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Regulation of Gill Cytosolic Corticosteroid Receptors in Juvenile Atlantic Salmon: Interaction Effects of Growth Hormone with Prolactin and Triiodothyronine

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The potential effects of growth hormone (GH), prolactin (Prl), and triiodothyronine (T_3) on gill Na^+,K^+-ATPase activity and corticosteroid receptor (CR) concentration (B_{max}) and dissociation constant (K_d) were examined in juvenile Atlantic salmon (*Salmo salar*). Compared to controls, fish injected with GH (ovine, 5.0 $\mu g\ g^{-1}$) had significantly greater gill Na^+,K^+-ATPase activity after 7 and 14 days. Gill CR B_{max} and K_d were significantly elevated on day 7, but not day 14. T_3 also significantly increased CR B_{max}. The effect of GH on CR B_{max} was also additive with T_3 (5.0 $\mu g\ g^{-1}$) treatment. There was a synergistic effect on CR B_{max} when purified coho salmon GH (csGH, 0.1 $\mu g\ g^{-1}$) was injected in combination with T_3 (1.6 $\mu g\ g^{-1}$). Prl (ovine, 5.0 $\mu g\ g^{-1}$; purified coho salmon, 0.1 $\mu g\ g^{-1}$) did not significantly alter gill CR B_{max}. Although Prl limited the increase in CR B_{max} by GH, the effect was not signicant. T_3 and Prl did not have an effect on K_d. GH significantly increased gill Na^+,K^+-ATPase activity, T_3 administration did not have a significant effect, and Prl-treated fish had significantly lower gill Na^+,K^+-ATPase activity. The results indicate that T_3 acts additively with GH, while Prl has no effect in regulating CR B_{max}. An increase in cytosolic CR by GH and T_3, but not Prl, may regulate gill responsiveness to cortisol and be an important mechanism in the endocrine control of physiological changes during the parr–smolt transformation.

Key Words: gill; corticosteroid receptor; Na^+,K^+-ATPase activity; cortisol; growth hormone; prolactin; triiodothyronine; Atlantic salmon.

The development of saltwater tolerance during the parr–smolt transformation is regulated by endocrine changes that occur during the spring. There is considerable evidence that cortisol plays a major role in stimulating many of the physiological changes associated with the parr–smolt transformation. The increase in plasma cortisol concentration during the spring is closely correlated with an increase in saltwater tolerance (Specker and Schreck, 1982) and gill Na^+,K^+-ATPase activity (Shrimpton and McCormick, 1998). Cortisol has also been shown to stimulate gill Na^+,K^+-ATPase activity *in vivo* (Bisbal and Specker, 1991) and *in vitro* (McCormick and Bern, 1989). The action of cortisol is likely mediated by high-affinity corticosteroid receptors (CR) in the gills (Chakraborti *et al.*, 1987; Maule and Schreck, 1990). Gill CR concentration has been observed to change seasonally in conjunction with smolting in coho salmon (*Oncorhynchus kisutch*) (Shrimpton *et al.*, 1994; Shrimpton, 1996) and hybrid steelhead-rainbow trout (*Oncorhynchus mykiss*) (McLeese *et al.*, 1994). The number of hormone receptors is an important parameter in the control of physiological mechanisms as the responsiveness of cells is dependent on the receptor concentration (Danielsen and Stallcup, 1984; Vanderbilt *et al.*, 1987). Recently, we have shown that *in vitro* responsiveness of gill Na^+,K^+-ATPase activity to cortisol is directly related to the concentration of CR in the gills of rainbow trout (Shrimpton and McCormick, unpublished data). Any factors that affect the number of gill

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CR are likely to affect the responsiveness of gills to cortisol.

Cortisol is not alone in influencing saltwater tolerance. Changes in circulating growth hormone (GH) levels during the spring support a role for GH in smoltification (Sakamoto *et al.*, 1993; McCormick *et al.*, 1995). Salinity tolerance and gill Na^+,K^+-ATPase activity are also increased by treatment with exogenous GH (Madsen, 1990a,b; Boeuf *et al.*, 1994). Although GH and cortisol have an effect on gill Na^+,K^+-ATPase activity and saltwater tolerance individually, treatment of salmonids with both hormones together has a synergistic effect (Madsen, 1990a,b; McCormick, 1996). GH has been shown to increase corticosteroid receptor concentration in the gills of coho salmon (Shrimpton *et al.*, 1995), which may be a mechanism for the interaction between cortisol and GH.

Thyroid hormones are also elevated during smoltification (McCormick *et al.*, 1987) and have been shown to promote some of the changes characteristic of smolting such as silvering (Milne and Leatherland, 1978, Miwa and Inui, 1985a). Evidence for thyroid hormones promoting salinity tolerance during smolting is conflicting. It does appear, however, that thyroid hormones may enhance the role of other hormones that are directly implicated in regulating salinity tolerance. For example, thyroxine enhanced the effect of cortisol on stimulating gill Na^+,K^+-ATPase activity in tilapia (Dangé, 1986) and may intensify the changes associated with smolting due to some other endocrine stimulus (Hoar, 1988).

Conversely, prolactin (Prl) decreases during smolting, is important in hyperosmoregulation, and has been shown to decrease the ability of salmonids to osmoregulate in salt water (Boeuf *et al.*, 1994). Prl antagonizes the effect of GH on the development of saltwater tolerance (Madsen and Bern, 1992).

Our first objective in this study was to determine whether GH increased gill cytosolic CR in juvenile Atlantic salmon (*Salmo salar*) as has been previously shown by Shrimpton *et al.* (1995) for coho salmon and to determine a time course of GH action. In addition we wanted to see if the effects of GH, triiodothyronine (T_3), and Prl on development of saltwater tolerance in juvenile salmonids were associated with CR concentration (B_{max}) in the gill. We examined combinations of these hormones to determine if the synergistic and antagonistic actions of their effects could be accounted for by changes in the number or affinity of gill CR. We also used purified coho salmon GH and Prl to determine whether the effects observed using the mammalian hormones could be substantiated using salmonid hormones.

MATERIALS AND METHODS

Fish and Rearing Conditions

Juvenile Atlantic salmon were obtained from White River National Fish Hatchery (Bethel, VT) and brought to the Anadromous Fish Research Center (Turners Falls, MA). Fish were maintained in 2-m tanks with fresh water at a flow rate of 4 liters min^{-1} while under natural photoperiod and fed to satiation twice daily (Ziegler Bros., Gardners, PA).

Experimental Design

Time course of GH action on CR (Experiment 1). On September 27, 42 parr were removed from a common holding tank and placed in a 1-m tank (13.3 ± 0.1 cm, 27.8 ± 0.7 g). On October 4, six fish were rapidly removed from their tank and placed in 200 mg $liter^{-1}$ tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0). Length (L) and weight (W) were measured and blood was collected from the caudal vasculature into heparinized syringes. Collection of blood was complete within 5 min of first disturbing the fish to ensure that a stress-associated rise in cortisol did not occur (Sumpter *et al.*, 1986). Blood was stored on ice for less than 30 min and centrifuged at 3000*g* for 5 min, and plasma was removed and frozen at −80°C. A gill biopsy (approximately six to eight primary gill filaments) was taken and placed in 100 µl of SEI (150 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) on ice for determination of Na^+,K^+-ATPase activity. Samples were frozen at −80°C within 30 min. The rest of the gill tissue was removed and placed in 2 ml of TEMS (10 mM Tris–HCl, 1 mM Na_2EDTA, 12 mM monothioglycerol, 20 mM sodium molybdate, 10% v/v glycerol, pH 7.4) and frozen immediately at −80°C for later analysis of corticosteroid receptor concentration and affinity.

The remaining fish were removed from their holding tank and anesthetized with 100 mg l^{-1} tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0). Once the fish were anesthetized, L and W were measured. Eighteen fish were given a single intraperitoneal injection of ovine GH (oGH; National Institutes of Health, Bethesda, MD) suspended in vegetable oil (5.0 μg g^{-1} body wt) and 18 fish were given a single intraperitoneal injection of vehicle only. Water temperature over the duration of the experiment ranged from 16.0 to 16.4°C. After 3, 7, and 14 days, 6 fish from each group were sampled for plasma cortisol, gill Na^+,K^+-ATPase activity, and gill corticosteroid receptors as described above.

Effect of GH, T_3, and Prl on CR (Experiment 2). On October 9, 72 Atlantic salmon parr (12.5 ± 0.07 cm, 19.3 ± 0.35 g) were anesthetized with 100 mg $liter^{-1}$ tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0). L and W were measured and the fish were injected with one of nine treatments: vegetable oil (vehicle), 2.0 μg g^{-1} oGH (GH2), 5.0 μg g^{-1} oGH (GH5), 5.0 μg g^{-1} T_3 (T_3, Sigma, St. Louis, MO), 2.0 μg g^{-1} oGH + 5.0 μg g^{-1} T_3 (GH2+T_3), 5.0 μg g^{-1} oGH + 5.0 μg g^{-1} T_3 (GH5+T_3), 5.0 μg g^{-1} ovine Prl (oPrl; National Institutes of Health), 2.0 μg g^{-1} oGH + 5.0 μg g^{-1} oPrl (GH2+Prl), and 5.0 μg g^{-1} oGH + 5.0 μg g^{-1} oPrl (GH5+Prl). Groups were identified by colored acrylic paint injected between the fin rays of the anal fin in combination with clipping of the adipose fin. After recovery, the fish were randomly divided into two identical 1-m circular tanks. Water temperature throughout the experiment was 12.1 to 12.5°C. On October 16 (day 7), fish were sampled as described above, except blood was collected in heparinized capillary tubes after the caudal peduncle was severed.

Effect of coho salmon GH and Prl on CR (Experiment 3). We also examined whether salmonid hormones had the same effect on CR as the mammalian hormones. Purified coho salmon GH (csGH) and Prl (csPrl) were used to determine their effect on CR in juvenile Atlantic salmon (17.3 ± 0.17 cm, 50.9 ± 1.66 g). Hormones were purified by high-performance liquid chromatography and frozen at −80°C until used (gift from Dr. P. Swanson). Hormones were solubilized in 0.01 N NaOH (1 μg μl^{-1}) and once dissolved were neutralized by dilution with 0.2 M sodium phosphate containing 0.2% BSA. On January 18, 30 Atlantic salmon parr were anesthetized with 100 mg $liter^{-1}$ tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0). L and W were measured and the fish were injected with one of the following: 0.2% BSA in 0.2 M sodium phosphate (vehicle), 0.1 μg g^{-1} csPrl, 0.1 μg g^{-1} csGH, 1.6 μg g^{-1} T_3, or 0.1 μg g^{-1} csGH + 1.6 μg g^{-1} T_3. Fish were individually identified by visual implant tags injected behind the eye. After recovery, the fish were randomly divided into two identical 1-m circular tanks. Water temperature during the study was 7.1 to 7.5°C. On January 22 (day 4), fish were reinjected, and on January 26 (day 8), fish were sampled as described above.

Determination of Plasma Cortisol

Plasma cortisol was quantified using a competitive solid-phase microtiter enzyme immunoassay (EIA) following a protocol similar to that of Munro and Stabenfeldt (1984) as outlined by Carey and McCormick (1998). Rabbit anti-cortisol antibodies (Cat F3–314, lot 345–10–22–80, Endocrine Science Products, Calabasas Hills, CA) were coated to microtiter plates. Cortisol-horseradish peroxidase conjugate (Gift from Coralee Munro, University of California, Davis, CA) was used as the label. Color development was with 3,3′,5,5′ tetramethylbenzidine (TMB) containing 0.01% hydrogen peroxide. The reaction was terminated with 0.5 M HCl and absorbance read at 450 nm. Sensitivity was 0.2 ng ml^{-1} and coefficients of variation ranged from 1.2 to 4.0 % for the cortisol assay.

Analysis of Gill Na^+,K^+-ATPase Activity

Gill Na^+,K^+-ATPase activity was measured according to the microassay protocol of McCormick (1993). Gill filaments were homogenized in SEI buffer containing 0.1% sodium deoxycholate. Following centrifugation (3000*g* for 0.5 min) to remove large debris, Na^+,K^+-ATPase activity was determined by linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), measured at 340 nm for 10 min at 25°C, in the presence and absence of 0.5 mM ouabain. Protein content in the gill homogenate was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Specific activities were expressed as μmol ADP mg^{-1} of protein h^{-1}.

Corticosteroid Receptor Analysis

Corticosteroid receptors were measured using the method of Maule and Schreck (1990) as modified by Shrimpton and Randall (1994). All procedures were carried out with samples on ice. Thawed gill tissue was scraped away from the cartilage and then homogenized in 2.0 ml of TEMS using an IKA-Ultra Turrax TP 18/10S1 homogenizer for two 10-s bursts. Homogenates were centrifuged in a Beckman GPKR knee-well centrifuge at 3000*g* for 15 min. The supernatant was removed and placed on ice. The pellet was resuspended with an equal volume of TEMS containing 50 μg ml^{-1} bacitracin, 20 μg ml^{-1} benzidine, 0.5 μg ml^{-1} aprotinin, 10 μg ml^{-1} *o*-phenanthroline to wash more CR from the pellet and recentrifuged at 3000*g*. The supernatants were combined and centrifuged at 48,000*g* for 2 h in a Beckman J2–21M centrifuge with a JA-21 rotor. The supernatant was removed and mixed with TEMS (1:1) containing 10% (w/v) activated charcoal and 1.0% (w/v) dextran and incubated for 10 min to remove endogenous steroids. To separate the charcoal from the liquid, the samples were centrifuged at 3000*g* for 15 min. The final supernatant was used to quantify cortisol binding. Protein content of this fraction was assayed with Bradford reagent (Bradford, 1976) using bovine serum albumin as standard.

Cortisol binding receptor studies were conducted with [^{3}H]triamcinolone acetonide (TA; 1,4-pregnadien-9α-fluoro-11β,16α,-17α,21-tetrol-3,20-dione-16,17 acetonide) with a specific activity of 43.8 Ci $mmol^{-1}$ (Dupont-NEN). Similar to the findings of Maule and Schreck (1990), we found that in binding and competition studies on duplicate gill homogenates, TA and cortisol bound to the same number of receptors, but TA had a higher affinity. To determine the number of cortisol receptors, 100 μl of the final supernatant was incubated in aliquots with 100 μl of buffer containing [^{3}H]TA with or without a 500-fold excess of cold TA. Final concentration of [^{3}H]TA ranged from 0.1 to 6 nM. Duplicate aliquots were incubated when enough tissue could be obtained. For Experiment 2, single aliquots were run as the fish were less than 20 g. The tubes were vortexed and incubated for 2 h on ice. After incubation, 0.5 ml of TEMS containing 2.5% (w/v) activated charcoal and 0.25% (w/v) dextran was added and vortexed. After 10 min, the charcoal containing unbound ligand was separated from bound ligand by centrifuging at 3000*g* for 15 min in a Beckman GPKR refrigerated centrifuge. Supernatant (0.5 ml) was added to 3 ml of Scintisafe Econo 2 (Fisher Scientific) aqueous counting scintillant. Samples were counted on a Beckman LS 6000IC liquid scintillation counter. Specific binding was determined by subtracting nonspecific bound from the total bound.

Using the protocol outlined above, gill CR are found in the cytosolic fraction. We have been unable to quantify CR in the nuclear fraction, as has also been reported by Pottinger *et al.* (1994). Although the source of the CR measured could be from the cytoplasm or nucleus, we refer to them as "cytosolic" due to their presence in this fraction following centrifugation. The equilibrium dissociation constant (K_d) and the concentration of corticosteroid receptor sites (B_{max}) were calculated according to Scatchard (1949). B_{max} was divided by the homogenate protein concentration, and CR concentration was expressed as fmol mg^{-1} protein. To estimate whether cooperativity between CR and ligand existed, the Hill coefficient was calculated according to Sandor *et al.* (1984).

Statistical Analysis

A two-way analysis of variance (ANOVA) was used to determine differences between GH and vehicle treatment over time (Experiment 1), followed by a Tukey's test to determine differences between treatment groups and time interval. In Experiment 2, a two-way ANOVA was used to determine the effects of the two tanks and treatment groups. A two-way ANOVA was also used to examine for effects of hormone and GH concentration, followed by a Tukey's test. For the Tukey's test, the treatments were grouped by hormone: vehicle (vehicle, GH2, and GH5), T_3 (T_3, GH2+T_3, and GH5+T_3), and Prl (Prl, GH2+Prl, and GH5+Prl). Conversely the treatments were grouped by GH concentration: GH0 (vehicle, T_3, and Prl), GH2 (GH2, GH2+T_3, and GH2+Prl), and GH5 (GH5, GH5+T_3, and GH5+Prl). For Experiment 3, a two-way ANOVA was used to compare vehicle, csGH, T_3, and csGH+T_3 groups followed by a Tukey's test. The Prl group was excluded from the ANOVA to test for an interaction effect between csGH and T_3. Statistical significance was taken at a level of $P = 0.05$. All values are expressed as means ± 1 SEM.

RESULTS

Experiment 1

Following a single injection of GH, there was a slight, but not significant increase in gill Na^+,K^+-ATPase activity by 3 days. Seven days postinjection, there was a significant twofold increase in gill Na^+,K^+-ATPase activity compared to day 0, which was also significantly greater than the vehicle-injected fish (Fig. 1). Gill Na^+,K^+-ATPase activity remained significantly elevated 14 days after GH treatment. Vehicle-injected fish did not exhibit a significant change in gill Na^+,K^+-ATPase activity over the duration of the experiment.

GH treatment did not significantly affect plasma cortisol concentration. Mean plasma cortisol levels of vehicle- and GH-injected fish pooled for all time intervals were 4.9 ± 1.5 ng ml^{-1} and 5.9 ± 2.2 ng ml^{-1}, respectively.

GH treatment significantly affected gill corticosteroid receptor concentration and dissociation constant (Fig. 1). Three days after GH injection, there was no change in gill CR B_{max}. By 7 days, gill CR B_{max} had increased significantly (twofold increase over $T = 0$ levels) and was also significantly greater than in vehicle-injected values. Gill CR declined by 14 days after GH injection and was not significantly different from the vehicle-injected fish or the $T = 0$ levels. Gill CR K_d of fish injected with GH also increased. Unlike B_{max}, there was an 18% increase in K_d by 3 days after treatment. This increase in K_d, however, was not significantly different from the preinjection levels. K_d was significantly higher (28%) than the $T = 0$ level on day 7 and was also significantly greater than the vehicle-injected values. By 14 days, K_d had declined in the GH-treated group to a level not significantly different from the $T = 0$ levels and the vehicle-injected fish.

Experiment 2

Treatment of juvenile Atlantic salmon with a single injection of GH, Prl, and T_3 had a significant effect on gill Na^+,K^+-ATPase activity. Two-way ANOVA indicated that there was a significant effect due to GH concentration ($P = 0.047$) and hormone ($P = 0.013$) (Fig. 2). In general, higher GH concentration resulted in higher gill Na^+,K^+-ATPase activity. Tukey's test on the pooled data for each GH concentration indicated that the GH5-treated groups were significantly greater than the GH0 groups ($P = 0.040$). There was little difference between the pooled vehicle- and T_3-treated groups at each level of GH, whereas Prl-injected groups were consistently lower. Tukey's test on the pooled data for each hormone indicated that Na^+,K^+-ATPase activity was not significantly different between vehicle and T_3 groups ($P = 0.998$), but Prl-injected fish exhibited significantly lower levels of Na^+,K^+-ATPase ($P = 0.025$). A tank effect did not exist for Na^+,K^+-ATPase activity, gill CR B_{max}, or K_d.

B_{max} was also significantly affected by GH treatment ($P < 0.01$) and hormone ($P < 0.01$). Within each hormone group there was a positive correlation between B_{max} and dose of GH injected (Fig. 2). There was a 23% and a significant 36% increase in B_{max} over the vehicle-injected fish for GH2- and GH5-treated fish, respectively. T_3 treatment increased CR B_{max} and was additive to the effect of GH. T_3 treatment increased CR B_{max} by 26, 21, and 17% over the corresponding vehicle-, GH2-, and GH5-treated fish. In comparison, Prl treatment alone did not appear to affect CR B_{max} compared to vehicle-injected fish, but appeared to oppose the effect of GH on CR B_{max}, as there was little increase in B_{max} with increasing doses of GH in Prl-injected fish. Tukey's test on the pooled data for each hormone group indicated that vehicle was significantly different from T_3 ($P = 0.027$), but not Prl ($P = 0.985$). Pooling the data for each GH concentration indicated that GH had a significant effect on CR B_{max}; GH0 did not differ from GH2 significantly ($P = 0.112$), but there was a highly significant difference between GH0 and GH5 ($P = 0.008$).

There was a general trend for the dissociation constant to increase with GH treatment, whether injected alone or in combination with T_3. The groups injected with Prl exhibited K_d values that were very similar to each other. Two-way ANOVA indicated that GH concentration ($P = 0.789$) and Hormone ($P = 0.270$) did not significantly affect K_d.

Experiment 3

Two injections of purified native hormones at the doses tested did not have a significant effect on gill Na^+,K^+-ATPase activity. Two-way ANOVA indicated

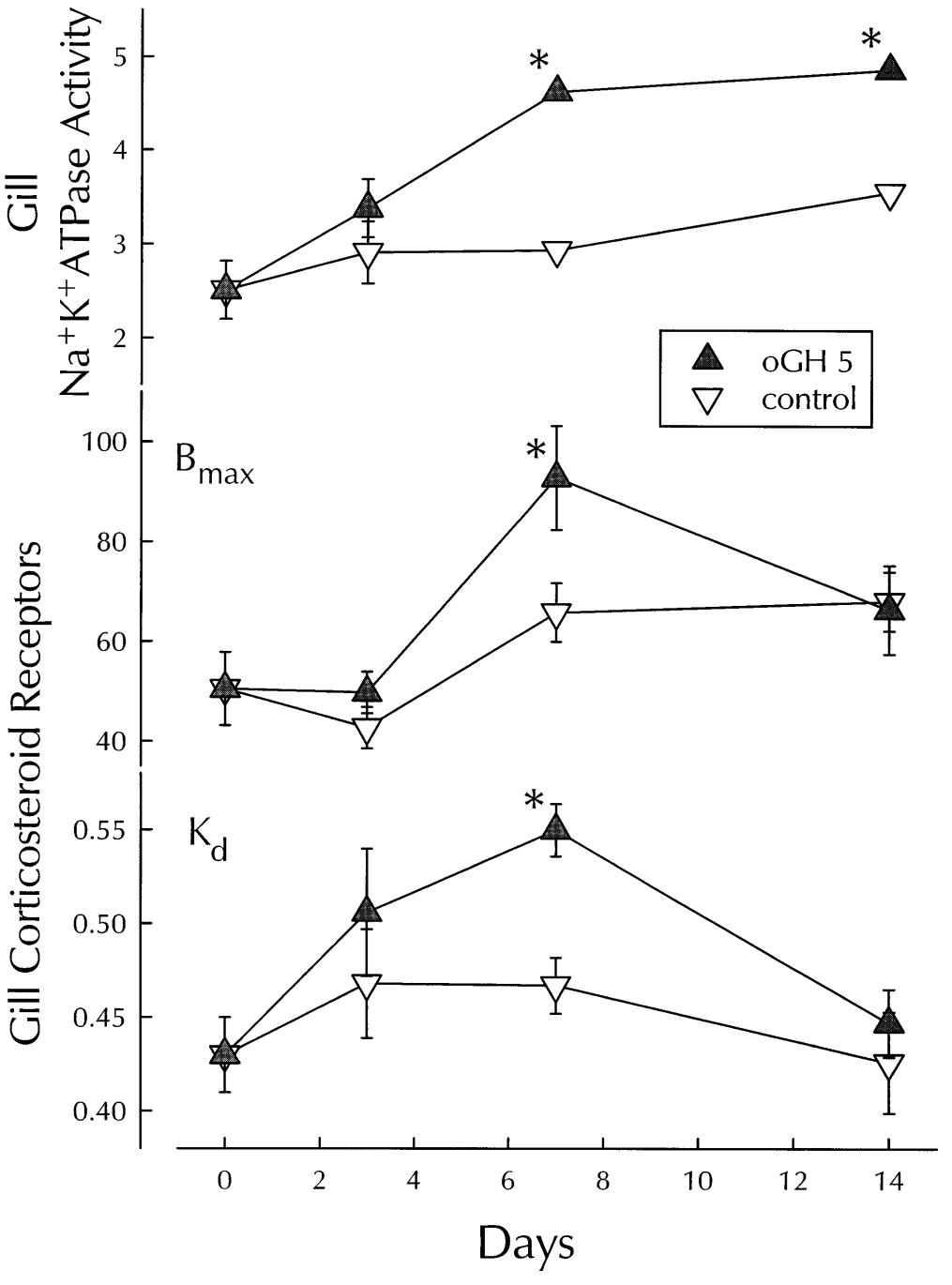

FIG. 1. Gill Na^+,K^+-ATPase activity (μmol ADP × mg^{-1} of protein × h^{-1}) and corticosteroid receptor concentration (B_{max}, fmol × mg^{-1} of protein) and dissociation constant (K_d, nM) of juvenile Atlantic salmon parr following a single injection of oGH (5 μg g^{-1})(Experiment 1). Two-way ANOVA indicated a significant effect of time ($F_{3,34} = 6.21$, $P = 0.02$) and GH treatment ($F_{1,34} = 5.11$, $P = 0.03$) and an interaction effect ($F_{3,34} = 3.36$, $P = 0.03$) on gill Na^+,K^+-ATPase activity, a significant effect of time ($F_{3,34} = 8.98$, $P<0.001$) and GH treatment ($F_{1,34} = 6.92$, $P = 0.01$) and an interaction effect ($F_{3,34} = 2.92$, $P = 0.05$) on CR B_{max}, and a significant effect of time ($F_{3,34} = 4.76$, $P <0.01$) and GH treatment ($F_{1,34} = 6.48$, $P <0.02$), but no interaction effect ($F_{3,34} = 0.50$, $P = 0.69$) on CR K_d. * indicates value for GH-injected fish is significantly different from that for the vehicle-injected fish for the same sampling interval. $n = 6$. Values are mean ± 1 SEM. Error bars are not shown when they are smaller than the symbol.

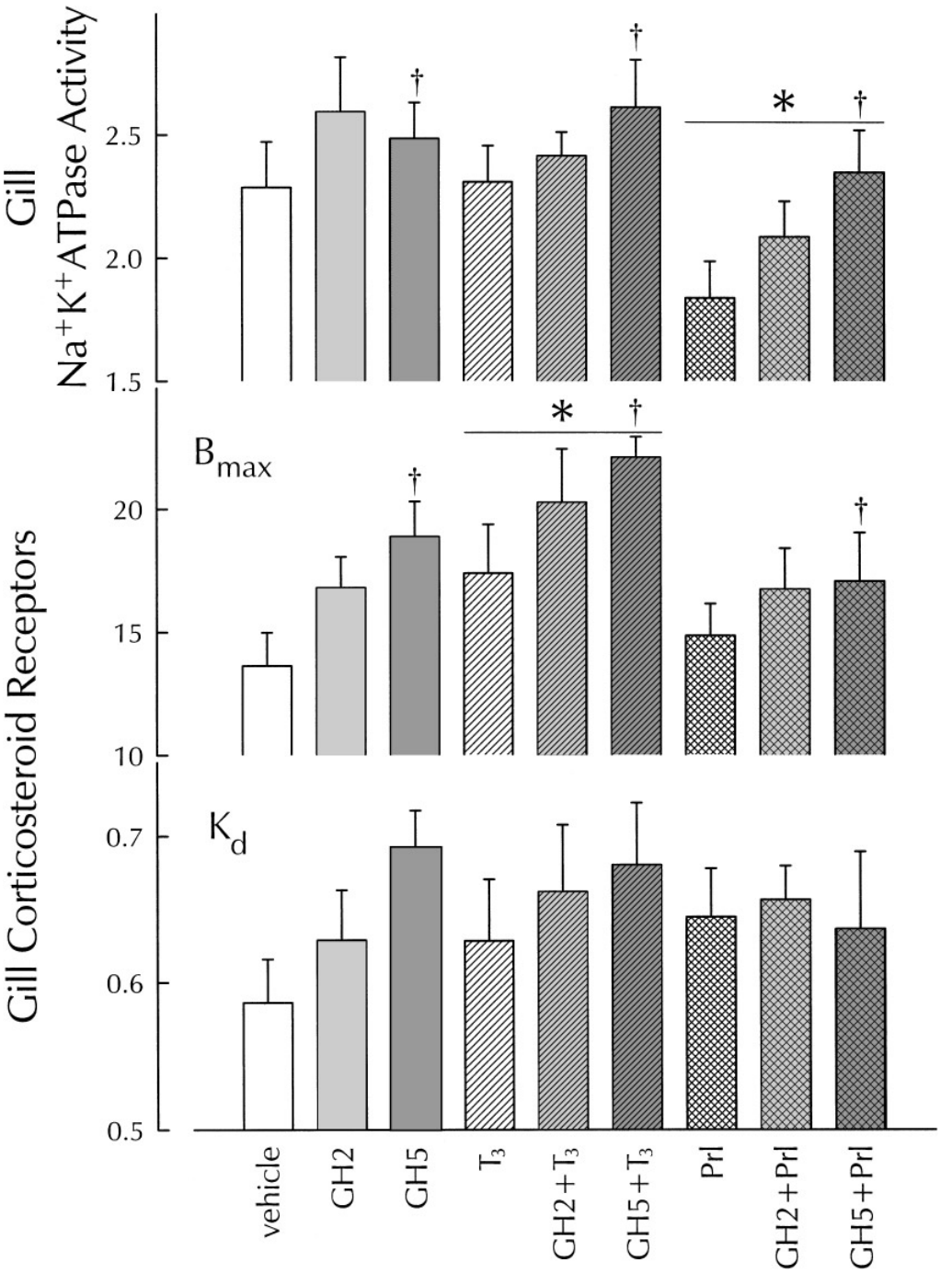

FIG. 2. Gill Na^+,K^+-ATPase activity (μmol ADP × mg^{-1} of protein × h^{-1}) and corticosteroid receptor concentration (B_{max}, fmol × mg^{-1} of protein) and dissociation constant (K_d, nM) of juvenile Atlantic salmon parr following a single injection of oGH, T_3, oPrl, oGH$^+T_3$, or oGH$^+$oPrl (Experiment 2). Two-way ANOVA indicated a significant effect of hormone ($F_{2,63} = 4.67$, $P = 0.013$) and GH treatment ($F_{2,63} = 3.20$, $P = 0.047$) on gill Na^+,K^+-ATPase activity, a significant effect of hormone ($F_{2,63} = 5.02$, $P = 0.01$) and GH treatment ($F_{2,63} = 4.98$, $P = 0.01$) on CR B_{max}, and no effect of hormone ($F_{2,63} = 0.24$, $P = 0.79$) or GH treatment ($F_{2,63} = 1.34$, $P = 0.27$) on CR K_d. There was no interaction effect observed on any of these parameters. * indicates pooled values are significantly different from the pooled vehicle injected groups. † indicates pooled values are significantly different from the pooled GH0 groups. $n = 8$. Values are means ± 1 SEM.

that gill CR B_{max} was significantly affected by T3 treatment ($P = 0.002$), but not csGH ($P = 0.312$). The interaction between the two hormones, however, was almost significant ($P = 0.051$). Tukey's test of all pairwise comparisons indicated that csGH ($P = 0.908$) and T_3 ($P = 0.699$) did not differ significantly from the BSA-injected fish, but csGH+T_3-injected fish differed significantly ($P = 0.020$) from the BSA-injected controls. Although K_d increased with csGH treatment, it was not significant ($P = 0.061$). T_3 had no effect on K_d ($P = 0.537$). Treatment with csPrl had no effect on gill Na^+,K^+-ATPase activity, CR B_{max}, or K_d.

DISCUSSION

Smolting is dependent on a series of changes in circulating levels of cortisol, GH, thyroid hormones, and Prl in the plasma (Hoar, 1988). The endocrine changes during the spring that are associated with smolting do not occur synchronously, but are temporally offset. Plasma prolactin levels decline, followed by an increase in plasma T_4, GH, and cortisol during the spring in Atlantic salmon (Prunet *et al.*, 1989) and coho salmon (Young *et al.*, 1989). Our present study has shown that hormones involved in the parr–smolt transformation have an effect on CR concentration in the gills which has implications to the endocrine mechanisms behind the transformation from parr to smolt.

Endogenous changes in GH are highly correlated with gill Na^+,K^+-ATPase activity (McCormick *et al.*, 1995), and an increase in circulating GH levels has been reported during the spring in a number of studies on Atlantic salmon (Bjornsson *et al.*, 1989; Boeuf *et al.*, 1989; Prunet *et al.*, 1989; McCormick and Bjornsson, 1994; McCormick *et al.*, 1995). Exogenous treatment with GH has been shown to increase gill Na^+,K^+-ATPase activity in Atlantic salmon (Komourdjian *et al.*, 1976; Boeuf *et al.*, 1990; McCormick, 1996) and brown trout (Madsen, 1990b). Previous studies have demonstrated that GH treatment generally must occur for a week or more before increased gill Na^+,K^+-ATPase activity is detected (Sakamoto *et al.*, 1993). In Experiment 1, we saw a significant increase in gill Na^+,K^+-ATPase activity following a single injection of GH by 7 days. In Experiment 2, however, an increase in Na^+,K^+-ATPase activity in the gills following GH administration for 7 days was indicated by two-way ANOVA, but the magnitude of the increase was less than that seen in Experiment 1. Gill Na^+,K^+-ATPase activity did not increase significantly in Experiment 3. The protocol of Experiment 1 was the same as that for Experiment 2, whereas hormones were dissolved in saline for Experiment 3. The differences seen between the three experiments may be related to temperature. Mean water temperature for Experiment 1 was 16.2°C compared to 12.3 and 7.3°C for experiments 2 and 3, respectively. Seven days appears to be near the minimum length of time for GH to have an effect on gill Na^+,K^+-ATPase activity for Atlantic salmon held in fresh water. A longer interval following injection would likely have resulted in significant changes in gill Na^+,K^+-ATPase activity. The time course of the present studies, however, was chosen to see maximal differences in gill CR.

GH significantly increased gill CR B_{max}. The timing of CR change from a single injection of GH is shown in Fig. 1, with significant changes in gill CR K_d, $B_{max,}$ and Na^+,K^+-ATPase activity occurring by day 7. The increase in K_d would lead to a decrease in gill sensitivity to cortisol. Conversely, the increase in CR B_{max} would indicate that gill sensitivity to cortisol had increased. The change in K_d of approximately 0.1 nM is not very large, whereas a twofold increase in B_{max} was observed. A synergistic interaction between GH and cortisol on gill Na^+,K^+-ATPase activity has been shown in rainbow trout (Madsen, 1990a), Atlantic salmon (McCormick, 1996), and brown trout (Madsen, 1990b). Shrimpton *et al.* (1995) suggested the increase in CR B_{max} was a potential mechanism for the synergistic interaction between GH and cortisol.

Plasma cortisol levels were unchanged following GH treatment in the present study. The increase in gill Na^+,K^+-ATPase activity can be attributed to two possible mechanisms. The increase in CR B_{max} enhanced gill responsiveness to cortisol, resulting in higher enzyme activities. The increase in Na^+,K^+-ATPase activity may not have been mediated exclusively by cortisol receptors. The presence of GH receptors in the gill suggest a possible direct action of GH to increase Na^+,K^+-ATPase activity. GH has been shown to bind to membrane receptors in the liver, gill, and kidney (Fryer and Bern, 1979; Sakamoto and Hirano, 1991). The increase in hypoosmoregulatory ability due to GH

may also be associated with production of IGF 1. McCormick *et al.* (1991) showed IGF 1 significantly increased hypoosmoregulatory ability in rainbow trout. Recently IGF 1 has also been shown to augment the effect of cortisol in increasing gill Na^+,K^+-ATPase activity and saltwater tolerance (McCormick, 1996). IGF 1 may also interact with cortisol in a manner similar to GH.

T_3 treatment did not have a significant effect on gill Na^+,K^+-ATPase activity (Figs. 2 and 3). T_3 treatment in rainbow trout did not increase gill Na^+,K^+-ATPase activity (Omeljaniuk and Eales, 1986). T_4 treatment has failed to show an increase in saltwater tolerance above that of vehicle-treated fish in rainbow trout (Madsen, 1990c), amago salmon (Miwa and Inui, 1985b), and Atlantic salmon (Saunders *et al.*, 1985). In hypophysectomized coho salmon, T_4 treatment actually inhibited gill Na^+,K^+-ATPase activity (Bjornsson *et al.*, 1987). A lack of effect of T_3 on gill Na^+,K^+-ATPase activity in the present study is, therefore, consistent with previously published findings.

T_3 treatment augmented the response seen with GH administration on CR B_{max} (Figs. 2 and 3). This permissive or synergistic role for thyroid hormones conforms with the proposal by Hoar (1988) that thyroid hormones intensify changes associated with smolting that are stimulated directly by other hormones. During the parr–smolt transformation, endogenous endocrine changes support the hypothesis for thyroid hormones, enhancing responsiveness of the gills to other hormones. Dickhoff *et al.* (1982) showed that coho and chinook salmon entering seawater after a well-defined pulse in plasma T_4 concentration had a better survival rate than groups transferred to seawater prior to the peak in plasma T_4 levels. A peak in T_4 has been observed 2–3 weeks prior to the peak in gill Na^+,K^+-ATPase activity in Atlantic salmon (Prunet *et al.*, 1989; McCormick *et al.*, 1987, 1995), whereas rises in GH (McCormick *et al.*, 1995) and cortisol (Shrimpton and McCormick, 1998) are correlated more closely with increases in gill Na^+,K^+-ATPase activity.

Reports of interactions between GH and T_3 are limited. T_3 has been shown to be necessary for GH to increase saltwater tolerance in brown trout and rainbow trout. Blocking the deiodination of T_4 to T_3 impairs saltwater tolerance even when GH is administered (Leloup and Lebel, 1993). In amago salmon, gill Na^+,K^+-ATPase activity was increased only when T_4 and GH were administered in combination, resulting in significantly greater saltwater tolerance in these fish than for fish injected with either hormone alone (Miwa and Inui, 1985b). We did not find T_3 to have a synergistic effect with GH on gill Na^+,K^+-ATPase activity, but there was a synergy between GH and T_3 on CR B_{max}. Cortisol levels, therefore, may need to be elevated to observe a synergy between GH and T_3 on gill Na^+,K^+-ATPase activity. Without GH, there appears to be a lack of synergism between cortisol and thyroid hormones. Madsen (1990c) found that T_4 treatment did not affect Na^+,K^+-ATPase activity or alter the stimulatory effect of cortisol in rainbow trout.

Prolactin had a slight but significant negative effect on gill Na^+,K^+-ATPase activity following a single injection of oPrl (Fig. 2). At the dose tested csPrl had no effect on gill Na^+,K^+-ATPase activity (Fig. 3). Prl has been found to have a slight negative, but statistically insignificant effect (Seidelin and Madsen, 1997) or no effect (Madsen *et al.*, 1995) on gill Na^+,K^+-ATPase activity in brown trout. In rainbow trout, however, Madsen and Bern (1992) showed that Prl significantly decreased gill Na^+,K^+-ATPase activity. The differences found in these studies may reflect variation of response to Prl among species. Levels of gill Na^+,K^+-ATPase activity in the rainbow trout, however, were elevated above those common for parr. Seidelin and Madsen (1997) proposed that Prl does not affect basal levels of gill Na^+,K^+-ATPase activity, but depresses Na^+,K^+-ATPase activity when elevated. The antagonism between GH and Prl on gill Na^+,K^+-ATPase activity (Madsen and Bern, 1992) supports this proposal.

Prl did not have a significant effect on gill CR B_{max} when injected alone (Figs. 2 and 3). Although the increase in CR B_{max} is less when GH is injected in combination with Prl than in fish injected with GH alone, the difference is not significant. Prl, therefore, does not appear to oppose the action of cortisol in stimulating gill Na^+,K^+-ATPase activity by affecting CR B_{max}. Seidelin and Madsen (1997) found that increases in gill Na^+,K^+-ATPase activity stimulated by cortisol administration are not affected by Prl treatment. These findings suggest that Prl opposes only the GH-induced stimulation of gill Na^+,K^+-ATPase activity and responsiveness of the gill to cortisol is not

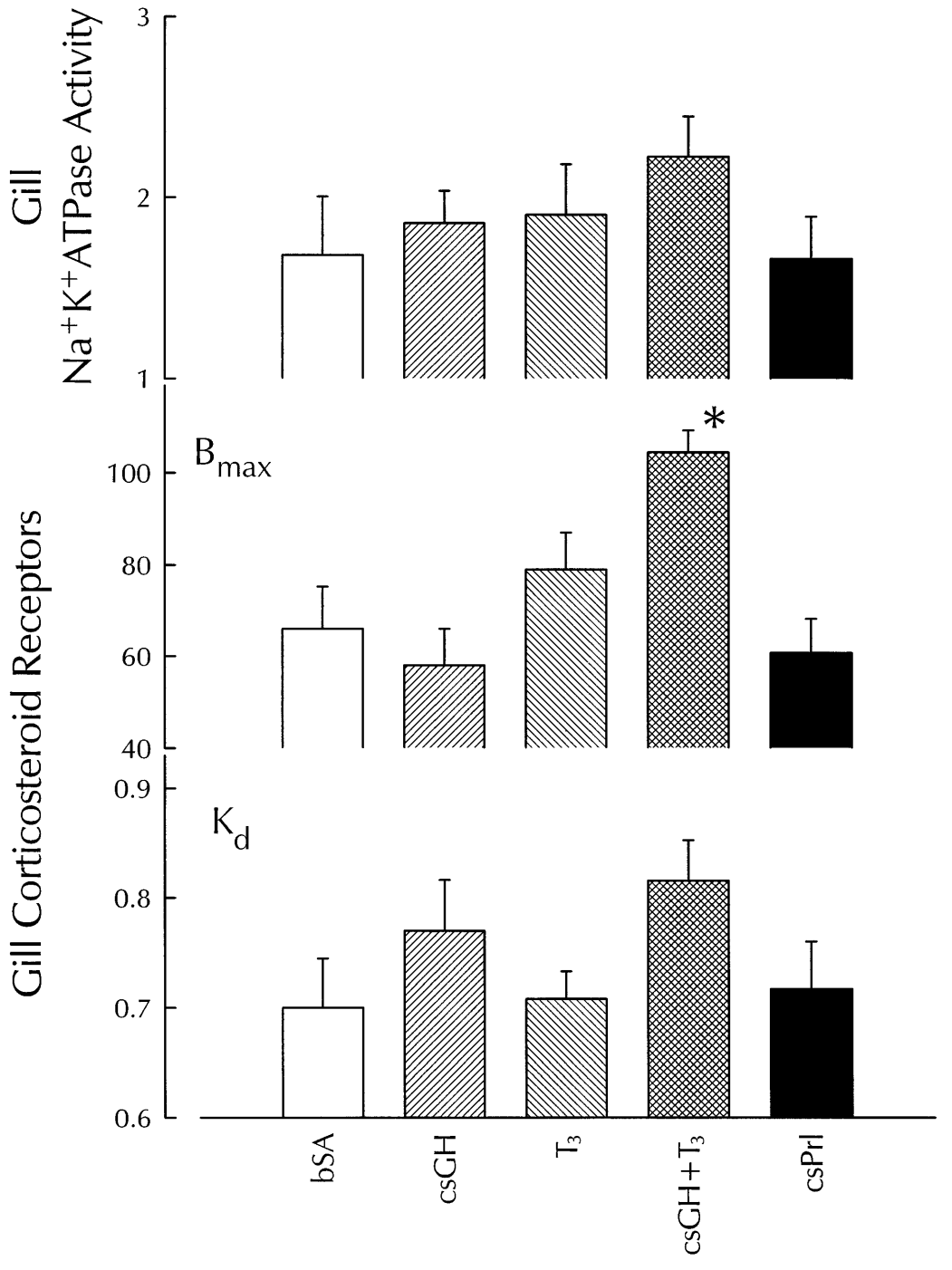

FIG. 3. Gill Na^+,K^+-ATPase^{++} activity (μmol ADP × mg^{-1} of protein × h^{-1}) and corticosteroid receptor concentration (B_{max}, fmol × mg^{-1} of protein) and dissociation constant (K_d, nM) of juvenile Atlantic salmon parr following two injections of csGH, T_3, csGH$^+T_3$, or csPrl (Experiment 3). Two-way ANOVA on the GH- and T_3-treated groups indicated that there was no effect of GH ($F_{1,20} = 0.80$, $P = 0.38$) or T_3 ($F_{1,20} = 1.12$, $P = 0.30$) and no interaction effect ($F_{1,20} = 0.03$, $P = 0.80$) on gill Na^+,K^+-ATPase activity. There was a significant effect of T_3 treatment ($F_{1,20} = 12.43$, $P = 0.002$), but not GH treatment ($F_{1,20} = 1.08$, $P = 0.31$) on CR B_{max}, and the interaction effect was almost significant ($F_{1,20} = 4.29$, $P = 0.051$). There was no significant effect of GH ($F_{1,20} = 3.93$, $P = 0.061$), or T3 treatment ($F_{1,20} = 0.54$, $P = 0.54$), and no interaction between the two hormones ($F_{1,20} = 0.20$, $P = 0.66$) on CR K_d. csPrl-treated fish did not differ significantly from the vehicle-treated fish. * indicates value for hormone-treated fish is significantly different from the saline-injected fish. $n = 6$. Values are means ± 1 SEM.

affected. The mechanism by which Prl opposes the action of GH is not known. Competition between oPrl and oGH at the receptor level appears unlikely as displacement studies on the teleost GH receptor indicate that oPrl has less than 1% of the affinity of oGH (Gray *et al.*, 1990; Sakamoto and Hirano, 1991).

The first two experiments in the present study used mammalian hormones. A similar response was seen for mammalian hormones compared to the salmonid hormones, particularly the interaction between T_3 and GH. Ovine GH and Prl are known to bind specifically to their respective teleost receptors, although with lower affinity (e.g., Yao *et al.*, 1991; Auperin *et al.*, 1994). This is reflected in the higher doses of oGH that were used to see an interaction with T_3 compared to csGH.

A significant increase in K_d of gill CR was observed following GH treatment. Changes in gill CR affinity have also been observed seasonally in Atlantic salmon (Shrimpton and McCormick, 1998) and in coho salmon (Shrimpton *et al.*, 1994; Shrimpton, 1996). There is a general increase in K_d in these studies during the spring associated with the parr–smolt transformation. Cortisol treatment has been shown to increase CR K_d in the gills of coho salmon (Maule and Schreck, 1991; Shrimpton and Randall, 1994) and rainbow trout liver (Pottinger *et al.*, 1994). Shrimpton *et al.* (1995) found that in coho salmon given a single injection of bovine placental lactogen (bPL), K_d was significantly elevated. The increase in K_d may not have been a function of bPL directly as cortisol was elevated in this group. There was a general trend for fish treated with bGH and bPL to have slightly, but not significantly, higher values of K_d than their vehicle-treated counterparts (Shrimpton *et al.*, 1995). The seasonal changes in GH may contribute to increases in K_d that are observed when plasma cortisol levels remain unchanged.

Whether the seasonal increase in thyroid hormones and GH and the decrease in Prl function to increase CR B_{max} in all salmonids is not known. Several studies have reported seasonal changes in CR B_{max}. Shrimpton and McCormick (1998) found a seasonal increase in CR B_{max} in both upper mode and lower mode juvenile Atlantic salmon. McLeese *et al.* (1994) observed a slight increase and then a decrease in CR B_{max} during the spring in steelhead / rainbow trout hybrids. Shrimpton (1996) found that from November to March there was a significant increase in gill CR B_{max} of coho salmon, followed by a significant decline by May. Shrimpton *et al.* (1994), however, documented a consistent decline in CR B_{max} from February to May in coho salmon. T_4, GH, and Prl levels were not measured in these studies. It is difficult, therefore, to determine whether a causal relationship exists between hormones implicated in the parr–smolt transformation and changes in CR B_{max}. Plasma cortisol, however, was quantified in these studies, and there is a clear inverse relationship between plasma cortisol and gill CR B_{max} (Shrimpton and Randall, 1994). As cortisol levels cause a decrease in gill CR B_{max}, circulating cortisol cannot account for the increases that are seen in gill CR B_{max} early in smolting. The peak in cortisol associated with smolting likely drives the decline in gill CR B_{max} (Shrimpton *et al.*, 1994; Shrimpton, 1996; Shrimpton and McCormick, 1998). A role for GH and T_3 may be to oppose the seasonal decline in CR B_{max}. We have shown that several hormones are involved in the regulation of CR B_{max} and K_d. The changes in CR, however, appear to oppose each other as GH treatment resulted in increases in B_{max} and a decrease in affinity. The magnitude of the changes in B_{max} were much greater than those observed for K_d; therefore, the net result of these changes will be a more responsive tissue to cortisol. The seasonal changes in hormone levels associated with the parr–smolt transformation may function to achieve high responsiveness of the gill to cortisol in preparation for the spring time increase in cortisol.

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