and *igf2* was similar (Fig. 5A and B). For *igf1*, the value in the Ct+RU treatment group was significantly lower ($P<0{\cdot}05$) than those of the two non-RU486 treatment groups, whereas for *igf2*, the value in the Ct+RU treatment groups was significantly lower ($P<0{\cdot}01$) than the values of the three other treatment groups (Fig. 5A and B).

By 7 dpf, the relative expression levels of the two genes were significantly higher ($P<0{\cdot}05$ or $<0{\cdot}01$) than in the comparable treatment groups sampled at t0 and 1 dpf (Fig. 5A and B). For *igf1*, the *in ovo* cortisol treatment elicited a significant increase ($P<0{\cdot}05$) in the Ct treatment group relative to the Con group but a significant decrease ($P<0{\cdot}05$) in the Ct+RU treatment group relative to the RU treatment group (Fig. 5A and B).

The among-treatment relative expression of both the *igf1* and the *igf2* genes in 13-dpf embryos exhibited a similar pattern, with a significantly higher ($P<0{\cdot}01$) value in the Ct treatment group relative to the other three treatment groups and significantly lower ($P<0{\cdot}01$) values for the RU and Ct+RU treatment groups than the embryos reared from oocytes that had not been incubated with RU486 (Fig. 5A and B).

The effects of cortisol and/or RU486 treatment of oocytes on the relative expression of igf-rIa and igf-r1b genes

The relative levels of mRNA encoding for *igf-rIb* were >50-fold more than those for *igf-rIa* in the t0 eggs and 1-dpf embryos (Fig. 6A and B). However, the expression levels of the two genes were essentially similar from 1- to 13-dpf embryos (Fig. 6A and B). The overall relative expression levels of *igf-rIa* in the t0 and 1-dpf stages were in the same range; there were ∼7·5- and ∼20·0-fold increases in the expression levels between these stages and 7- and 13-dpf embryos respectively; these increases were significantly different ($P<0{\cdot}01$) for all treatment groups at each stage (t0/1, <7, and <13 dpf; Fig. 6A). The relative expression of *igf-rIb* was in a similar range at all the developmental stages examined (Fig. 6B).

At the t0 stage, the among-treatment pattern of the relative expression of *igf-rIa* and *igf-Ib* was essentially similar, with significantly lower ($P<0{\cdot}01$) values evident in the Ct+RU treatment group compared with the other three treatment groups (Fig. 6A and B). In the case of *igf-rIa*, the values were not significantly different in the Con, Ct, and RU treatment groups (Fig. 6A), whereas for *igf-rIb*, the Con, Ct, and RU treatment groups differed significantly ($P<0{\cdot}05$) from one another (Ct>Con>RU; Fig. 6B).

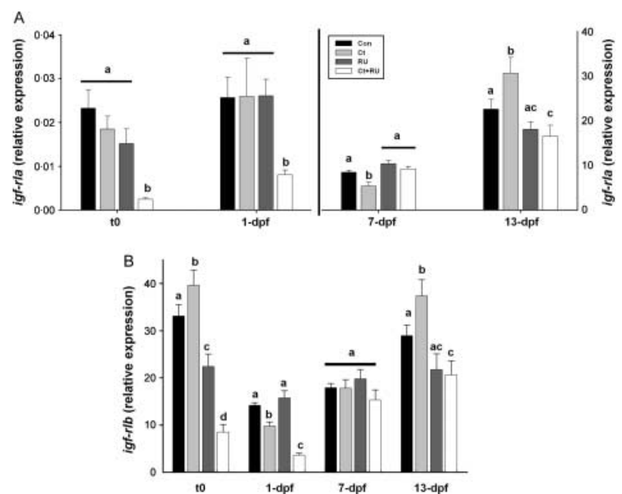

Figure 6 The effects of incubation of oocytes in ovarian fluid enriched with cortisol with and without RU486 on the relative expression of *igf-rIa* (A) and *igf-rIb* (B) by zygotes and early embryo. See the legend to Fig. 3. Data are shown as mean ± S.E.M. ($n=4$). Bars with similar subscript letters are not significantly different from one another. See the text for differences between age groups.

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Table 3 The effects of *in ovo* cortisol and RU486 treatment on the correlation between the expressions of *igf* system genes and *gr* genes in 7-days post-fertilization (dpf) embryos

	Treatment groups							
	Con		Ct		RU		Ct+RU	
	gr1	*gr2*	*gr1*	*gr2*	*gr1*	*gr2*	*gr1*	*gr2*
igf1	+0·20	+0·19	+0·06	**+0·85**†,‡	+0·20	**+0·60***,‡	+0·33	**+0·95**†,‡
igf2	+0·69†	+0·74†	**+0·40**‡	+0·83†	**+0·01**‡	+0·85†	**+0·29**‡	+0·92†
igf-rIa	+0·02	+0·14	−0·07	**+0·91**†,‡	**−0·57***,‡	+0·12	−0·37	**+0·97**†,‡
igf-rIb	+0·69†	+0·56†	+0·46‡	+0·63†	**+0·41**‡	+0·94†	**−0·12**‡	+0·91†

R values are shown (n=16); significances ($^{*}P<0·05$ or $^{\dagger}P<0·01$ respectively) correlations. *R* values shown in bold and marked '‡' are correlation results that differ from the control treatment group (Con).

Among the 1-dpf embryos, the relative expression of *igf-rIa* in the Ct+RU treatment group was significantly lower ($P<0·01$) than in the other three groups, which did not differ significantly from one another (Fig. 6A). In the same age group, the relative expression of *igf-rIb* in both the Ct and the Ct+RU treatment groups was significantly ($P<0·05$) lower than that in the Con and RU treatment groups (Fig. 6B). In the 7-dpf embryos, the relative expression of *igf-rIa* was significantly lower ($P<0·05$) in the Ct treatment group relative to the three other treatment groups, which had similar values (Fig. 6A); for *igf-rIb*, there were no significant differences in the relative expression of the gene among the four treatment groups (Fig. 6B).

By 13 dpf, a similar among-treatment pattern of expression was found for both *igf-rIa* and *igf-rIb*; the values in the Ct treatment group were significantly higher ($P<0·05$) than those in the other three groups, and the value in the Ct+RU treatment group was significantly lower ($P<0·05$) than that in the Con group (Fig. 6A and B).

The effects of cortisol and/or RU486 treatment of oocytes on the correlations between the relative expression of gr1 and gr2 and several IGF system genes in 7-dpf embryos

The *R* values of the correlation analyses of specific genes are shown in Table 3; the values shown in bold indicate different correlation outcomes for either *gr1* or *gr2* correlations with other genes than were found in the Con group. The findings can be summarized as follows: a) The expression of *gr1* was significantly correlated ($P<0·01$) with the expression of *igf1* in the Con group, but this correlation was not found in the other three treatment groups; b) the expression of *gr1* was significantly correlated ($P<0·01$) with the expression of *igf-rIb* in the Con group, but this correlation was not found in the other three treatment groups; c) there was no correlation between the expression of *gr1* and *igf-rIa* in the Con group, but a significant negative correlation ($P<0·05$) was found in the RU treatment group; d) there was no correlation between the expression of *gr2* and *igf1* in the Con group, but significant correlations were found in the Ct ($P<0·01$), RU ($P<0·05$), and Ct+RU ($P<0·01$) treatment groups; and e) there was no correlation between the expression of *gr2* and *igf-rIa* in the Con group, but significant correlations ($P<0·01$) were found in the Ct and Ct+RU ($P<0·01$) treatment groups.

Discussion

Because of the practical limitations imposed by the necessity to pool oocytes from several fish before the prefertilization incubations of oocyte treatment groups (described in detail in the Materials and Methods section), the experimental design did not permit within-treatment random replication. Consequently, it was not possible to estimate accurately within a single reproductive period the biological variation of the population in response to the various treatments. However, as indicated in the Materials and Methods section, with the exception of the measurements of total RNA and gene expression, the remaining parameters were examined over more than one reproductive season, with similar patterns of among-treatment responses.

The patterns of responses to the various treatments found in this study argue strongly for a role for GRs during zygotic activation and the expression of growth-related genes during early embryogenesis in rainbow trout. These observations support our previous findings showing that increasing the cortisol content of rainbow trout oocytes from ~3 ng.oocyte^{-1} (untreated oocytes) to ~5 ng.oocyte^{-1} significantly enhanced the growth of the embryos reared from these oocytes (Li *et al.* 2010). In addition, the expression of *igf1* in the zygote and embryos between 1- and 21-dpf and *igf2* in 21-dpf embryos was enhanced (Li *et al.* 2010). This study provided evidence that suggests a key role for GR in the modulation of growth, possibly via changes in the expression of several IGF system-related genes, including both the genes that encode for the peptide hormones, IGF1 and 2, and their protein receptors. An important component of the study was the use of the progesterone receptor (PR) and GR antagonist, RU486 (mifepristone), which has been used previously for the evaluation of GR function in fish and mammals (Vijayan *et al.* 1994, Pariante *et al.* 2001, Aluru *et al.* 2004, Scott *et al.* 2005, Veillette *et al.* 2007, Vizzini *et al.* 2007, Peeters *et al.* 2008, Du *et al.* 2009, Alderman *et al.* 2012).

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The antagonistic properties of RU486 on PRs and GRs are complex and still not fully understood; the PR antagonistic action of RU486 has received most attention (reviewed by Leonhardt & Edwards (2002)). RU486 appears to interact with the receptors at several levels of the ligand–receptor-gene activation/repression process. These include some nonexclusive competitive interaction with the receptor ligand binding domain (LBD), enhanced dimerization of the receptor protein, an increased bonding of the dimer with the DNA hormone response elements in the promoter regions of responsive genes, inhibition of co-activator recruitment, interference with the direct interaction of receptors with proteins involved in gene transcription, and impaired interaction of the receptor protein with several other transcriptional factors. Moreover, different isoforms of the receptor have differing affinities for and interactions with RU486 (Leonhardt & Edwards 2002, Bury *et al.* 2003). Consequently, tissue and species variability in the responses to RU486 may also be a factor. Thus, interpretation of the responses of the embryonic cells to complex and partial antagonists such as RU486 needs to be made with caution; however, as discussed below, in this study, several of the responses to cortisol were curtailed if the oocytes were co-incubated with RU486, suggesting that cortisol was exerting at least some of its actions via an interaction with GR. Notwithstanding, as will be discussed below, some of the observed effects of RU486 with regard to the suppression of maternal mRNA transcripts cannot be attributed to its GR antagonistic properties.

There were clear interactions of cortisol with GR proteins during zygote activation after fertilization at 2 hpf, whereby the glucocorticoid appeared to mediate the movement of GR toward the nucleus in the two cortisol-treated groups of zygotes (with and without co-incubation with RU486; Fig. 2). Interestingly, despite the presence of ∼ 3·5 ng per organism of cortisol in the Con zygotes, there was no such GR translocation evident as might be expected (Fig. 2). This observation suggests that there may be an intracellular threshold cortisol level for the activation of GR; however, a more likely explanation relates to the amount of cortisol that is ‘accessible’ to GR in the zygote and blastomeres. Although the intracellular distribution of the glucocorticoid in the oocyte and zygote is not known, it is likely that a major fraction is associated with the lipid-rich content of the cytoplasmic vesicles, rather than in solution in the aqueous medium of the ooplasm. If this is the case, the cortisol concentration in the aqueous medium of the Con oocytes may not have been sufficient to activate the GR; conversely, following the incubation of the oocytes in cortisol-enriched ovarian fluid, the aqueous cortisol component may have been sufficient to facilitate GR activation. Further, the absence of GR translocation in the Con zygotes also suggests that the translocation that was observed in this study was probably not part of the range of post-fertilization molecular migratory events involved in the relocation of ooplasm organelles toward the animal pole.

In this study, there was no evidence of either an inhibitory or stimulatory effect of RU486 on GR translocation (Fig. 2), which suggests that the binding of RU486 to the GR protein does not bring about translocation in the rainbow trout zygote as it appears to do in several mammalian cell lines (murine fibroblast cells: Pariante *et al.* (2001), primate kidney and osteosarcoma cells: Lewis-Tuffin *et al.* (2007), and murine pituitary cells: Peeters *et al.* (2008) and Spiga *et al.* (2011)). Moreover, RU486 does not appear to inhibit cortisol-activated translocation. It is thus possible that if RU486 does associate with the salmonid GR LBD, it does not activate the receptor, as it appears to do in mammals. This argument is supported by the observation that RU486 on its own did not significantly enhance cell numbers (relative to the controls) during the 48-hpf period, whereas in the Ct and Ct+RU treatment groups, there was a significant increase in cell numbers (Table 2); these findings suggest that cortisol acting as the ligand for GR elicited this increased cell proliferation at a very early stage in embryogenesis. The apparent absence of a GR translocation response to RU486 might be that the RU486-associated GR is not recognized by the antiserum, but since RU486 did not appear to decrease the GR immunofluorescence signal, the absence of a response was not a decrease in antigenicity. The observations presented herein suggest actions of the GR antagonist that are currently not defined; whether these are related to GR protein degradation (Alderman *et al.* 2012), affecting intracellular GR levels and thus reducing the extent of agonistic actions of RU486 (Zang *et al.* 2007), the agonism of RU486 for GR being affected by competition with other receptors (Hawkins & Thomas 2010), has yet to be determined.

An interesting and unanticipated finding was the apparent partial suppression of the total RNA content of the zygotes (t0) following the *in ovo* RU486 treatment (Fig. 3). Evidence of an inhibition of RNA synthesis by RU486 was less pronounced in the 1-dpf embryos and not found in the 7-dpf embryos; however, the effect was again evident in the 13-dpf embryos, in which the total RNA content of the two RU486-treated groups was significantly lower ($P<0·01$) than that of the Con and Ct treatment groups (Fig. 3). Because the total RNA measurements include both coding and noncoding RNA, the relative composition of coding vs noncoding RNA is not known; however, the apparent loss of the suppressive action of RU486 on total RNA during the transition from the zygotic to embryonic genome and reappearance of RU486 suppression when the embryonic genome is established may reflect a broad-based regulatory role for glucocorticoids in RNA synthesis. As for other findings, it suggests that nongenomic programming occurs in the zygote in response to the presence of RU486 and that the programming of the one-time exposure to RU486 is still in effect during early embryonic genome expression, an indicator of the plasticity of the epigenomic response to environmental influences (in this case, the presence of a GR antagonist) during early development.

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The among-treatment patterns of the relative abundance of mRNA transcripts of the six genes considered in this study in the t0 samples (Figs 4, 5 and 6) suggest that RU486 may destabilize components of the zygotic transcriptome. It is not clear at this point how that may occur, and it would appear to contradict the currently held view that the zygotic transcriptome is relatively stable and necessary for the regulation of the first cell division. However, there is evidence in several species for differential destabilization of a subset of mRNAs (Bashirulla *et al.* 1999, 2001, Tandros *et al.* 2003, 2007, Ferg *et al.* 2007, Shiokawa *et al.* 2008, Tandros & Lipshitz 2009). Therefore, the data presented in this paper may reflect an involvement of RU486 in destabilizing of some components of the zygotic transcriptome. Of significance was the evidence of a reoccurrence of the suppressive action of RU486 on the transcriptome of 13-dpf embryos: *gr2*, *igf1*, *igf2*, *igf-rIb*, and to a lesser extent, *igf-rIa* (Figs 4, 5 and 6) were significantly affected, and only *gr2* mRNA transcript abundance, which was very low in all treatment groups in both 7- and 13-dpf embryos, was not affected by RU486 treatment (Fig. 4). Furthermore, co-incubation of oocytes with a combination of RU486 and cortisol did not overturn the suppressive action of RU486 on the transcriptome. On the contrary, the relative abundance of mRNA encoding for *gr2*, *igf2*, *igf-rIa*, and *igf-rIb* in the Ct+RU treatment group of zygotes was significantly ($P<0{\cdot}05$) lower than those of the RU treatment group (Figs 4, 5 and 6); there were no significant differences between these two treatment groups for any of the genes of interest in the 13-dpf embryos (Figs 4, 5 and 6). These data are suggestive of an initial suppressive action of cortisol, compounding the suppressive action of the GR antagonist, and a loss of that additional action of cortisol by the 13-dpf stage of embryo development. However, comparisons of the Con and Ct treatment groups showed a stimulatory action of cortisol on the expression of the four IGF-related genes in the 13-dpf embryos (Figs 5 and 6). Thus, in the absence of the GR antagonist, cortisol stimulates the relative expression of these genes, but in the presence of the GR antagonist, gene expression is suppressed relative to the control and may be suppressed further by the concerted actions of RU486 and cortisol.

The information obtained from the multiple correlation analysis of the relative expression of *gr1* and *gr2* and the IGF system genes in the 7-dpf embryos provided additional indications of complex interactions between these genes during the critical period of transition from the influence of the zygotic genome to that of the embryonic genome (and epigenome). In the Con treatment group, there were significant ($P<0{\cdot}01$) direct correlations between the relative expression of both *gr1* and *gr2* with the relative expression of *igf2* and *igf-rIb* (Table 3), suggesting a link between the two systems. Moreover, the significant correlations (or lack thereof) between the relative expression of *gr1* and *gr2* with other genes that were evident in the Con treatment group were radically changed in the three other treatment groups (Table 3). Specifically, the relationships between the relative expression of *gr1* and *igf2*, *igf-rIa* and *igf-rIb* were changed, as were the relationships between *gr2* and *igf1*, *igf2* and *igf-rIa* (Table 3). Taken together, these observations support the hypothesis of a complex interaction between the GR and IGF system, one that is sensitive to changes in cortisol levels and the altered status of the GR (as brought about by the presence of the GR antagonist).

The dynamic developmental processes of embryogenesis involve extremely complex and highly coordinated cell proliferation and growth, cellular migration, and apoptosis. Some of these processes have been shown to be influenced by IGF signaling (Wood *et al.* 2005, Huang *et al.* 2007, Schlueter *et al.* 2007, Reinecke 2010). The growth promoting effects of cortisol on embryonic cell proliferation and embryo growth in this study (Table 2), together with the changes in the expression of IGF system gene expressions in early embryos (Figs 5 and 6), are consistent with the proposition that cortisol activates the IGF system signaling pathway in early embryonic cells. IGF system activation may then contribute to the promotion of early embryonic cell proliferation and embryonic growth. In post-embryonic fish, cortisol-activated stimulation of the expression of IGF- and GH system-related genes has been described in *Mozambique tilapia* (*Oreochromis mossambicus*) ovarian follicles (Huang *et al.* 2007), hepatocytes (Pierce *et al.* 2011), and rainbow trout lymphocytes (Yada *et al.* 2005). In mammalian embryos, the role of GR activation in cellular maturation and growth, most notably stem cell differentiation, is well established (Derfoul *et al.* 2006, Phillips *et al.* 2006, Mikami *et al.* 2008, Stewart *et al.* 2008). Similarly, GR has been associated with the regulation of some aspects of the development of ovine cardiomyocytes (Giraud *et al.* 2006) and the complex patterns of expression of myogenic genes in zebrafish (*Danio rerio*; Nesan *et al.* 2012). These and other studies demonstrate that cortisol plays numerous biological functions by interactions with multiple cellular signaling pathways, that include, but are not limited to, the direct transcription factor actions of cortisol-activated GR on target genes. These interactions are still imperfectly understood, as is the mode of action of cortisol during prenatal stressor-related epigenomic programming (reviewed by Phillips (2007), Gluckman *et al.* (2009), Godfrey *et al.* (2010) and Hochberg *et al.* (2011)).

In summary, a one-time *in ovo* exposure to cortisol by immersion of rainbow trout oocytes, before fertilization, in cortisol-enriched ovarian fluid had lasting effects on the growth characteristics of the embryo and juvenile progeny (Li *et al.* 2010). This study demonstrated that at least some of these effects are related to multiple and complex interactions between GR activation of changes in the expression of IGF system genes. The studies also point to novel actions of cortisol that cannot be explained on the basis of the currently understood modes of action and roles of the glucocorticoids. Similarly, the studies also provide evidence of an action of the RU486 GR antagonist on the destabilization of maternal mRNA transcripts.

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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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