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Effects of hexazinone and atrazine on the physiology and endocrinology of smolt development in Atlantic salmon

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Abstract

Exposure to hexazinone (HEX) and atrazine (ATZ), highly mobile and widely used herbicides along rivers in the United States, is potentially harmful to Atlantic salmon, which have been listed as an endangered species. To determine the effects of these contaminants on smolt development, juvenile Atlantic salmon were exposed under flow-through conditions to 100 $\mu g\,l^{-1}$ HEX, 10 and 100 $\mu g\,l^{-1}$ ATZ in fresh water (FW) for 21 days at 10 °C beginning in mid-April. Twelve fish per treatment were sampled in FW, following a 24 h seawater (SW) challenge and after growth for 3 months in SW. Exposure to 100 $\mu g\,l^{-1}$ HEX or 10 $\mu g\,l^{-1}$ ATZ caused no mortalities of smolts in FW or after SW challenge, while 9% of the fish exposed to 100 $\mu g\,l^{-1}$ ATZ died during exposure. Fish exposed to 100 $\mu g\,l^{-1}$ ATZ reduced feeding after 10 days of exposure and had an impaired growth rate in FW and during the first month in SW; compensatory growth occurred in the second and third month in SW. HEX and ATZ at 10 $\mu g\,l^{-1}$ exposure had no effect on plasma levels of cortisol, growth hormone (GH), insulin growth factor I (IGF-I), thyroxine (T_4) and plasma 3,5,3′-triiodo-L-thyronine (T_3), Cl^-, Mg^{2+}, Na^+, Ca^{2+} in FW or after SW challenge. FW smolts exposed to 100 $\mu g\,l^{-1}$ ATZ had decreased plasma Cl^-, Mg^{2+}, Na^+ and Ca^{2+} ions and increased cortisol. No effect on plasma levels of GH, IGF-I, T_4 or T_3 was found in FW smolts exposed to 100 $\mu g\,l^{-1}$ ATZ. Following SW challenge, fish previously exposed to 100 $\mu g\,l^{-1}$ ATZ had significant increases in hematocrit, plasma cortisol, Cl^-, Mg^{2+}, Na^+, Ca^{2+} and a decrease in T_4 and T_3. It is concluded that under the conditions imposed in this study, HEX does not affect salinity tolerance of Atlantic salmon smolts, while ATZ causes ionoregulatory, growth and endocrine disturbance.

Keywords: Atlantic salmon; Hexazinone; Atrazine; Osmoregulation; Smolt; Hormone

1. Introduction

The number of adult salmon returning to rivers in Europe and North America has been declining for more than a century, and river contamination has been implicated as a causative factor for these population declines (Parrish et al., 1998). As part of their anadromous life history, salmon undergo a transformation from stream-dwelling parr to downstream migrating smolts. This transformation includes a number of morphological, behavioral and physiological changes that are adaptive for downstream migration and seawater entry (Hoar, 1988). Prominent among these changes is an increase in salinity tolerance, which is the result of a reorganization of the major osmoregulatory organs, the gill, gut and kidney. The development of salinity tolerance is accompanied by an increase in the scope for growth in seawater. Gill Na^+, K^+-ATPase activity is a widely used marker for these ion regulatory changes and is strongly correlated with the development of seawater tolerance in smolts (Hoar, 1988).

A number hormones are involved in controlling the parr-smolt transformation. Cortisol, growth hormones (GH) and insulin like growth factor I (IGF-I) are known to be directly involved in the development of seawater tolerance, as well as other aspects of smolt development (McCormick, 2001). Thyroid hormones (thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3)) have an indirect role in ion regulation, but more directly control morphological and behavioral changes. Recent studies have shown that this endocrine driven process is sensitive to contaminants that act as endocrine disruptors (Madsen et al., 2004; McCormick et al., 2005; Lerner et al., 2007).

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Hexazinone (HEX) and atrazine (ATZ) are non-selective herbicides in the triazine family that are often applied during spring to control undesirable plants. Due to its high application and water solubility, both pesticides can be detected in run-off water at concentrations up to 275 $\mu g\,l^{-1}$ (Huber, 1993). In Maine, this is especially problematic because HEX and ATZ are used primarily in areas immediately adjacent to rivers where Atlantic salmon breed and spend their early life history.

No published data are available on the effects of HEX on parr-smolt transformation or SW tolerance. Documented effects of ATZ in fish include a slow down in reflexes, swimming activity and feeding (Hussein et al., 1996). ATZ can also cause damage to gill epithelium and kidneys, increasing the renal excretion of sodium, chloride and proteins in rainbow trout (*Oncorhynchus mykiss*) (Fisher-Scherl et al., 1991) and carp (*Cyprinus carpio* L.) (Neskovic et al., 1993). Furthermore, ATZ reduces plasma testosterone and olfactory sensitivity in mature male Atlantic salmon (Moore and Waring, 1998). Waring and Moore (2004) found that atrazine exposure for 7 days reduces salinity tolerance of Atlantic salmon. However, the mechanism by which ATZ impacts salinity tolerance has yet to be established. In particular, the effects of ATZ and HEX on the hormones involved in smolt development have not been examined.

The objective of this study was to determine the effect of environmentally relevant concentrations of HEX and ATZ on, survival, growth and ion regulation in fresh water and seawater, gill Na^+, K^+-ATPase activity and the major hormones that promote smolt development (cortisol, GH, IGF-I, T_4 and T_3) in Atlantic salmon. We also examined whether ATZ and HEX were stressors by examining the primary and secondary stress response (plasma cortisol and glucose).

2. Materials and methods

2.1. Fish rearing and treatment

Atlantic salmon parr were obtained from White River National Fish Hatchery (Bethel, VT) in the fall of 2002 and transported to the Conte Anadromous Fish Research Center (Turners Falls, MA). Parr were raised in 1.6-m diameter tanks with Connecticut River water at a flow rate of 6–8 $l\,min^{-1}$ under natural photoperiod and supplemental aeration. Fish were fed to satiation twice daily with a specific salmon formulated dry feed (Zeigler Bros., Gardners, PA, USA).

2.2. Chemical exposure

Atrazine concentrations were based on field and laboratory levels reported in previous studies (Moore et al., 2003; Spano et al., 2004; Waring and Moore, 2004; Alvarez and Fuiman, 2005). Hexazinone concentrations were based on preliminary studies (Nieves-Puigdoller and McCormick, unpublished data) where no significant effect of HEX was found on survival, ion regulation and plasma hormones of Atlantic salmon smolts exposed to environmentally relevant concentrations of 2, 20 and 200 $\mu g\,l^{-1}$ HEX. Fish were exposed to chemicals for 21 days, a time period that has been widely used as a proxy for long-term exposure (Davies et al., 1994; Baatrup and Junge, 2001; Spano et al., 2004; Lerner et al., 2007). Exposure began approximately 1-month prior to the beginning of normal downstream migration and the peak of smolt development based on previous lab studies (McCormick et al., 1995).

On April 24, 2003, 1-year-old Atlantic salmon parr large enough to become smolt (>25 g) were exposed for 21 days at 10 °C to 0, 10, 100 $\mu g\,ATZ\,l^{-1}$ (purity 98%, Chemservice) and 100 $\mu g\,HEX\,l^{-1}$ (generic name for Velpar, 75% HEX and 25% inert ingredients, Dupont Company) in 300 l tanks (2 tanks per treatment, 18 fish per tank, 144 fish total). All the tanks had supplemental, constant aeration and oxygen measured twice per week were always greater than 90% saturation. Both compounds were prepared daily by dissolving them in water. A peristaltic pump (Cole-Palmer Instrument Co., Vernon Hills, IL, USA) was used to add the stock solutions of ATZ and HEX to a head tank. ATZ and HEX were mixed by a water pump with dechlorinated tap water and delivered to the fish tanks at a rate of 1 $l\,min^{-1}$. Water samples were taken and stored at −80 °C before analysis of HEX or ATZ concentrations. Fish were sampled immediately following exposure as describe below.

2.3. Fish sampling

After 21 days of exposured, 12 fish per treatment (6 per tank) were sampled in FW, another 12 smolt per treatment were exposed to a 24 h SW challenge (30 ppt), and another 12 smolt per treatment (9 for 100 $\mu g\,l^{-1}$ ATZ) were reared in SW (30 ppt) tank at 10 ± 1 °C for 3 months. For the salmon reared in SW for 3 months, 10 fish per treatment were individually marked with intraperitoneal passive integrated transponder (PIT) tags and then transferred to a 1.6-m diameter SW tank. The SW tank contained 25 ppt SW for the first week and then the salinity was increased to 30 ppt. Fish were fed to satiation daily. Every 4 weeks, all the fish were dip-netted, anesthetized, blot-dried, and fork length and weight were measured.

Specific growth rate (SGR) for weight and length were calculated each month using the following formula (Brett and Groves, 1979):

$$\mathrm{SGR} = [(\log_e W_t - \log_e W_0) \times t^{-1}] \times 100 \quad (1)$$

where W_t is the final weight or length, W_0 the initial weight or length and t is the time in days.

At the time of sampling, fish were anesthetized in 100 $mg\,l^{-1}$ tricaine methanesulfonate (MS-222, pH 7.0) and weight and fork length measured to the nearest 0.1 g and mm. Fish were bled from the caudal blood vessels into heparized syringes within 6 min of the first tank disturbance. Hematocrit was measured using microhematocrit capillary tubes. The remaining blood was centrifuged at 3000 × g for 5 min, and the plasma was aliquoted and stored at −80 °C until analysis. Brains were removed, weighed and frozen at −80 °C for measurement of cholinesterase activity. Gill biopsies of five or six filaments were placed in 100 μl of ice-cold SEI (250 mM sucrose, 10 mM Na_2EDTA and 50 mM imidazole, pH 7.3) buffer and stored at −80 °C for measurements of gill Na^+, K^+-ATPase activity. As

an index of possible impacts on the reproductive axis, liver and gonads were weighed and expressed relative to body weight (gonadosomatic index, GSI, and hepatosomatic index, HSI).

Estimates of food consumption were made on days 10 and 15 by weighing out 3.0 g of feed (estimated maximum daily ration) for each tank. Small increments of this were fed to each tank until fish were sated. The observer could see both the movement of fish toward feed and any accumulation of food on the bottom of the tank. Once feeding behavior stopped and more than one or two pellets were observed on the bottom of the tank, fish were deemed to be sated. The amount of food eaten was expressed as grams food per kilogram fish.

2.4. Analytical methods

Plasma sodium, calcium and magnesium ions were measured in duplicate with a flame atomic absorption spectrophotometer (AAnalyst 100, Perkin-Elmer, Norwalk, CT, USA). Plasma chloride was measured using a digital Buchler-Cotlove Chloridometer (Model 442-5000, Labconco). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose-6-phosphate dehydrogenase (Stein, 1963). Plasma protein was determined with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Plasma cortisol was determined with a enzyme immunoassay as outlined in by Carey and McCormick (1998). Plasma thyroxine (T_4) and plasma 3,5,3′-triiodo-L-thyronine (T_3) were measured by radioimmunoassay (Dickhoff et al., 1978) as modified by McCormick et al. (1995). Plasma growth hormone was determined with a specific double-antibody salmon growth hormone radioimmunoassay (Bolton et al., 1986) as modified and validated for Atlantic salmon by Björnsson et al. (1994). Plasma IGF-I was measured by radioimmunoassay validated for salmonids (Moriyama et al., 1994).

Gill Na^+, K^+-ATPase activity was determined using a microassay method as outlined in McCormick (1993). Gill tissue was homogenized in 125 μl of SEI buffer with a Kontes pellet pestle motor for 10–15 s, then centrifuged at 5000 × *g* for 0.5 min. Ten microliters of homogenate in quadruplicate were pipetted into a 96-well plate. Each sample had two wells containing an assay mixture with ouabain (0.5 mM) and two wells containing an assay mixture without ouabain. The kinetic assay was read at a wavelength of 340 nm at 25 °C for 10 min. Ouabain-sensitive ATPase activity was detected by the enzymatic coupling of ATP dephophorylation to NADH oxidation. The gill homogenate protein concentration was determined using the BCA protein assay using bovine serum albumin as standard. Na^+, K^+-ATPase activity was expressed as micromoles ADP per milligram protein per hour.

Brain cholinesterase activity was measured in order to determine whether the observed lethargy at high doses of ATZ was due to changes in this enzyme. Brain cholinesterase activity was determined by a colorimetric method of Ellman et al. (1961) as modified by Ceron et al. (1996). Whole brains were homogenized in a dilution of 1:50 with 0.1 M phosphate buffer, pH 8 using a manual Potter-Elvehjem homogenizer. Five microliters of homogenate in quadruplicate were pipetted into a 96-well plate. Each sample had two wells containing 200 μl assay mixture of acetylthiocholine iodide (1 mM) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (80 nM) and two wells containing 200 μl DTNB. The kinetic assay used was read at a wavelength of 405 nm at 25 °C for 7 min. Cholinesterase activity was expressed as micromoles acetylthiocholine hydrolyzed per milligram protein per hour. The brain homogenate protein concentration was determined using the BCA protein.

2.5. Water analysis

Water levels of ATZ and HEX were analyzed using solid phase extraction C18 cartridge followed by gas chromatography–mass spectrophotometer (GC–MS) determination (Lyytikäinen et al., 2003; Palma et al., 2004). ATZ and HEX were not detected in the control tanks. Water was analyzed in duplicates and data represent mean values ± S.E.

2.6. Statistics

All data are expressed as mean ± standard error. No tank effects were found for any parameter ($P > 0.05$, one-way ANOVA). Therefore, data for replicate tanks were pooled for further analyses. Analyses were run on ranked data because data for some parameters failed to meet the assumptions of parametric statistics. The treatment effect in FW or SW was analyzed by a one-way analysis of variance (ANOVA). Two-way ANOVA was used to examine the effect of treatment and sex on HSI and GSI. No significant sex differences were found for other parameters (two-way ANOVA). Significant ANOVA effects were followed by a Dunnett's test. Statistical significance was taken at a level of $P < 0.05$.

3. Results

Water levels of ATZ at the end of the treatment period were 8.5 ± 1.1 and $84.3 \pm 1.3\ \mu g\,l^{-1}$, compared with nominal concentrations of 10 and $100\ \mu g\,l^{-1}$, respectively. The mean value of HEX was $79.8 \pm 2.1\ \mu g\,l^{-1}$ for nominal concentration of $100\ \mu g\,l^{-1}$.

Exposure to ATZ ($100\ \mu g\,l^{-1}$) caused a 9% mortality of the fish in FW over the 21 days of treatment. There were no other mortalities in FW or SW in smolt treated with HEX or $10\ \mu g\,l^{-1}$ ATZ. In control tanks, fish would move rapidly around tank in response to daily observations for mortality. In contrast, 2 weeks of exposure to ATZ $100\ \mu g\,l^{-1}$ resulted in decreased responsiveness to this external disturbance. Fish exposed to $100\ \mu g\,l^{-1}$ ATZ had reduced food consumption after 10 and 15 days of exposure (Table 1). There was no significant difference in weight or length in FW or after SW challenge in smolt exposed to $100\ \mu g\,l^{-1}$ HEX, $10\ \mu g\,l^{-1}$ ATZ or $100\ \mu g\,l^{-1}$ ATZ (Table 2). An increase in hematocrit was observed for smolts exposed to 10 and $100\ \mu g\,l^{-1}$ ATZ after the SW challenge. No significant difference in hematocrit was observed for HEX-treated fish (Table 2). Exposure to ATZ and HEX did not affect plasma levels of GH or IGF-I in FW or after 24 h SW challenge (Table 2).

Table 1
Estimated feed intake at days 10 and 15 of exposure to 100 μg l^{-1} HEX, 10 and 100 μg l^{-1} ATZ in fresh water

Treatment	Day 10 Feed intake (g)/fish (kg) Tank 1/Tank 2	Day 15 Feed intake (g)/fish (kg) Tank 1/Tank 2
Control	81/93	81/93
100 μg l^{-1} HEX	93/81	93/81
10 μg l^{-1} ATZ	79/78	79/78
100 μg l^{-1} ATZ	26/11*	0/0*

Values are expressed per kg of fish (18 fish/tank) measured at the end of exposure period. Asterisks (*) indicate a significant difference from control ($P<0.05$, one-way ANOVA, Dunnett's test).

Exposure to HEX or ATZ did not affect the levels of brain cholinesterase (Table 2). A significant increase of HSI was found in females exposed to 100 μg l^{-1} ATZ (Table 3). No significant difference in HSI was observed in the other treatments. Male GSI decreased significantly in smolt exposed to 100 μg l^{-1} ATZ (Table 3). No significant changes in GSI were found in the other treatments.

Plasma Na^+ increased significantly in FW smolts treated with 10 μg l^{-1} ATZ, but decreased in fish exposed to 100 μg l^{-1} ATZ (Fig. 1). Plasma Na^+, Cl^-, Ca^{2+} and Mg^{2+} decreased in fish exposed to 100 μg l^{-1} ATZ in FW and increased significantly in SW challenged smolts (Fig. 1). HEX exposure had no effect on plasma levels of Cl^-, Mg^{2+}, Na^+ or Ca^{2+} in FW or after 24 h SW challenge. Gill Na^+, K^+-ATPase activity decreased significantly in FW smolts treated with 100 μg l^{-1} HEX in FW and 100 μg l^{-1} ATZ after 24 h SW challenge (Fig. 1).

Plasma glucose and cortisol were significantly elevated in smolts treated with 100 μg l^{-1} ATZ in FW and after SW challenge (Table 2). No significant changes in levels of cortisol were found in other treatments (Fig. 2). Plasma T_4 was reduced in smolt exposed to 10 μg l^{-1} ATZ in FW (Fig. 2). Smolts treated with 100 μg l^{-1} ATZ had reduced plasma T_4 and T_3 levels after 24 h SW exposure (Fig. 2). Plasma protein levels were significantly higher in fish exposed to 100 μg l^{-1} ATZ in FW and after the SW challenge (Table 2).

Significant reduction in weight and length were found in smolt exposed to 100 μg l^{-1} ATZ on May 15 and June 4 in SW (Fig. 3). No significant differences from control were found in smolt treated with 100 μg l^{-1} HEX and 10 μg l^{-1} ATZ. Specific growth rate for weight were lower in fish exposed to 100 μg l^{-1} ATZ, during the first month in SW (Table 4). A significant increase in weight and length SGR was observed in smolts treated with 100 μg l^{-1} ATZ during the second and third month (Table 4). The growth rate of control, as well as fish exposed to 100 μg l^{-1} HEX and 10 μg l^{-1} ATZ did not differ form controls after transfer to SW.

4. Discussion

The present study demonstrates that exposure to 100 μg l^{-1} ATZ (actual 84 μg l^{-1}) can cause osmoregulatory disturbance, physiological stress and reduction in food intake and growth

Table 2
Weight, length, plasma growth hormone (GH), insulin-like growth factor I (IGF-I), glucose, hematocrit and brain cholinesterase in salmon exposed to 100 μg l^{-1} HEX, 10 and 100 μg l^{-1} ATZ for 21 days in fresh water and after 24 h seawater challenge

Treatments	Weight (g)	Length (cm)	GH (ng ml^{-1})	IGF-I (ng ml^{-1})	Plasma glucose (mM)	Hematocrit (%)	Brain cholinesterase (μmol/mg protein/h)
Fresh water							
Control	37.0 ± 1.4	16.0 ± 0.2	14.0 ± 2.5	54.0 ± 4.2	5.3 ± 1.1	ND	39.7 ± 2.2
100 μg l^{-1} HEX	37.6 ± 1.4	16.1 ± 0.2	8.2 ± 2.5	58.9 ± 4.5	6.3 ± 1.0	ND	46.3 ± 2.2
10 μg l^{-1} ATZ	40.1 ± 1.4	16.4 ± 0.2	14.6 ± 4.1	61.8 ± 4.5	5.4 ± 1.6	ND	46.6 ± 2.2
100 μg l^{-1} ATZ	35.8 ± 1.5	15.8 ± 0.2	21.6 ± 5.0	44.3 ± 3.6	20.4 ± 1.5*	ND	49.9 ± 2.2
Seawater							
Control	35.4 ± 2.1	15.8 ± 0.3	21.8 ± 2.6	39.6 ± 2.5	3.6 ± 0.5	33.5 ± 1.5	ND
100 μg l^{-1} HEX	40.2 ± 2.1	16.4 ± 0.3	18.4 ± 2.8	47.9 ± 4.1	4.2 ± 0.2	37.5 ± 1.4	ND
10 μg l^{-1} ATZ	38.7 ± 2.1	16.5 ± 0.3	10.1 ± 2.7	46.6 ± 2.9	3.9 ± 0.5	50.1 ± 1.8*	ND
100 μg l^{-1} ATZ	32.0 ± 2.1	15.4 ± 0.3	17.9 ± 3.1	42.3 ± 2.1	6.3 ± 0.5*	44.7 ± 2.0*	ND

Data represent mean values ± S.E. of $n=12$ fish per treatment. ND, No determined data. Significant difference from controls is indicated by an asterisk ($P<0.05$, one-way ANOVA, Dunnett's test).

Table 3
Hepatosomatic and gonadosomatic index in salmon exposed to 100 μg l^{-1} HEX, 10 and 100 μg l^{-1} ATZ for 21 days in fresh water

Treatments	HIS (%)		GSI (%)	
Fresh water	Male	Female	Male	Female
Control	0.56 ± 0.03	0.57 ± 0.08	0.031 ± 0.004	0.221 ± 0.019
100 μg l^{-1} HEX	0.66 ± 0.01	0.71 ± 0.02	0.029 ± 0.001	0.206 ± 0.030
10 μg l^{-1} ATZ	0.72 ± 0.03	0.65 ± 0.05	0.030 ± 0.004	0.235 ± 0.028
100 μg l^{-1} ATZ	0.73 ± 0.04	0.92 ± 0.12*	0.019 ± 0.005*	0.169 ± 0.054

Data represent mean values ± S.E. of $n=5$ or 6 fish per treatment. Significant difference from controls are indicated by an asterisk ($P<0.05$, One-way ANOVA, Dunnett's test).

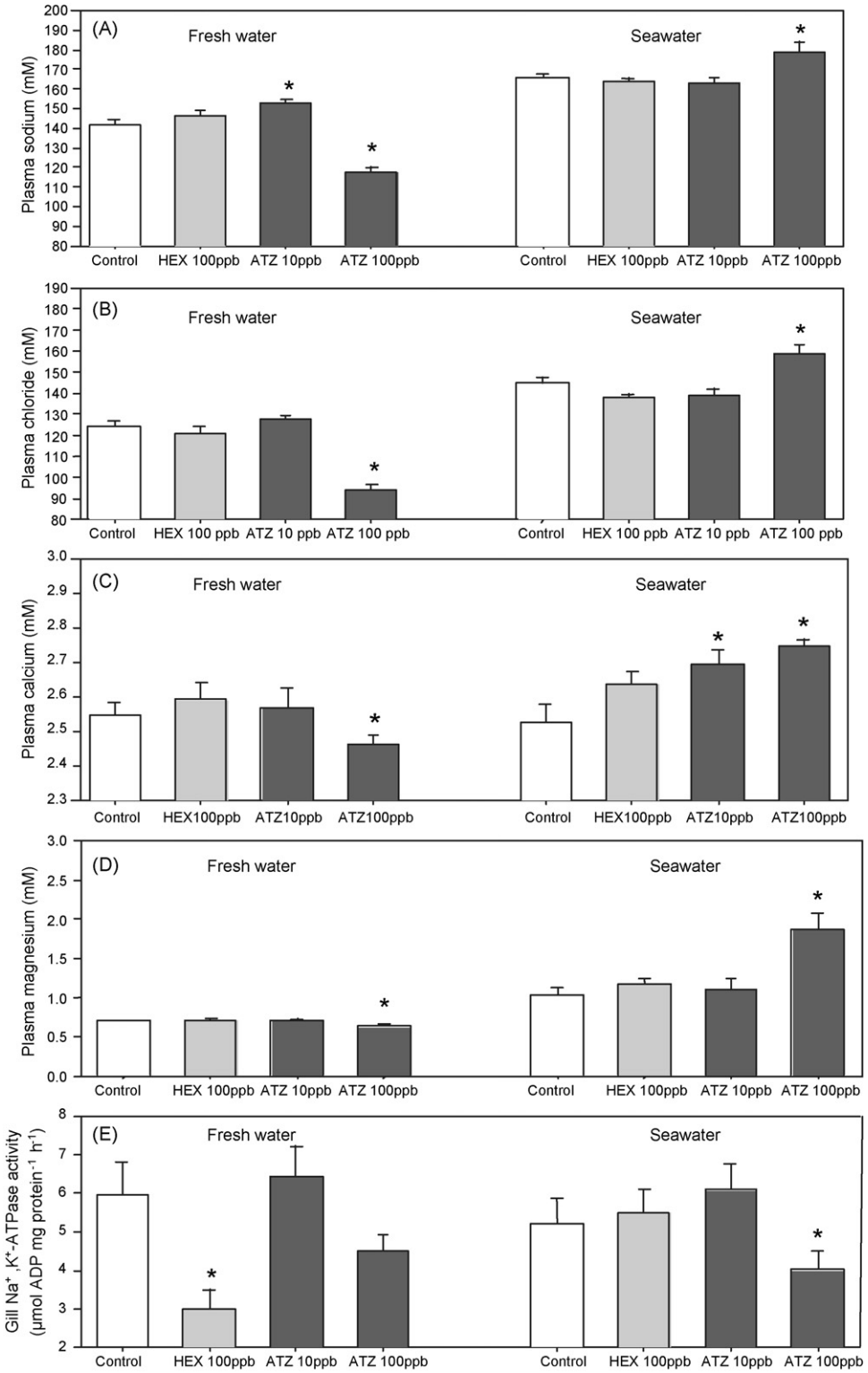

Fig. 1. Plasma sodium (A), plasma chloride (B), plasma calcium (C) and plasma magnesium (D) in smolt exposed to 100 $\mu g\,l^{-1}$ hexazinone, 10 and 100 $\mu g\,l^{-1}$ atrazine for 21 days in fresh water and after 24 h seawater challenge. Gill Na^+, K^+-ATPase activity (E) in smolt exposed to 100 $\mu g\,l^{-1}$ hexazinone, 10 and 100 $\mu g\,l^{-1}$ atrazine for 21 days in fresh water and after 24 h seawater challenge. Significant differences from controls are indicated by an asterisk ($P < 0.05$, Dunnett's test). Values are means ± S.E., $n = 12$ fish per treatment.

of Atlantic salmon smolts. Smolts exposed to 100 $\mu g\,l^{-1}$ ATZ for 21 days in FW had lower plasma levels of monovalent and divalent ions. Similar decreases in plasma Na^+ and Cl^- have been observed in Nile tilapia (*Oreochromis niloticus*) and catfish (*Chrysichthyes auratus*) after being exposed to high concentrations of ATZ (3 and 6 $mg\,l^{-1}$; Hussein et al., 1996). After a 14-days exposure, high concentrations (1.5–6 $mg\,l^{-1}$) of ATZ can cause hyperplasia and fusion of the secondary lamellae in

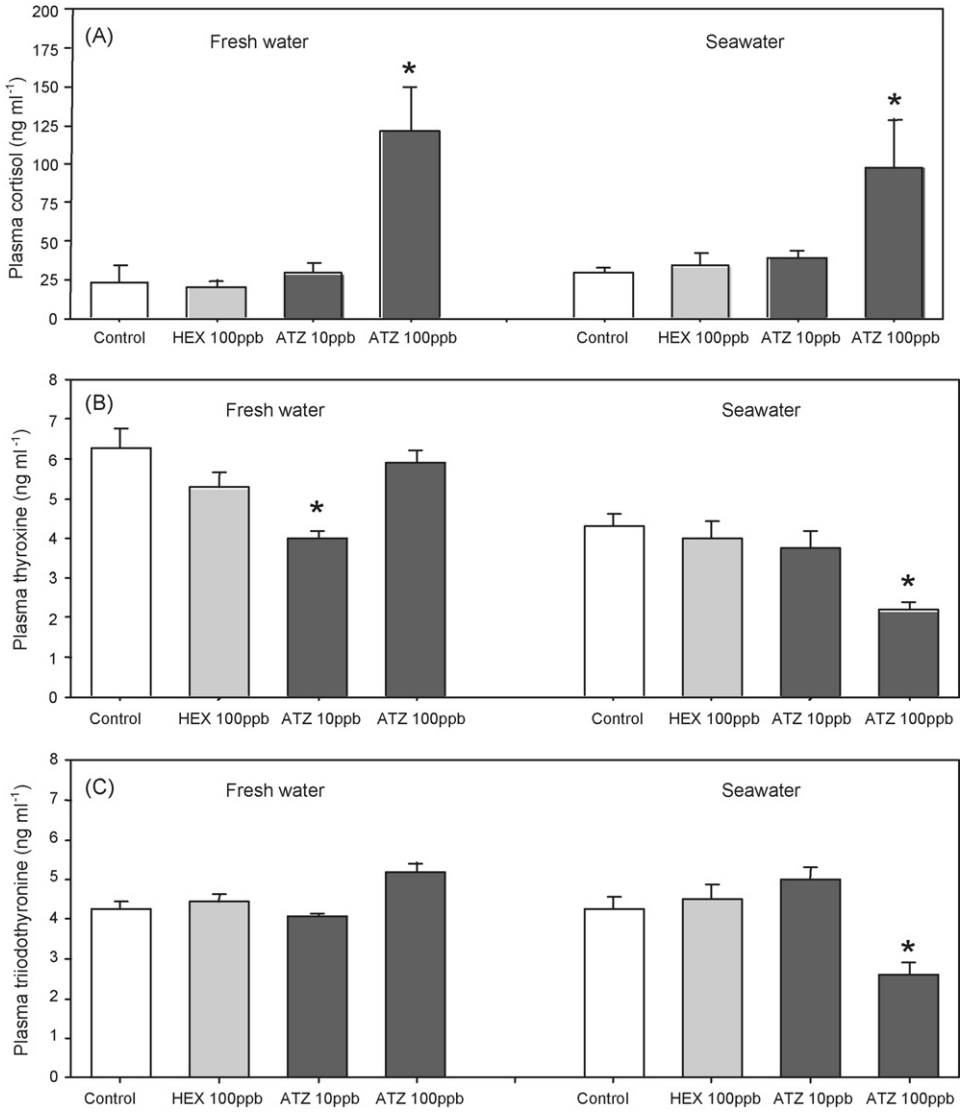

Fig. 2. Plasma cortisol (A), thyroxine (B) and 3,5,3′-triiodo-L-thyronine (C) in smolt exposed to 100 μg l^{-1} hexazinone, 10 and 100 μg l^{-1} atrazine in fresh water and after 24 h seawater challenge. Significant differences from controls are indicated by an asterisk ($P < 0.05$, Dunnett's test). Values are means ± standard errors, $n = 12$ fish per treatment.

the gill epithelium of carp that, in turn, affects osmoregulation (Neskovic et al., 1993). In addition to effects on the gill, impairment of the kidney function may also play a role in the observed loss of plasma ions. Fisher-Scherl et al. (1991) and Oulmi et al. (1995) showed that ATZ concentrations between 10 and 80 μg l^{-1} can cause degeneration in the proximal tubules of the kidneys of rainbow trout, which may increase the renal excretion of ions and proteins.

Elevated plasma Na^+ and Cl^- following SW challenge indicate that exposure to 100 μg l^{-1} ATZ decreases salinity tol-

Table 4
Three months specific growth rate (SGR) in seawater smolts previously treated for 21 days with 10 and 100 μg l^{-1} ATZ, and 100 μg l^{-1} HEX

Treatments	May 15–June4		June 4–July 1		July 1–July31	
Fresh water	Weight (% day^{-1})	Length (% day^{-1})	Weight (% day^{-1})	Length (% day^{-1})	Weight (% day^{-1})	Length (% day^{-1})
Control	0.67 ± 0.14	0.26 ± 0.03	1.23 ± 0.08	0.31 ± 0.03	1.14 ± 0.08	0.38 ± 0.03
100 μg l^{-1} HEX	0.62 ± 0.12	0.19 ± 0.03	1.09 ± 0.10	0.32 ± 0.03	1.19 ± 0.09	0.38 ± 0.03
10 μg l^{-1} ATZ	0.46 ± 0.14	0.23 ± 0.16	1.06 ± 0.12	0.31 ± 0.03	1.01 ± 0.13	0.37 ± 0.03
100 μg l^{-1} ATZ	0.27 ± 0.16[#]	0.17 ± 0.09	1.72 ± 0.13*	0.44 ± 0.03*	1.43 ± 0.05*	0.47 ± 0.04*

Data represent mean values ± S.E. of $n = 12$ (100 μg l^{-1} ATZ $n = 9$) fish per treatment. Significant differences from controls are indicated by an asterisk ($P < 0.05$, one-way ANOVA, Dunnett's test).
[#] $P = 0.06$.

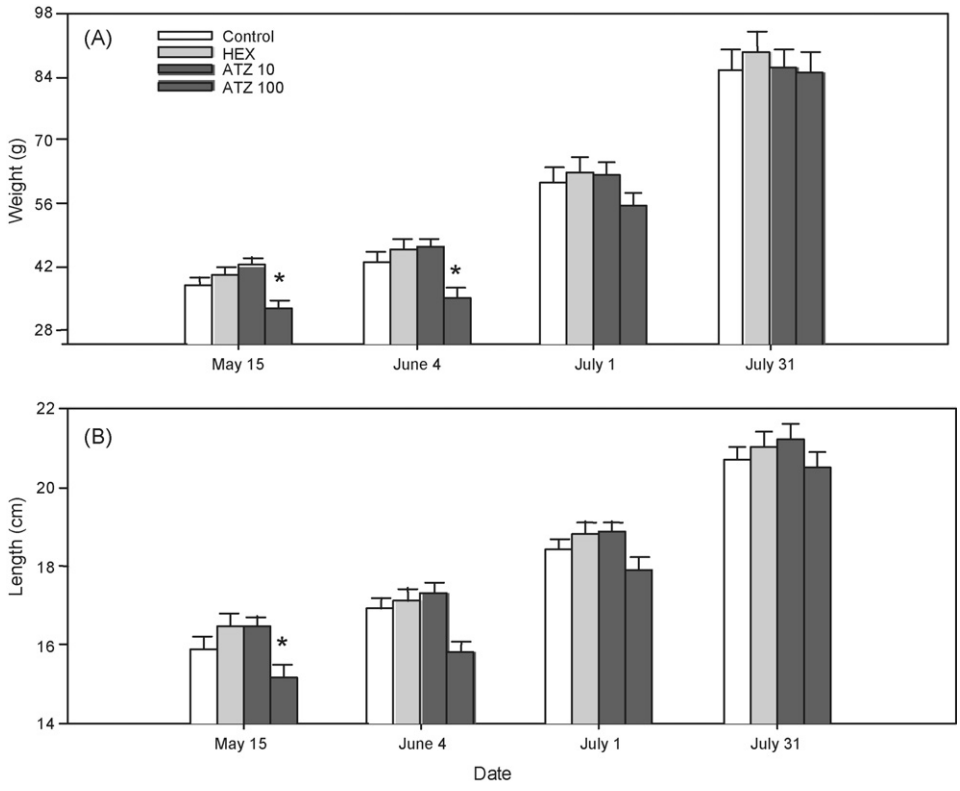

Fig. 3. Weight (A) and length (B) for smolt growth in seawater for three month that has been previously treated for 21 days with 10 and 100 $\mu g\,l^{-1}$ ATZ, and 100 $\mu g\,l^{-1}$ HEX. Significant differences from controls are indicated by an asterisk ($P < 0.05$, Dunnett's test). Values are means ± standard errors, $n = 12$ (100 $\mu g\,l^{-1}$ ATZ $n = 9$) fish per treatment.

erance of Atlantic salmon smolts. This is further supported by the reduced growth in SW of smolts previously exposed to 100 $\mu g\,l^{-1}$ ATZ. Fish treated with 100 $\mu g\,l^{-1}$ ATZ and exposed to seawater for 24 h also had lower gill Na^+, K^+-ATPase activity relative to controls. Na^+, K^+-ATPase is directly involved in salt secretion by marine fish, and its reduction may explain the observed decrease in SW tolerance. Smolts exposed to both 10 and 100 $\mu g\,l^{-1}$ ATZ had elevated plasma Mg^{2+} levels after SW exposure. As the kidney is primarily responsible for divalent ion regulation in fish (Marshall and Grosell, 2006) this finding suggests kidney damage following ATZ exposure.

Waring and Moore (2004) found that exposure to 10 and 22.7 $\mu g\,l^{-1}$ ATZ (actual 8.6 and 16.6 $\mu g\,l^{-1}$) for 7 days reduced salinity tolerance in Atlantic salmon smolts. To some degree this contrasts with the present results, in that 21-day exposure to 100 $\mu g\,l^{-1}$ ATZ (actual 84.3 $\mu g\,l^{-1}$) reduced salinity tolerance, but 10 $\mu g\,l^{-1}$ (actual 8.5 $\mu g\,l^{-1}$) did not. It is possible that the longer exposure used in the present study allows for compensatory mechanism(s) to develop, negating the influence of 10 $\mu g\,l^{-1}$ ATZ. Alternatively, differences in stock of fish used, prior rearing, developmental stage or other factors may account for this difference.

In the present study, the lowest dose of ATZ (10 $\mu g\,l^{-1}$) elevated plasma Na^+ levels in fresh water while not affecting plasma Cl^- or gill Na^+, K^+-ATPase activity. These results are identical to those of Waring and Moore (2004) at a similar dose and 7 days of treatment. Cassano et al. (2006) demonstrated that doses as low as 2 $\mu g\,l^{-1}$ ATZ can stimulate the short-circuit current of the ventral skin of frog (*Rana esculenta*), resulting in stimulated Na^+ absorption. Increases of plasma Na^+ at intermediate doses of ATZ may be a compensatory response to moderate damage of ion regulatory tissue. We propose that the observed decrease in SW tolerance after exposure to 100 $\mu g\,l^{-1}$ ATZ, may also be a compensatory response to the impacts of on the mechanisms of ion uptake. The toxic action of acid and aluminum exposure in fish is loss of ion uptake ability, and in smolts exposure to sublethal levels results in loss of salinity tolerance, presumably due to a trade-off between competing mechanisms of ion uptake and salt secretion in the gill (Saunders et al., 1983). A similar effect may occur following ATZ exposure; as smolts compensate for impacts on ion uptake they must reduce their capacity for salt secretion in SW.

In the present study, we observed a decreased feeding after 10 days exposure to 100 $\mu g\,l^{-1}$ ATZ, and the fish were less responsive to external disturbance. Increased lethargy and decreased food consumption have been observed in Nile tilapia and catfish after exposure to 3 and 6 mg l^{-1} ATZ (Hussein et al., 1996). These authors suggest that these behavioral changes were the result of decreased acetylcholinesterase activity. However, at the lower concentrations of ATZ used in the present study, no effect of ATZ on brain cholinesterase activity was found. Furthermore, several studies conclude that ATZ alone does not affect cholinesterase activity in insects and annelids (Belden and Lydy, 2000; Anderson and Lydy, 2002; Jin-Clark et al., 2002; Lydy and Linck, 2003).

Food deprivation can lower thyroid activity in teleost fish (Leatherland, 1982; McCormick and Saunders, 1990), but in the present study, plasma T_4 and T_3 levels were only affected in smolts exposed to 100 $\mu g\,l^{-1}$ ATZ after 24 h SW challenge. In salmon, thyroid hormones are involved in several aspects of smolt development including migration, morphological changes and imprinting (Hoar, 1988), but there is little evidence that thyroid hormones have a direct role in controlling salinity tolerance in salmonids (McCormick, 2001). The response of the thyroid axis to changes in environmental salinity appears to be highly variable (Leatherland, 1982), and the precise nature of the interaction between ATZ and SW exposure in affecting the thyroid axis will require further investigation.

Although not statistically significant, exposure to 100 $\mu g\,l^{-1}$ ATZ resulted in a 50% increase in plasma GH levels and 20% decrease in plasma IGF-I levels. This trend is likely linked to the ATZ-induced decrease in feed intake, as food deprivation elevates plasma GH levels and suppresses plasma IGF-I levels in salmon (Björnsson, 1997; Pierce et al., 2005; Takei and Loretz, 2006). In salmonids and other teleosts, GH and IGF-I facilitate hypo-osmoregulation in SW by increasing of gill Na^+, K^+-ATPase activity and the number gill chloride cells (Sakamoto et al., 1993). The present results suggest that the impact of ATZ on salinity tolerance of smolts may not work through GH and IGF-I.

Prior exposure to ATZ 100 $\mu g\,l^{-1}$resulted in a decrease in growth rate during the first month in SW (Table 4). It has been suggested that reallocation of energy toward detoxification, restoring homeostasis and tissue repair following contaminant exposure has an impact on growth (Wendelaar Bonga, 1997; Levesque et al., 2002). Fish exposed to 100 $\mu g\,l^{-1}$ ATZ had reduced food intake during the exposure period, and thus may have had to replenish energy stores during the first month in SW at the expense of growth rate.

During the second and third month in SW, a significant compensatory increase in growth rate took place in the 100 $\mu g\,l^{-1}$ ATZ group (Table 4). To our knowledge this is the first demonstration of compensatory growth following exposure to contaminants, and is thus an important observation with several implications. This delayed, but substantial recovery from ATZ exposure may indicate a return to normal homeostasis and endocrine status. However, there may be costs associated with compensatory growth. An increase in feeding may expose fish to increased predation resulting in decreased survival (Morgan and Metcalfe, 2001). Recent studies indicate that compensatory growth may also result in subtle developmental deformities and reduced investment in tissue maintenance (Ali et al., 2003).

In the present study, ATZ raised hematocrit in fish after SW challenge. Hematocrit increases under stressful conditions (Pierson et al., 2004), and this can be attributed to red blood cell recruitment from the spleen (Jensen, 1987), red blood cell swelling (Wang et al., 1994) and/or hemoconcentration due to reduced plasma volume (Wilson and Taylor, 1993). Prasad et al. (1991) found that damage of the gill lamellae causes decreased respiratory capacity in *Tilapia mosambica* exposed to 1.1 $mg\,l^{-1}$ ATZ. The increase in hematocrit we observed may therefore represent a compensatory response due to a respiratory surface reduction. Structural changes of the fish gill epithelia such as epithelial lifting, cell hypertrophy and proliferation may represent a defense response to pollutants, increasing pollutant-blood diffusion distance but reducing respiratory surface (Mallatt, 1985; Cengiz and Unlu, 2003; Oropesa-Jimenez et al., 2005).

Salmon exposed to 100 $\mu g\,l^{-1}$ ATZ in FW and after SW challenge were stressed as indicated by elevated levels of plasma cortisol and glucose. Plasma glucose and cortisol levels in fish increase after exposure to many pesticides (Bhavan and Geraldine, 1997; Aguiar et al., 2004; Waring and Moore, 2004; Hori et al., 2006), including ATZ exposure (100 $\mu g\,l^{-1}$) of carp (Gluth and Hanke, 1985). Increased cortisol may be important for mobilizing energy for responding to and repairing damage caused by contaminants, but may have negative consequences for disease resistance and growth (Wendelaar Bonga, 1997). The elevated levels of cortisol in smolts exposed to 100 $\mu g\,l^{-1}$ ATZ in FW and 24 h in SW may be also causal for the reduced growth of these fish in the first month in SW.

A reduction in male GSI was observed after exposure to 100 $\mu g\,l^{-1}$ ATZ for 21 days. The smolts in the present study were not reproductively mature, so the significance of this observation is not clear. Decreases in GSI are known to occur in reproductive female and male fish exposed to contaminants such as mercury, cadmium, copper and pesticides (Gimeno et al., 1998; Levesque et al., 2002; Cardinali et al., 2004). Exposure of goldfish to 1000 $\mu g\,l^{-1}$ ATZ decreased plasma testosterone (Spano et al., 2004). Moore and Waring (1998) found that mature male Atlantic salmon exposed to 6 $\mu g\,l^{-1}$ ATZ had decreased plasma testosterone and 11-ketotestosterone concentrations. In the present study, another possible explanation for the observed reduction in GSI is a high demand for energy and reduced food consumption of fish exposed to 100 $\mu g\,l^{-1}$ ATZ. Reduction in reproductive investment has been associated with the reallocation of energy in response to natural and anthropogenic stressors (Wendelaar Bonga, 1997; Maltby, 1999; Power, 2002; Ali et al., 2003).

Relative liver weight increased in females and males treated with 100 $\mu g\,l^{-1}$ ATZ. Changes in liver weight have been associated with vitellogenesis in both sexes (Jalabert, 2005), and with increased detoxification of xenobiotics (Zhou et al., 1999; Sepulveda et al., 2001). The observed increase in HSI following ATZ exposure is most likely due to the enhanced activity of xenobiotic biotransformation. Exposure to ATZ did not induce hepatic vitellogenin (VTG) production in male or female goldfish (100 $\mu g\,l^{-1}$ ATZ; Spano et al., 2004) or in male carp (30 μM ATZ; Sanderson et al., 2001). Also, estrogens and xenoestrogens that induced plasma VTG levels are positively correlated to total and protein-bound plasma Ca^{2+} levels (Björnsson et al., 1989; McCormick et al., 2005). In the present study, total plasma Ca^{2+} was reduced by ATZ, indicating that the elevated HSI is not likely due to ATZ-induced VTG.

Atlantic salmon smolts exposed to HEX did not show any effects on plasma ions, glucose, protein, cholinesterase activity or hormones involved in smolting, except for a reduction in gill Na^+, K^+-ATPase activity. The physiological relevance of this is unclear, as no effect on salinity tolerance was observed. Under the conditions imposed in this study, there was no effect of

HEX on salinity tolerance or other osmoregulatory parameters in Atlantic salmon smolts.

5. Conclusion

In the present study, HEX was generally without effect, whereas ATZ at 100 $\mu g\,l^{-1}$ (actual 84 $\mu g\,l^{-1}$) caused perturbations of ion homeostasis in FW, and loss salinity tolerance and decreased growth in SW. Reductions in salinity tolerance are associated with reduced performance in SW and lower adult return rates (Handeland et al., 1996; McCormick et al., 1998). Levels as high as 100 $\mu g\,l^{-1}$ ATZ (actual 84 $\mu g\,l^{-1}$) are relatively rare in nature, especially in flowing water. It should be noted, however, that the present study were carried out for only 21 days and under relatively benign conditions. In the wild, fish are likely to be exposed prolonged periods as ATZ is relative persistent in FW with a half-life up to 350 days depending on the environmental conditions (Tavera-Mendoza et al., 2002). The presence of other stressors such as changes in temperature, food availability, water flow, pH or other contaminants may lower the threshold for impacts of ATZ. The present research indicates that ATZ at sublethal levels under laboratory conditions has the capacity to alter normal physiological function of Atlantic salmon smolts that may compromise their competitive ability and predator avoidance leading to a higher mortality in nature.

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