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Inhibition of cortisol metabolism by 17α,20β-P: Mechanism mediating semelparity in salmon?

Terence P. Barry [a,*], Ashok Marwah [b], Scott Nunez [c]

[a] Department of Animal Sciences, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI 53706, USA
[b] Department of Biochemistry – Enzyme Institute, University of Wisconsin-Madison, 1710 University Ave., Madison, WI 53726, USA
[c] Mathematics, Engineering and Sciences Division, Lee College, P.O. Box 818, Baytown, TX 77522-0818, USA

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ABSTRACT

In vitro experiments were conducted to test the hypothesis that 17α,20β-dihydroxy-4-prengnen-3-one (17,20-P) regulates cortisol metabolism in Pacific salmon. In both rainbow trout and coho salmon, cortisol metabolism was significantly higher in the kidney compared to the liver. The rainbow trout kidney converted cortisol primarily into an unidentified water-soluble metabolite with a molecular mass of 354. The coho salmon kidney converted cortisol primarily into cortisol-21-sulfate. High physiological concentrations of 17,20-P had no effect on cortisol metabolism by the rainbow trout kidney, but almost completely inhibited the production of cortisol-21-sulfate by the coho salmon kidney. This was accompanied by a coincident increase in the production several neutral cortisol metabolites, including cortisone. Cortisone was also found to inhibit renal sulfotransferase (SULT) activity suggesting that there could be a local positive feedback mechanism initiated by the rise in 17,20-P that quickly reduces SULT activity as follows: the pre-spawning rise in 17,20-P inhibits SULT, cortisol is metabolized to cortisone instead of cortisol-21-sulfate, cortisone further inhibits SULT, more cortisone is produced, and so on. If SULT normally acts as a gatekeeper enzyme to protect the cell from cortisol excess, this mechanism would rapidly remove enzymatic protection and expose tissues to high local concentrations of cortisol. In addition, the inhibition of peripheral cortisol metabolism by 17,20-P could increase circulating concentrations of the corticosteroid. These events could be a part of the mechanism that leads to the symptoms of cortisol excess associated with the post-spawning mortality of semelparous Pacific salmon.

1. Introduction

The die-off of Pacific salmon shortly after they spawn is one of the most dramatic but poorly understood phenomena in biology (Dickhoff 1989). Cortisol excess of unknown etiology mediates this programmed death by causing tissue degeneration, suppressing the immune system, and impairing various homeostatic mechanisms (Robertson and Wexler, 1960; reviewed by Dickhoff, 1989; Stein-Behrens and Sapolsky, 1992). Evidence that gonadectomy blocks the normal increase of cortisol in pre-spawning salmon suggests that a gonadal factor regulates cortisol excess (Fagerlund and Donaldson, 1970; Donaldson and Fagerlund, 1972). Given the temporal disassociation between elevated androgen and estrogen levels with pre-spawning cortisol excess, it is unlikely that these steroids are the responsible factors. The steroid 17α,20β-dihydroxy-4-prengnen-3-one (17,20-P) is produced by the gonads of salmonid fish in response to a pre-spawning rise in gonadotropin, and regulates final gamete maturation in both sexes (reviewed by Scott and Canario, 1987; Nagahama, 1987; Nagahama et al., 1993). In salmonids, blood levels of 17,20-P rise markedly prior to spawning, and in some species remain elevated for several days thereafter (Scott et al. 1983). Typically, peak 17,20-P levels are higher in females (~300 ng/ml) than males (10–100 ng/ml) (Ueda et al., 1984). Only very low concentrations of 17,20-P are required to stimulate final gamete maturation (100-fold less than peak plasma concentrations) in both sexes, and thus it has been postulated that 17,20-P plays other physiological roles at spawning (Scott and Canario 1987).

Given the close temporal relationship between the rise in 17,20-P and the death of spawning salmonids, we hypothesize that cortisol excess in Pacific salmon is regulated by the pre-spawning rise of 17,20-P. Specifically, we postulate the following: Under normal physiological conditions, even in the face of elevated stress-induced cortisol levels, peripheral targets are protected from cortisol excess by cortisol-metabolizing enzymes, which inactivate cortisol before it can bind to cellular receptors and initiate a biological response. At spawning, however, 17,20-P inhibits peripheral cortisol metabolism, and thus, cortisol receptors in various targets are exposed to high concentrations of cortisol only when 17,20-P levels

* Corresponding author. Fax: +1 608 262 0454.
E-mail address: tpbarry@wisc.edu (T.P. Barry).

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are elevated at the time of spawning. The purpose of the present investigation was to use an *in vitro* tissue culture system to characterize cortisol-metabolizing activity in the liver and kidney of the semelparous coho salmon (*Oncorhynchus kitsuch*) and the iteroparous rainbow trout (*Oncorhynchus mykiss*), and investigate the effects of 17,20-P, and other sex steroids, on cortisol metabolism in these species. If our hypothesis regarding the role of cortisol-metabolizing enzymes in mediating post-spawning mortality is correct, we expected to observe differences in cortisol metabolism between these two species of Pacific salmon.

2. Materials and methods

2.1. Fish

Kamloops strain rainbow trout were obtained from commercial sources in Wisconsin. Coho salmon were obtained from the Lake Mills State Fish Hatchery, Lake Mills, Wisconsin. The fish were reared in 750-L flow-through tanks under ambient temperature conditions (11–16 °C) and a constant 16-h light/8-h dark photoperiod. The fish were fed Silver Cup trout feed (Murray, UT) once daily at approximately 1% of their body weight. Food was withheld 24 h prior to sacrifice. The experimental fish were mixed-sex and ranged in size from 80 to 263 g. All were sexually immature as evidenced by macroscopic examination of the gonads following sacrifice.

2.2. In vitro cultures

In vitro cultures of rainbow trout and coho salmon liver and kidney tissues were conducted using a modification of the method of Barry et al. (1997). In brief, three to five fish were sacrificed using a lethal dose of MS-222. The livers and kidneys were removed and pooled in a 50 ml polypropylene culture tube containing 20 ml of ice-cold physiological saline (Kobayashi et al., 1986). The tissues were minced with a Polytron homogenizer set at the lowest speed until the tissue was finely fragmented. The tissue fragments were washed for 30s by gentle vortexing in 10 ml of physiological saline, centrifuged at 2000*g*, and the supernatant withdrawn with a glass pipette. This washing procedure was repeated a total of four times using fresh saline. After the final wash, the saline was withdrawn and fresh culture medium (approximately 5× tissue vol.) was added to the tube. The tissue fragments were allowed to loosely settle to the bottom of the tube, and 50-μl aliquots of tissue were removed and added to individual 150 × 20-mm culture tubes. Tissue was transferred using a 1-ml pipette tip whose tip was cut off to increase the size of the orifice to ~4 mm. Culture medium and 10 μl of concentrated test solution(s) were then added to each well to a final incubation volume of 1 ml. Cultures were conducted under ambient air at 15 °C for 1–24 h with constant shaking in a temperature-controlled incubator. All treatments were carried out in duplicate or triplicate within each experiment, and each experiment was repeated at least twice with tissues from different pools of fish. To obtain sufficient cortisol metabolites for identification by LC–MS, some larger-scale (10-fold the conditions described above) incubations were conducted with rainbow trout and coho salmon kidney tissues.

2.3. Cortisol metabolism by the liver and kidney

Cortisol metabolism was analyzed by measuring the formation of ether- and water-soluble products after incubation of kidney and liver fragments of both species in the presence of [4-^{14}C]-cortisol (150 nM total cortisol concentration) for 1–24 h at 15 °C. [4-^{14}C]-Cortisol (53.5 mCi/mmol) was purchased from NEN-Perkin-Elmer (Boston, MA). Cultures were conducted in triplicate in pre-weighed (±0.1 mg), 150 × 20-mm test tubes. Controls were incubates without tissue. The effects of various steroid hormones on cortisol metabolism was evaluated by conducting the cultures in the presence of 17,20-P, estradiol-17β (E_2), testosterone (T), 11-ketotestosterone (11-KT), 17α,20α-dihydroxy-4-prengnen-3-one (17,20α-P), cortisol, cortisone, or the 11β-hydroxsteroid dehydrogenase inhibitor, 18β-glycyrrhetinic acid (GA). All steroids and GA were tested at 1.5×10^{-6} M except for 17,20-P, which was tested at various doses (detailed below). Steroids were obtained from Sigma Chemical Co. (St. Louis, MO) or Steraloids (Newport, RI). Concentrated steroid stock solutions were prepared in ethanol and subsequently diluted in physiological saline.

Neutral steroid metabolites were extracted from the medium three times with diethyl ether (5× vol.). The ether fractions were collected by rapidly freezing the aqueous layer in a dry ice/ethanol bath, pouring the ether into a clean 16 × 120-mm test tube, and dried under a stream of nitrogen gas at 40 °C. The products were separated using 20 × 20 cm, 60 Å, 19-channel, preadsorbent plates with fluorescent indicator (Whatman No. 4866 821, Clifton, NJ), and the solvent system chloroform:methanol (97:3). Radioactivity on the plates was imaged and quantified using an InstantImager (Perkin-Elmer, Wellesley, MA). Recovery of [^{14}C]-cortisol from the no tissue control incubations was used to estimate and correct for procedural loss. The radioactivity remaining in the culture medium after ether extraction was quantified by liquid scintillation counting. Cultures were standardized by dry tissue weight, which was determined by drying the test tubes in a vacuum oven overnight and weighing to the nearest 0.1 mg.

2.4. Characterization of cortisol metabolites

In both rainbow trout and coho salmon, cortisol was primarily converted into water-soluble metabolites (WSMs), which were characterized both by liquid chromatography–mass spectroscopy (LC–MS) and biochemically.

2.4.1. LC–MS

Total metabolites (neutral and conjugated) were extracted using Oasis C-18 cartridges (Waters, Inc., Milford, MA). Chromatography of cortisol and its neutral metabolites was performed according to Marwah et al. (2002) on C_{18} analytical columns (Zorbax-SB, 3.0 × 150 mm, 3.5 μm, Mac-Mod, and Novapack, 3.9 × 75 mm, 4.0 μm, Waters Inc., Milford, MA) protected with matching C_{18} guard columns at a flow rate of 0.4–0.8 ml/min and a temperature of 20–40 °C. The solvent system was acetonitrile/water with a linear gradient of 20–45% acetonitrile in 25 min, 94% at 32 min and back to 20% at 34 min, followed by a 10 min post-run time. Water-soluble conjugates were resolved on a C_{18} analytical column (Zorbax-SB, 3.0 × 150 mm, 3.5 μm) at a flow rate of 0.4 ml/min using acetonitrile–water and acetonitrile containing 3% acetic acid and 3% acetic acid gradients. The gradients started with 10% acetonitrile to reach 40% in 30 min, and 96% acetonitrile in 38 min and back to 10% at 40 min. Radioactivity was quantified using an inline radioactivity detector.

2.4.2. Biochemical characterization

Ether-extracted culture media was dried under a stream of nitrogen gas at 40 °C, and incubated for 18 h at 50 °C in 5 ml trifluoroacetic acid/ethyl acetate (1/100, v/v) to cleave glucuronic acid and/or sulfate groups from the steroid conjugates and release free steroids (Scott and Canario, 1992). The trifluoroacetic acid/ethyl acetate solution was dried under a stream of nitrogen at 40 °C. The free steroids were dissolved in methanol and separated and quantified by TLC as described above. Further analysis was conducted to determine if the WSMs were primarily steroid glucuronides or sulfates. Acetylation transforms water-soluble glucuronides

into benzene-soluble triacetyl glucuronides, but has no effect on sulfates (Scott and Canario, 1992). Steroid conjugates in the ether-extracted culture medium were concentrated on Oasis C-18 cartridges, and eluted with 3 ml methanol. The methanol was evaporated under a stream of nitrogen gas at 40 °C, and the residue treated overnight with 2 ml of a pyridine:acetic anhydride (50:50, v:v) solution. The reaction mixture was dried under a stream of nitrogen gas at 40 °C, and partitioned between 200 µl water and 4 ml benzene. Radiation in each fraction was quantified by liquid scintillation counting.

The neutral compounds were analyzed in the mass detector using electro-spray ionization in positive mode. Operating conditions were optimized by flow injection analysis of cortisol. Conjugated cortisol metabolites were analyzed in negative ion mode using only electro-spray ionization (conjugates are poor substrates for chemical ionization). Operating conditions were: drying gas (N_2), 8 L/min; drying gas temperature, 350 °C; nebulizer pressure, 40 psi; fragmentor, 80 V; and capillary voltage, 3000 V. Some unknown metabolites were positively identified by comparing their retention times under two different chromatographic conditions, and their mass spectral data, with pure chemically synthesized compounds. The identification of all unknown metabolites, however, was beyond the scope of this investigation.

2.5. Statistics

The *in vitro* studies were analyzed by ANOVA and means comparisons were made using protected LSD tests. For every experiment, the duplicate tissue pools gave essentially identical results, and only the results from one duplicate are shown. The units of comparison shown, therefore, are the pseudo-replicates from an individual tissue pool. All data are shown as mean ± standard error of the mean (SEM).

3. Results

3.1. Cortisol metabolism by liver and kidney

The livers of both rainbow trout and coho salmon had very low cortisol metabolic activity. Under the experimental conditions used (i.e., 20 h cultures, 15 °C, average dry tissue weight of 6.2 ± 1.0 mg per incubation, the livers of both species never converted more than 6% of added [^{14}C]-cortisol to cortisol metabolites. No species differences were detected in hepatic cortisol metabolism.

The kidneys of both rainbow trout and coho salmon had significantly higher cortisol metabolic activity than the liver. Under similar experimental conditions used to evaluate hepatic cortisol metabolism (i.e., 20 h cultures, 15 °C, average dry tissue weight of 4.3 ± 1.1 mg per incubation), the kidneys of rainbow trout and coho salmon converted 19% and 48% of added cortisol into cortisol metabolites, respectively.

In both species, over 95% of the radioactivity that was not accounted for by unmetabolized cortisol was in the form of water-soluble metabolites. Time-course studies indicated that WSM production by the kidneys of both rainbow trout and coho salmon increased steadily over a 24-h period, and that the coho salmon kidney had significantly higher cortisol metabolic activity than the rainbow trout kidney (Fig. 1).

3.2. Major renal cortisol metabolites

Total cortisol metabolites (both neutral and water-soluble products) were extracted from large-scale incubations using solid-phase cartridges, and quantified by LC–MS. Cortisol had a retention time of approximately 15.25 min (Figs. 2 and 3). The rainbow trout kidney converted cortisol into one major metabolite with a retention time of 13.6 min (Fig. 2). Mass spectroscopy showed that this major peak was a single product with a molecular mass of 353. This cortisol metabolite was water soluble when partitioned between diethyl ether and water. Based on its mass, it is not a conjugated metabolite, and is likely a 5α or 5β reduced, tetrahydroxylated (e.g., 3, 11, 17 and 21) compound. There are many steroids with a molecular mass of 353, and positive identification would have required the synthesis and testing of multiple steroids for comparison with the unknown. This effort was beyond the scope and funding limitations of the present investigation.

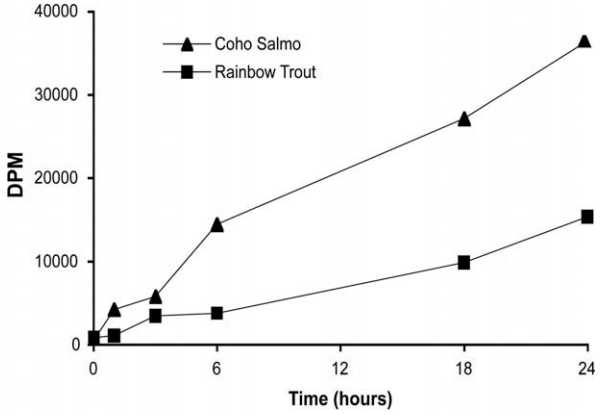

Fig. 1. Production of water-soluble cortisol metabolites (WSMs) by the kidneys of rainbow trout and coho salmon. Tissue was incubated *in vitro* for 24 h at 15 °C in the presence of [^{14}C]-cortisol (150 nM total cortisol concentration). Data shown are mean ± SEM of triplicate incubations. DPM = disintegrations per minute.

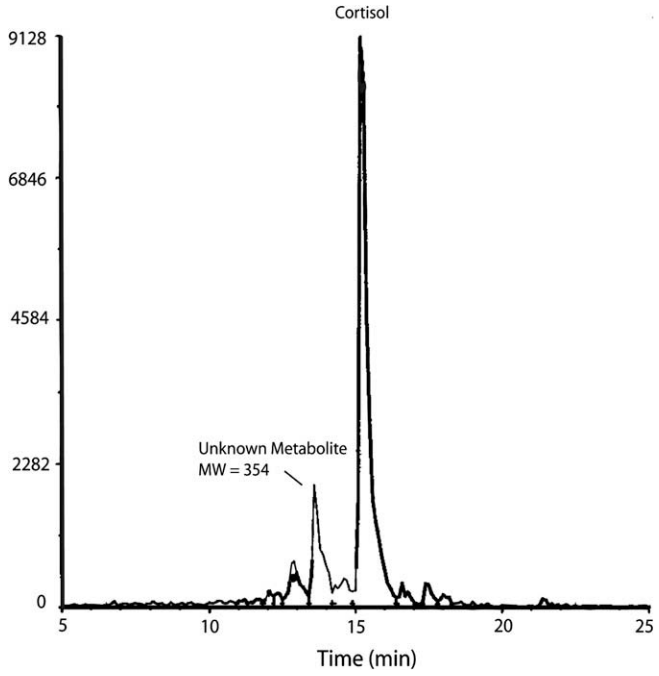

Fig. 2. Chromatogram showing production of cortisol metabolites produced by the rainbow trout kidney. Tissue was incubated *in vitro* for 24 h at 15 °C in the presence of cortisol. Both water-soluble and neutral-cortisol metabolites were analyzed by HPLC and mass spectrography; the mass of each peak was quantified. The large peak with an elution time of 15.2 min is unmetabolized cortisol. The predominate metabolite, with an elution time of 13.6 min, represents a single unidentified metabolite with a molecular mass of 354.

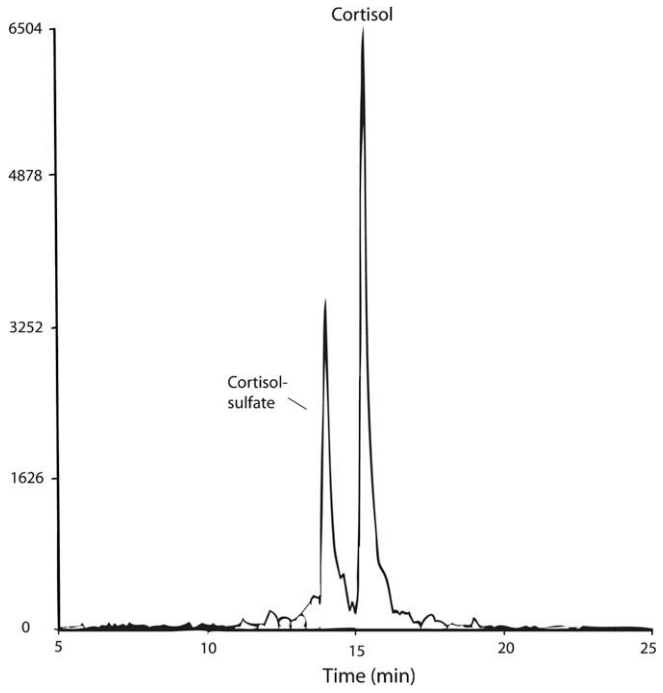

Fig. 3. Chromatogram showing production of cortisol metabolites produced by the coho salmon kidney. Tissue was incubated *in vitro* for 24 h at 15 °C in the presence of cortisol. Both water-soluble and neutral-cortisol metabolites were analyzed by HPLC and mass spectrography; the mass of each peak was quantified. The large peak with an elution time of 15.3 min is unmetabolized cortisol. The predominate metabolite, with an elution time of 14.0 min, represents a single metabolite with a molecular mass of 442. Subsequent analysis showed that this metabolite is cortisol-21-sulfate.

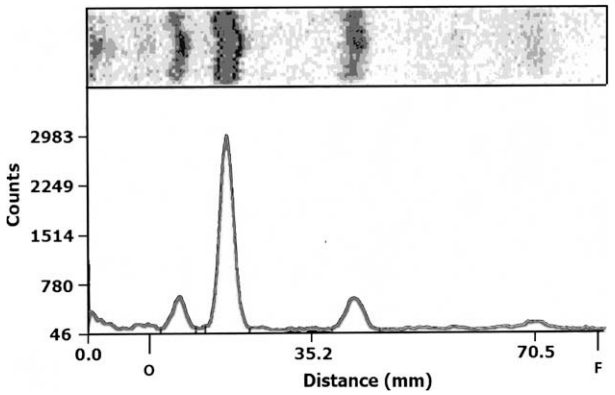

Fig. 4. The coho salmon kidney was incubated *in vitro* for 24 h at 15 °C in the presence of [^{14}C]-cortisol. The culture was extracted with diethyl ether, and the water-soluble, conjugated cortisol metabolites remaining in the medium were treated with trifluroacetic acid to release the free steroids by acid hydrolysis. Free steroids were separated by thin layer chromatography and the radioactivity on the plate was imaged and quantified using an InstantImager. The spots from left to right indicate the elution positions and radioactive counts for an unknown steroid, cortisol, and cortisone, respectively. O and F indicate the origin and solvent front, respectively.

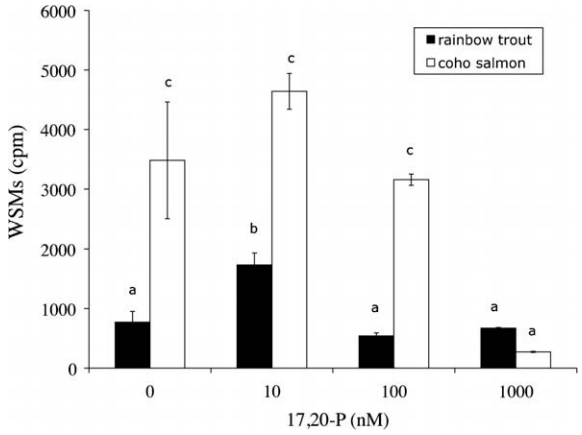

Fig. 5. Dose–response effects of 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) on the production of water-soluble metabolites (WSMs) by the rainbow trout and coho salmon kidneys. Tissue was incubated *in vitro* for 21 h at 15 °C in the presence of [^{14}C]-cortisol. The cultures were extracted with diethyl ether, and the water-soluble cortisol metabolites remaining in the medium were quantified by liquid scintillation counting. The principal WSM produced by the rainbow trout is an unidentified multi-hydroxylated product with a molecular mass of 354. The principal WSM produced by the coho salmon kidney is cortisol-21-sulfate. Data shown are mean ± SEM of triplicate incubations. CPM = counts per minute.

The rainbow trout kidney also produced several minor cortisol metabolites with retention times of 12.3, 14.6, 16.6 and 17.4 min (Fig. 2). None of these compounds were positively identified, although they were all ether soluble when partitioned between water and ether, indicating that they are neutral, non-conjugated metabolites (data not shown).

The coho salmon kidney converted cortisol into one major metabolite with a retention time of 14.0 min (Fig. 3). Mass spectroscopy showed that this compound was a single product with a molecular mass of 442. Further LC–MS analysis using authentic standards confirmed that the product was cortisol-21-sulfate (MS data not shown). The coho salmon kidney also produced several minor cortisol metabolites with retention times of 12.1, 13.6, 17.5 and 19.0 min (Fig. 3). In contrast to the rainbow trout kidney, where products were partitioned between diethyl ether and water, all were associated with the water fraction. None of these metabolites was positively identified. Acid solvolysis of the radiolabelled WSMs produced by coho salmon kidney released free steroids that co-eluted with cortisol (95% of the total radioactivity), cortisone (2%), and an unknown (2%) following TLC (Fig. 4). When the WSMs produced by coho salmon kidney were acetylated and partitioned between benzene and water, 87% of the radioactivity was found in the water-soluble fraction indicating that they were sulfates (data not shown).

3.3. Effects of 17,20-P on renal cortisol metabolism

17,20-P inhibited the production of cortisol-sulfate by the coho salmon kidney, but had no effect on the production of water-soluble cortisol metabolites by the rainbow trout kidney (Fig. 5).

In coho salmon, the inhibition of cortisol-sulfate production by 17,20-P resulted in the production of several ether-soluble (neutral) cortisol metabolites that were not produced in the absence of 17,20-P (Fig. 6). Of these compounds, only cortisone was identified (Fig. 6).

Additional dose–response studies indicated that relatively high doses of 17,20-P (300 ng/ml or higher) were required to significantly inhibit the production of cortisol-sulfate by the coho salmon kidney (Fig. 7). Moreover, there was a dose-dependent increase in the production of each of the neutral-cortisol metabolites produced in response to 17,20-P. The data for cortisone are shown in Fig. 7.

3.4. Effects of steroids on the production of WSMs

In this 21 h experiment, the kidneys of coho salmon and rainbow trout converted 28.2 ± 2.1% and 24.6 ± 2.8% of added [^{14}C]-cortisol into WSMs, respectively. This species difference was not significant.

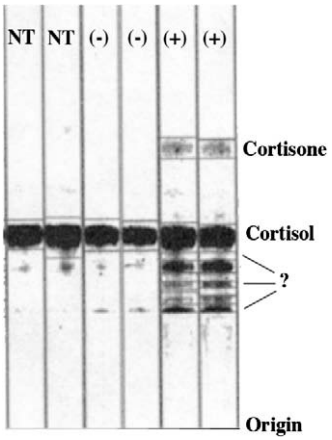

Fig. 6. Thin-layer chromatograph showing the effects of 17,20-P on the production of neutral-cortisol metabolites by the kidney of coho salmon. Kidney tissue was incubated *in vitro* with [^{14}C]-cortisol at 15 °C in the absence (−) or presence (+) of 1000 nM (332.5 ng/ml) 17,20-P. Steroids were visualized and quantified using an InstantImager. NT = no tissue control; ? = Unidentified cortisol metabolites.

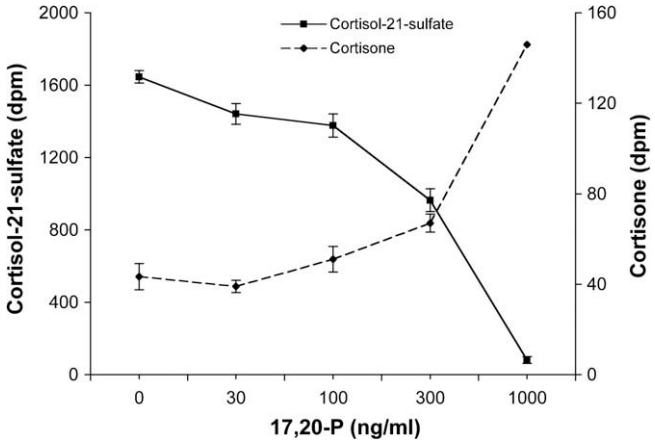

Fig. 7. Dose–response effects of 17,20-P on the production of cortisol-21-sulfate and cortisone by coho salmon kidney. Tissue was incubated for 24 h at 15 °C in the presence of [^{14}C]-cortisol. Data shown are means ± SEM of triplicate incubations. CPM = counts per minute. There was a significant dose-dependent inhibition of cortisol-21-sulfate production ($P < 0.001$), and a corresponding increase in cortisone production by 17,20-P ($P < 0.001$). Note: the radioactivity associated with cortisol-21-sulfate was determined by LSC and that associated with cortisone was determined using the InstantImager, and therefore the absolute CPM data are not directly comparable.

In coho salmon, the following steroids (in order of potency) significantly inhibited the conversion of [^{14}C]-cortisol to [^{14}C]-cortisol-21-sulfate (the primary WSM produced by coho salmon): 17,20β-P, cortisone, 17,20α-P, cortisol, and 11-KT (Table 1). Estradiol-17β, T and GA had no effect on the production of WSMs by the coho salmon kidney (Table 1).

In rainbow trout, the following steroids (in order of potency) significantly inhibited conversion of [^{14}C]-cortisol to radiolabelled WSMs (the principal WSM produced by rainbow trout kidney is an unknown multi-hydroxylated product with a molecular mass of 354): cortisone, cortisol, 17,20β-P, and 11-KT (Table 1). As in coho salmon, E_2, T, and GA had no effect on the production of WSMs by the rainbow trout kidney. In contrast to coho salmon, 17,20α-P had no significant effect on the production of WSMs by the rainbow trout kidney.

The steroids 17,20β-P and 17,20α-P were approximately 7-fold more potent, and 11-KT was slightly less potent, at inhibiting cortisol metabolism in coho salmon compared to rainbow trout (Table 1).

Table 1
Effects of steroids on the production of water-soluble metabolites (WSMs) by the kidney of coho salmon and rainbow trout.

Production of WSMs (% of control)		
Treatment	Coho salmon	Rainbow trout
No steroid control	100 ± 7.4	100 ± 11.8
17,20β-P	6.4 ± 0.2***	43.7 ± 0.9*
17,20α-P	9.8 ± 0.9***	73.7 ± 11.8
Cortisone	9.4 ± 0.5***	30.1 ± 10.2**
Cortisol	53.7 ± 7.6**	32.9 ± 8.0**
Estradiol-17β	115.6 ± 12.3	65.7 ± 6.3
Testosterone	76.7 ± 0.6	101.3 ± 13.1
11-Ketotestosterone	63.4 ± 3.9*	48.8 ± 0.9*
18β-Glycyrrhetinic acid	77.5 ± 4.5	89.9 ± 32.7

Note. Levels are expressed as percentage of control (mean ± SEM, $n = 3$). Asterisks indicate significant differences within species between the steroid treatment group and the corresponding no steroid control (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$). Italics indicate steroids where a significant difference between species was observed ($P < 0.05$).

3.5. Effects of steroids on the production of cortisone

In coho salmon, the following steroids (in order of potency) significantly enhanced the conversion of [^{14}C]-cortisol to [^{14}C]-cortisone: 17,20α-P, 17,20β-P, and cortisone (Table 2). The 17,20-hydroxylated progestins increased cortisone production 3- to 4-fold relative to the "no steroid" controls (Table 2). The production of [^{14}C]-cortisone was inhibited by the addition of radioinert cortisol (Table 2).

In rainbow trout, only 17,20α-P significantly enhanced the conversion of [^{14}C]-cortisol to [^{14}C]-cortisone (Table 2); in contrast to coho salmon, there was no effect of 17,20-P. Cortisone, cortisol and 11-KT all inhibited cortisone production by the rainbow trout kidney (Table 2).

Significant differences between coho salmon and rainbow trout were found in the steroid regulation of the metabolism of cortisol

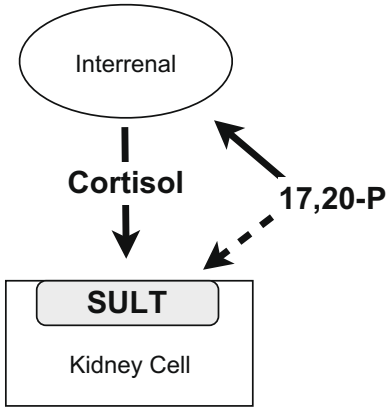

Fig. 8. Model depicting the roles of sulfotransferase (SULT) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in regulating the responsiveness of kidney cells to cortisol in semelparous Pacific salmon. Under normal physiological conditions, the cell is protected from cortisol excess by SULT, which converts cortisol into biologically inactive cortisol-sulfate. The pre-spawning surge in 17,20-P inhibits SULT activity, and also leads to increased interrenal cortisol production of cortisol (Barry et al., 1997 showed the 17,20-P can be rapidly converted into cortisol by the interrenal), which results in very high cortisol levels – both locally and in the peripheral circulation. All of these events are regulated by the pre-spawning surge in 17,20-P, which can explain why death in salmon only occurs after spawning. Dashed lines indicated negative control/feedback, and solid lines indicated stimulatory control.

Table 2
Effects of steroids on the production of cortisone by the kidney of coho salmon and rainbow trout.

Production of cortisone (% of control)		
Treatment	Coho salmon	Rainbow trout
No steroid control	100 ± 3.2	100 ± 3.8
17,20β-P	291.9 ± 16.4*	110.1 ± 5.3
17,20α-P	414.4 ± 62.6*	127.0 ± 2.5*
Cortisone	150.0 ± 11.4*	66.7 ± 18.1*
Cortisol	61.3 ± 3.3*	52.4 ± 7.3*
Estradiol-17β	112.9 ± 11.5	87.8 ± 8.2
Testosterone	162.9 ± 21.5	96.7 ± 3.7
11-Ketotestosterone	82.2 ± 2.8	46.0 ± 1.6*
18β-Glycyrrhetinic acid	145.9 ± 12.9	76.2 ± 13.8

Note. Levels are expressed as percentage of control (mean ± SEM, $n = 3$). Asterisks indicate significant differences within species between the steroid treatment group and the corresponding no steroid control (*$P < 0.001$). Italics indicate steroids where a significant difference was observed between species ($P < 0.05$).

to cortisone. Both 17,20α-P and 17,20β-P enhanced cortisone production 2.5- to 3-times more effectively in coho salmon than rainbow trout. Cortisone enhanced the conversion of [^{14}C]-cortisol to [^{14}C]-cortisone in coho salmon, but inhibited this conversion in rainbow trout. Finally, 11-KT significantly inhibited the conversion of [^{14}C]-cortisol to [^{14}C]-cortisone in rainbow trout, but had no effect in coho salmon.

4. Discussion

Our data support the hypothesis that the mechanism mediating the post-spawning death of coho salmon involves, at least in part, the regulation of peripheral cortisol-metabolizing enzymes by 17,20-P. The kidney of coho salmon converts cortisol primarily into corisol-21-sulfate, and this renal cortisol sulfotransferase activity is almost completely inhibited by high physiological concentrations of 17,20-P. These results suggest that SULT normally acts as a "gatekeeper" to protect the kidney from high circulating concentrations of cortisol by converting it into an inactive metabolite before it can exert its biological effects. This enzymatic protection and its elimination by the pre-spawning surge in 17,20-P can explain why (1) cortisol excess that is not associated with spawning (e.g., during stress) does not result in tissue degeneration and death, and (2) the death of salmon only occurs after spawning.

In contrast to the coho salmon, the kidney of the closely related, iteroparous rainbow trout converts cortisol primarily into a multihydroxylated metabolite whose production was not inhibited by 17,20-P. Thus, the kidney of the rainbow trout, like that of the coho salmon, appears to be protected from cortisol excess by "gatekeeper" enzymes. In contrast to coho salmon, however, the gatekeeper enzymes in rainbow trout do not appear to be regulated by 17,20-P. Thus, rainbow trout tissues remain protected from cortisol excess at spawning. These differences in the regulation of peripheral cortisol-metabolizing activity may explain why iteroparous salmonids like rainbow trout, that also show cortisol excess during the periovulatory period, show little or no tissue degeneration and do not die after spawning.

Sulfotransferases are known gatekeeper enzymes in mammals, and act by transferring a sulfonate group (SO_3^{-1}) from the cofactor 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to active steroids. Sulfotransferases generally produce metabolites that are water-soluble and biologically inactive (Strott, 2002). Estrogen sulfotransferase, for example, is expressed or induced in several estrogen target tissues in humans, including the testis, where it acts to control local estrogen concentrations and prevent the deleterious effects of chronic estrogen stimulation (Falany and Falany, 1996; Song et al., 1997; Qian et al., 1998, 2001; Falany et al., 1998; Qian and Song, 1999). All known sulfotransferases are members of an enzyme/gene superfamily termed SULT. In general, SULTs are high-affinity, low-capacity enzymes that react at relatively low concentrations of steroid substrate, an essential characteristic for a gatekeeper enzyme. To our knowledge, no study has specifically documented the tissue distribution of SULTs in fish, although they are likely to be ubiquitously distributed, as in mammals. SULT activity has been reported in the catfish liver and intestine where it was shown to sulfonate the steroids dehydroepiandrosterone (DHEA), estrone, and estradiol-17β (Tong and James, 2000). SULT activity has been reported in the gonads of Atlantic salmon (Scott and Canario, 1992), and coho salmon.

An unexpected finding in the present study was the relatively low SULT activity in the coho salmon liver. The physiological significance of low cortisol-metabolizing enzyme activity in the liver is unknown, but may explain why the liver is one of the first tissues in spawning salmon to show early and extensive degeneration during reproductive maturation and spawning (Robertson and Wexler, 1960). Another explanation for low cortisol-metabolizing activity in the liver is that this tissue lost viability in culture, and the kidney did not.

Cortisol sulfotransferase activity in the coho salmon kidney was inhibited in a dose-dependent manner by high physiological concentrations of the gonadal steroid 17,20-P. In Atlantic salmon, 17,20-P is itself a substrate for sulfonation (Scott and Canario, 1992), suggesting that 17,20-P could out-compete cortisol for access to SULT. When cortisol-sulfate production by the coho salmon kidney is inhibited by 17,20-P, there was a corresponding increase in the production of cortisone and at least three other neutral steroid metabolites. This suggests that 17,20-P causes a shift in the cortisol metabolic pathway from cortisol-sulfate production to the production of neutral steroids. The enzyme that converts cortisol to cortisone is 11-hydroxysteroid dehydrogenase (11-HSD). Of the two mammalian isoforms (11-HSD1 and 11-HSD2), 11-HSD2 has a much higher affinity for cortisol and is specifically expressed in aldosterone responsive tissues where it confers aldosterone specificity upon the mineralocorticoid receptor (Monder and White, 1993). The only 11-HSD isoform identified in teleosts to date is similar to the mammalian 11-HSD2 (Kusakabe et al., 2003; Jiang et al., 2003; Nunez, unpublished). Rainbow trout 11-HSD mRNA is strongly expressed in gill, heart, intestine, spleen and ovary, with lower expression in other tissues, including the kidney and liver (Kusakabe et al., 2003). Preliminary studies indicate 11-HSD is expressed in the heart, kidney, liver, pituitary, gill, muscle and ovary of coho salmon (Nunez, unpublished). However, it does not appear that 11-HSD expression is sufficient to act as a gatekeeper in coho salmon kidney or liver, as cortisone was synthesized by the kidney tissue *in vitro* only when SULT was inhibited, even in the presence of excess cortisol. Therefore, tissues that lose the protection of SULT are probably not protected by 11-HSD.

The physiological roles played by cortisone and the other neutral steroid metabolites that appeared in the *in vitro* coho salmon kidney cultures when cortisol sulfonation was inhibited by 17,20-P are unknown. However, these steroids were not produced by the rainbow trout kidney in response to 17,20-P, and therefore, one possibility is that they may regulate the degenerative effects observed in the kidney of post-spawned salmon.

Like 17,20-P, cortisone was a potent inhibitor of SULT activity in the coho salmon kidney. Cortisone has a low binding affinity for the glucocorticoid and mineralocorticoid receptors in fish (Ducouret et al., 1995; etc.), and we postulate that cortisone probably acts by competing with cortisol for SULT (i.e., cortisone is a competitive inhibitor). The inhibition of SULT activity by cortisone could be part of a local positive feedback mechanism initiated by

the rise in 17,20-P that quickly reduces SULT activity. The data support the following model. The pre-spawning rise in 17,20-P inhibits SULT, cortisol is metabolized to cortisone instead of cortisol-21-sulfate, cortisone further inhibits SULT, more cortisone is produced, and so on (Fig. 8). If SULT normally acts as a gatekeeper enzyme, this mechanism would rapidly remove enzymatic protection and expose tissues to high local concentrations of cortisol, or biologically active neutral cortisol metabolites that are not normally produced when SULT activity is high.

Further research is required to test this hypothesis and identify and test the biological effects of the unknown cortisol metabolites discovered in the present investigation. Moreover, studies are required to identify other tissues in addition to the kidney that have high cortisol-metabolic activity that may also be regulated by 17,20-P and/or cortisone. Additional studies are also required to assess sex differences and the effects of reproductive maturity on the regulation of SULT by 17,20-P. Male salmon generally have lower peak serum 17,20-P levels than females (10–100 vs. 300 ng/ml, respectively), and only high 17,20-P concentrations, such as those found in females, inhibited SULT. Thus, if our model is correct, the lower 17,20-P levels in males might contribute to their longer in-stream life compared to females (Barry et al., 2001). Alternatively, SULT in mature male fish may be more sensitive to inhibition by 17,20-P than females or immature fish. Until studies are conducted using sexually mature animals of both sexes to establish that the kidney of males and females differ in sensitivity to 17,20-P, the interpretations of the data generated using immature fish have to be regarded as tentative.

Besides inhibiting "gatekeeper" protection, the inhibition of kidney SULT activity by 17,20-P will likely decrease the excretion rate of cortisol, and thereby increase circulating concentrations of the corticosteroid. In addition, 17,20-P can serve as a substrate for cortisol biosynthesis, further contributing to systemic cortisol excess (Barry et al., 1997). Because the peripheral conversation of 17,20-P to cortisol bypasses the normal, regulated steps of interrenal steroidogenesis, this alternate pathway can increase cortisol levels even if normal negative feedback control still occurs at the hypothalamus and pituitary. The result is that all tissues, not just those normally protected by "gatekeeper" enzymes, will be exposed to high concentrations of cortisol when 17,20-P levels are elevated at spawning. Another possible mechanism by which 17,20-P could contribute to cortisol excess and the death of spawning salmon is by competing with cortisol for binding to corticosteroid binding protein and increasing the concentrations of biologically active "free" cortisol (Barry et al., 2001). The combination of "gatekeeper" inhibition, and an increase in both total and free plasma cortisol concentrations likely lead to the symptoms of cortisol excess associated with the post-spawning mortality of semelparous Pacific salmon.

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