The *in vitro* metabolism of cortisol by ovarian follicles of rainbow trout (*Oncorhynchus mykiss*): comparison with ovulated oocytes and pre-hatch embryos

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Abstract

Mid-vitellogenic stage rainbow trout (*Oncorhynchus mykiss*) ovarian follicles (both intact and yolk free (YF)), ovulated oocytes and embryos were co-incubated with [2,4,6,7-³H]cortisol for 18 h to determine the degree and nature of the metabolism and biotransformation of the glucocorticoid. There was evidence of the conversion of cortisol to the less biologically potent glucocorticoid, cortisone, and the formation of glucocorticoid sulphates (both cortisol and cortisone) for all cell and tissue samples, suggesting the presence of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) and glucocorticoid sulphotransferase (GST) activity at all stages; however, GST activity was particularly marked in both intact and YF ovarian follicles, suggesting an important role of follicles in limiting the exposure of oocyte to maternal cortisol. As there was no evidence of 11 β -HSD or GST activity in ovarian fluid, the findings affirm that ovarian follicles (probably the thecal and granulosa cells) provide a barrier against the transfer of cortisol to the oocytes by forming sulphated steroids, whereas ovulated oocytes and early embryos have a more limited capacity to either metabolize or conjugate cortisol and are therefore more vulnerable at the post-ovulatory and early embryonic stages to increases in exposure to the glucocorticoid.

Reproduction (2012) 144 713–722

Introduction

Antenatal maternal stress in mammals that is associated with elevated maternal cortisol secretion has been linked to reproductive dysfunction and epigenomic reprogramming in embryos that is associated with certain adult-onset diseases (see reviews by Mulder *et al.* (2002), Phillips (2007), Talg *et al.* (2007), Field & Diego (2008), Tegethoff *et al.* (2009), Matthews & Phillips (2010) and Li & Leatherland (2012)). The relationship between the maternal stress response, maternal hypercortisolism and reproductive and embryonic outcomes is complex and still the object of ongoing research; however, some of the outcomes are most likely caused by metabolic or immune system-related actions of cortisol that indirectly impact the maternal involvement in the reproductive process; however, other responses can be attributed to the direct actions of cortisol on the developing embryo or fetus. In mammals, the antral fluid of the ovarian follicle regulates the exposure of the pre-ovulatory oocyte to cortisol, and the placental tissues act as a partial barrier to the transfer of maternal cortisol to the embryo and fetus. Because these protective barriers are not present in oviparous vertebrate species, there is the potential for the transfer of maternal cortisol to the

oocytes. Consequently, if the oocytes and early embryos are unable to metabolize cortisol, embryogenesis may be compromised (Leatherland *et al.* 2010, 2012).

The antral fluid of the ovarian follicles in mammals contains relatively high concentrations of unconjugated cortisol, the levels of which are regulated within narrow limits by the actions of two forms of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) that are co-expressed in the mural granulosa cells (Michael *et al.* 2003, Sunak *et al.* 2007, Thurston *et al.* 2007, Michael & Papageorgiou 2008, Webb *et al.* 2008). The maintenance of a stable high concentration of cortisol suggests that the glucocorticoid plays an important role in the 'normal' development and maturation of the oocyte. This hypothesis is supported by evidence of a significant reduction in the meiotic competency of the oocyte when antral fluid cortisol concentrations are elevated (Yang *et al.* 1999, Thurston *et al.* 2002, 2003a, 2003b, Andersen 2003).

Far less is known about the role of cortisol on oocyte development and maturation in oviparous fishes, although there is evidence of the involvement of cortisol in several aspects of ovarian function (reviewed by Leatherland *et al.* (2010)). The ovarian follicles of

oviparous fishes differ anatomically from those of mammals in that the oocyte is enveloped by two layers of steroidogenic cells that overlay the acellular zona pellucida. The outer discontinuous cell layer comprises fusiform thecal cells and the inner layer a cuboidal epithelium of granulosa cells that is in direct contact with cytoplasmic extensions of the ooplasm that traverse the zona pellucida (Leatherland *et al.* 2010). The granulosa cell layer is the sole physicochemical barrier that separates the oocyte from the (maternal) extracellular fluid. In rainbow trout, *11 β -hsd* transcripts are present in the thecal and granulosa cells, and the expression of these transcripts increases between early and late stages of vitellogenesis (Kusakabe *et al.* 2003), suggesting that the thecal and granulosa cells can modulate the exposure of the oocytes to fluctuations in maternal cortisol levels; however, there is currently no direct evidence that they play such a role.

Some form of modulation of the exposure of oocytes to maternal glucocorticoids in migratory Pacific (*Oncorhynchus* sp.) salmonid fishes is to be anticipated in light of the relatively high maternal plasma levels of cortisol in these species (ranging from 140 to 650 ng/ml) during the critical late stages of ovarian maturation (Morrison *et al.* 1985, Kubokawa *et al.* 1999, Carruth *et al.* 2000, Westring *et al.* 2008, McConnachie *et al.* 2012). While the maternal total cortisol concentrations during gonadal maturation are somewhat lower for non-migratory oncorhynchid salmonids, such as rainbow trout (20–70 ng/ml) (Bry 1985, Caldwell *et al.* 1991, Koldjær *et al.* 2004), ~45% of that steroid is in the form of unconjugated hormone (Caldwell *et al.* 1991).

Cortisol of maternal origin is translocated from ovarian interstitial fluid to the oocytes during ovarian maturation in salmonid fishes (Schreck *et al.* 2001, Eriksen *et al.* 2006, Norris & Hobbs 2006), and increases in maternal plasma cortisol concentrations are reflected in increases in oocyte cortisol content (Stratholt *et al.* 1997, Eriksen *et al.* 2006, Leatherland *et al.* 2010, M Li & J F Leatherland, unpublished data). Moreover, the presence of glucocorticoid receptor (Gr) protein and *gr* mRNA transcripts in oocytes and zygotes of fish (Milla *et al.* 2009, Li *et al.* 2012) suggests multiple roles (as yet poorly understood) of glucocorticoids during early ontogeny (Eriksen *et al.* 2006, Milla *et al.* 2006, Auperin & Geslin 2008, Hayashi *et al.* 2010, Li *et al.* 2010, 2011, 2012, Yamaguchi *et al.* 2010). If that is the case, some form of buffering mechanisms that protect the oocyte and embryos from the transfer of excessive amounts of cortisol from maternal sources would be anticipated, particularly as pre-fertilization increases in oocyte cortisol content have been shown to have marked effects on the expression of growth-related (Li *et al.* 2010, 2012) and innate immune system-related genes and embryo lysozyme content (Li *et al.* 2011) and on the behavioural traits of juveniles reared from the cortisol-enriched oocytes (Espmark *et al.* 2008, Sloman 2010, Eriksen *et al.* 2011). The primary

goal of this study was to determine whether the ovarian follicle acts as a partial barrier to the translocation of cortisol from the maternal circulation to the oocyte, by converting the hormone into less biologically active forms of steroids.

Very little is known about the catabolism of glucocorticoids by ovarian follicles in fish or by fish embryos before the formation of the organ systems. Even for juvenile and adult fish, only a very few reports of glucocorticoid catabolic products have been published, and for the most part, these studies identified water-soluble (conjugated) steroids that are the products of catabolism of cortisol by hepatocytes (cortisone, 11 β -hydroxyandrostenedione (11 β OHA₄) androstenedione, 20 β -cortolone, tetrahydrocortisol, 5 β -dihydrocortisone and tetrahydrocortisone) (Kime 1978, Truscott 1979, Pottinger *et al.* 1992, Khan *et al.* 1997a, Vijayan *et al.* 1997). In the absence of a functional liver, these products would probably not be found for ovarian follicles, oocytes and early-stage embryos incubated *in vitro*.

In order to address some of these issues, a series of *in vitro* incubations were carried out using vitellogenic stage ovarian follicles, naturally ovulated oocytes and embryo tissues to look for evidence of cortisol biotransformation, either catabolic metabolites or conjugated steroids. A primary goal of this study was to examine the capacity of ovarian follicles *in vitro* to metabolize or conjugate cortisol, specifically looking for evidence of 11 β -HSD and glucocorticoid sulphotransferase (GST) activity, and, if present, to locate the cellular source of these enzymes in the follicles. In addition, the study was undertaken to determine whether there was evidence of the conjugation and catabolism of glucocorticoids by the oocytes and by pre- and post-hatched embryos that might allow them to regulate their glucocorticoid homeostasis.

Results

Radioactivity in the eluates of solid-phase extraction

Table 1 shows the radioactive counts in each of the three fractions following solid-phase extraction (SPE) of ³H-labelled steroids from the media in which ovarian follicles, oocytes or embryo tissues were co-incubated with [³H]cortisol. In addition, the fractionation of medium 199 (M199) and ovarian fluid (OvF) incubated without tissue (medium blanks) is shown for comparison (Table 1). The three eluate fractions were as follows: i) the unconjugated (free) fraction containing [³H]cortisol and other steroid metabolites; ii) the conjugated fraction containing ³H-labelled steroid sulphates and glucuronides and iii) the flow-through (water) (FT) fraction (Table 1); the radioactive counts in the FT fraction is a measure of the ³H in the form of ³H₂O, which is formed during hydroxylation of the [³H]cortisol backbone at positions 2, 4, 6 or 7 (Petkam *et al.* 2003b).

Table 1 SPE for steroids from the culture media; the data show the percentage of total tritium counts in the non-steroid flow-through ($^3\text{H}_2\text{O}$) eluate and the unconjugated and conjugated eluate fractions following the co-incubation of ovarian follicles, oocytes and embryo tissues of rainbow trout with $[1,2,6,7-^3\text{H}]$ cortisol *in vitro* in medium 199 (M199) or ovarian fluid (OvF).

	$^3\text{H}_2\text{O}$ (%)	Unconjugated fraction (%)	Conjugated fraction (%)
Follicles (intact) in M199	3.5 ± 0.3	67.4 ± 1.4	26.2 ± 1.4
Follicle (yolk free) in M199	5.0 ± 0.5	78.7 ± 0.9^a	14.7 ± 0.7^a
Follicle (intact) in OvF	4.7 ± 0.4	70.9 ± 0.9	20.9 ± 1.2
Follicle (yolk free) in OvF	4.3 ± 0.2	80.2 ± 0.8^a	12.4 ± 0.8^a
OvF only	4.1 ± 0.3	89.5 ± 5.3	8.5 ± 1.0
M199 only	3.4 ± 0.2	92.8 ± 5.9	7.8 ± 0.6
Oocytes	4.1 ± 0.4	91.5 ± 0.1	3.0 ± 0.5
Embryos (26 dpf) in M199	2.1 ± 0.3	91.7 ± 0.2	3.5 ± 0.5
Embryos (40 dpf) in M199	1.0 ± 0.2	88.2 ± 0.8	9.1 ± 0.8
Embryos (58 dpf) in M199	0.8^b	82.0 ± 1.6	16.2 ± 1.6

The data are shown as mean $\pm$ S.E.M. ($n=5-6$ for follicles; $3-4$ for oocytes and embryos).

^aSignificantly different ($P<0.01$) from values for intact follicles. ^bAll three values were the same.

The percentage of SPE fractionation of the ^3H -labelled products in the medium following the incubation of $[^3\text{H}]$ cortisol in M199 or OvF alone (i.e. without tissue) was essentially similar, with low percentage counts in the FT fraction ($\sim 3-4\%$) and the conjugated fraction ($\sim 7.5-10\%$), and most counts ($\sim 90-95\%$) being present in the unconjugated fraction. HPLC examination of each of these fractions in the two incubation media showed only a single ^3H -labelled peak. It was present only in the extract of the unconjugated fraction, and it co-eluted with the authentic cortisol standard (see below). The absence of tritium-labelled peaks in either the FT or the conjugated fraction suggests that the low tritium count levels in the FT and conjugated SPE fractions (Table 1) probably represent carry-over trace background contamination that is inherent to the SPE protocol.

Table 1 shows the SPE fractionation following the co-incubation of intact ovarian follicles with $[^3\text{H}]$ cortisol in either M199 or native OvF. The relative distribution of the tritium counts in the three fractions was essentially similar in both media used. The values in the FT fraction were similar to those found in the respective medium blanks for OvF and M199 and probably represent background values. The ratio of the mean counts in the unconjugated and conjugated fraction were similar for both M199 (68:27) and OvF incubations (71:21), indicating marked conjugation of $[^3\text{H}]$ cortisol or of its metabolites. Taking into account the background values of the medium blank incubations, these counts in the conjugated fraction represent 13–19% conversion of cortisol into conjugated steroids; these were subsequently found to be predominantly associated with sulphated steroids (85:15 ratio of sulphate:glucuronide).

Co-incubation of $[^3\text{H}]$ cortisol with ovarian follicles from which the ooplasm had been removed (yolk free (YF) follicle) also resulted in a marked production of conjugated steroids (Table 1), suggesting that at least some of the conjugation activity of the intact follicles was associated with the thecal and granulosa cell layers. However, the relative levels of tritium in the conjugated fraction for both M199 and OvF were significantly

($P<0.05$) lower than those for comparable incubations with intact follicles (Table 1).

For oocytes and all embryo stages, $\sim 85-95\%$ of the radioactive counts were in the unconjugated (free) steroid fraction (Table 1); there was no net percentage change in the FT fraction relative to the medium blanks. Similarly, for oocytes, 26 dpf embryos and 40 dpf embryos, there was no net increase in the percentage of counts in the conjugated fraction (Table 1), but there appeared to be a net increase relative to the medium blanks of $\sim 6\%$ in the medium of the 58 dpf embryos (Table 1).

HPLC separation of unconjugated and conjugated steroids in the incubation medium following the co-incubation of ovarian follicles, oocytes and embryos with $[^3\text{H}]$ cortisol

A two-stage HPLC process was used to separate the ^3H -labelled steroids present in the unconjugated and conjugated fractions following SPE extraction. Stage 1 used a binary acetonitrile:water gradient at a flow rate of 0.7 ml/min for 40 min. Stage 2 used the pool of the eluates from 0 to 12 min following stage 1 (containing $>95\%$ of the radioactivity) that was subjected to further SPE followed by a further HPLC separation on an acetonitrile:water gradient for 25 min (Table 2). A 21-steroid cocktail of reference standards was run concurrently in each HPLC run; a representative chromatogram is shown in Fig. 1.

Table 2 HPLC protocols used to separate unconjugated and conjugated tritium-labelled steroids following the co-incubation of ovarian follicles, oocytes and embryo tissues of rainbow trout with $[1,2,6,7-^3\text{H}]$ cortisol *in vitro*.

	Acetonitrile:water gradient (collection time)	Flow rate (ml/min)	Frequency of eluate collections
Protocol I	Binary gradient from 28:72 to 90:10 (40 min)	0.7	Every 1 min
Protocol II	Single isocratic gradient 25:75 (25 min)	0.5	Every 15 s

Figure 1 HPLC profiles of reference standards run using two different mobile phases (Table 2). (A) Stage 1: 28–90% acetonitrile/water binary gradient run over 40 min. Only the glucocorticoid steroid reference standards in the reference standard cocktail are numbered: (1) cortisol, (2) cortisone, (3) corticosterone and (4) 11-deoxycorticosterone. The other reference standards (androgens, oestrogens and progestogens) are grouped together under horizontal lines: (a) androgens, (b) a range of androgens and oestrogens and (c) progestogens; for further details, see Khan *et al.* (1997a, 1997b). (B) Stage 2: 25% acetonitrile:water isocratic gradient over 25 min. Reference steroid standards: (1) cortisol and (2) cortisone.

Glucuronidated steroids

There were no discernable ^3H -labelled peaks in the glucuronide fraction following the incubation of oocytes or embryos with ^3H -cortisol, suggesting that glucocorticoid glucuronidation does not occur at a high level in these cells and tissues. There was a single ^3H -labelled peak eluting at ~ 12 min following the stage 1 HPLC protocol after the co-incubation of intact ovarian follicles with ^3H -cortisol (not shown). However, this represented $<5\%$ of the total counts, suggesting that if glucuronidation of cortisol or of its metabolites does occur in the follicles, it is not a major pathway of cortisol metabolism. Consequently, this study focused on the unconjugated and sulphated steroids that were present in the incubation media of the various tissues examined.

Unconjugated and sulphated steroids

Ovarian follicles

The type of the medium used for the incubation had no effect on the radiolabelled steroid profiles of the unconjugated and conjugated fractions for both intact and YF follicles. Moreover, only unconjugated ^3H -cortisol was present in the eluates of the SPE of OvF

incubated without tissue (medium blank), indicating that OvF itself has no inherent $11\beta\text{-HSD}$ or GTS activity.

Figure 2 shows the unconjugated and sulphated ^3H -labelled steroids following the co-incubation of intact ovarian follicles with ^3H -cortisol using the sequential stage 1 (A) and stage 2 (B) HPLC separation of steroids. After stage 1, in both the unconjugated and the conjugated (predominantly sulphate) fractions, there was a single major ^3H -labelled peak eluting between 6 and 8 min that accounted for $\sim 40\%$ of the total counts (Fig. 2A). In addition, there were several minor, as yet unidentified ^3H -labelled peaks eluting at ~ 3 –4, ~ 8 , ~ 12 and ~ 22 min representing ~ 6 , ~ 6 , ~ 6 and $\sim 3\%$ of the total radioactivity counts respectively (Fig. 2A). The stage 2 separation protocol confirmed that the major peak evident in the unconjugated fraction (Fig. 2A) was a combination of predominantly ^3H -cortisol together with a smaller peak of ^3H -cortisone; major peak in the conjugated fraction was largely ^3H -cortisone sulphate together with a smaller amount of ^3H -cortisol sulphate (Fig. 2B).

The cortisol metabolic products identified in the incubation medium following the co-incubation of YF ovarian follicles with ^3H -cortisol were identical in nature and relative abundance to those found in media following the incubation of intact ovarian follicles.

Figure 2 HPLC profiles of the products of metabolism of ^3H -cortisol by rainbow trout (*Oncorhynchus mykiss*) intact ovarian follicles incubated in M199. Both the unconjugated (free) and the sulphated steroid fractions after stages 1 (A) and 2 (B) of the HPLC protocol are shown. Cortisone is present in both the free and the sulphate SPE fractions; most of the cortisone appears as a sulphate. (Reference standards were included in each HPLC run (unconjugated steroids – solid line related to the left y-axis; sulphated steroids – dotted line related to the right y-axis). * unidentified ^3H -labelled steroids. See the legend to Fig. 1 for labels.

Figure 3 HPLC profiles of the products of metabolism of [^3H]cortisol by rainbow trout (*Oncorhynchus mykiss*) ovulated oocytes incubated in ovarian fluid (OvF). Both the unconjugated (free) and the sulphated steroid fractions after stages 1 (A) and 2 (B) of the HPLC protocol are shown. Cortisone is present only in the sulphated steroid SPE fraction. (Reference standards were included in each HPLC run (unconjugated steroids – solid line relates to the left y-axis; sulphated steroids – dotted line relates to the right y-axis). See the legend to Fig 1 for labels. * Minor ^3H -labelled peaks of unknown chemistry.

Ovulated oocytes

After stage 1 HPLC separation of steroids present in the OvF medium following the co-incubation of ovulated oocytes in OvF with [^3H]cortisol, there was a single major ^3H -labelled steroid peak in both the unconjugated and the conjugated fractions (>60 and $\sim 15\%$ of the total counts respectively) (Fig. 3A). In addition, there was again evidence of an additional ^3H -labelled peak (sulphated fraction), representing $\sim 6\%$ of the total counts eluting at ~ 3 – 4 , ~ 14 min (Fig. 3A). After stage 2 of the HPLC separation of the steroids, the single major peak identified at stage 1 was found to be predominantly [^3H]cortisol in the unconjugated fraction, much smaller peaks ($\sim 10\%$) of [^3H]cortisol sulphate and [^3H]cortisone sulphate in the conjugated fraction (Fig. 3B).

Embryo tissues

After stage 1 of the HPLC steroid separation protocol, there was a single major ^3H -labelled steroid peak in the unconjugated (free) fraction (>60 , >50 and $>30\%$ of the total counts in the 26-, 40- and 58 dpf embryo tissues respectively) and conjugated fraction (<10 , >10 and $\sim 7\%$ of the total counts – predominantly sulphate in the 26-, 40- and 58 dpf embryo tissues respectively) (Fig. 4A, B and C).

Following stage 2 of the HPLC protocol, the unconjugated ^3H -labelled steroids present for all three stages of embryo tissues were primarily [^3H]cortisol together with traces of [^3H]cortisone (Fig. 5). The sulphated

glucocorticoids were primarily [^3H]cortisone sulphate in the 40 ($>10\%$) and 58 dpf ($>15\%$) embryos) together with small amounts of [^3H]cortisol sulphate; for the 26 dpf embryos, there were approximately equal amounts ($\sim 4\%$) of [^3H]cortisol sulphate and [^3H]cortisone sulphate (Fig. 5A). In addition, there was evidence of minor peaks ($<5\%$ of the total counts) of ^3H -labelled unidentified products in the sulphate fraction (Fig. 4B). Depending on the age of the embryo, these eluted at ~ 3 – 4 , ~ 8 , ~ 12 and ~ 20 min (Fig. 4B).

Although the profiles of the major products were similar in all three embryo age groups, there was a progressive decrease of unconjugated [^3H]cortisol from $>70\%$ of the total counts for the 26 dpf embryos, $\sim 55\%$ for the 40 dpf embryos and $<40\%$ for the 58 dpf embryo tissues. Conversely, whereas there was a relatively similar level of [^3H]cortisol sulphate for all three ages of embryos, the levels of [^3H]cortisone sulphate increased from $\sim 4\%$ of total counts for the 26 dpf embryos to ~ 12 and $\sim 15\%$ of the total counts for the 40 and 58 dpf embryo tissues respectively (Fig. 5A, B and C).

Discussion

This study provided direct evidence of the metabolism of the potent glucocorticoid, cortisol, to form the less potent glucocorticoid, cortisone, by rainbow trout intact

Figure 4 HPLC profiles of the products of metabolism of [^3H]cortisol by rainbow trout (*Oncorhynchus mykiss*) embryos (26 (A), 40 (B) and 58 (C) days post-fertilization (dpf)) incubated in M199 after stage 1 of the HPLC protocol. Both the unconjugated (free) and sulphated steroids are shown. A single peak overlapping the elution time of the cortisol and cortisone reference standards is found for all stages of embryos. (Reference standards were included in each HPLC run (unconjugated steroids – solid line relates to the left y-axis; sulphated steroids – dotted line relates to the right y-axis). See the legend to Fig 1 for labels. * Minor ^3H -labelled peaks of unknown chemistry.

Figure 5 HPLC profiles of the products of metabolism of [3 H]cortisol by rainbow trout (*Oncorhynchus mykiss*) embryos (26 (A), 40 (B) and 58 (C) days post-fertilization (dpf)) incubated in M199 after stage 2 of the HPLC protocol. Both the unconjugated (free) and sulphated steroids are shown. At all developmental stages, cortisol is predominantly present as an unconjugated steroid, with traces (4–6% of the total counts) as a sulphated steroid, whereas cortisone is largely present in the form of a sulphate. (Reference standards were included in each HPLC run (unconjugated steroids – solid line relates to the left y-axis; sulphated steroids – dotted line relates to the right y-axis). See the legend to Fig 1 for labels.

and YF ovarian follicles, naturally ovulated oocytes and embryo tissues; in addition, the study provided evidence of sulphation of these glucocorticoids, which was particularly marked for the intact and YF ovarian follicles. The findings are indicative of the presence of 11 β -HSD and GTS activity from ovum to late-stage embryos. The study also demonstrated that the incubation medium used did not affect the extent of steroid conjugation or steroid biotransformation. *In vivo*, this is particularly relevant with regard to OvF in which the oocytes are retained in the body cavity after ovulation, and which may contain steroid metabolic enzymes released from the follicle during ovulation. Based on the distribution of counts among the various SPE fractions (Table 1) and the HPLC separation of steroids (data not shown), there was no metabolism of [3 H]cortisol following incubation in native OvF.

The findings suggest a role for the ovarian follicles of fish in protecting the oocyte from the unregulated translocation of cortisol from maternal (ovarian) extracellular fluid to the oocyte. Not only were the ovarian follicles able to convert cortisol to cortisone but they also had a marked glucocorticoid conjugation capacity, largely to form steroid sulphates. The net increase (i.e. the increase over values present in medium blanks containing no tissue) in the tritium counts in the unconjugated fraction was 13–20% in the medium

following the incubation of intact ovarian follicles (Table 1). The net increases in counts in the conjugated fraction were considerably lower (in the 4–7% range) following the incubation of an equal number of YF ovarian follicles (Table 1). The findings suggest that at least some of the steroid metabolic enzymatic/sulphation activity was associated with the thecal or granulosa cells of the follicle. This is consistent with the demonstration of 11 β -hsd expression in ovarian follicles that was reported by Kusakabe *et al.* (2003). However, the findings that show a reduced percentage of conjugation by the YF follicles relative to the intact follicles (Table 1) might suggest that the oocyte also participates in the biotransformation of [3 H]cortisol by the intact follicles *in vitro*; however, there was negligible sulphation or biotransformation of [3 H]cortisol by naturally ovulated oocytes incubated in OvF (Table 1), which suggests that the contribution of the oocyte to glucocorticoid metabolism is considerably less than that of the ovarian follicle steroidogenic tissues. Although it is possible that there is a change in the capacity of glucocorticoid metabolism from the late-stage intrafollicular oocyte to the newly post-ovulation stage, it is more likely that the lower conjugation values in the YF ovarian follicle incubations relative to those of the intact follicles are due to the loss of integrity (and therefore of enzyme content) of the thecal or granulosa cell layers during the puncture, manipulation and repeated washing of the follicle tissues that was necessary to ensure the complete extrusion of the ooplasm.

The data from the SPE fractionation suggest that relative to the ovarian follicles, there appeared to be no net gain in counts in the conjugated fraction for ovulated oocytes or early-stage embryos (Table 1); however, there was some evidence of a net gain of ~8% of the radioactive counts in the conjugation fraction following incubation of the 58 dpf embryo tissues with [3 H]cortisol (Table 1). However, the HPLC profiles of the medium following the co-incubation of oocytes and all three stages of embryos with [3 H]cortisol consistently showed the presence of low levels of [3 H]cortisol sulphate, [3 H]cortisone and [3 H]cortisone sulphate (in addition to the major steroid peak, [3 H]cortisol), which suggests that there is some 11 β -HSD and GST activity in these cells and tissues.

The findings presented herein suggest a role for the ovarian follicle in protecting the oocyte from the transfer of undue amounts of cortisol by ovarian follicle cells, both by the production of the less biologically potent cortisone and by steroid conjugation. However, in light of the reported increases in oocyte cortisol content following on from increases in maternal plasma cortisol concentrations (Stratholt *et al.* 1997, Contreras-Sánchez *et al.* 1998, Eriksen *et al.* 2006, Leatherland *et al.* 2010), it would appear that the maternal blood–oocyte barrier is only partially effective. Furthermore, as increases in oocyte cortisol levels before their fertilization affect the

expression patterns of several genes (Li *et al.* 2010, 2011, 2012), the rate of replication of early embryonic cells (Li *et al.* 2012) and the behavioural phenotype of the juvenile fish (Espmark *et al.* 2008, Sloman 2010, Eriksen *et al.* 2011), it would appear that the capacity of the oocyte, zygote and early embryo to metabolize cortisol is limited making these developmental stages more vulnerable to changes in their cortisol milieu.

In this study, there was no net gain (compared with the medium blanks) in the radioactive counts in the FT fraction following SPE (Table 1), which suggests no catabolic pathway involving the hydroxylation of any of the four points of labelling of the [2,4,6,7-³H]cortisol backbone. The only catabolic pathway that was identified in this study was the metabolism of [³H]cortisol to form [³H]cortisone, which would not involve any of the four points of labelling.

The relatively limited cortisol metabolic capacity of embryos was not anticipated, particularly that of late-stage embryos in which the major organ systems (including the liver) were present (Table 1 and Fig. 5). There was an apparent progressive increase with embryo age in steroid conjugation (Table 1 and Fig. 5), but this may be explained on the basis of the increasing amount of embryonic tissue (particularly hepatic tissue) present as the embryos increased in age and size.

In summary, the study shows that although ovarian follicles, oocytes (in OvF) and early embryo tissues were able to metabolize cortisol to cortisone and to produce cortisol and the cortisone sulphates, the potency of the ovarian follicles was markedly higher than that of the oocytes and early-stage embryos, suggestive of a protective role for the ovarian follicle (probably by the action of the thecal and granulosa cells) in restricting the translocation of cortisol from the maternal tissues before ovulation. However, increases in oocyte cortisol content are known to occur in salmonid fishes, suggesting that the protection has its limits. Consequently, any increase in the translocation of the glucocorticoid from maternal tissues during ovarian maturation has the potential to be carried forward into the early stages of embryogenesis in which there appears to be only limited capacity to metabolize or sulphate the glucocorticoid. As a result, this 'carry forward' of maternal cortisol may impact the phenotype of the animal at a later developmental stage. The severity of maternal stress during the critical stages of oocyte maturation likely determines the extent of the within-cohort embryonic phenotypic variation, depending on the extent to which the level of cortisol transfer exceeds the limits of the protective role of the 11 β -HSD and GSTs.

Materials and Methods

Animal care protocols

The University of Guelph Animal Care Committee approved the experimental protocols used in this study. The fish used

were obtained either from the Alma Aquaculture Research Station (Alma, ON, Canada) or Cedar Crest Trout Farm (Hanover, ON, Canada).

Chemicals

M199 was purchased from Invitrogen as the culture medium. Steroids used for HPLC reference standards were purchased from Steraloids (Newport, RI, USA). BSA, L-glutamine, β -D-glucose, bovine β -glucuronidase and streptomycin sulphate were purchased from Sigma-Aldrich Canada Ltd. [1,2,6,7-³H]Hydrocortisone (cortisol) was purchased from Perkin Elmer (Boston, MA, USA).

Cell and tissue incubations

Incubation media

M199, supplemented with 0.1% BSA, 0.01% L-glutamine and 0.1% β -D-glucose, was used for the incubations of ovarian follicles and embryos, and OvF harvested during the collection of naturally ovulated oocytes was used for incubation of ovarian follicles and naturally ovulated oocytes. OvF was used specifically for incubations of oocytes because this is the medium in which the ovulated oocytes are immersed in the peritoneal cavity of the female before ovulation. OvF maintains the flexible characteristic and permeability of the zona pellucida, whereas synthetic aqueous media cannot be used because of the rapid hydration (and thereby the loss of permeability) of the zona pellucida. Comparative incubation of ovarian follicles was made using M199 and OvF to determine whether OvF has effects of cortisol metabolism.

Intact and YF ovarian follicles

For each trial, two or three mature rainbow trout (3+ year class) were used as a source of ovarian follicles for the first series of studies. Each animal was killed by a blow to the head, and its ovaries were removed for the collection of mid-vitellogenic stage follicles. In a series of studies, the relative effects of M199 and OvF on cortisol catabolism were examined. Briefly, intact follicles were removed from the ovary and allocated randomly, ten follicles per well, to 24-well tissue culture plates (Corning Incorporated, Corning, NY, USA) containing 2 ml ice-cold medium (at least four replicates); 10 μ l of a stock solution of [³H]cortisol were then added to each well to make a final concentration of 0.25 μ Ci/ml and the preparation was incubated for 18 h at 8 °C on a tilt shaker.

An additional set of follicle samples taken from the same ovary was pierced with a fine needle and the follicle was gently pressed to express the ooplasm. The YF follicles were then washed several times in fresh medium to remove the oocyte cell debris before being incubated in either M199 or OvF in the same manner as that described for the intact follicles. An additional 2 ml of each medium and 10 μ l of a stock solution of [³H]cortisol were incubated without follicles as a medium blank.

Ovulated oocytes and embryos

A pool of naturally ovulated oocytes was obtained by manually stripping five rainbow trout. Some of these oocytes were used

for co-incubation with [^3H]cortisol in OvF and others were fertilized to produce embryos for the future co-incubation of embryo tissues with [^3H]cortisol.

Fifteen oocytes were placed in 24-well tissue culture plates (ten replicates) containing 2 ml OvF, and [^3H]cortisol was added to each well as described earlier. The remaining oocytes were maintained in OvF until they were fertilized with aliquots of a sperm pool harvested from five fish. The oocytes and sperm were gently mixed together and a small volume of water was added to activate the sperm and promote the penetration of the sperm through the micropyle of the oocytes. The eggs were then washed in running water to allow the *zona pellucida* to water harden, and the pre-zygotic oocytes were transferred to Heath incubators supplied with aerated, running artesian well water at 8 °C. 'Eyed stage' (26 dpf), late pre-hatch (40 dpf), and late yolk absorption (58 dpf) stage embryos were used to examine the capacity of embryos to metabolize [^3H]cortisol. For each developmental stage, the body of each embryo tissues was separated from the yolk-sac and cut into pieces as described previously (Petkam *et al.* 2003a, 2003b) to allow penetration of cortisol. Tissue from ten 26 dpf embryos, eight 40 dpf embryos and two 58 dpf embryos ($\times 12$ replicates) were co-incubated with [^3H]cortisol in 2 ml supplemented M199 (described earlier) for 18 h at 8 °C on a tilt shaker.

Solid-phase extraction

Following the various incubations, the medium was removed from the tissue and the steroids present in the incubation medium were extracted by solid-phase chromatography to separate the unconjugated and conjugated steroids before separation of the steroids in the conjugated and unconjugated fractions by means of reverse-phase HPLC.

Sep-Pak C_{18} cartridges (Waters Corp., Milford, MA, USA) were used for the SPE procedure as described previously (Khan *et al.* 1997a, 1997b, Petkam *et al.* 2003a, 2003b, Barkataki *et al.* 2011, 2012). Each cartridge was primed by adding 5 ml methanol followed by 5 ml ddH₂O wash; each sample was mixed with double-distilled water (ddH₂O) to make 5 ml, and this was then added to a primed cartridge and allowed to elute. Each cartridge was then washed with 5 ml ddH₂O, which was then displaced with 5 ml hexane. The unconjugated (free) steroid fraction was eluted from the cartridge with 5 ml diethyl ether, and the conjugated steroid fraction was then eluted with 5 ml methanol. The amount of radioactivity in the water (FT) eluate (which contained no steroid) and the unconjugated and conjugated eluates was measured by adding 50 μl of each eluate fraction to 4 ml scintillation cocktail (Ecolite, MP Biomedicals, Solon, OH, USA); a Searle Delta 300 scintillation counter was used to measure radioactivity as counts per minute. The diethyl ether (unconjugated) and methanol fractions (conjugated) were dried under nitrogen at <45 °C in a water bath, for further processing.

For each sample, the sulphate and glucuronide conjugates in the methanol fraction were sequentially separated first by acid solvolysis (sulphate) and then by glucuronidase hydrolysis (glucuronide). The dried methanol fractions were incubated with trifluoroacetic acid/ethyl acetate (1:100, v:v) overnight at 45 °C to remove the sulphate from the sulphated steroids; the

fractions were then evaporated under nitrogen at <45 °C in a water bath and redissolved in 5 ml ddH₂O. The aqueous solution was then submitted to SPE as described earlier to separate the previously sulphated steroid fraction (in diethyl ether) from the glucuronidated steroids (in methanol). The methanol fraction containing the glucuronidated steroids was then evaporated under nitrogen at <45 °C, redissolved in 500 μl 0.5 M sodium acetate (pH 5) containing 2000 U bovine hepatic glucuronidase and incubated overnight at 37 °C; 5 ml ddH₂O was then added and the mixture was subjected to SPE to extract the previously glucuronidated steroids in the diethyl ether fraction. The radioactive counts in each elute fraction at each stage of the processing were measured as described earlier. The radioactive counts of the SPE eluate samples (unconjugated and conjugated steroid fractions, and the FT water eluate) were calculated as a per cent of the total count (Table 1).

To evaluate the extent of residual counts in the tissues, the intact and YF follicles incubated in M199 were subjected to further extraction. Following the removal of the medium after incubation, the tissues were transferred to glass tubes, crushed, sonicated in 1 ml 80% ethanol and left to stand at room temperature for 48 h. The supernatant was then transferred to a clean tube, and the sonication was repeated using 1 ml 100% methanol and then 1 ml ether. The pooled supernatants were left at room temperature in uncapped tubes for 18 h to allow the ether to evaporate. The radioactive counts in the extracts were measured as described earlier. The values for intact and YF follicles were 7.5 ± 0.2 and $2.4 \pm 0.1\%$ respectively, which approximates the ratio of the initial volume of tissues for intact follicles ($\sim 50 \mu\text{l}$) and YF follicles ($\sim 20 \mu\text{l}$).

Reverse-phase HPLC protocol

A Waters Model 2690 Alliance Separation Module HPLC system fitted with a Waters 996 Photodiode Array Detector, and using Millennium 32 Software, was used to create an acetonitrile:water (mobile phase) binary gradient (Waters # 8). Two HPLC stages were used to separate the ^3H -labelled steroids present in the incubation media after the co-incubation of ovarian follicles, oocytes and embryo tissues with [^3H]cortisol (Table 2). Stage 1 employed a binary gradient from 28:72 to 90:10 over 40 min, on a Waters Nova-Pak C_{18} (150 $\times$ 39 mm, 4 μm diameter core) column; the flow rate was 0.7 ml/min, and fractions were collected each minute for 40 min. The radioactivity in each fraction was measured by adding a portion of the fraction to a scintillation cocktail as described earlier. At this stage of the HPLC protocol, there appeared to be a single ^3H -labelled steroid peak eluting between 5 and 7 min, overlapping the period when cortisol and cortisone elute from the column. To determine whether this represented a single peak or overlapping peaks, stage 2 of the HPLC protocol involved the pooling of the fractions of 5–7 min peaks following stage 1, drying them under N_2 , reconstituting them in 20% acetonitrile and then use a mobile phase of acetonitrile:water 25:75 on the same column run over 25 min with a flow rate of 0.5 ml/min; fractions were collected every 15 s; and the radioactivity in each fraction was measured as described earlier. Similar HPLC profiles using a sequential

combination of stages 1 and 2 were obtained for medium blanks ($[^3\text{H}]$ cortisol incubated in medium alone (no tissue) for 18 h at 8 °C), and for the $[^3\text{H}]$ cortisol stock (without incubation) to confirm that the radioactivity was restricted to a single peak (present only in the unconjugated fraction) that co-elutes with the authentic cortisol references. A mixture of 21 authentic steroid reference standards (Khan *et al.* 1997b, Petkam *et al.* 2003a, 2003b) was included in all HPLC runs to compare the elution times of the ^3H -labelled steroids with the standards; the absorbance of the standards was monitored at a light wavelength of 254 nm (representative chromatographs of the reference standards using the two protocols are shown in Fig. 1).

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.

Funding

The project was funded by the by a Discovery Grant from the National Research Council of Canada, a Research Grant from the Ontario Ministry of Agriculture, and a Doctoral Scholarship from the National Research Council of Canada.

Acknowledgements

The authors wish to thank the staff at the Alma Aquaculture Research Centre, ON, for their assistance in the collection of oocytes and rearing of the embryos. Thanks are extended to Cedar Crest Trout Farm (Hanover, Ontario Canada) who donated some of the fish that were used for ovarian follicle samples.

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Received 13 September 2012

Accepted 15 October 2012