Silver in Fish

SILVER IN FRESHWATER AND SEAWATER FISH: TOXICITY, BIOACCUMULATION, AND PHYSIOLOGY

By

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A thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1998) McMASTER UNIVERSITY
(Biology) Hamilton, Ontario

TITLE: Silver in Freshwater and Seawater Fish: Toxicity, Bioaccumulation, and Physiology

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NUMBER OF PAGES: xv, 197

ABSTRACT

Freshwater rainbow trout were exposed to 9.2 μg/L total Ag (as $AgNO_3$, a level approximately equal to the 168 h LC50) for 144 h to clarify the toxic mechanism of silver in freshwater teleosts. It was found that silver inhibits active Na^+ and Cl^- uptake at the gills, resulting in a net loss of both ions from the fish and creating a metabolic acidosis. This leads to a classic stress response (mobilization of cortisol and glucose into the blood plasma), and hyperventilation as a respiratory response to decreased blood pH. Plasma ammonia levels rise without any decrease in ammonia excretion; ammonia excretion later increases. This suggests that the increased plasma levels are due to increased metabolic production. Increased $[H^+]$ (decreased pH) results in excess H^+ ions in the internal fluids, which are either complexed with ammonia to form NH_4^+ or are buffered in muscle tissue. The latter results in increased movement of K^+ ions into the plasma, which are then excreted at the gills, preventing hyperkalemia. In the end, freshwater teleosts probably die from iono- and osmo-regulatory failure and associated cardiovascular collapse.

Seawater teleosts (rainbow trout, tidepool sculpins, English sole, and plainfin midshipmen) and elasmobranchs (Pacific spiny dogfish and long nose skate) were exposed to constant concentrations on total Ag (as $AgNO_3$) ranging from 1.5 to 50.0 μg/L for periods of up to 21 d at salinities of 18 ppt or 30 ppt. These exposure levels are well below those causing acute toxicity

in seawater. Silver appears to enter marine teleosts and marine elasmobranchs differently. Seawater teleosts drink the seawater, so the intestines are a major site of silver uptake, along with the gills. Since marine elasmobranchs do not drink, the gills appear to be the sole site of silver uptake from the water. As in freshwater, the liver is the main site for silver accumulation in all marine fish studied. Despite similar terminal liver silver concentrations, marine elasmobranchs have a higher rate of silver accumulation since the livers in elasmobranchs are 10-20 fold larger than in teleosts.

Both environmental salinity and exposure concentration play direct roles in determining silver bioaccumulation in marine teleosts. Increasing salinity alters the speciation of silver in the water, which decreases the amount of silver able to enter the fish. Increased silver concentrations mean more silver is available to enter the fish and subsequently cause sublethal toxic effects. Oxygen consumption decreased during the first 7 d of chronic exposure to sublethal silver levels in marine teleosts, with the decrease being more pronounced at higher (still sublethal) silver levels. Ammonia excretion, unaltered during acute exposure (48 h) to high silver levels (250 μg/L), was decreased during the first 7 d of exposure to sublethal silver levels (14.5-50.0 μg/L). This suggests that silver interferes with energy demanding processes such as protein synthesis or iono-regulation. Activity levels of the main enzyme involved in iono-regulation, namely Na^+/K^+-ATPase, was affected

differently in different fish. In a marine teleost that lives solely in seawater (plainfin midshipmen), silver inhibited the gill ATPase activity after 7 d of exposure, with the inhibition being more effective at higher silver levels. In the tidepool sculpin, a truly euryhaline species, gill ATPase activity increased as the silver levels increased, the latter probably representing a compensatory strategy. Similarly, intestinal ATPase activity was unchanged in the midshipmen, but was increased in the sculpins. Drinking rate in tidepool sculpins, which is involved with both iono- and osmo-regulation, was unaffected by salinity, but was decreased in fish exposed to silver for 8 d.

Overall, Ag is far less toxic in seawater than in freshwater, but the mechanisms of toxicity are similar. In both waters, Ag interferes with iono- and osmo-regulation. In seawater, Ag exhibits a significant potential for bioaccumulation and interference with physiological processes during long term low level exposures of marine fish, especially at lower salinity levels.

ACKNOWLEDGMENTS

Well, it's done. There were times when I didn't know if I would get this far, and I'm pretty sure similar thoughts went through other peoples' minds. Thank you for not voicing them.

Despite it all, I really enjoyed my stay here at MAC, especially the times that I was off gallivanting across the country. My time here has been filled with various high points and some nefarious low ones, but all in all, it was worth it.

Being at MAC has taught me a few good lessons to live by. Sleeping on a freezer is not good for the neck, the couch in the women's washroom is much more comfortable. When it is 4 am, and you have been working all night, DO NOT listen to Pink Floyd. Rainbow trout are great animals to work with, controls can be rather tasty. There is nothing so bad with your data that a good chat with Chris can't cure. The Phoenix is a good place to be. And thank god, but I make an ugly woman.

I would be remiss if I didn't bring up Bamfield, the place that kept me sane, I learned a fair amount out there as well. Probably the first thing I learned, was how to fillet a fish. The second was, don't drive through the mountains in the winter, and don't drive with two flats, a broken back window, cracked engine mount, broken alternator, no shocks and no rear view; other than that the drive across Canada is great! I learned a few other things as well. Work, despite long days, can be relaxing and enjoyable.

When tossing fish, do not turn around to pick up speed, just swing your arms. Sharks ain't so tough. And when away from home, make sure the Research Coordinator is your friend.

Fortunately, there have been a great deal of people that have helped me these last couple of years, and I could fill a whole other thesis just thanking them all personally.

To all my friends from the Wood and McDonald labs: Thank you for nights (and afternoons) at the phoenix, for hours of conversation when procrastination was the subject of the day, for Rice Lake and various other conferences, and for support and knowledge when I needed either. Some of you taught me about science, while others taught me about life, I thank you all.

To my friends from Bamfield: Thank you for nights at the pub (and days), for fishing trips (both for science and eating), for lessons on cooking, billiards, and surfing, and for Happy Hour, I thank you all.

To the ones that I relied on more than most, Kevin and Joelle, two people on either side of the country, who made me feel at home wherever and whenever. You listened, and talked and knew when to pass a cold beer. Thank you.

To my parents, who believed in me more than I believed in myself. I thank you for understanding.

To the people from Kodak, Tom Gorham (Kodak Canada), Joe Gorsuch, Tom Bober, and Ken Robillard (Kodak USA), who helped fund my research and made it possible for me to attend the 4th International Argentum on Silver and the SETAC 1998 annual meeting, thank you for your input and assistance.

To my committee members, that WILL read this entire document, and probably understand it better than I do, and to the various professors and scientist that I have had the pleasure and opportunity to work with and learn from, I thank you for your patience and perseverance.

And last of all, Dr. Chris Wood. A true believer of miracles. Thank you for pushing and prodding and the odd swift kick when I needed it. What I have learned from you will never go to waste.

Nathan A. Webb, B.Sc.
McMaster University
May, 1998.

THESIS FORMAT

This thesis is organized into four chapters. Chapter one presents introductory background information and provides a general overview and summary of the following three chapters, all of which report experimental work. These have been written as individual manuscripts for journal publication. Literature cited in each chapter has been amalgamated in one list and follows the last chapter.

Chapter 1: General Introduction

Chapter 2: Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*)

Authors: N.A. Webb and C.M. Wood

Comments: Experimental work performed by NAW with instruction and supervision by CMW. This paper is published and appears in the journal Environmental Toxicology and Chemistry.

Environ. Toxicol. Chem. (1998) 17: 579-588.

Chapter 3: Bioaccumulation and distribution of silver in four marine teleost and two marine elasmobranchs: comparison between fish species, water salinity, and exposure concentration

Authors: N.A. Webb and C.M. Wood

Comments: Experimental work performed by NAW with some initial assistance and supervision by CMW This paper will be submitted to the journal *Aquatic Toxicology*.

Chapter 4: Effects of acute and chronic silver exposure on physiological parameters in two marine teleosts

Authors: N.A. Webb, I.J. Morgan, and C.M. Wood

Comments: Exposure experiments and metabolic parameter analysis performed by NAW, with assistance from IJM for tissue dissections and enzyme analysis, all under the supervision of CMW. This paper will be submitted to the journal *Comparative Biochemistry and Physiology* in a slightly modified form once some final data points have been collected.

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CHAPTER 1: General Introduction

OSMOTIC AND IONIC REGULATION

All fish, with the exception of the hagfishes and marine elasmobranchs, have plasma osmotic pressures that differ substantially from the water in which they live. Therefore, they must regulate their salt and water content, to maintain cell volume and ionic homeostasis. Elasmobranchs also regulate their salt and water content closely, but retain nitrogenous wastes so as to match internal osmotic pressures with that of the external seawater.

The main organs involved in osmotic and ionic regulation differ between species living in different environments. Freshwater teleosts have a well developed renal system (kidney and urinary bladder), which, in conjunction with the important role of the gills, allows these fish to live in a dilute media. The intestine is not involved in osmoregulation in freshwater teleosts. Seawater teleosts have a much smaller renal system which produces a small volume of isoosmotic urine. The intestine and gills of these fish both play major roles in osmoregulation. Elasmobranchs, which are largely confined to the marine environment, have a fully functional and extremely complex kidney, which although important, is tertiary to the rectal gland and gills (Evans, 1979, 1982 and 1993).

Freshwater Teleosts

Freshwater teleosts have plasma salt levels that are roughly 1/3 that of seawater (200 times that of freshwater), thus they are hyperosmotic to the very dilute media in which they live. This means that they face an osmotic influx of water and a diffusional loss of salt. To counteract the salt loss and water gain, these fish generally have gills with low ionic and osmotic permeabilities. These low permeabilities reflect the nature of the tight junctions between epithelial cells and the channels on the apical surfaces of the cells (Evans, 1979; Karnaky, 1992; Reuss, 1992).

The kidneys of freshwater fish play a very important role in osmo- and ionoregulation. The average glomerular filtration rate (GFR) is about 4 ml/kg/h with urine flow rates only slightly less (Evans, 1979). However, Na^+ and Cl^- are strongly reabsorbed, resulting in copious amounts of very dilute urine. The final site of urine processing occurs in the urinary bladder. Na^+ and Cl^- ions are actively taken up across a relatively impermeable bladder wall, thereby forming a resorbate that is hyperosmotic to the urine. This reabsorption of salt and excretion of excess water, helps to balance the osmotic gain at the gills. Typical excreted urine osmolarities are less than 10% of plasma levels (Demarest, 1984; Dawson and Frizzell, 1989).

Although this allows the fish to stay in relative osmotic balance, the fish must still overcome an overall loss of salt. The primary site of salt uptake from the water is at the gills. The movement of ions at the gills has

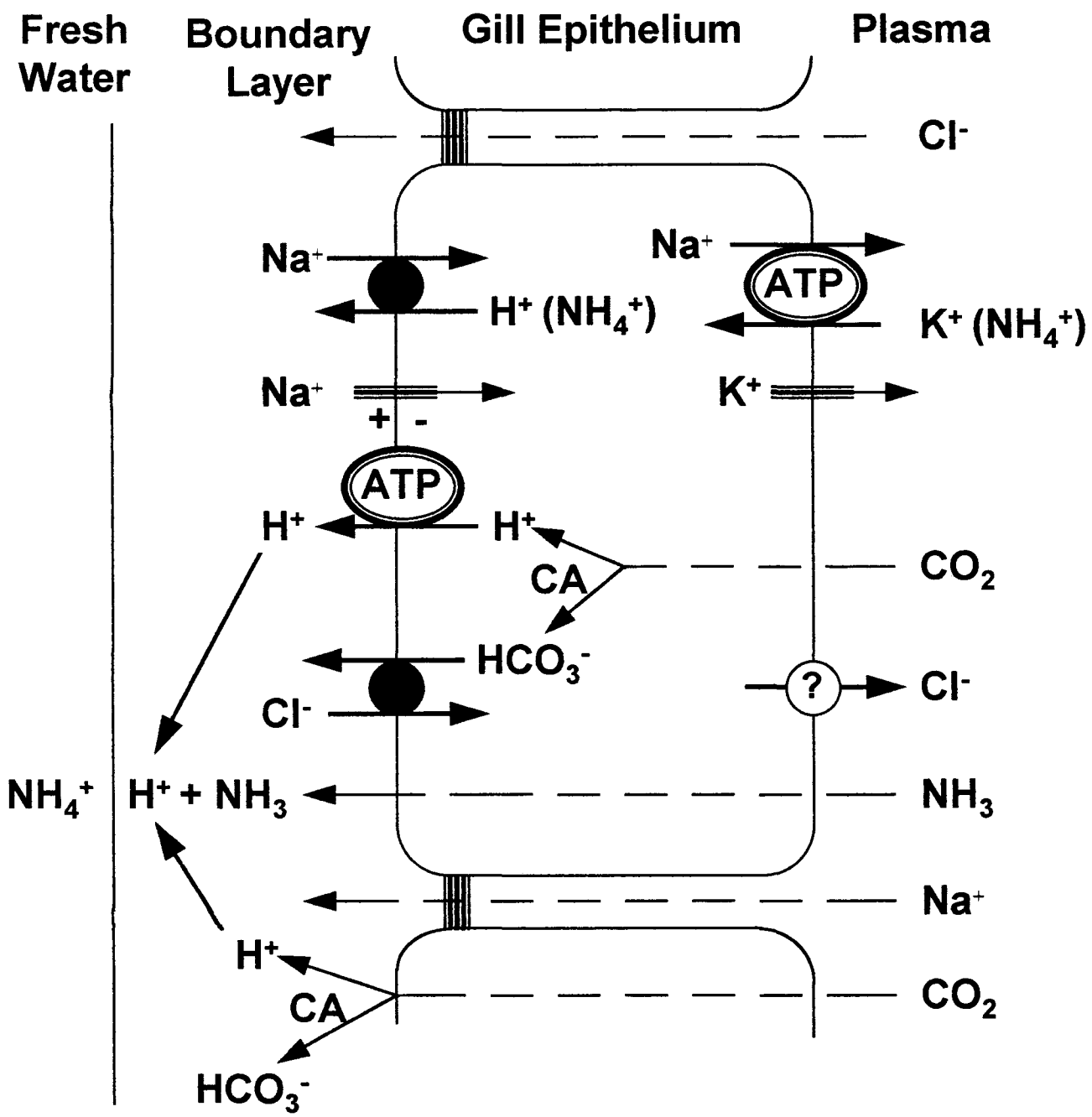
been studied extensively, with the current model of the freshwater gill (Fig. 1.1) suggesting that ionic regulation is closely linked to acid-base regulation (Wood, 1991, 1992; Perry, 1997; Wilkie, 1997). Na^+ enters the gill epithelium via either a sodium channel coupled to a H^+-ATPase or in exchange for H^+ (or NH_4^+). Once in the cell, the Na^+ is transported into the blood stream by Na^+/K^+-ATPase enzymes on the basolateral membrane in exchange for K^+ (or NH_4^+). Chloride ion uptake from the water into the gill epithelium is thought to occur via Cl^-/HCO_3^- exchange. Once inside the cell, the transport mechanism of Cl^- across the basolateral membrane and into the blood stream is unknown (Fig. 1.1).

H^+ ions needed to drive Na^+ uptake (J_{in}) across the apical membrane are provided either by NH_4^+ conversion to $NH_3 + H^+$ in the cell, or by carbonic anhydrase (CA) catalyzed hydration of CO_2 to form H^+ and HCO_3^-. CO_2 hydration is thought to be the primary source of HCO_3^- for Cl^- exchange. The continuous removal of reaction end products from the cell to the water, NH_3 by diffusion and HCO_3^- in exchange for Cl^- uptake, ensures a continuous supply of H^+ in epithelial cells (Wood, 1991, 1992).

Marine Teleosts

Marine fish maintain a blood NaCl concentration that tends to be only slightly higher than freshwater species, thus they are hypoosmotic in relation to their environment (seawater). The result is that they face the problems of

Fig. 1.1. The current model for ionic uptake at the freshwater gill. Na^+ enters the gill epithelium through a sodium channel (≡) connected to an H^+-ATPase or via a Na^+/H^+ (NH_4^+) exchanger, down an electrochemical gradient created by the Na^+/K^+-ATPase, which transports the Na^+ into the plasma. Cl^- enters the epithelium in exchange for HCO_3^- formed via CO_2 hydration by carbonic anhydrase (CA). NH_3 diffuses down the partial pressure gradient into the boundary layer where it is trapped as NH_4^+.


Fresh Water
Boundary Layer
Gill Epithelium
Plasma
Cl-
Na+
H+ (NH4+)
Na+
ATP
K+ (NH4+)
Na+
+ -
K+
ATP
H+
H+
CA
CO2
HCO3-
Cl-
?
Cl-
NH4+
H+ + NH3
NH3
Na+
H+
CA
CO2
HCO3-

volume depletion and salt loading, the opposite of the problems to living in freshwater. The problem of salt loading is exacerbated by the fact that they have a much higher branchial permeability to monovalent ions than freshwater teleosts. This is due to leakier intercellular junctions between chloride and accessory cells (Karnaky, 1992; Reuss, 1992). However, the problem of volume depletion, due to the hyperosmotic environment, is counteracted by the low permeability of the seawater gill, which is even lower than that of freshwater teleosts gill (Evans, 1979).

In order to counter the loss of water, marine teleosts must drink the seawater. Reported drinking rates vary considerably among species (2 to 10 ml/kg/h; see Fuentes and Eddy, 1997), and depend greatly on water salinity and osmotic pressure (Evans, 1979; Fuentes and Eddy, 1997). Drinking seawater is a main contributor to salt loading, with over 95% of the Na^+, K^+, and Cl^- ingested being absorbed in the intestines, and the water following passively (Hickman, 1968). Divalent ions (Mg^{2+}, Ca^{2+}, and SO_4^{2-}) are generally not absorbed in the intestine, and therefore are the main contributors to the osmotic pressure of the rectal fluid (approximately isoosmotic to blood), which is excreted through the anus at about 10% of the drinking rate (Evans, 1979; Demarest, 1984).

To counteract excessive influx of salt via leaky gills and efficient gut uptake, Na^+ and Cl^- are actively excreted at the gills via chloride cells in a

manner similar to the shark rectal gland (see Fig. 1.2; substitute seawater for lumen; Wood and Marshall, 1994; Marshall, 1995).

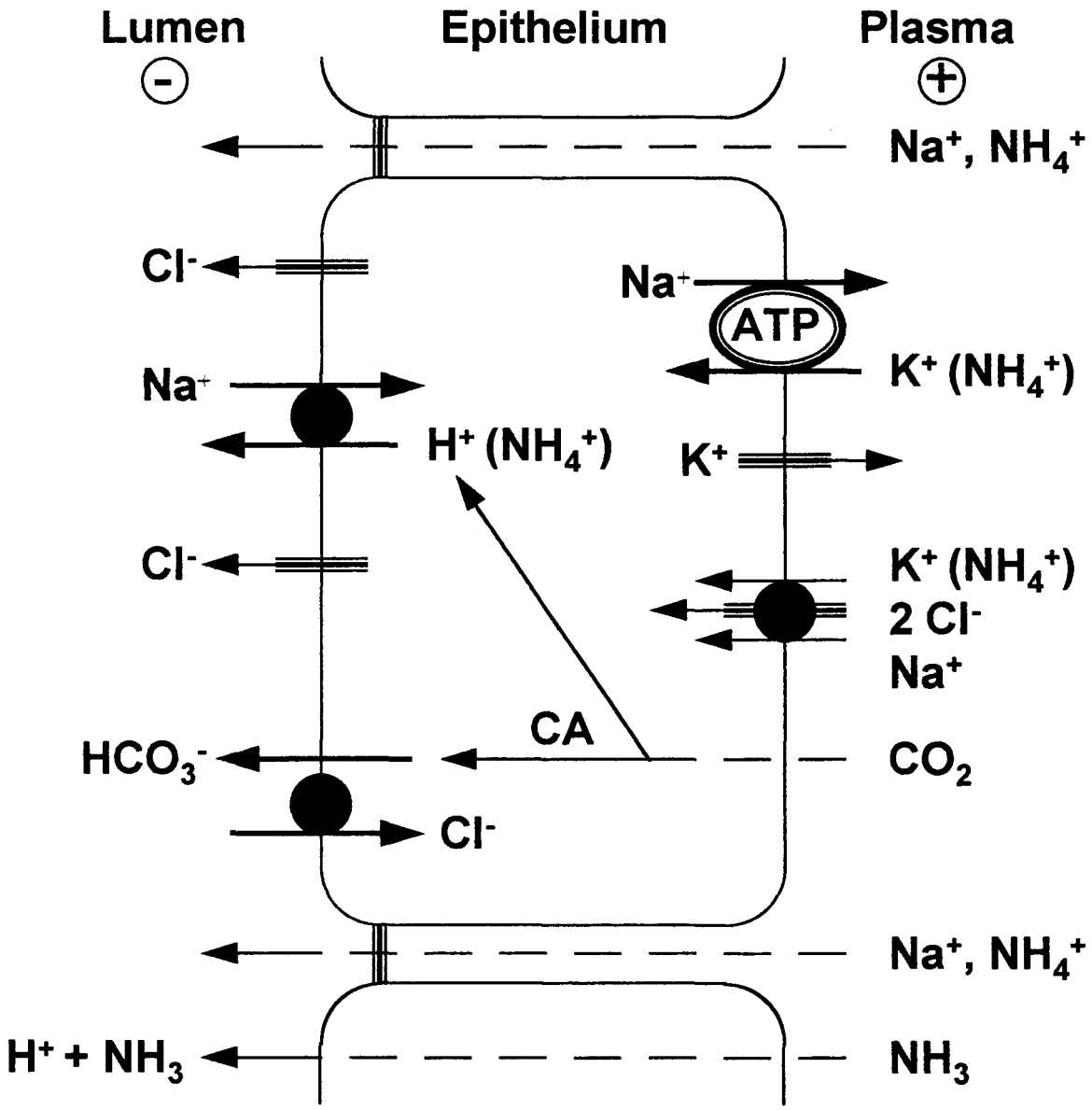
Marine Elasmobranchs

Sharks, skates, and rays have evolved a different method, compared to teleosts, of coping with life in the sea. They have blood Na^+ and Cl^- levels that are 40 to 50% of seawater (160 to 180 μMol), roughly 25 to 40% higher than marine teleosts, with the concentration of secondary electrolytes (K^+, Mg^{2+}, Ca^{2+} and SO_4^{2-}) being only slightly higher (Forster et al., 1972; Evans, 1979).

Despite having lower salt levels than seawater, elasmobranchs are very slightly hyperosmotic to seawater. This is accomplished by the presence of urea and trimethlyamine oxide (TMAO) in the plasma and intracellular fluid (Evans, 1979, 1993). Although urea can be fatal at these levels, TMAO, when present at about 50% of the urea concentration, counteracts the toxic effects, and allows the fish to use urea and TMAO to maintain high osmotic pressures (Yancey and Somero, 1980). This means that marine elasmobranchs are faced with both a volume and salt load (Evans, 1979).

Elasmobranchs have evolved gills that are extremely permeable to water, but less so to ions (Evans, 1979). The kidney, although fully functional, does not appear to play a very significant role in the excretion of

Fig. 1.2. The current model of the shark rectal gland and seawater teleost gill (for the latter substitute seawater for lumen). Na^+ is pumped out of the cell, into the plasma via the Na^+/K^+-ATPase pump, creating an electrochemical gradient that drives the $Na^+/K^+/2Cl^-$ cotransporter. This pulls Cl^- and K^+ against their electrochemical gradients. K^+ exits passively through basolateral potassium channels (≡) and Cl^- exits passively through apical chloride channels, creating a net potential difference across the epithelium (inside positive relative to the lumen). This transepithelial gradient pulls Na^+ into the lumen through leaky paracellular junctions. While NH_3 can diffuse across epithelial cells, NH_4^+ can only diffuse through paracellular junctions. NH_4^+ may also enter the cell by replacing K^+ on basolateral transporters. Once in the cell, it can either dissociate into NH_3 + H^+ and diffuse into the lumen, or be exchanged for Na^+ via apical Na^+/H^+ exchangers. Apical Na^+/H^+ and Cl^-/HCO_3^- exchangers are thought to be primarily involved in intracellular pH regulation.


Lumen
Epithelium
Plasma
⊖
⊕
Na+, NH4+
Cl-
Na+
ATP
K+ (NH4+)
Na+
H+ (NH4+)
K+
Cl-
K+ (NH4+)
2 Cl-
Na+
CA
HCO3-
CO2
Cl-
Na+, NH4+
H+ + NH3
NH3

salt (Hickman, 1969; Evans, 1979). However, it does form a small volume of urine (~1 ml/kg/h).

The most important organ involved in salt excretion in elasmobranchs is the rectal gland (Fig. 1.2; Greger et al., 1986; Shuttleworth, 1988). It forms a solution that is isoosmotic with the plasma, but lacking TMAO and urea. It therefore has a higher NaCl concentration than seawater. The driving force for salt excretion is the Na^+/K^+-ATPase pump (as in the freshwater teleost gill), which is functionally coupled to a $Na^+/K^+/2Cl^-$ cotransporter. Na^+ is pumped out of the cell via the ATPase, creating a gradient that drives the cotransporter. K^+ diffuses back into the plasma via K^+ channels, while Cl^- exits into the lumen via Cl^- channels that are controlled via cAMP. This creates a transepithelial electrical gradient that pulls Na^+ into the lumen through leaky paracellular junctions (Greger et al, 1986; Shuttleworth, 1988).

The gills of elasmobranchs also play a role in salt excretion, although to a lesser extent than the rectal gland. The branchial epithelium is most likely the site of acid-base regulation (Evans and More, 1988) but can take a primary role in salt excretion when fish have had their rectal gland experimentally removed (Burger, 1965; Evans, 1979).

AMMONIA EXCRETION

Sites of excretion

In 1929, Smith managed to separate the outputs of the gills and kidney to show that the gills accounted for at least 85% of ammonia excretion (J_{Amm}) in freshwater carp and goldfish. This has since been shown to be the case for most freshwater fish, with the kidney and skin being responsible for the remainder (Wood, 1993). The gills of marine fish (both teleost and elasmobranchs) however, account for only 50 to 80% of J_{Amm}. With less than 1% being excreted by the kidney and bladder, the remaining 20 to 50% of excretion is thought to occur across the skin (Sayer and Davenport, 1987; Wood, 1993).

Mechanism of excretion

The mechanism(s) for branchial ammonia excretion is highly debated and too large a subject to be dealt with here (see reviews Wood, 1993; Wilkie, 1997), and therefore only a brief overview of the current theories is presented below.

In freshwater, the majority of J_{Amm} probably occurs as NH_3 diffusion down its partial pressure gradient (ΔP_{NH3}) from blood to water. This gradient is maintained via the trapping of NH_3 as NH_4^+ in the gill boundary layer. Protons in this layer are thought to be formed by the catalyzed hydration of CO_2 via carbonic anhydrase on the apical surface of the gill epithelium

following CO_2 diffusive excretion. Branchial H^+-ATPase's may also aid in acidification of the boundary layer. Passive diffusion of NH_4^+ does not appear to play a role in freshwater ammonia excretion, however, the coupled exchange of NH_4^+ for Na^+ may play a role, particularly in environments where ΔP_{NH3} is unfavorable for diffusion (Fig. 1.1; Wood, 1993; Wilkie, 1997).

In marine fish, branchial J_{Amm} occurs via NH_3 diffusion but NH_4^+ diffusion appears to be important as well. The increased cationic permeability of seawater gills allows for NH_4^+ to diffuse through paracellular channels. NH_4^+ exchange for Na^+ may also occur due to the presence of Na^+/H^+ exchangers and the high $[Na^+]$ in the water. As in freshwater gills, NH_3 movement from blood to gill cells can occur by diffusion and/or NH_4^+ can enter by replacing K^+ in Na^+/K^+-ATPase pumps or $Na^+/K^+/2Cl^-$ exchangers (replace lumen with seawater in Fig. 1.2; Wilkie, 1997).

HEAVY METALS IN THE AQUATIC ENVIRONMENT

There are many sources of heavy metals in the aquatic environment. Water is the final sink for runoff from agriculture and mining sites, leaching from waste sites, and dumping from industrial sources. Some of these metals are essential to the health and survival of fish (Cu, Fe, Co, and Zn), and are involved in many physiological processes, particularly as cofactors in enzymatic reactions. However, at high concentrations, even essential metals can be toxic to aquatic organisms. Other metals, that are mainly present in

effluents and runoff, can be toxic at very low levels (Ag, Hg, and Cd) (Bryan, 1976; Wittmann, 1983; Sorensen, 1991). The differing toxic abilities of metals has been related to the mechanisms by which they interact at their specific site of toxicity. Nieboer and Richardson (1980) designed a classification system for metals based on the metals' toxic action, that depends on the metals' preference for oxygen, nitrogen, and sulfur. Class A metals have a higher affinity for oxygen than sulfur (O > N > S), while Class B metals have a higher affinity for sulfur (S > N > O). Borderline metals fit in between Class A and B metals, with the lines of separation being distinct between Class A and Borderline metals, but blurred between Borderline and Class B metals. Based on this system, the metals of special interest to aquatic toxicologists are the Borderline (i.e.: Cd, Cu, Zn and Fe) and Class B (i.e.: Ag and Hg) metals, since they are the most toxic at low levels.

The toxicity of a metal is directly related to the metal's speciation in the environment, and its ability to bind to the site(s) of toxic action. The most important species of metal, when considering aquatic toxicity, is thought to be the free metal ion (M^{n+}). The M^{n+} is attracted to anionic sites on the fish, where it can either exert its toxic action or be taken up into the fish (Pagenkopf, 1983; Evans, 1987; Campbell, 1995). Not all of the metal in the water column is present as M^{n+}. The majority of metal atoms in the water are bound to various ligands (anions, sediment, mucous particles, algae, etc.) and never reach the fish. The portion that does reach the fish,

and is able to interact with the sites of uptake/toxicity, must then compete with other cations for these sites. The fraction that does bind to the sites of uptake/toxicity is referred to as the bioavailable fraction, because this is the fraction of metal in the environment, that can exert toxic effects on the fish. Some factors that affect the bioavailable fraction of a metal include, adsorption to particulate matter, dissolved organic carbon molecules (DOC), pH, alkalinity, salinity, and hardness (Playle et al., 1993a, b; Janes and Playle, 1995; Kramer et al., 1997).

In freshwater, an environment that is low in salt content, and prone to fluctuations in pH, salinity and hardness, the bioavailable fraction, and thus potentially toxic fraction of a metal (M^{n+}), can change dramatically. However, in seawater, there is usually very little of the free metal ion in the water, due to high [Cl^-], [Ca^{2+}], and DOC levels.

Sites of metal uptake

Metal absorption from the environment may occur across the epithelial barriers of gills, intestines, or skin. Many studies have shown that for both freshwater and seawater fish, the main site of uptake/toxicity is the gills (Evans, 1987; Spry and Wood, 1989; Harris, 1991; Roesijadi and Robinson, 1994; Janes and Playle, 1995). The intestines, while contributing a small portion of uptake via metal biologically incorporated in food, does not appear to be important in toxicity of freshwater fish (Williams and Giesy, 1978;

Verbost et al., 1989; Schoenmakers et al., 1992). Current evidence of intestinal metallothionien induction in seawater teleosts (Cousins, 1985; Schoenmakers et al., 1992; Roesijadi and Robinson, 1994), and increased accumulation of Cu (Kuroshima, 1992), Cd (C.H. Hogstrand, unpublished observations), and Ag (C.H. Hogstrand and C.M. Wood, unpublished observations) in the intestines, suggests that the gastro-intestinal tract may be an important site of metal uptake and toxicity in these fish. These findings may be due to the fact that marine teleosts drink to obtain water. It must be remembered however, that the volume of water passing over the gills per unit time, is at least 3 orders of magnitude greater than that passing through the intestines. Thus, the gills remain the major site of exposure in marine teleosts (Evans, 1987). Although little is little is known about the mechanism(s) or site(s) of toxicity in marine elasmobranchs, it can be reasoned, that since these fish do not drink, the gills are probably the main site of metal uptake from the water.

The importance of silver in the environment

The primary sources of silver, other than mineral deposits, are land fill sites, atmospheric deposition from smelting and coal burning activities, and erosion, mining, industrial, and sewage discharges into the aquatic environment. In 1978, it was estimated that of the total amount of silver entering the aquatic environment, 17% was due to the photographic industry,

10% came from sewage treatment plants, and 10% from urban runoff; more than 62% was due to natural sources (Purcell and Peters, 1998).

Although the only effect silver has on humans is cosmetic (US EPA, 1991), it is one of the most toxic metals to freshwater teleosts, with LC50 values ranging from 6.5 to 65 μg/L (60 to 600 nMol) total Ag, when present as $AgNO_3$ (Nebeker et al., 1983; LeBlanc et al., 1984; Hogstrand et al., 1996; Galvez and Wood, 1997). This has led the regulatory agencies to restrict the amount of silver permitted to be present in effluent that is released into the environment. The current EPA limits on effluent release are based on a "hardness" equation (US EPA, 1980), which is designed to protect aquatic life against acute toxicity due to silver:

$$\text{maximum total recoverable Ag } (\mu g/L) = e^{(1.72[\ln \text{hardness}]-6.52)} \qquad (1.1)$$

where hardness is expressed as mg/L of $CaCO_3$.

While the idea of using water chemistry to determine silver toxicity is useful, recent evidence suggests that the sole consideration of hardness for Ag is too simplistic, in that it fails to take [Cl^-] and DOC (the two main determinants of silver toxicity) into account (Brooke et al., 1994; Klaine et al., 1995; Galvez and Wood, 1997; Ferguson and Hogstrand, 1998).

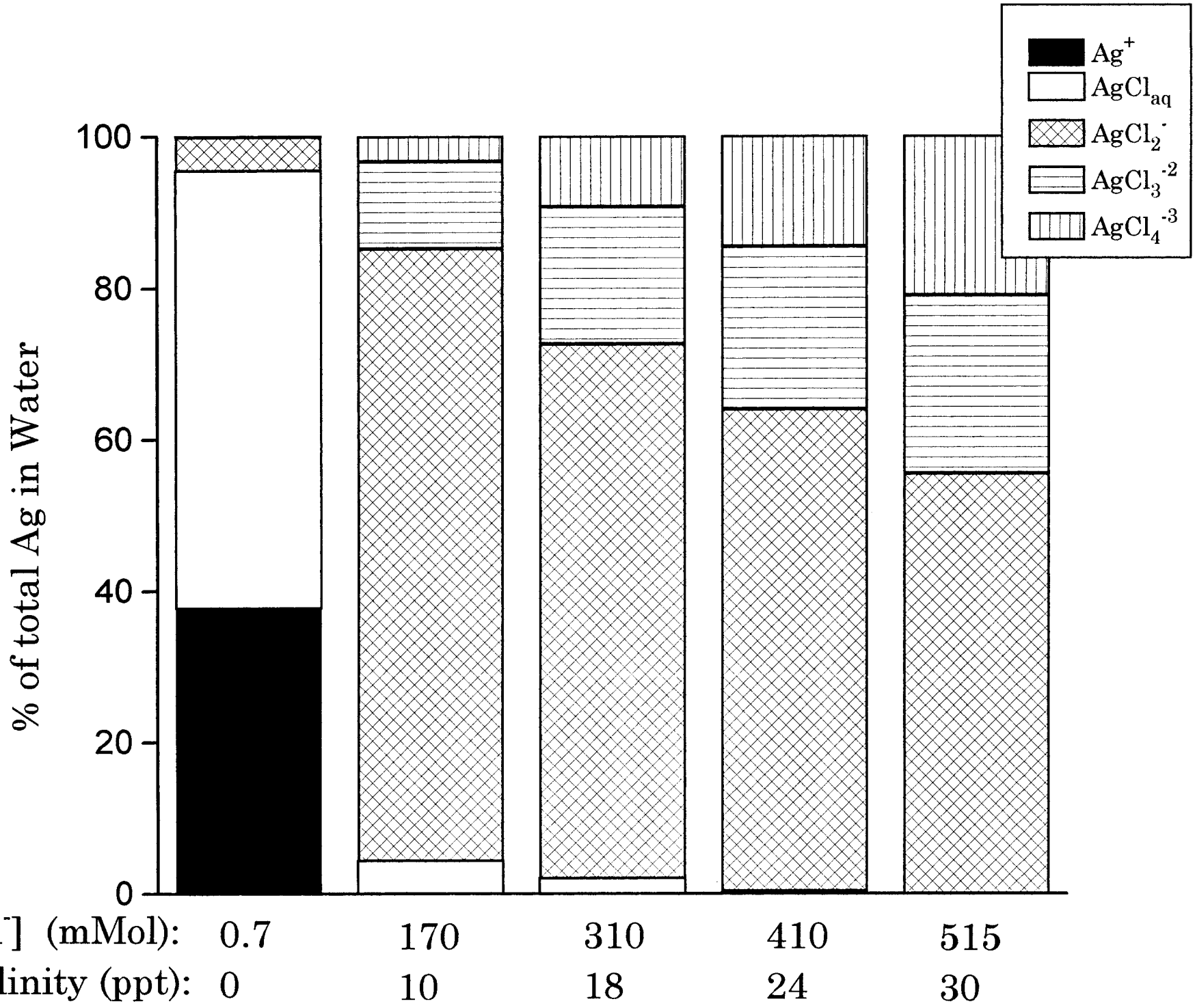
In Canada, the provinces of Ontario and Manitoba have adopted a total silver concentration limit of 0.1 μg/L, without regard to hardness (CCME, 1995). This is also the IJC (1982) limit for the Great Lakes. The province of British Columbia has set criteria for both freshwater (hardness >

100 mg/L) and marine water (open ocean and estuaries) of 1.5 μg/L and 3.0 μg/L for the 30 d average and daily maximum, respectively. In water of lower hardness (≤ 100 mg/L), these levels are reduced to 0.05 and 0.1 μg/L respectively (Warrington, 1995). These levels are based on natural levels in both fresh and marine waters. It is estimated that uncontaminated waters in non-industrialized areas have natural silver levels ≤ 3.0 μg/L, with concentrations increasing near cities and at depth in the open ocean (Warrington, 1995).

SILVER TOXICITY

As with other metals, the most toxic species of Ag in the water is the free ion, Ag^+ (Wood et al., 1996a; Morgan et al., 1997). The concentration of Ag^+ in natural freshwater is highly dependent on DOC and complexing ions (Cl^- and S^{2-}). In laboratory test waters, the free [Ag^+] can be as much as 40% of the total Ag in the water, although in natural conditions it is generally much less (Wood et al., 1996a). With [Cl^-] being one of the main factors affecting Ag speciation, the increase of salinity from freshwater to seawater has a dramatic affect on silver toxicity (Hogstrand et al., 1996; Hogstrand and Wood, 1998). The LC50's for silver in seawater are 50 to 100 times higher (i.e. lower toxicity) than those of freshwater (Eisler, 1996; Hogstrand and Wood, 1998). This can be related back to the concentration of Ag^+ in the

Fig. 1.3. The change of predominant Ag species as [Cl^-] increases from freshwater (0.7 mMol [Cl^-]) to full strength seawater (550 mMol [Cl^-]). In freshwater, nearly 40% of the silver exists as Ag^+, with nearly 60% present as $AgCl_{aq}$. As the [Cl^-] increases, Ag^+ disappears as more Cl^- binds to silver complexes, until, at full strength seawater, only charged $AgCl_n^{1-n}$ species are present. It should be noted that this model assumes a DOC concentration of 0, an [S^{2-}] = 0, and a [Ag] = 10 μg/L (as $AgNO_3$).


Ag+
AgClaq
AgCl2-
AgCl3-2
AgCl4-3
% of total Ag in Water
100
80
60
40
20
0
[Cl-] (mMol): 0.7 170 310 410 515
Salinity (ppt): 0 10 18 24 30

water. As the Cl^- level increases, the Ag^+ concentration becomes nonexistent and $AgCl_n^{1-n}$ species dominate (Fig. 1.3; Cowan et al., 1985; Galvez and Wood, 1997; Ferguson and Hogstrand, 1998). Even in brackish waters (0.8 to 15% salinity), the Ag^+ ion has disappeared, and an insoluble species of silver forms ($AgCl_s$, ceragarite). This may however only be important in laboratory studies in pristine water (i.e., free of DOC) since the high levels of silver and low levels of Cl^- and DOC needed for formation of ceragarite, tend not occur in nature (Galvez and Wood, 1997; Ferguson and Hogstrand, 1998, Hogstrand and Wood, 1998).

MECHANISM OF SILVER TOXICITY

Freshwater

In recent investigations on the physiological mechanisms of silver toxicity (Wood et al., 1996a; Morgan et al., 1997), silver, at levels between 10 and 50% of the 96 h LC50 (1 to 5 μg/L Ag), has been seen to block active Na^+ and Cl^- uptake(J_{in}), by profound inhibition of gill Na^+/K^+-ATPase activity. This results in large declines in plasma Na^+ and Cl^- levels, and associated decreases in plasma volume and hemoconcentration, and an elevation of plasma glucose, indicative of a stress response. All of these responses have also been seen in fish acutely exposed to either copper (Lauren and McDonald, 1985, 1986; Wilson and Taylor, 1993a; Taylor et al., 1996) or low environmental pH (Wood, 1989, 1992; Reid, 1995), suggesting similarities in

the toxic mechanism(s) of action. Both Cu^{2+} and H^+ have also been shown to increase the diffusive loss (J_{out}) of Na^+, K^+, and Cl^- due to increased paracellular leakage. This is probably due to the displacement of Ca^{2+} from tight junction complexes.

Elements of silver toxicity that differ from copper and acid exposure, are an absence of increased diffusional ion losses at the gills and an absence of red blood cell swelling (Wood et al., 1996a; Morgan et al., 1997). It remains to be seen if silver affects K^+ fluxes, plasma cortisol levels, plasma ammonia levels, or branchial ammonia fluxes, and acid-base exchanges.

Seawater

In contrast to freshwater, much less is known about the physiological response to silver in seawater. However, recent studies have shown that silver causes an increase in plasma Na^+ and Cl^- levels (Hogstrand et al., 1996 ; Hogstrand and Wood, 1996, 1998). This result, along with a concomitant rise in plasma ammonia and glucose levels, suggests that the effects of silver exposure in seawater are also similar to those of copper exposure in seawater (Stagg and Shuttleworth, 1982a, b; Wilson and Taylor, 1993b). As in freshwater, copper exposure in seawater interferes with iono-regulation by inhibiting branchial Na^+/K^+-ATPase. NaCl excretion is inhibited and plasma Na^+ and Cl^- levels rise. Plasma cortisol and glucose levels also rise, as does the plasma ammonia concentration. Copper has also been seen to increase

been seen to increase oxygen consumption and ventilation rate in marine fish (O'Hara, 1971; Sorensen, 1991).

Although the toxic mechanisms of silver and copper in seawater appear to be similar, it is not known if silver elicits a fluid shift, or a mobilization of cortisol. The increased plasma ammonia seen in starry flounder, during silver exposure, was only transitory and was not dependent on exposure concentration (Hogstrand and Wood, 1996). Recent observations suggest that increased hematocrit and plasma ion levels in silver exposed fish may be due to an inhibition of drinking rate (Hogstrand and Wood, 1998; Wood et al. unpublished results).

OVERVIEW OF CHAPTER 2

These experiments were designed to complement the previous studies by Wood et al. (1996a) and Morgan et al. (1997). The first objective of the study was to confirm the mechanism of Ag^+ action on freshwater rainbow trout (*Oncorhynchus mykiss*), namely the changes in iono-regulation at the gills, plasma ionic and acid-base status, and the cause of hemoconcentration. The second was to document responses in elements which had not been measured in the previous two studies, specifically K^+ fluxes, plasma cortisol levels, plasma ammonia levels, branchial ammonia fluxes, and branchial acid-base exchanges. To achieve this, fish were exposed to a total Ag concentration of 9.2 μg/L (as $AgNO_3$) for 6 d in moderately hard freshwater.

This Ag concentration was close to the 168 h LC50 (Hogstrand et al., 1996; Galvez and Wood, 1997). Depending on the group, blood samples or flux measurements were taken at the start and again at 4, 24, 48, 96, and 144 h during the exposure. In another group of fish, ammonia flux measurements were taken intensively over the first 12 hours of exposure to determine if increased plasma ammonia levels were due to inhibition of J_{Amm} or increased metabolic production.

This study confirmed that silver dramatically disrupts iono-regulation causing a net loss of ions by blocking active Na^+ and Cl^- uptake, without increasing passive efflux. Ion uptake rates did not recover over the 6 d of exposure, while unidirectional effluxes were gradually reduced below control levels. Despite the reduced diffusive ion loss, ionic balance remained negative. Decreased plasma volume and hemoconcentration probably resulted from declines in plasma [Na^+] and [Cl^-], but red blood cells did not swell. A substantial plasma acidosis of metabolic origin appeared to be due to a net uptake of acidic equivalents from the water. This uptake greatly exceeded the measured acid load in the extracellular fluid, suggesting that acidosis also occurred in the intracellular compartment. This may explain the continual loss of K^+ to the water without any change in plasma [K^+]. The dramatic disruption in iono-regulation and acid-base balance, led to a general stress response with the mobilization of glucose and cortisol. Plasma ammonia levels also rose throughout the exposure, along with ammonia

excretion. This suggests that the increase in plasma ammonia is due to elevated metabolic production rather than inhibited excretion. The cause is probably the stress-induced mobilization of cortisol. The increased plasma ammonia, in conjunction with hyperventilation, helps to counteract metabolic acidosis. However, since the fish is unable to counteract the loss of plasma ions, death eventually results from a severe iono-regulatory disturbance.

OVERVIEW OF CHAPTER 3

This series of experiments was done to monitor the chronic sublethal effects of silver on a variety of adult fish in the marine environment, since the majority of previous work on silver effects on fish in seawater has been either acute studies, or early life stage analysis (Eisler, 1996; Hogstrand and Wood, 1996, 1998; Shaw et al, 1998).

The first specific objective was to monitor how species differences affect silver accumulation and distribution in marine fish. One previous study has shown that the rate and quantity of silver accumulation varies between marine teleosts (plaice) and elasmobranchs (thornback ray) (Pentreath, 1977). In the present study, four marine teleosts; rainbow trout (*Oncorhynchus mykiss*), tidepool sculpin (*Oligocottus maculosus*), English sole (*Parophrys vetulus*), and plainfin midshipman (*Porichthys notatus*), and two marine elasmobranchs; the Pacific spiny dogfish (*Squalus acanthius*) and long nose skate (*Raja rhina*), were exposed to 14.5 μg/L Ag (as $AgNO_3$) for a

period of three weeks at a salinity of 31% ± 1%. A comparison of elasmobranchs with teleosts was of interest because of the different ionic transport mechanisms in the gills and the absence of drinking in the elasmobranchs (see above).

The second objective of the marine study was to analyze the effects of salinity on the bioaccumulation and distribution of silver. Tidepool sculpins, acclimated to either 18 or 30 ppt salinity for one week, were exposed to several different silver concentrations (as $AgNO_3$) for 21 days.

The third objective was to determine the effects of exposure concentration on bioaccumulation and distribution. Rainbow trout, plainfin midshipman, and tidepool sculpin were exposed to silver concentrations of, 0 (control), 1.5, 14.5, and 50 μg/L Ag (as $AgNO_3$) for 21 days.

From the first series, it was found that the route of silver uptake plays a decisive role in determining internal distribution. Drinking fish (marine teleosts), accumulated more silver in the intestines and gut, than the gills, while the reverse was true for non-drinking fish (marine elasmobranchs). In both, the major site of internal accumulation was the liver. Surprisingly, non-drinking fish (elasmobranchs) had a higher rate of accumulation, since these fish have liver sizes 10-fold larger than teleosts, and the terminal liver silver concentrations were the same. This suggests that in the same time span, 10 times the amount of silver entered the elasmobranchs than the teleosts.

The main factor affecting bioavailability appears to be speciation of silver in the water. At lower salinities, neutral charged $AgCl_{aq}$ is present and can enter the fish, allowing for increased bioaccumulation. At the higher salinity, only negatively charged $AgCl_n^{1-n}$ complexes are present, which greatly decreases the bioavailability of silver, and prevents silver from entering the fish.

Silver bioaccumulation does not appear to cause toxicity, but it does appear to increase a fishes' susceptibility to other toxicants in the environment. Once in the fish, it may interfere with metabolism (ammonia production, oxygen consumption), Na^+/K^+-ATPase activity, and potentially other aspects of a fish's' physiology that could decrease fitness.

OVERVIEW OF CHAPTER 4

The final set of experiments examined the physiological effects of silver on marine fish during both acute high level exposure, and chronic low level exposure. This study was concerned with the effects of both salinity and exposure concentration on physiological parameters. In an acute exposure, tidepool sculpins acclimated to 10, 18, 24, and 30 ppt seawater were exposed to 250 μg/L Ag (as $AgNO_3$) for 48 h. Silver accumulations in the gills, intestines, and livers were measured, as was the ammonia excretion. A chronic exposure of tidepool sculpins in 18 and 30 ppt, and plainfin midshipmen in 30 ppt, to low silver levels was performed as in Chapter 3. In

brief, tidepool sculpins acclimated to 18 and 30 ppt were exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag for 21 d. Ammonia excretion, oxygen consumption, gill and intestine Na^+/K^+-ATPase activity, and drinking rates were measured over the first week of exposure. Midshipmen were exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag for 21 d, with gill and intestine Na^+/K^+-ATPase activity being measured on days 0, 7, and 21.

It was found that an acute exposure to high silver levels led to increased accumulation of silver in the gills and intestines, but not in the liver of sculpins at all salinities. Ammonia excretion was unaffected by silver exposure during the first 48 h, but was altered by the salinity of acclimation, with J_{Amm} increasing as the external salinity decreased.

Long term exposure of tidepool sculpins to low silver levels decreased ammonia excretion by the seventh day, in fish at both 18 and 30 ppt, although, there was no salinity effect. The routine metabolic rate in these fish was also decreased, with fish at 18 ppt having lower oxygen consumption rates than 30 ppt fish. Careful documentation of oxygen consumption over the first few days showed that this depression was a gradual event, eventually becoming significant by the fourth day.

Measurement of Na^+/K^+-ATPase in the gills of midshipmen showed that chronic exposure to silver significantly inhibited activity at high levels with the inhibition being proportional to exposure concentration. Intestinal

Na^+/K^+-ATPase levels in midshipmen showed no effect of silver exposure at any silver level during the 21 d exposure.

Enzyme activity levels in tidepool sculpins at 18 ppt were lower than in sculpins at 30 ppt, and the activity levels increased in silver exposed fish. Activity levels in gills of 30 ppt sculpins exposed to 50.0 μg/L Ag increased up to day 6, but were significantly lower than controls on day 21. Activity levels in sculpin intestines at 18 ppt were generally lower than in fish at 30 ppt, and were increased in sculpins exposed to 50.0 μg/L Ag. Activity levels in the intestines of sculpins at 30 ppt were generally lower than in controls for all fish exposed to silver on all test days.

Drinking rate in tidepool sculpins showed no difference between salinities, but was inhibited in silver exposed fish by the eighth day of exposure.

These data suggest that the silver which enters the fish, may interfere with metabolic processes involved in ammonia production and energy metabolism. The fish also face an interference with iono-regulation (decreased Na^+/K^+-ATPase activity) which is linked to changes in the drinking pattern. Fish at the lower salinity are able to cope with the iono-regulatory problems better than those at higher salinities, but they face an increased accumulation of silver.

CHAPTER 2: Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*)

ABSTRACT

Rainbow trout were exposed to $AgNO_3$ (9.2 μg/L total Ag, of which 35% was as the free ion, Ag^+) for a 6 d period in dechlorinated Hamilton tapwater. Our findings suggest that the primary toxic mechanism of Ag is an interruption of ionoregulation at the gills, stopping active Na^+ and Cl^- uptake, without increasing passive efflux, thereby causing net ion loss. There is no recovery of influxes over 6 d, whereas effluxes are gradually reduced below control levels, and ion balance remains negative. The resulting fall in plasma $[Na^+]$ and $[Cl^-]$ leads to a decrease in plasma volume and hemoconcentration, but the red blood cells do not swell. A substantial metabolic acidosis with partial respiratory compensation occurs in the blood, due to a net uptake of acidic equivalents from the environmental water. This uptake greatly exceeds the measured acid load in the extracellular fluid, suggesting that acidosis also occurs in the intracellular compartment, which in turn explains the continual loss of K^+ to the water in the absence of any change in plasma $[K^+]$. Plasma ammonia, glucose, and cortisol rise. As there is no reduction, but rather a progressive rise in ammonia excretion, the increase in plasma ammonia is due to elevated metabolic production rather than inhibited excretion. The cause is

probably the stress-induced mobilization of cortisol. This increased plasma ammonia, in conjunction with hyperventilation, helps to counteract metabolic acidosis. However, because the fish is unable to counteract the loss of plasma ions, death eventually results from a severe ionoregulatory disturbance.

Keywords - Silver, Toxicity, Ionoregulation, Rainbow trout

INTRODUCTION

While various forms of complexed Ag are relatively benign, free ionic Ag^+, as liberated from dissolved $AgNO_3$, is extremely toxic to freshwater fish, with 96 h 50% lethal concentrations (LC50s) in the range of 6.5 to 65 µg/L total Ag (Nebeker et al., 1983; LeBlanc et al., 1984; Hogstrand et al., 1996a; Galvez and Wood, 1997). Recent data (Wood et al., 1996a; Morgan et al., 1997) suggest that these toxic effects of ionic Ag^+ are similar to those of both copper (Laurén and McDonald, 1985, 1986, 1987; Wilson and Taylor, 1993a; Pilgaard et al., 1994; Taylor et al., 1996) and acid exposure (see Wood, 1989, 1992; and Reid, 1995 for reviews of the extensive low-pH literature). Both Cu^{2+} and H^+ cause net ion losses (J_{net}) from the fish by inhibition of active branchial Na^+ and Cl^- influxes (J_{in}) and increases in diffusive Na^+ and Cl^- effluxes (J_{out}). In addition, net K^+ losses have also been reported. Decreased uptake appears to be caused by the inhibition of gill basolateral Na^+/K^+-ATPase in the case of Cu^{2+}, whereas in the case of H^+, the decrease is due to the inhibition of apical ion-exchange processes. For both toxicants, increased efflux appears to be mainly due to a rise in paracellular leakage, probably by displacement of Ca^{2+} from the junctional complexes. The resulting drop in plasma ions causes internal fluid shifts that decrease plasma volume, exacerbating the hemoconcentration caused by red blood cell swelling and splenic contraction. Plasma glucose and cortisol levels rise, indicative of a typical stress response. Plasma ammonia levels also rise, but it is unclear whether this is due to an

inhibition of branchial ammonia excretion that has been reported in several studies and/or due to the metabolic effects of cortisol mobilization ("proteolysis"; van der Boon et al., 1991). Plasma acidoses of diverse origins, including a disturbance of acid-base exchange at the gills, have also been seen in some studies.

In the two investigations to date on the physiology of Ag^+ toxicity (Wood et al., 1996a; Morgan et al., 1997) both on adult rainbow trout (*Oncorhynchus mykiss*), elements of similarity to the effects of Cu^{2+} and/or H^+ include blockade of active Na^+ and Cl^- influxes, profound inhibition of gill Na^+/K^+-ATPase activity, large declines in plasma Na^+ and Cl^- levels, associated decreases in plasma volume, splenic contraction, hemoconcentration, elevated plasma glucose, and plasma acidosis. Elements of difference include an absence of stimulated diffusive effluxes at the gills and an absence of red cell swelling. Elements that have not yet been examined include K^+ fluxes, plasma cortisol levels, plasma ammonia levels, branchial ammonia fluxes, and branchial acid-base exchanges.

The present study was designed as a comprehensive examination of the stress response to Ag^+ in adult rainbow trout under the same exposure conditions as those used by Wood et al. (1996a) and Morgan et al. (1997). Fish were exposed to total Ag = 9.2 µg/L as $AgNO_3$ (approximately 1/3 as Ag^+, close to the 168 h LC50; Hogstrand et al., 1996a; Galvez and Wood, 1997) for 6 d in moderately hard freshwater. The first specific objective was to confirm key

responses of the previous studies, including effects on Na^+ and Cl^- exchanges at the gills, plasma ionic and acid-base status, and the nature of hemoconcentration. The second was to document responses, or lack thereof, in the elements listed above that were not examined in the two previous studies. Inasmuch as the occurrence of profound ionoregulatory disturbance and a complex plasma acidosis were confirmed, and elevations of both plasma ammonia and cortisol were documented for the first time, the final objective was to understand the mechanisms behind the acid-base and ammonia responses. In particular, because both ammonia excretion and acid-base exchange are known to be intimately linked to ion-exchange processes at the gills (Wood, 1992), we tested whether the observed responses were due to disturbances of ammonia excretion and acid-base exchange with the environment.

MATERIALS AND METHODS

Experimental animals

Adult rainbow trout (*O. mykiss,* 200 - 400 g) were obtained from Humber Valley Trout Farm (Orangeville, ON, Canada) and held for at least 2 weeks prior to experimentation in flowing, aerated dechlorinated Hamilton tapwater (Ca^{2+} = 1.0, Mg^{2+} = 0.2, Na^+ = 0.5, Cl- = 0.7, K^+ = 0.05, in mM; titratable alkalinity to pH 4.0 = 1.9; total hardness = 140 ppm as $CaCO_3$ equivalents; pH

= 8.0 ± 0.2; 15 ± 1°C). They were fed a ration of 1% body weight of commercial trout pellets three times a week.

To standardize metabolic conditions, all fish were starved for 7 d prior to experimentation. On the seventh day, the fish were either fitted with indwelling dorsal aortic catheters (Wood et al., 1996a) while under MS-222 anesthesia (group A) or were left intact (groups B and C). The catheters consisted of PE50 tubing filled with Cortland saline heparinized at 50 IU/ml with sodium heparin (Sigma, Oakville, ON, Canada). All fish were then weighed and transferred to individual darkened Plexiglas chambers (volume = 4 L, McDonald and Rogano, 1986) served with 800 ml/min of flow through water, for 48 h of recovery. The chambers were individually aerated and could be closed to allow measurements of ammonia excretion and ionic and acid-base exchanges with the environment. By slowing the flow, ammonia excretion could also be continually monitored from measurements of differences in concentration between inflow and outflow (group B). The three groups of fish underwent similar exposure protocols with group A fish being used for blood parameter analysis over 6 d of exposure, group B fish being used to monitor the fine time course of ammonia excretion during the first 12 h of exposure, and group C being used for whole body ionic, acid-base, and ammonia fluxes over the 6 d period. Tests were performed on control and experimental fish simultaneously to yield a total N of 8 control (240.6 ± 14.4 g) and 14 experimental (216.3 ± 8.8 g) in group A, 5 control (228.7 ± 16.9 g) and 6

experimental (216.9 ± 6.6 g) in group B, and 13 control (380.8 ± 35.2 g) and 13 experimental (344.6 ± 29.5 g) in group C.

Experimental protocol

At the start of the exposure (T = 0), $AgNO_3$ was added to a well-mixed head tank by a peristaltic pump, from a light shielded stock bottle (20.0 µg/ml Ag as $AgNO_3$, BDH) to give a nominal total Ag concentration in the inflow water of 10 µg/L. At time 0, each flux box was spiked with the stock solution to bring the water immediately to the exposure level. Water samples were taken from the fish boxes throughout the experiment to verify the exposure concentration for each fish. Water leaving the fish boxes exited to waste; there was no recirculation.

Group A

For both control and experimental fish, blood samples (0.8 ml) were taken via the dorsal aortic catheters prior to the exposure (control or "C" sample) and again at 4, 24, 48, 96, and 144 h during the exposure. Blood was drawn anaerobically into ice-cold gas-tight Hamilton syringes for analysis of arterial blood pH (pH_a), total plasma CO_2 content (Ca_{CO2}), hematocrit, hemoglobin, and plasma levels of protein, total ammonia, glucose, cortisol, Na^+, Cl^-, K^+ and total Ag. This volume was replaced by reinfusion of nonheparinized Cortland saline plus the resuspended red blood cells not used in analysis.

After the final blood sample on day 6, the fish were immediately sacrificed by an overdose of MS-222 (1 g/L, neutralized with NaOH; Syndel Labs, Vancouver, BC, Canada). Gill, liver, and white muscle samples were dissected, frozen in liquid N_2, and stored at -80°C for future analysis.

Group B

Ammonia excretion rates were monitored hourly on a flow through basis prior to and throughout the first 12 h of exposure for both control and experimental fish. The water inflow to all boxes was decreased to 200 ml/min. Water samples (10 ml) from both inflow and outflow were taken and analyzed for pH and total ammonia concentration. The goal of these fine timescale measurements was to test whether there was any initial inhibition of ammonia excretion.

Group C

Measurements of ionic exchange, acid-base exchange, and ammonia excretion in both control and experimental fish were carried out over 3-h periods prior to the start of exposure ("C" sample) and also starting at 4, 24, 48, 96, and 144 h. For each measurement period, the water flow was stopped and the volume of the box was set to exactly 4 L. At this time, 4 μCi of ^{22}Na (as NaCl; NEN-Dupont, Boston, MA, USA) and 5 μCi of ^{36}Cl (as NaCl; ICN Radiochemicals, Irvine, CA, USA) were added to the water and allowed to mix

for 5 min. Vigorous aeration was used to ensure thorough mixing and to maintain the partial pressure of O_2 at >85% of air-saturation levels. A 50-ml water sample was taken at this time and again at the end of 3 h. The boxes were then reopened to a flow through system. Each water sample was analyzed for gamma (^{22}Na only) and total radioactivity (^{22}Na plus ^{36}Cl), pH, and total water levels of Na^+, Cl^-, K^+, Ag, ammonia, and titratable alkalinity. After the final sample period (day 6) the fish were immediately sacrificed by an overdose of MS 222 (1 g/L), and a terminal blood sample was taken by caudal puncture to monitor internal ^{22}Na and ^{36}Cl, and plasma Na^+, and Cl^- levels. Gill, liver and white muscle samples were dissected, frozen in liquid N_2, and stored at -80°C for future analysis.

Analytical Methods

Blood and tissue parameters

Arterial blood pH was determined at the experimental temperature by a Radiometer microelectrode system (E-5021) connected to a Radiometer PHM72 meter. Hemoglobin was measured colorimetrically using the cyanmethemoglobin method and Sigma reagents. Total carbon dioxide concentration (C_{aCO_2}) in the plasma was determined with a Corning 965 CO_2 analyzer. Plasma glucose and ammonia were determined enzymatically using Sigma kits (HK 16-UV and 171-UV, respectively). Cortisol levels were determined by a radioimmunoassay kit from Immunocorp (^{125}I RIA kit, ICN

Biomedical, Irvine, CA, USA). Cl^- levels were measured with a Radiometer CMT10 chloridometer, while Na^+ and K^+ concentrations were determined by atomic absorption spectrophotometry (AAS, Varian 1275). All silver levels were determined as detailed by Hogstrand et al. (1996a) using graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer).

Water parameters

Water pH was measured with Radiometer electrodes (GK 2401C) connected to Radiometer PHM84 meters. Water P_{O2} was monitored with a Radiometer O_2 electrode (E-5046) connected to the PHM72 meter. Ammonia levels were determined using the colorimetric assay of Verdouw et al. (1978), which employs the reaction of ammonia with salicylate and hypochlorite. Water Cl^- was measured colorimetrically by the liberation of thiocyanate from mercuric thiocyanate to form mercuric chloride (Zall et al., 1956). Na^+ and K^+ levels were determined by atomic absorption spectrophotometry (Varian 1275). Water silver levels were determined by graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer), again as described by Hogstrand et al. (1996). Titratable alkalinity was determined as described by McDonald and Wood (1981) by titrating continually aerated 10-ml water samples to pH 4.00 with 0.02 N HCl using Gilmont digital microburettes and Radiometer electrodes (GK 2401C) attached to Radiometer PHM82 meters.

^{36}Cl is a pure β-emitter, while ^{22}Na is a mixed γ- and β-emitter. Dual-labeled 5-ml water samples (and 100-µl terminal plasma samples) were prepared in duplicate. ^{22}Na radioactivity alone was measured by γ-counting in a well counter (Canberra-Packard Minaxi Auto-Gamma 5000) and ^{22}Na plus ^{36}Cl radioactivity by scintillation counting (LKB Rackbeta 1217). ^{36}Cl radioactivity was obtained by subtraction after accounting for differences in efficiency of ^{22}Na counting by the two instruments, as described by Wood (1988).

Calculations

Group A

Acid-base calculations employed the Henderson-Hasselbalch equation, as detailed in Playle et al. (1989), to determine Pa_{CO2} and plasma $[HCO_3^-]$ from measured values of pH_a and Ca_{CO2}. CO_2 solubility (αCO_2) and pK' values at experimental temperatures for trout plasma were taken from Boutilier et al. (1984). The metabolic acid load (ΔH^+_m) in the blood plasma was calculated from changes in plasma HCO_3^- concentrations and pH_a using the formula of McDonald et al. (1980). In this equation, the whole blood buffer capacity (β) for true plasma was estimated from the measured Hb concentration and the regression relationship determined for rainbow trout blood by Wood et al. (1982). Mean cell Hb concentration (MCHC) was calculated as the ratio of Hb to hematocrit. The inverse ratio of the plasma protein concentration at day 6

to the initial (time "C") value was used as an index in the change of plasma volume (Wood et al., 1996a; McDonald et al., 1980). Ag speciation in the exposure water was calculated using the determined water chemistry and the aquatic geochemical equilibrium program MINEQL+ (Schecher and McAvoy, 1991).

Partial pressures of ammonia (P_{NH3}) in the plasma and water were determined from the measured total ammonia concentrations (T_{Amm}) and pH by the equations detailed in Wright and Wood (1985), using NH_3 solubility (αNH_3) and pK' values for trout plasma and freshwater at the experimental temperature from Boutilier et al. (1984).

Group B

Ammonia excretion rates during the first 12 h of exposure were calculated by the Fick principle from measurements of body weight, water flow rate, and inflowing and outflowing concentrations, as detailed by Booth et al. (1988).

Group C

Detailed rationales for net and unidirectional flux calculations are given by Wood (1988). In brief, net flux rates of ammonia and ions were calculated

from changes in water concentrations over the 3-h period of box closure:

$$J_{net} = \frac{([initial] - [final]) \times V}{W \times t} \quad (2.1)$$

where [initial] and [final] are the initial and final concentrations in the water respectively (μmol/L), V is the volume of the water in the flux chamber (L), W is the weight of the fish (kg), and t is the duration of the flux period (h). Thus net losses from the fish have a negative sign, and net gains a positive sign. By reversing the [initial] and [final] terms, the net titratable acid flux was calculated from the titratable alkalinity measurements. The sum of titratable acid and ammonia fluxes, signs considered, yields the net flux of acidic equivalents. As pointed out by McDonald and Wood (1981), this method does not distinguish between ammonia movements in the NH_3 versus NH_4^+ forms nor between the net excretion of acidic equivalents and the net uptake of basic equivalents, or vice versa. Fortunately, this does not matter in terms of the net acid-base balance of the fish, which is still faithfully represented.

Unidirectional influx rates for sodium and chloride were determined by monitoring the disappearance of radiotracers from the external water into the fish:

$$J_{in} = \frac{(cpm_i - cpm_f) \times V}{MSA \times W \times t} \quad (2.2)$$

where cpm_i and cpm_f are the initial and final concentrations of counts per minute (cpm/L) respectively, MSA is the mean specific activity of the test water during the flux period (cpm/μmol), and the other variables are as above.

Unidirectional efflux rates were determined by subtraction:

$$J_{out} = J_{net} - J_{in} \quad (2.3)$$

By convention, unidirectional influx rates and net uptake rates by the fish have a positive sign, while unidirectional efflux rates and net loss rates have a negative sign.

Repeated radioisotope flux determinations may lead to internal accumulation and associated "backflux" of radiotracers, that would require a correction of the calculated J_{in} values. This becomes important when the internal SA of the isotope is ≥10% of the external specific activity (Wood, 1988). However, as the final SA measured from terminal plasma samples for all fish was ≤6% of the external SA on day 6 in the present study, correction was not required.

Data have been expressed as means ± SEM. Differences between control and experimental treatment means at the same time were analyzed by the Student's unpaired two-tailed *t*-test. Comparisons of changes over time within treatments were assessed with Student's two-tailed paired *t*-test using the Bonferroni procedure to adjust the *t*-value for multiple comparisons. Significant differences ($p < 0.05$) between treatments are indicated by asterisks

(*), while significant differences within treatments are indicated by a plus sign (+).

RESULTS

Water chemistry and survival

The mean total water Ag concentration during the exposure was 9.2 ± 0.2 µg/L. Ag speciation calculated by MINEQL+ showed similar species distribution as in Wood et al. (1996a) for Hamilton tapwater, indicating that 35% of total Ag was in the free ionic form (Ag^+), with the rest being bound up in various forms of silver chloride (AgCl (aq) = 60%, $AgCl_2^-$ = 5%). The lack of any $AgNO_3$ remaining in bound form is directly due to the dissociation constant of NO_3^- being extremely high in freshwater (LeBlanc et al., 1984; Schecher and McAvoy, 1991; Janes and Playle, 1995).

Of the fish exposed for 6 d (groups A and C), three experimental fish died after 4 d and two fish after the fifth day (i.e., 5/27). Only one of the control fish died (i.e., 1/21) immediately before the sixth day of sampling. Data from all individuals that died were included in calculations as they did not bias any of the trends seen. None of the short-term exposed fish (group B) died.

Tissue silver burden

Gill silver levels were found to be approximately 1,300 µg/kg (or about 12 µmol/kg), 8 times those of control fish by the end of 6 d exposure. Silver

levels in liver samples from exposed fish were much higher (~26,000 µg/kg), but only 3.5 times those of control fish. White muscle concentrations were intermediate (6,000 µg/kg) and there was no difference seen between the mean values for control and exposed fish (Table 2.1).

Group A - blood chemistry

Plasma total Ag concentration in exposed fish increased continually throughout the exposure (Fig. 2.1A). This increase was significant by day 1 and reached about 7 µmol/L (750 µg/L), again nearly eight times the pre-exposure levels by day 6. The Ag levels in the plasma of control fish remained less than 1 µmol/L and did not change during the treatment (Fig. 2.1A).

Plasma K^+ levels remained unchanged throughout the exposure (Table 2.2), while both Na^+ and Cl^- levels dropped by 16% over the 6 d (Fig. 2.1B and C). Ion concentrations in control fish remained unchanged throughout the experiment.

The hematocrit values for both exposed and control fish did not differ significantly from pre-exposure levels (Fig. 2.2A). However, Ag exposure prevented the decrease in Hb seen in control fish that resulted from repetitive blood sampling (Fig. 2.2B). MCHC did not change significantly (Table 2.2). A significant increase in plasma protein levels to nearly twice the control value was seen by the end of the exposure (Fig. 2.2C). This corresponded to a marked 40% drop in relative plasma volume by the sixth day (Table 2.2).

Table 2.1. Tissue Ag burdens of rainbow trout exposed to 9.2 µg/L Ag (as $AgNO_3$) for 6 d. [a]

Total Ag (µg/kg wet weight)	Control	$AgNO_3$
Gill	164 ± 10	1,305 ± 202[b]
Liver	7,450 ± 352	26,150 ± 7,240[b]
White Muscle	5,420 ± 1,280	6,320 ± 1,050

[a] Values are means ± 1 SEM (n = 8).

[b] $p < 0.05$.

Fig. 2.1. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (A) plasma total Ag levels, (B) plasma Na^+, and (C) plasma Cl^- in both control (■, n = 8) and Ag-exposed fish (▲, n = 14). Data points are means ± 1 SE. Asterisks indicate a significant difference ($p < 0.05$) between groups, while + indicates a significant difference from pre-exposure (day C) values.

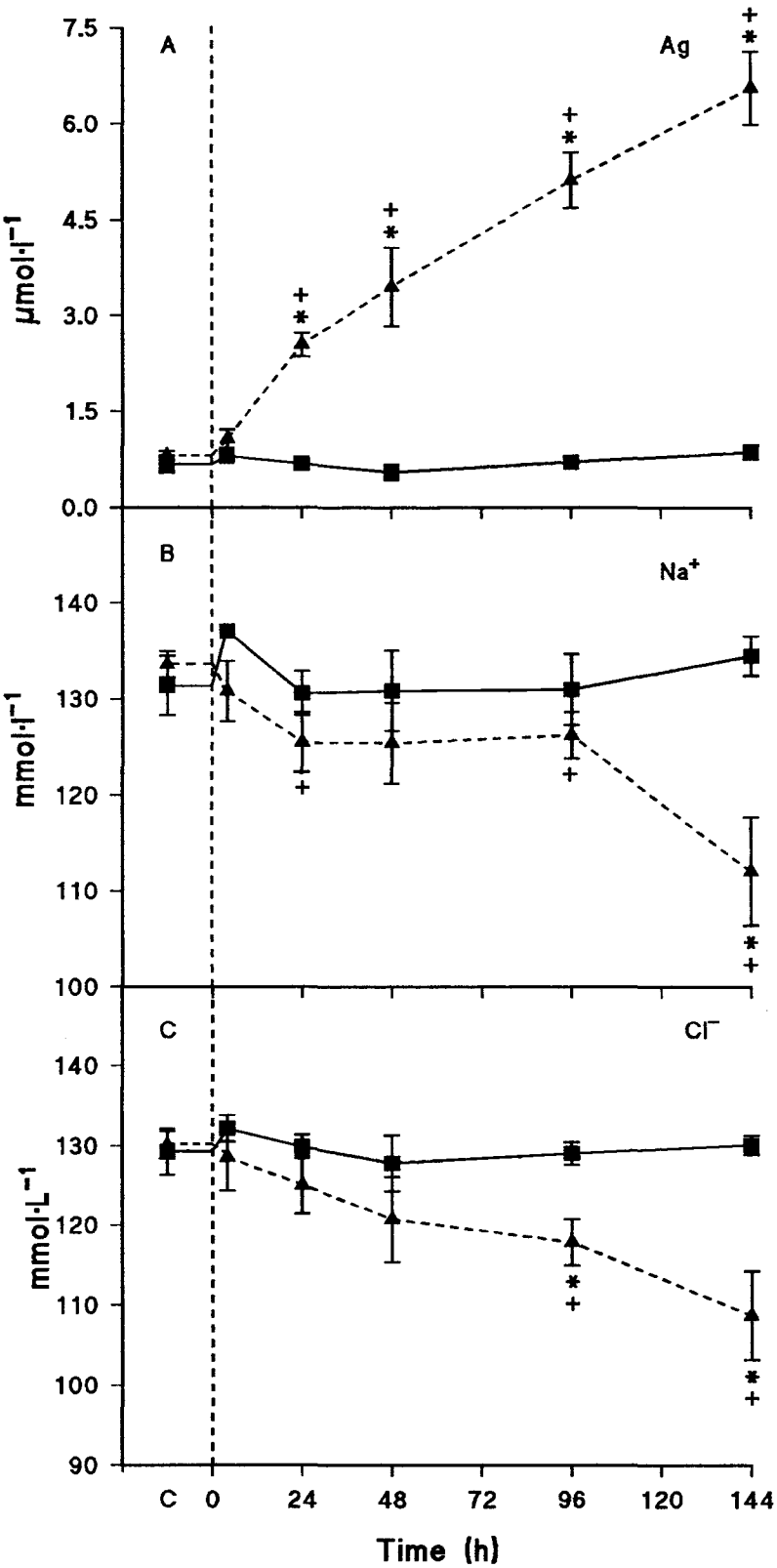

A
Ag
B
Na+
C
Cl−
µmol·l−1
mmol·l−1
mmol·L−1
7.5
6.0
4.5
3.0
1.5
0.0
140
130
120
110
100
90
C
0
24
48
72
96
120
144
Time (h)

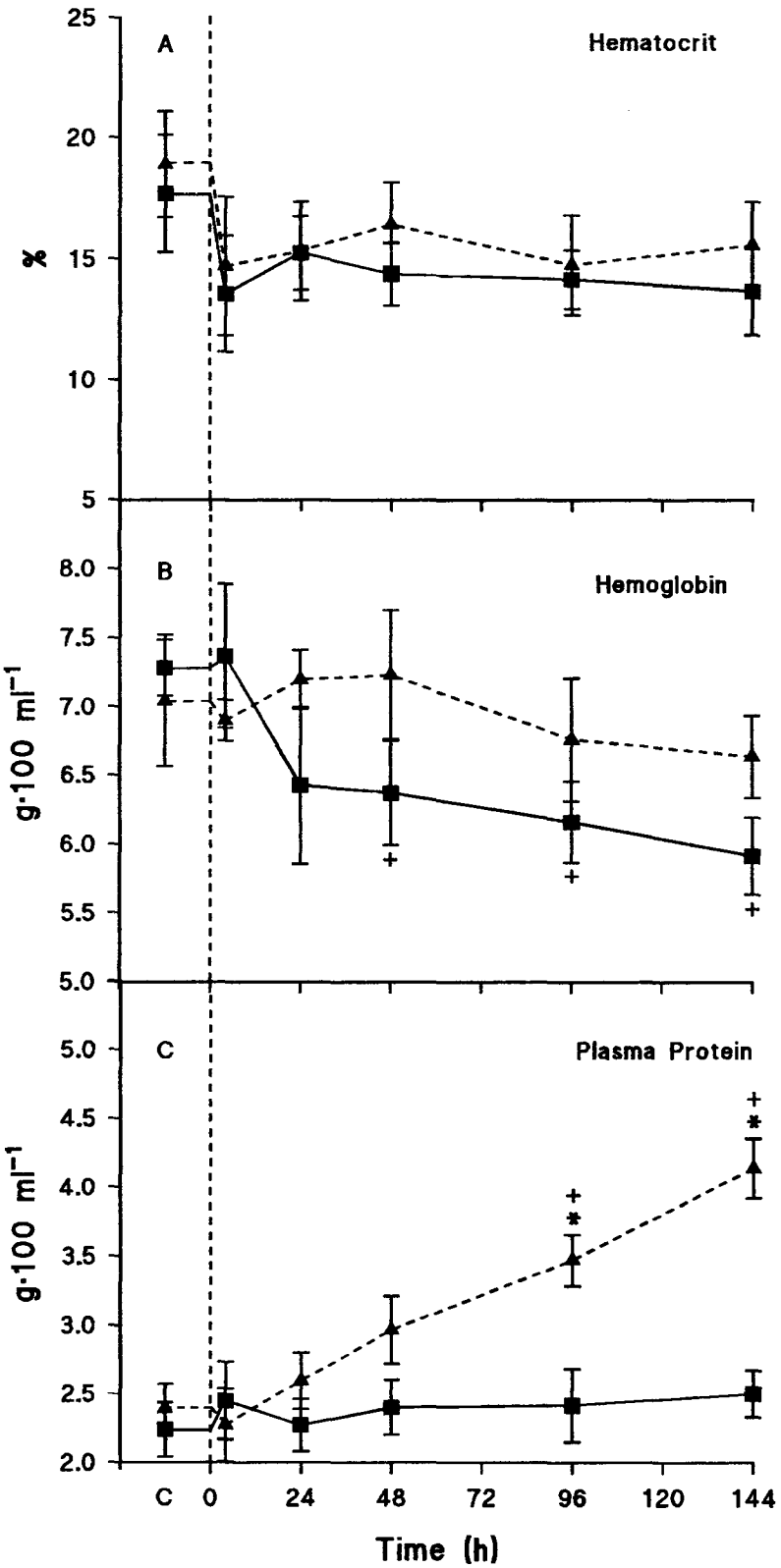
Table 2.2. Plasma K^+ and hematological parameters of rainbow trout exposed to 9.2 µg/L Ag (as $AgNO_3$) for 6 d. [a]

	Control	$AgNO_3$
Plasma K^+ ($mmol \cdot L^{-1}$)	3.418 ± 0.509 (6)	3.900 ± 0.227 (9)
MCHC (g Hb·ml RBC^{-1})	0.464 ± 0.083 (6)	0.475 ± 0.063 (14)
Relative plasma volume (%)	89.0 ± 3.4 (6)	62.6 ± 2.9[b] (14)

[a] Values are means ± 1 SEM (*n*).

[b] $p < 0.05$.

Fig. 2.2. The effects of 6 d exposure to 9.2 µg/L Ag (as $AgNO_3$) on (A) hematocrit, (B) hemoglobin, and plasma protein (C) levels in rainbow trout. Other details as indicated in legend to Figure 2.1.


A
Hematocrit
%
25
20
15
10
5
B
Hemoglobin
g·100 ml⁻¹
8.0
7.5
7.0
6.5
6.0
5.5
5.0
C
Plasma Protein
g·100 ml⁻¹
5.0
4.5
4.0
3.5
3.0
2.5
2.0
C
0
24
48
72
96
120
144
Time (h)

Acid-base measurements demonstrated progressive, highly significant decreases in both P_{aCO2} (Fig. 2.3A) and plasma [HCO_3^-] (Fig. 2.3B). By day 6, both parameters had fallen to about 50% of pre-exposure values (Fig. 2.3A and B). Blood pH_a in these fish dropped by 0.2 units after 6 d of Ag exposure, with the drop becoming significant by the fourth day (Fig. 2.3C). The decreases in [HCO_3^-] and pH_a was associated with an increase in the metabolic acid load (ΔH^+_m) of the plasma, significant by day 4 (Fig. 2.3D). By this time, the Ag-exposed trout exhibited an increased depth of breathing indicative of hyperventilation.

Total ammonia (T_{Amm}, Fig. 2.4A) and P_{NH3} (data not shown) levels in the blood plasma increased in Ag-exposed fish by almost 150% and 75% respectively, by day 6, compared to control fish. T_{Amm} in control fish dropped at 4 h, perhaps an effect of blood sampling, but thereafter recovered to pre-exposure levels. Ag-exposed fish showed a progressive increase in plasma glucose and cortisol levels (150 - 230%; Fig. 2.4B and C), that were significantly different from controls by day 4. Cortisol levels did not change in control fish, but plasma glucose dropped below pre-exposure levels at 4 h and remained slightly depressed for the remainder of the experiment.

Changes in the partial pressure gradient for ammonia between blood and water (ΔP_{NH3}), thought to be the major driving force for ammonia excretion (see Discussion), are summarized in Table 2.3. ΔP_{NH3} increased significantly by day 2 in Ag-exposed trout, whereas it fell significantly over this same period

Fig. 2.3. The effects of 6 d exposure to 9.2 μg/L Ag (as $AgNO_3$) on (A) partial pressure of CO_2 in the blood (P_{CO2}), (B) plasma [HCO_3^-], (C) blood pH, and (D) metabolic acid load (ΔH^+_m) in rainbow trout. Other details as indicated in legend of Figure 2.1.

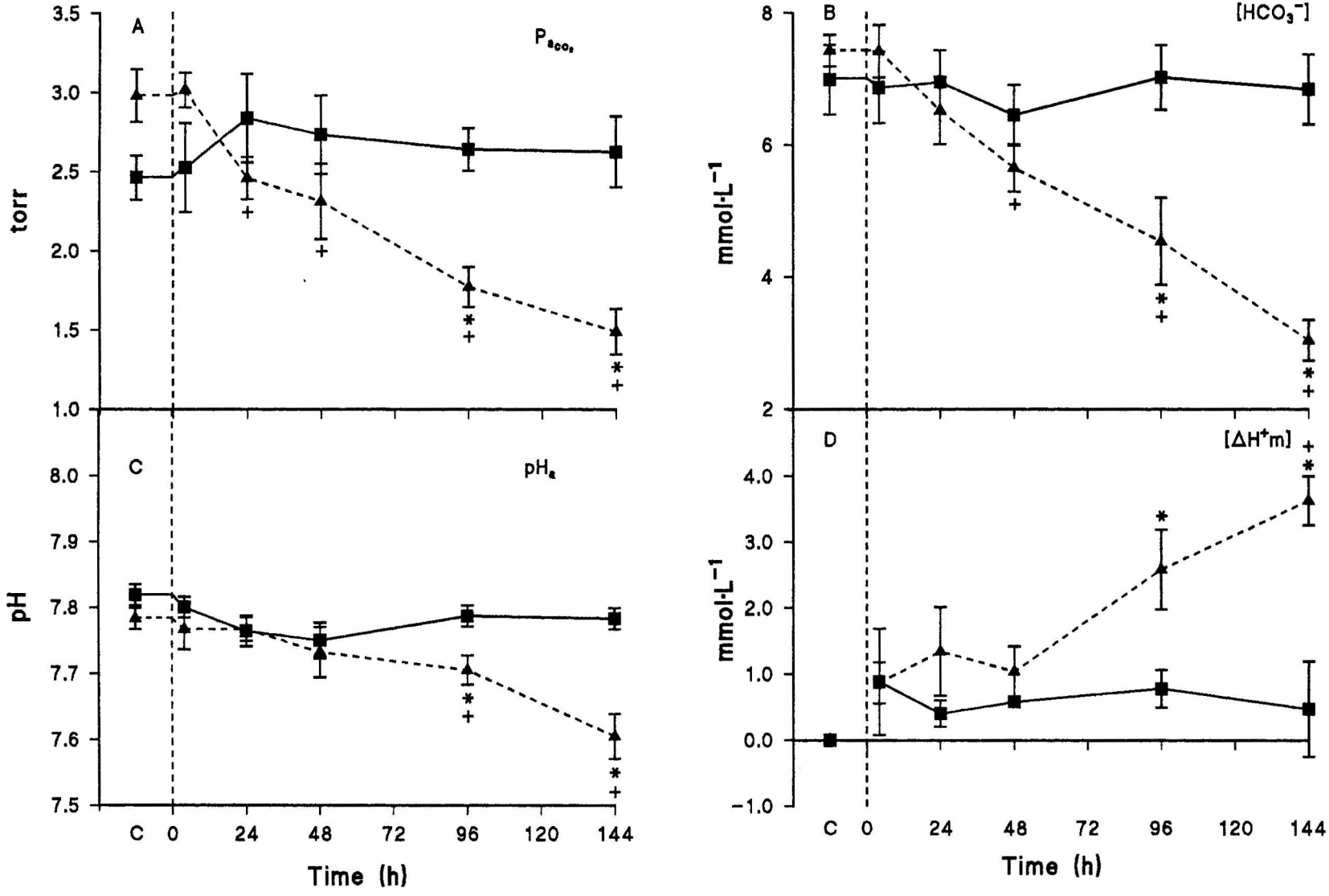

A
P_{sco_2}
torr
3.5
3.0
2.5
2.0
1.5
1.0
B
$[HCO_3^-]$
mmol·L^{-1}
8
6
4
2
C
pH_e
pH
8.0
7.9
7.8
7.7
7.6
7.5
D
$[\Delta H^+m]$
mmol·L^{-1}
4.0
3.0
2.0
1.0
0.0
−1.0
C 0 24 48 72 96 120 144
Time (h)

Fig. 2.4. The effects of 6 d exposure to 9.2 µg/L Ag (as $AgNO_3$) on (A) plasma ammonia, (B) glucose, and (C) cortisol levels in rainbow trout. Other details as indicated in legend of Figure 2.1.

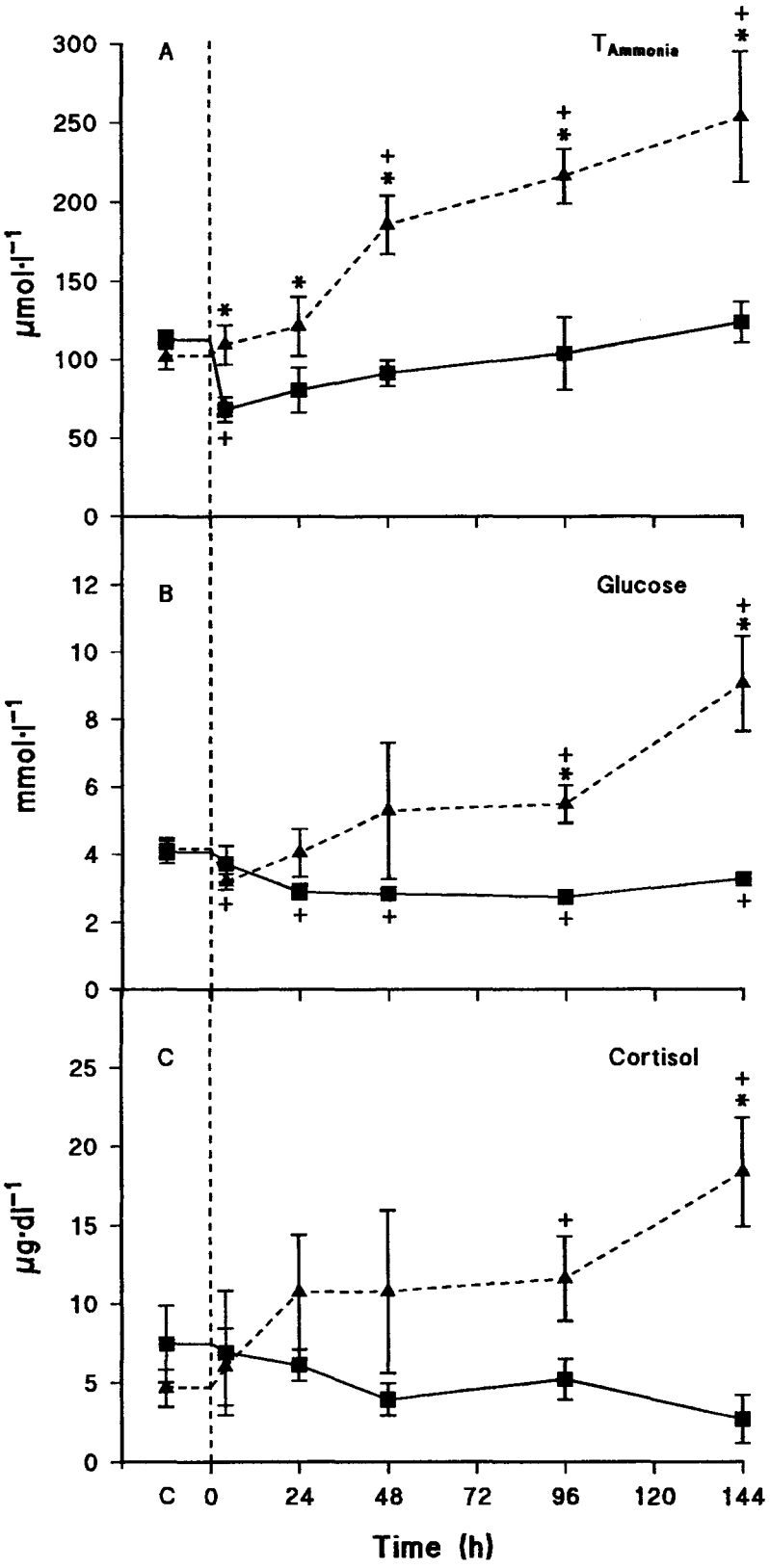

A
$T_{Ammonia}$
300
250
200
150
100
50
0
µmol·l⁻¹
B
Glucose
12
10
8
6
4
2
0
mmol·l⁻¹
C
Cortisol
25
20
15
10
5
0
µg·dl⁻¹
C
0
24
48
72
96
120
144
Time (h)

Table 2.3. Plasma to water ammonia gradients in control and fish exposed to 9.2 μg/L Ag (as $AgNO_3$) for 6 d. [a]

	ΔP_{NH3} (μTorr)	
Hour	Control	Exposed
C	33.17 ± 1.83	27.64 ± 3.62
4	16.69 ± 1.57[b]	23.88 ± 5.86
24	18.34 ± 2.18[b]	25.46 ± 4.67
48	21.44 ± 1.10[b]	40.67 ± 2.38[bc]
96	26.44 ± 4.87	41.20 ± 7.70
144	35.18 ± 3.61	45.23 ± 4.56[b]

[a] Values are means ± 1 SEM (n = 8).

[b] $p < 0.05$ from pre-exposure value of same group.

[c] $p < 0.05$ between groups.

in control fish. By day 6, the gradient in the exposed fish had increased by about 60%, whereas it had returned to pre-exposure levels in the control group.

Group B - initial ammonia excretion rates

Ammonia flux rates for both control and exposed fish were essentially the same over the first 12 h of exposure, although the exposed fish, on average, had a slightly higher (but nonsignificant) excretion rate compared to controls (Fig. 2.5A). These measurements demonstrated that the progressive build-up of plasma T_{Amm}, seen as early as 4 h (Fig. 2.4A), was not due to an inhibition of ammonia excretion to the environment.

Group C - ammonia, acid-base, and ionic flux rates

Measurements of ammonia fluxes over the longer term (Fig. 2.5B) demonstrated a dramatic increase in ammonia excretion, that became significant at 24 h and reached almost threefold control levels by day 6, in concert with the build-up of T_{Amm} in the blood plasma (Fig. 2.4A) and the increase in ΔP_{NH3} (Table 2.3). Ammonia excretion in the control fish remained constant over the 6 d of the exposure (Fig. 2.5B).

These ammonia excretion rates are shown in relation to the net acid-base exchange of the fish with the environment (Fig. 2.6). In control fish, there were no significant changes in the net uptake of titratable acidity or the net

Fig. 2.5. Ammonia excretion rates for both control (■, n = 5) and rainbow trout exposed to 9.2 μg/L $AgNO_3$ (▲, n = 6) during the first 12 h of exposure (A) and throughout 6 d of exposure (B; n = 13 for both control and Ag-exposed fish). Other details as indicated in legend of Figure 2.1.

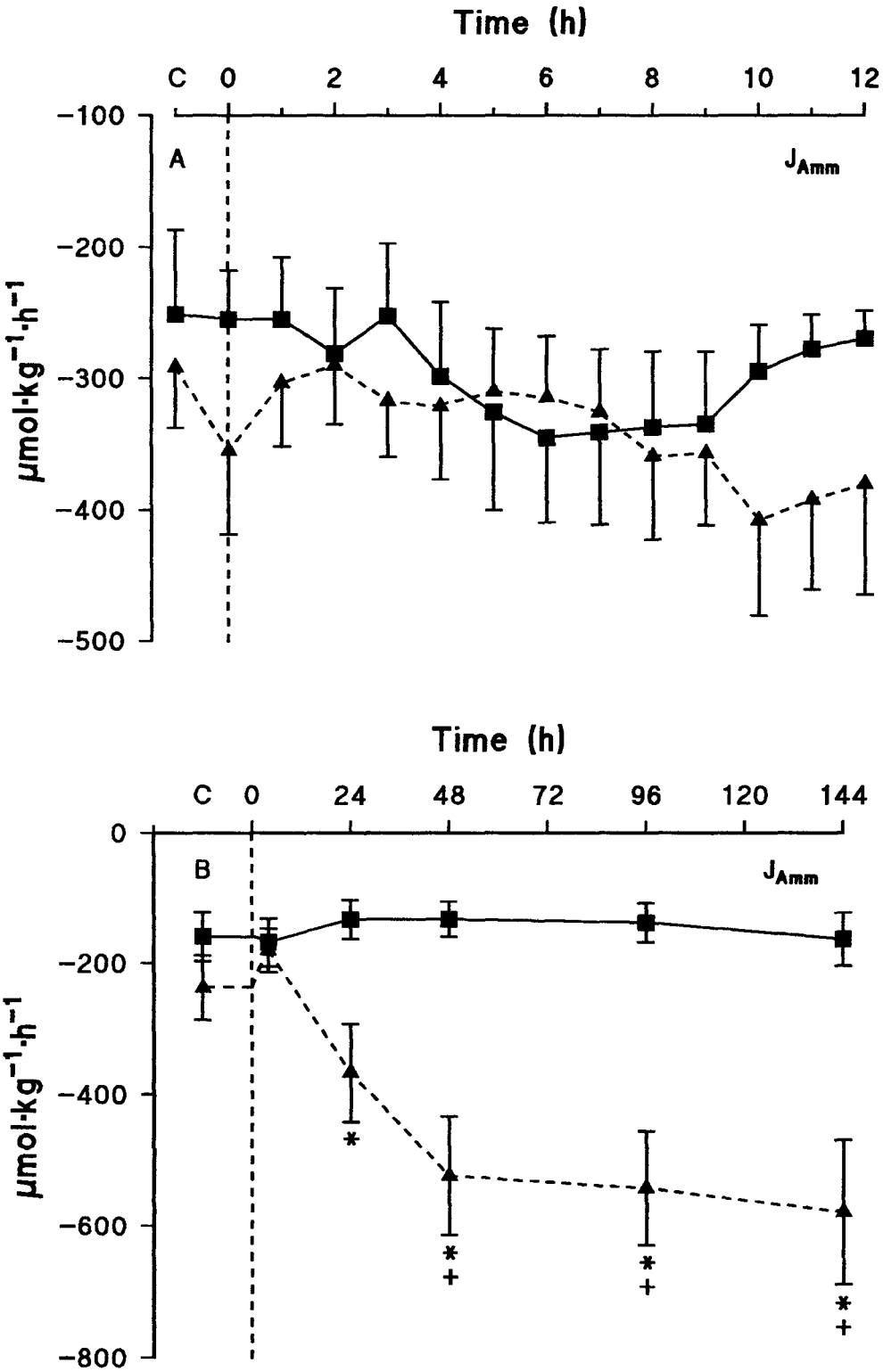
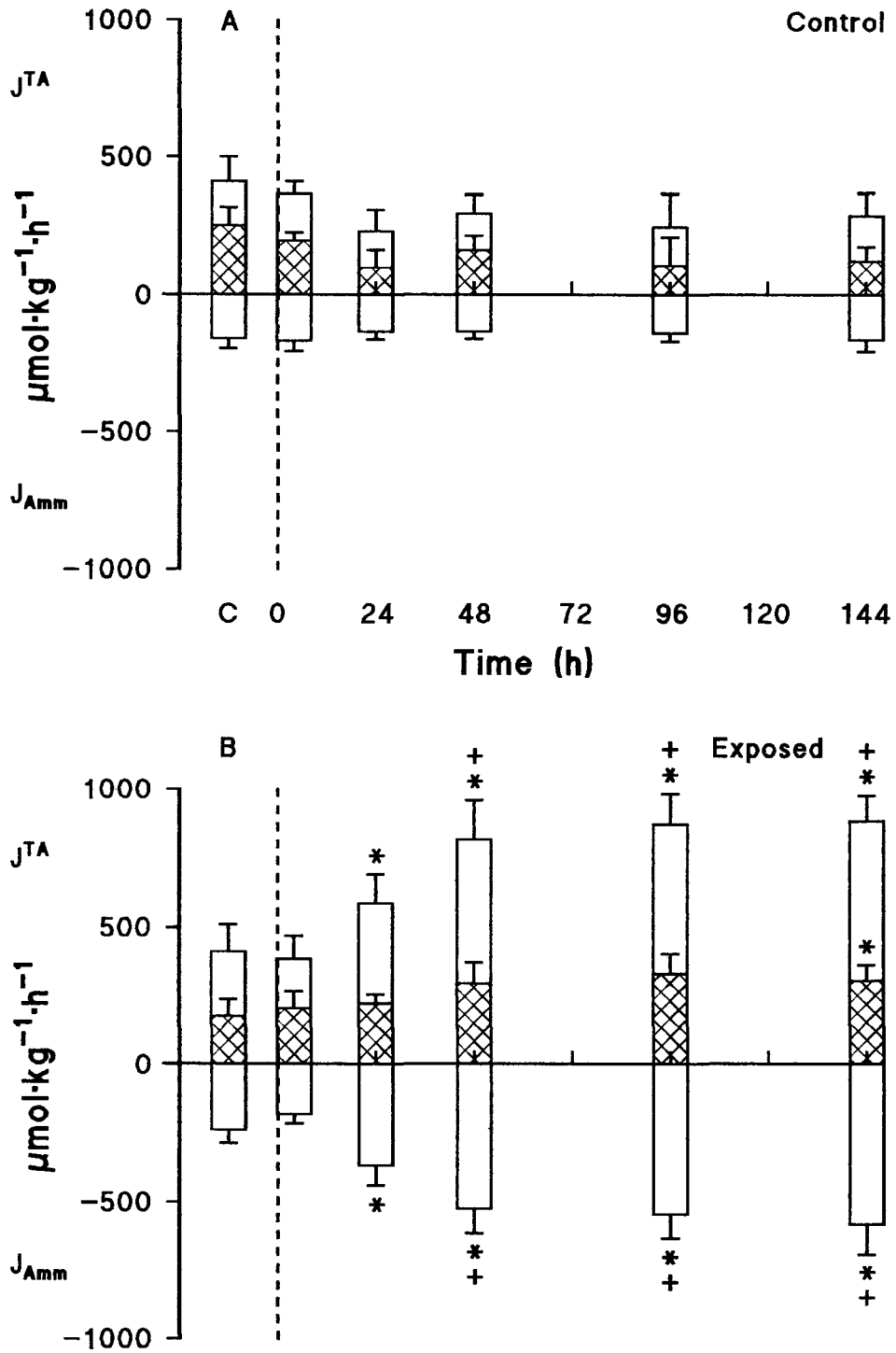

Time (h)
C 0 2 4 6 8 10 12
−100
−200
−300
−400
−500
A
J_{Amm}
µmol·kg⁻¹·h⁻¹
Time (h)
C 0 24 48 72 96 120 144
0
−200
−400
−600
−800
B
J_{Amm}
µmol·kg⁻¹·h⁻¹
*
*
+
*
+
*
+

Fig. 2.6. The movement of acid and base equivalents across the gills for control (A, n = 13) and Ag-exposed (B, n = 13) rainbow trout. Open bars above the x-axis represents titratable acidity (J^{TA}); open bars below the axis represent the ammonia excretion (J_{Amm}), with the hatched bars representing the net acid flux at the gills. Other details as indicated in legend of Figure 2.1.


A
Control
1000
500
0
−500
−1000
J^{TA}
J_{Amm}
$\mu mol \cdot kg^{-1} \cdot h^{-1}$
C
0
24
48
72
96
120
144
Time (h)
B
Exposed

excretion of ammonia over the 6 d of the experiment (Fig. 2.6A). The arithmetic sum of the two components remained positive and constant, indicating a continuing uptake of acidic equivalents (or loss of basic equivalents) over this period. However, in Ag-exposed trout, a significant rise in the net uptake of titratable acidity from the environment occurred concomitant with the rise in ammonia excretion (Fig. 2.6B). This increase in titratable acidity flux exceeded that in ammonia flux, so the net uptake of acidic equivalents increased significantly to approximately twice the control levels by day 6 (Fig. 2.6B). Thus, the internal acidosis seen in the blood plasma of Ag-exposed fish (Fig. 2.3) was associated with an increased net uptake of acidic equivalents or loss of basic equivalents by the fish.

The movements of major ions (Na^+, Cl^-, and K^+) between the fish and the environmental water were markedly affected by Ag exposure. Despite the constancy of plasma K^+ concentration (Table 2.2), K^+ balance changed from a net uptake rate to a net loss rate during the first 4-7 h of Ag exposure, and remained negative at about the same level (-25 μmol/kg/h) for the duration of the experiment (Fig. 2.7).

In addition to net flux rates (J_{net}), unidirectional influx (J_{in}) and efflux (J_{out}) components were measured for Na^+ and Cl^- balance (Fig. 2.8). Prior to exposure, J_{net} values were positive, as J_{in} exceeded J_{out} for both Na^+ and Cl^-.

Fig. 2.7. (A) Net flux rates of potassium across the gills in control (open bars, n = 8) and Ag-exposed fish (hatched bars, n = 8) during 6 d in 9.2 μg/L Ag (as $AgNO_3$). Other details as indicated in legend of Figure 2.1.

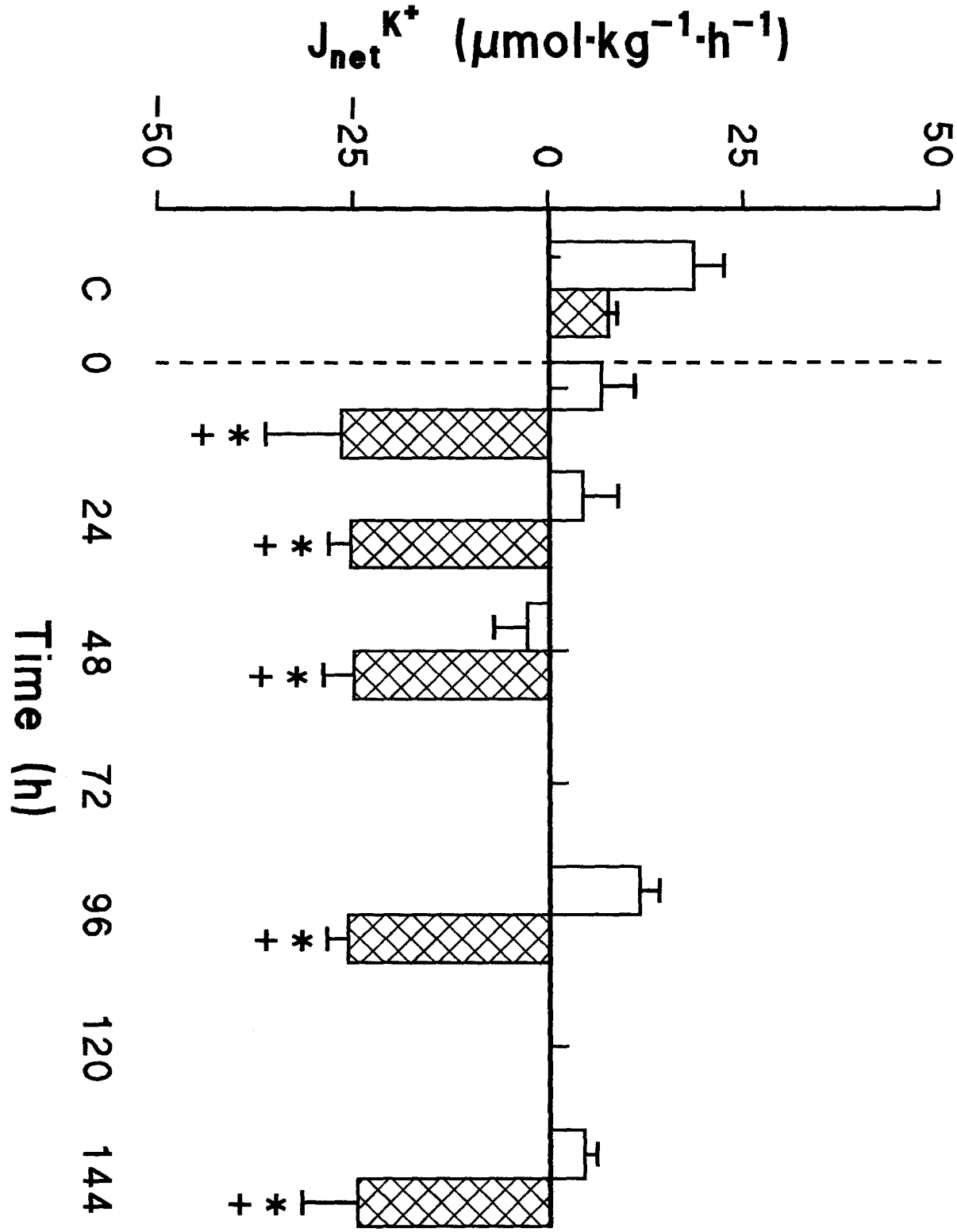
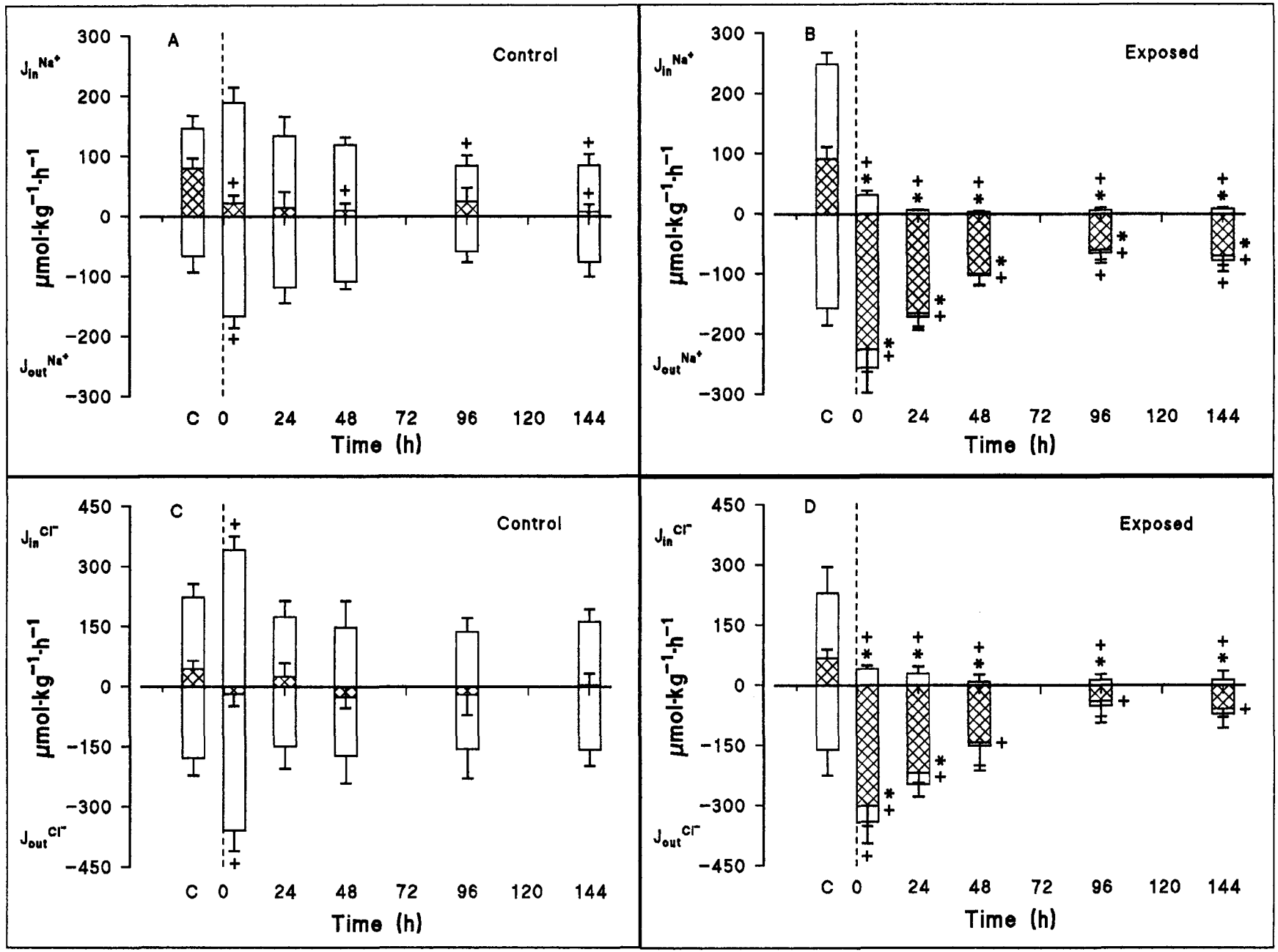

$J_{net}^{K^+}$ (μmol·kg^{-1}·h^{-1})
−50
−25
0
25
50
C
0
24
48
72
96
120
144
Time (h)
+ *
+ *
+ *
+ *
+ *

Fig. 2.8. Unidirectional Na^+ (A: control fish, n = 13; B: Ag-exposed fish, n = 13) and Cl^- (C: control fish, n = 13; D: Ag-exposed fish, n = 13) flux rates during 6 d of exposure to 9.2 µg/L Ag (as $AgNO_3$). Open bars above the axis indicate influx, open bars below the axis indicate efflux, and hatched bars indicate the net ion flux. Other details as indicated in legend of Figure 2.1.


A
Control
B
Exposed
C
Control
D
Exposed
$J_{in}^{Na^+}$
$J_{out}^{Na^+}$
$J_{in}^{Cl^-}$
$J_{out}^{Cl^-}$
µmol·kg⁻¹·h⁻¹
300
200
100
0
−100
−200
−300
450
150
−150
−450
C 0 24 48 72 96 120 144
Time (h)

Throughout the experiment, the control fish remained close to zero balance for both ions, with J_{in} and J_{out} values approximately equal. However, following only 4 h of Ag exposure, J_{in} values for Na^+ and Cl^- decreased by 88% (Fig. 2.8B) and 64% (Fig. 2.8D) respectively, and were completely abolished after 48 h. As a result, J_{net} became highly negative for both ions (-100 to -300 μmol/kg/h) over this period. These negative balances were largely due to the inhibitions of the active influx (J_{in}) components. Although J_{out} values also appeared to increase (significant only for Cl^- at 4-7 h; Fig. 2.8D), a similar phenomenon occurred in the controls (Fig. 2.8A and C). The absolute J_{out} values in the Ag-exposed trout were not significantly different from those of the control fish at this time. As time progressed, the net loss rates of Na^+ and Cl^- from the fish decreased due entirely to decreases of the J_{out} components that eventually reached values less than half the control levels. There was no significant recovery of influx (J_{in}) for either ion during the exposure (Figs. 2.8B and D).

DISCUSSION

The present study was successful in its first objective, that of confirming key responses to ionic Ag^+ exposure in rainbow trout. Thus, with only minor quantitative differences, the observed decreases in plasma Na^+ and Cl^- levels, associated increases in plasma protein and Hb (the latter relative to sampling-induced decreases in control fish), decreases in plasma volume, and complex acidosis in the blood plasma (see below) were very similar to those reported by

Wood et al. (1996a, b) for adult rainbow trout similarly exposed to 10 μg/L total Ag (as $AgNO_3$) for 6 d in moderately hard Hamilton tapwater. The results also confirm that the red cells do not swell (i.e., unchanged MCHC), which suggests that Ag in the plasma may in some way block the β-adrenergically stimulated Na^+/H^+ exchange on the erythrocyte membrane, which is thought to be responsible for this response during low pH or Cu^{2+} exposure (see reviews cited in the Introduction). Plasma [Ag] rose steadily over the 6 d, rather than levelling off after 2-4 d as reported by Wood et al., and by day 6 were twofold higher than the levels seen in the previous study (Wood et al., 1996a, b). However, gill and liver Ag concentrations were very comparable in the two investigations. Notably in white muscle, a tissue that has not previously been examined, there was a high background level of Ag in control samples but no further Ag accumulation during the exposure. In this regard, muscle appears very similar to the kidney tissue monitored by Wood et al. (1996a). These studies therefore confirm that nonexposed fish have considerable background levels of Ag in tissues, that they have presumably accumulated from trace levels in the water or the diet during their lifetimes.

Our study also confirms that the Na^+ and Cl^- losses are due to a rapidly developing inhibition of active Na^+ and Cl^- uptake processes (J_{in}) at the gills and are not due to a stimulation of diffusive effluxes (J_{out}), in agreement with the recent report of Morgan et al. (1997). Assuming that measured declines (~20 mmol/kg) in plasma Na^+ and Cl^- levels occurred throughout an internal

distribution space of about 270 ml/kg (as determined by Wood, 1988 - i.e., largely extracellular fluid), they would amount to about -5,400 μmol/kg, approximately 1/3 of the measured net loss rates to the water over 6 d (~-100 μmol/kg/h x 144 h = -14,400 μmol/kg). Considering that the flux measurements represent only isolated "snapshots" in time versus an integrated plasma measurement, agreement is not unreasonable. As for Cu^{2+} (Lauren and McDonald, 1986; Li et al., 1996), the influx blockade is undoubtedly related to the potent inhibition of branchial Na^+/K^+-ATPase caused by Ag^+ (Morgan et al., 1997). However, the absence of efflux stimulation (which is normally thought to be due to Ca^{2+} displacement from the gill; Wood, 1992) is a clear difference from the actions of both Cu^{2+} (Lauren and McDonald, 1985, 1986) and low pH (Wood, 1992), but is in accord with the very limited protective effect of water $[Ca^{2+}]$ against Ag^+ toxicity (Galvez and Wood, 1997) and the very weak affinity of the Ag^+ binding ligands on gills for Ca^{2+} (Janes and Playle, 1995). These observations all suggest that Ag^+ does not attack the Ca^{2+} binding sites that stabilize paracellular permeability. The later reductions in unidirectional Na^+ and Cl^- effluxes to less than 50% of control levels are much larger than can be explained by the drop in the gradient alone (i.e., falling plasma $[Na^+]$ and $[Cl^-]$). As with acid stress, hormonal factors such as prolactin mobilization are probably also involved (Reid, 1995).

The present observations confirm that the blood acid-base response to Ag exposure comprises a dominant metabolic acidosis (ΔH^+_m accumulation

leading to HCO_3^- reduction) and partially compensating respiratory alkalosis (Pa_{CO2} reduction, also leading to HCO_3^- reduction). An obvious hyperventilation is the probable cause of the latter, without which the drop in pH_a would be more severe. Earlier, Wood et al. (1996a) demonstrated all of these same responses and also reported that there was no appearance of lactate in the blood, thereby ruling out anaerobic metabolism as the cause of the metabolic acidosis.

Two completely new, and likely interrelated, findings of the present study are that stimulated uptake of acidic equivalents (or loss of basic equivalents) and steady net loss of K^+ to the water both occur during Ag exposure. The former is the probable cause of the marked build-up of metabolic acid (ΔH^+_m) in the blood. Applying the same sort of calculations as for Na^+ and Cl^- losses (above), the net uptake of acidic equivalents from the water over 6 d, relative to controls, was about 12,000 μmol/kg, or at least 10-fold the measured accumulation of ΔH^+_m in the extracellular compartment. This discrepancy is too large to be an artifact and strongly suggests that a considerable portion of the net acid uptake penetrated the intracellular compartment, that has about a 10-fold greater ability to tolerate ΔH^+_m loading because of its 5-fold larger buffer capacity and 2-fold larger compartmental size (Wood et al., 1982; Milligan and Wood, 1986). The major intracellular compartment lies in the white muscle (>50% of body mass). Strong corroborative evidence for this intracellular acid-loading was the steady loss of

K^+ to the water (~-5,000 μmol/kg relative to controls over 6 d) in the absence of any change in plasma [K^+]. Exchange of H^+ for K^+ is the classic mechanism by which metabolic acid enters muscle, accompanied by a net excretion of K^+ to prevent hyperkalemia from occurring in the blood. Exactly the same phenomenon of acid uptake from the water in excess of the extracellular load and K^+ loss to the environment has been seen during exposure of trout to low pH in the same water quality (McDonald et al., 1980; McDonald and Wood, 1981; Wood, 1989). We are unaware of any acid flux data for Cu^{2+}-exposed fish, but a similar loss of K^+ has been reported (Lauren and McDonald, 1985).

The mechanism responsible for this disturbance of acid-base exchange with the environment is unknown. One possibility is that it is a direct consequence of the observed disruption of Na^+ and Cl^- uptake at the gills, which are thought to involve Na^+/"acid" and Cl^-/"base" exchange processes (Wood, 1992). Another possibility, which may be synergistic, is an inhibition of branchial carbonic anhydrase, the enzyme that provides the acidic (H^+) and basic (HCO_3^-) counterions through the catalysis of CO_2 hydration. Indeed, Morgan et al. (1997) demonstrated that in fish exposed to 10 μg/L Ag for 48 h in vivo, there was a 30% reduction of carbonic anhydrase activity in the gills, as well as when added in vitro. Ag has also been reported to inhibit erythrocytic carbonic anhydrase (Christensen and Tucker, 1976).

The present observations on ammonia metabolism and cortisol mobilization are also entirely new. Ammonia excretion (J_{Amm}), more than 90%

of which occurs across the gills (Wood, 1993), was monitored throughout the exposure and no inhibition was seen at any time. J_{Amm} increased, in association with an increase in ΔP_{NH3}, the driving gradient for NH_3 diffusion across the gills. Thus, the situation appears to be different to that for low pH and Cu^{2+} exposure, where ammonia retention has been attributed to an initial reduction of J_{Amm} at the gills due to an inhibition of linked Na^+ uptake and NH_4^+ efflux (Wood, 1993; Taylor et al., 1996). The whole question of whether Na^+ influx is really coupled to NH_4^+ efflux at the gills or rather to H^+ efflux (with NH_3 diffusing passively) remains controversial (see Wilson et al., 1994) and beyond the scope of this Discussion. The key point here is that the increased plasma T_{Amm} observed during Ag exposure was due to an increased metabolic production, not retention due to a blockade of J_{Amm}.

The substantial mobilization of cortisol reported here for the first time had been earlier predicted on the basis of observed glucose mobilization (Wood et al., 1996a). These two parameters are standard "stress responses" in fish and usually occur simultaneously (Thomas, 1990). Cortisol has well-known proteolytic and gluconeogenic effects (van der Boon et al., 1991). The increased rate of ammonia production was therefore likely caused by the action of cortisol in stimulating protein catabolism, subsequent amino acid oxidation and gluconeogesis, and resulting ammoniogenesis. Some longer-term studies with both low pH (Audet and Wood, 1993) and Cu^{2+} (De Boeck et al., 1995a, b) have suggested a similar scenario.

The levels of plasma T_{Amm} accumulating during Ag exposure are well below those known to be acutely damaging in trout (Wilson et al., 1994), so it is unlikely that ammonia build-up directly contributes to toxicity. Indeed, from an acid-base standpoint, because ammonia is produced metabolically as NH_3 but exists almost entirely as NH_4^+ at physiological pH, its action in buffering H^+ ions may be helpful in reducing the extent of acidosis. This may be particularly important in alleviating intracellular acidosis. Tissue T_{Amm} levels in fish are usually far greater than plasma T_{Amm}. However, recent studies on Cu^{2+} (Taylor et al., 1996) have suggested that internal ammonia retention, especially in white muscle, may have an important sublethal action to reduce swimming performance in fish. In future studies it would be useful to measure exercise performance, muscle intracellular pH_i, and muscle T_{Amm} in Ag-exposed trout.

This study was designed to elucidate the acute toxic mechanism of silver in teleosts. Due to the level and type of Ag used (10 μg/L Ag as $AgNO_3$, 35% as Ag^+), the degree of toxicity seen here is severe compared to a natural environment. Ag levels in nature tend to be much lower, with concentrations being no more than a few percent of the levels used here (Wen et al., 1997). However, the parameters measured here (plasma Na and Cl, and ion flux rates) can serve as nonspecific indicators of sublethal stress in fish. This nonspecificity is due to the similarity of the toxic action of Ag^+ to that of acid (H^+) and other heavy metals (Cu^{2+}), as discussed earlier (see Introduction).

Clearly, the next step is to examine the mechanism of toxicity of these three toxicants at the cellular and molecular levels in greater detail and also to evaluate whether these sublethal stress effects result in impaired growth, reproduction, or long-term survival.

CHAPTER 3: Bioaccumulation and distribution of silver in four marine teleosts and two marine elasmobranchs: comparisons between fish species, water salinity, and exposure concentration

ABSTRACT

The bioaccumulation and internal distribution of waterborne silver was compared amongst drinking (teleosts) and non-drinking marine fish (elasmobranchs). Four marine teleosts (rainbow trout, tidepool sculpin, plainfin midshipman, and English sole), and two marine elasmobranchs (Pacific spiny dogfish and long nose skate), were exposed to 14.5 μg/L Ag (as $AgNO_3$) for a period of 3 weeks in 30 ppt seawater. The effects of salinity on the bioaccumulation and distribution of silver were studied with tidepool sculpins acclimated to 18 and 30 ppt salinity. Fish from each salinity were exposed to 0 (control), 1.5, 14.5, or 50 μg/L Ag (as $AgNO_3$) for 21 days. The effects of exposure concentration on bioaccumulation and distribution were studied by exposing rainbow trout, plainfin midshipman, and tidepool sculpin to the above four silver concentrations for 21 days.

Naive fish were seen to have measurable levels of silver in all tissue, suggesting that they accumulate silver from the natural environment throughout their lifetimes. After 21 d of exposure to 14.5 μg/L Ag, silver distribution in teleost tissues was liver > gills ≥ intestines > white muscle

(based on silver concentration). Distribution in elasmobranchs was gills > liver > white muscle > intestines.

The route of uptake appears to play a strong role in determining internal silver accumulation and distribution. Drinking fish (marine teleosts) accumulate more silver in the intestines, than the gills. The reverse is true for non-drinking fish (marine elasmobranchs). Net accumulation rates calculated from terminal liver silver concentrations showed no difference between teleosts and elasmobranchs, but when corrected for liver size, elasmobranchs had 10 fold higher uptake rates. Net accumulation rates were also higher in fish at lower salinity due to increased bioavailability of silver.

Silver bioaccumulation is markedly affected by speciation. At lower salinities, a neutral charged $AgCl_{aq}$ complex exists in the water, allowing for increased bioaccumulation to occur. At the higher salinity, only negatively charged forms of Ag ($AgCl_2^-$, $AgCl_3^{2-}$, $AgCl_4^{3-}$) are present, thereby reducing the amount of silver that enters the fish. Tissue silver accumulation was also dependent on exposure concentration, with internal levels rising as the external water silver concentration increased.

Keywords - Silver, Bioaccumulation, Seawater, Elasmobranchs, Teleosts

INTRODUCTION

The accumulation of metals in aquatic organisms has been linked to decreased survival rates and reduced reproductive ability; it may also present a significant pathway into the upper levels of the aquatic food chain (Thomann, 1981; Biddinger and Gloss, 1984; Dallinger et al., 1987; Sanders et al., 1990). For fish, metal uptake can occur via two pathways: uptake from the aqueous phase, and/or from metal biologically incorporated in food (Fowler, 1982; Sanders and Riedel, 1987; Fisher and Reinfelder, 1995). In marine teleosts, uptake from seawater can occur via the gills or via the gastro-intestinal tract from ingested water. At present, we are unaware of any published information on the relative importance of these two routes from the water. However, in marine elasmobranchs, uptake of dissolved metals from seawater, probably occurs solely at the gills, since these fish do not need to drink to maintain osmotic homeostasis (Evans 1979, 1993). A comparison of relative uptake rates in marine teleosts *versus* marine elasmobranchs may therefore prove informative.

The work of Pentreath (1977) has shown that even in full strength seawater, the accumulation of silver from the water can be highly variable. He found that accumulation of ^{110m}Ag was much higher for a marine elasmobranch (thornback ray) than for a marine teleost (plaice). However, he did not give an explanation as to the origin of the differences seen between the two species.

The amount of metal in an aquatic environment that is available to enter a fish represents only a small fraction of the total metal concentration. The majority of metal is bound to sediments and detritus, and other ligands in the environment, making the bound portion unavailable for uptake by fish. The portion of metal that is available, is termed the bioavailable fraction. While many factors such as hardness, pH, alkalinity, salinity, and dissolved organic carbon (DOC) can affect the bioavailability of a metal, the main factors involved in silver bioavailability, at least in freshwater, appear to be chloride ([Cl^-]) and dissolved organic carbon (DOC) (Janes and Playle, 1995; Galvez and Wood, 1997; Kramer et al., 1997; Hogstrand and Wood, 1998).

The much higher chloride levels present in seawater, than freshwater, have a dramatic effect on toxicity as well as bioaccumulation of silver. LC50's for silver are 50-100 times higher in seawater (lower toxicity) than those seen in freshwater fish (Eisler, 1996; Hogstrand and Wood, 1998). In 1998, Shaw et al. measured the LC50's and bioaccumulation of silver in euryhaline tidepool sculpins acclimated to 25 and 32 ppt salinity (430 and 550 mMol [Cl^-] respectively), showing that a small increase of 7 ppt salinity can lead to a doubling of the 96 h LC50, while the same increase in salinity resulted in a fourfold increase in the 168 h LC50. The increased salinity also led to a dramatic decrease of silver bioaccumulation in the fish (Shaw et al., 1998).

The current study was designed to address three objectives involved in silver accumulation from the water.

The first objective was to confirm the differing accumulation rates of silver between marine elasmobranchs and marine teleosts, with a more extensive survey of species than used by Pentreath (1977). Four teleosts (rainbow trout, plainfin midshipman, English sole, and tidepool sculpins) and two elasmobranchs (Pacific spiny dogfish and long nose skate) were exposed to 14.5 μg/L Ag (as $AgNO_3$) for 21 d in full strength seawater. Analysis of the gills and intestinal tract, in addition to liver and white muscle, provided some indication of the relative importance of branchial *versus* gastro-intestinal uptake in teleosts and elasmobranchs.

The second objective of the study was to confirm the salinity effect on silver bioaccumulation in marine teleosts. Tidepool sculpins were acclimated to 18 and 30 ppt seawater and exposed to silver at 1.5, 14.5, and 50.0 μg/L Ag (as $AgNO_3$) for 21 d. Terminal tissue silver levels were measured to either confirm or refute the findings of Shaw et al. (1998).

The third objective of this study was to understand the effect that the waterborne silver concentration has on the rate of silver bioaccumulation in tissues of marine teleosts. To determine this, rainbow trout, plainfin midshipmen, and tidepool sculpins were exposed to 1.5, 14.5, and 50.0 μg/L Ag (as $AgNO_3$) for 21 d in seawater.

Inasmuch as different Ag accumulation rates and routes were documented amongst different fish species, and the salinity effect was confirmed, the mechanisms underlying silver bioaccumulation and incorporation in marine fish are addressed and directions for future research are indicated.

MATERIALS AND METHODS

Experimental animals

Pacific spiny dogfish (*Squalus acanthius*; 1-2 kg), long nose skate (*Raja rhina*; 450-950 g), plainfin midshipman (*Porichthys notatus*; 50-250 g), and English sole (*Parophrys vetulus*; 150-350 g) were obtained as by-catch from local fishermen off the coast of Vancouver Island, BC, Canada, and held at Bamfield Marine Station (Bamfield, BC, Canada). Tidepool sculpins (*Oligocottus maculosus*; 0.5-3.5 g) were collected from local tidepools located near the marine station, and rainbow trout (*Oncorhynchus mykiss*; 300-500 g) were purchased from Rosenser Aquaculture, a local fish farm on Vancouver Island. Dogfish and skate were held in a large common tank (2 x 10^5 L), while midshipmen and sole were held in square fiberglass tanks (450 L) with sandy substrata. Trout were slowly acclimated to full strength seawater over a period of 2 weeks in 2,000 L fiberglass tanks. Tidepool sculpins were held in an 80 L tub containing rocks for shelter. All tanks were provided with aeration and constantly flowing seawater (pH ~ 7.9, 30-

32 ppt salinity, 11-13°C). Dogfish and skates were fed freshly dead fish, while midshipmen, sole, and sculpins were fed fresh shucked blue mussels on a daily basis. The rainbow trout were fed commercial trout pellets every other day at a ration of 1% total body weight at each feeding.

Experimental series

Three different experimental series were performed, with some overlap between series.

(i) *Fish species comparison.* All 6 species were exposed to 14.5 μg/L Ag, in full strength seawater, for 21 d, with respective control groups in Ag-free water.

(ii) *Salinity comparison.* Tidepool sculpins were exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag in either 18 or 30 ppt seawater, for 21 d.

(iii) *Exposure concentration comparison.* Rainbow trout, plainfin midshipmen, and tidepool sculpins were exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag in 30 ppt seawater for 21 d.

The 0 and 14.5 μg/L Ag groups in 30 ppt seawater were shared amongst all three series for sculpins, and between series i and iii for trout and midshipmen.

Experimental protocol

Dogfish, skate, and flounder were exposed to 14.5 ug/L Ag (134.4 nMol Ag, as $AgNO_3$, BDH) for 21 d in a well aerated flow-through seawater system. Water entered each 2,000 L tank at 8 L/min, with a turnover time of ~4 h and an initial loading density of 2.9, 1.3, and 2.8 g/L for the dogfish, skate, and flounder respectively. Silver was added from light shielded stock bottles (116 μg/ml Ag) by a peristaltic pump (1 ml/min). Water samples were taken periodically throughout the exposure to monitor water [Ag]. On day 21, control and exposed fish were sacrificed with MS222 (1 g/L, neutralized with NaOH; Syndel Labs, Vancouver, BC, Canada), and weighed. Gill, liver, and white muscle samples were dissected and rinsed for 1 min in silver free seawater, while the intestines were flushed with 50 ml of silver free seawater before rinsing. Samples were then frozen in liquid N_2 and stored at -80°C for future analysis.

Midshipmen and rainbow trout were exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag (0, 13.9, 134.4, and 463.5 nMol Ag, as $AgNO_3$, respectively) in well aerated seawater for 21 d. The midshipmen exposure was a static set-up in 150 L tanks with an initial loading density of 9.3 g/L wet weight. Ninety-five percent of the water was changed daily with Ag being added to the inflowing clean water for mixing from a stock bottle (1 g/L). Aeration was used for further mixing and to prevent oxygen depletion. Rainbow trout were exposed to silver in a well aerated flow-through seawater system. Water entered each

2,000 L tank at 8 L/min, for a turnover time of just over 4 h. Silver was added from light shielded stock bottles (12, 116, and 400 μg/ml Ag) by a peristaltic pump at 1 ml/min. Water samples from all tanks were taken periodically throughout to monitor water [Ag]. On day 21, control and exposed fish were sacrificed with MS222 and weighed. Terminal blood samples were taken from rainbow trout, while gill, liver, intestine, and white muscle samples were taken from all fish. Blood samples were spun, and plasma samples were frozen in liquid N_2 and stored at -80°C. Gill, liver, and muscle tissues were rinsed for 1 min in silver free seawater, while the intestines were flushed with 50 ml of silver free seawater before rinsing. Samples were then frozen in liquid N_2 and stored at -80°C for future analysis.

Tidepool sculpins were separated into 2 groups and acclimated to 18 or 30 ppt salinity (310 or 515 mMol [Cl^-], respectively). Fish from each salinity were separated into 4 groups and statically exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag (0, 13.9, 134.4, and 463.5 nMol Ag, as $AgNO_3$, respectively), for 21 d. Silver was placed into the exposure water from a stock solution of 1 g/L Ag. Fish were held in 50 L tubs at average initial loading densities of 2.4 g/L wet weight. Each tub was fitted with an airline for aeration and water mixing. The water was replaced every other day with the appropriate salinity and [Ag]. The tubs was held in a seawater bath to maintain exposure temperature. On days 0, 2, 6, and 21, fish were sacrificed by cephalic blow,

rinsed in silver free seawater and weighed. Gill and liver samples were taken and washed for 1 min, while intestines were flushed with 10 ml of seawater before rinsing. Tissue samples, along with the remaining carcass (representing mainly white muscle), were then frozen in liquid N_2 and stored at -80°C for future analysis.

For all exposures, simultaneous controls in Ag-free seawater were maintained in a similar matter to the exposed fish.

Water and tissue silver analysis

All silver levels were determined as detailed by Hogstrand et al. (1996a). Briefly, water samples were acidified with 1% HNO_3 (Fisher; trace metal grade), while tissue samples were weighed and digested over-night at 80°C in 5x (v/w) of concentrated HNO_3 (Fisher; trace metal grade). Tissue samples were then allowed to cool before adding H_2O_2 to remove any debris from the digests. The tissue digests were then heated to evaporate all liquid, and reconstituted with 5 ml of 1% HNO_3. Silver levels were read using graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer). Ag speciation in the exposure water was calculated using the determined water chemistry and the aquatic geochemical equilibrium program MINEQL$^+$ (Schecher and McAvoy, 1991).

Data have been expressed as means ± SEM (n). Differences between control and experimental treatment means were analyzed by the Student's

unpaired two-tailed *t*-test. Comparisons within groups were assessed by one-way ANOVA tests followed with Duncan tests to determine individual differences. Significant differences ($p < 0.05$) between treatments are indicated by asterisks (*) while significant differences within treatments are indicated by a plus sign (+). Significant differences determined by ANOVA plus Duncan testing are indicated by different letters.

RESULTS

Water chemistry and survival

Fish species comparison. The mean total water Ag concentration throughout the 21 d exposure was 14.7 ± 0.3 (17) μg/L, with a high of 15.2 and a low of 12.5 μg/L. Ag speciation calculated by MINEQL$^+$ showed that there was virtually no Ag^+ present in seawater. All of the silver was bound as various forms of $AgCl_n^{1-n}$ (Fig. 1.3).

Throughout the experiment, none of the exposed tidepool sculpins (n = 50), plainfin midshipman (n = 20), or dogfish (n = 8) died. One of the smallest English sole (n = 8) and the smallest long-nose skate (n = 6) died on the twelfth and seventeenth day, respectively. The rainbow trout appeared to be the least resistant to silver with 28 of the initial 40 fish dying throughout the 21 d exposure. Within the respective control groups, the only fish which died were 6 of 40 rainbow trout.

Salinity comparison. The mean total Ag concentration for this exposure is listed in Table 3.1. All of the concentrations were very close to nominal values for the experiment. Speciation for full strength seawater was similar to that of the above experiment, with all Ag being bound up as various forms of $AgCl_n^{1-n}$. At 18 ppt seawater, all the silver was bound with chloride as well; however, the lower [Cl^-] resulted in a small percent of $AgCl_{aq}$ being present, a form that was not seen at all in 30 ppt seawater (see Fig. 1.3).

None of the exposed or control sculpin (n = 50/tank) died throughout the experiment.

Exposure concentration comparison. The mean total Ag concentrations for this exposure are listed in Table 3.2. All of the concentrations were very close to nominal values for the experiment. The silver speciation for all concentrations did not vary from that seen at 10 μg/L Ag in 18 and 30 ppt seawater (see Fig. 1.3).

Of the exposed fish (n = 40/tank), 57.5% of trout exposed to 1.5 μg/L, 70% of trout exposed to 14.5 μg/L, and 85% of trout exposed to 50 μg/L died by the twenty-first day. None of the exposed or control sculpin (n = 50/tank) or midshipmen (n = 20/tank) died during the study, while 15% of the control rainbow trout (n = 40/tank) perished.

Table 3.1. Water Ag concentrations for salinity comparison exposure. [a]

Nominal [Ag] (µg/L)	Actual [Ag] (µg/L)	
	18 ppt	**30 ppt**
Control	0 (17)	0 (17)
1.5	1.3 ± 0.4 (20)	1.4 ± 0.2 (20)
14.5	15.2 ± 0.6 (18)	14.8 ± 0.5 (18)
50.0	51.0 ± 0.8 (25)	50.2 ± 0.7 (25)

[a] Values are means ± SEM (*n*).

Table 3.2. Water Ag concentrations for the concentration comparison exposure. [a]

Nominal [Ag] (μg/L)	Actual [Ag] (μg/L)	
	Rainbow Trout	**Midshipman**
Control	0 (15)	0 (15)
1.5	1.4 ± 0.3 (18)	1.3 ± 0.2 (18)
14.5	13.4 ± 0.5 (23)	13.8 ± 0.7 (23)
50.0	48.9 ± 0.8 (25)	49.5 ± 0.5 (25)

[a] Values are means ± SEM (*n*).

Tissue silver burden

Fish species comparison. In virtually all tissues of all species, there were detectable levels of Ag (10-200 μg Ag/kg wet weight) in control fish (fish not exposed to Ag in the experiments).

Gill silver levels (Fig. 3.1) in control fish were fairly uniform (50-100 μg Ag/kg wet weight) among all species. Only midshipmen gills had a silver level that was significantly different (lower) from the other species tested. After 21 d of exposure to 14.5 μg/L Ag, gill silver levels increased significantly in all species except the English sole. This increase was roughly 2 to 3 fold in the teleost fish gills, and 5 to 6 fold in the elasmobranch gills (Fig. 3.1).

Silver levels in the intestines (Fig. 3.2) of control fish were also quite uniform (50-75 μg Ag/kg wet weight). The midshipmen intestines were again significantly lower than the other fish, and indeed, not significantly different from zero. Ag levels in all exposed teleost intestines rose significantly by 2 to 3 fold by the end of 21 d of Ag exposure. There was no change in the intestine levels of silver in exposed elasmobranchs (Fig. 3.2).

Liver silver levels (Fig. 3.3) of control fish were significantly lower in both tidepool sculpin and midshipmen (< 30 μg Ag/kg wet weight), while significantly higher in the English sole (~200 μg Ag/kg wet weight). Liver levels in control rainbow trout, dogfish, and skate were intermediate (60-100 μg Ag/kg wet weight). After 21 d exposure, liver silver levels were

Fig. 3.1. Gill silver levels in fish exposed to 14.5 μg/L Ag for 21 d. Open bars are control values while hatched bars are exposed values. Data points are means ± SEM (6). Asterisks (*) indicate a significant difference ($p < 0.05$) from control values of the same fish. Control values that have a different lower case letter are significantly different from other control values, while upper case letters indicate differences between exposed tissue levels.

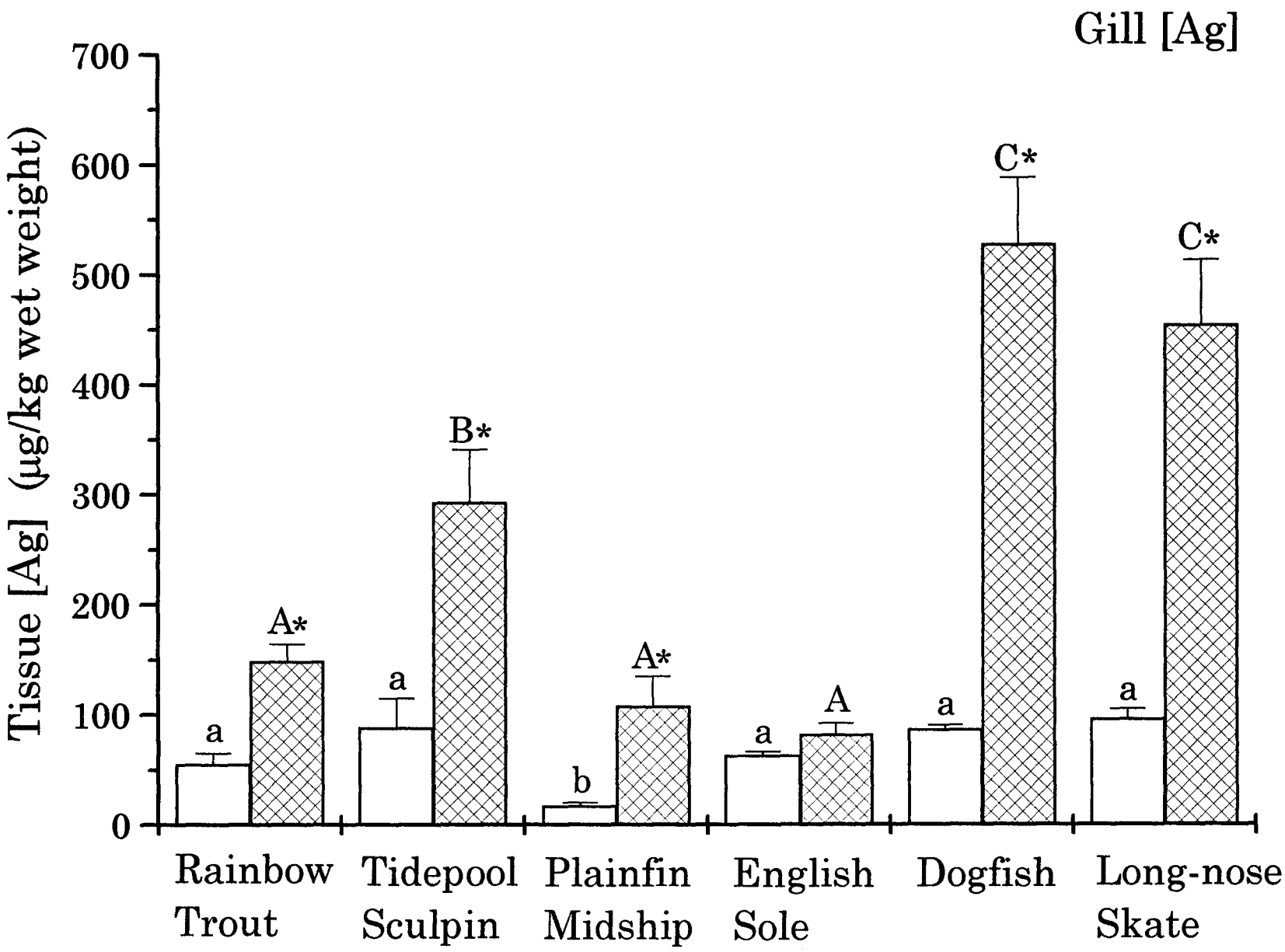

Gill [Ag]
Tissue [Ag] (µg/kg wet weight)
700
600
500
400
300
200
100
0
a
A*
a
B*
b
A*
a
A
a
C*
a
C*
Rainbow Trout
Tidepool Sculpin
Plainfin Midship
English Sole
Dogfish
Long-nose Skate

Fig. 3.2. Intestine silver levels in fish exposed to 14.5 μg/L Ag for 21 d. Other details are as indicated in legend to Figure 3.1.

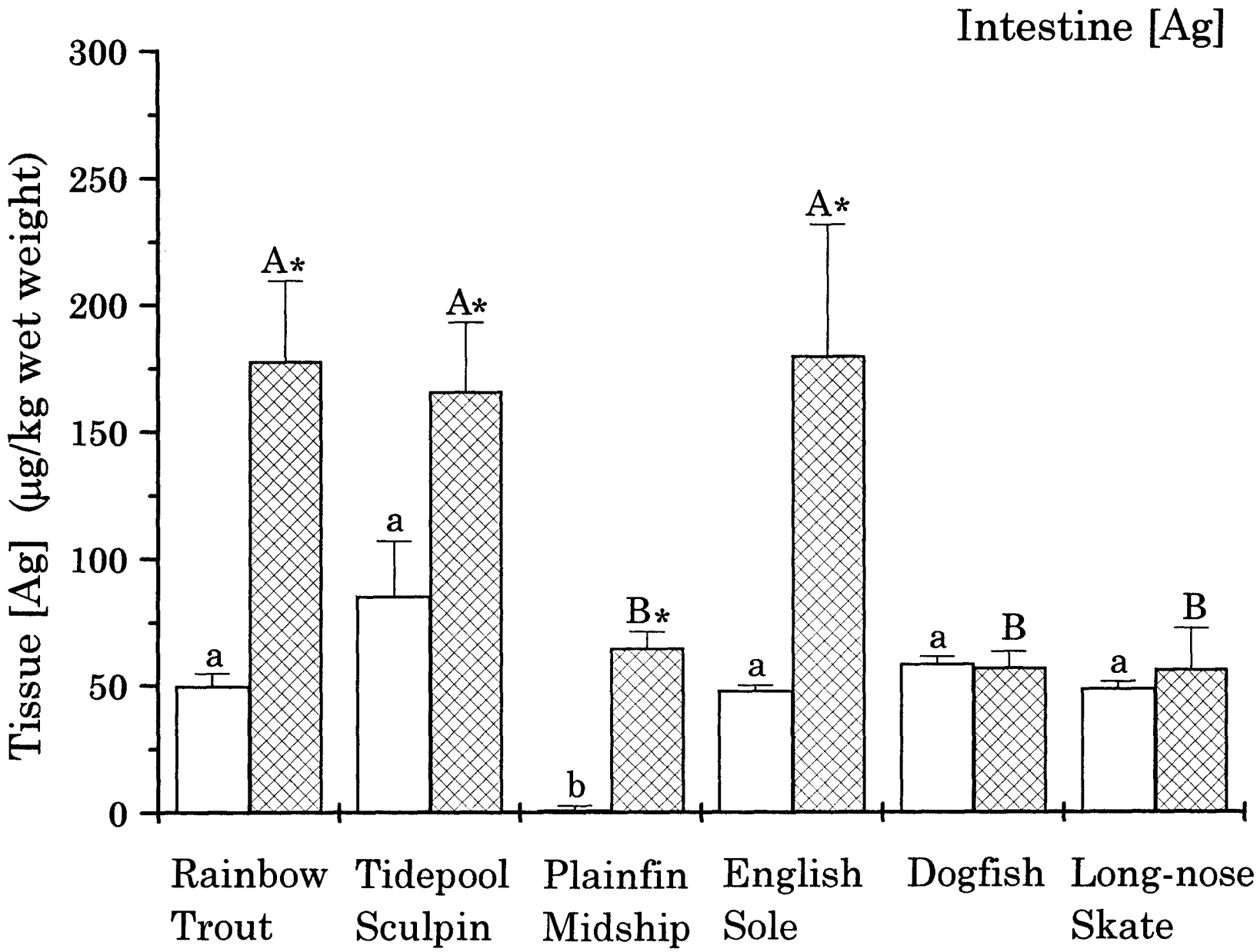
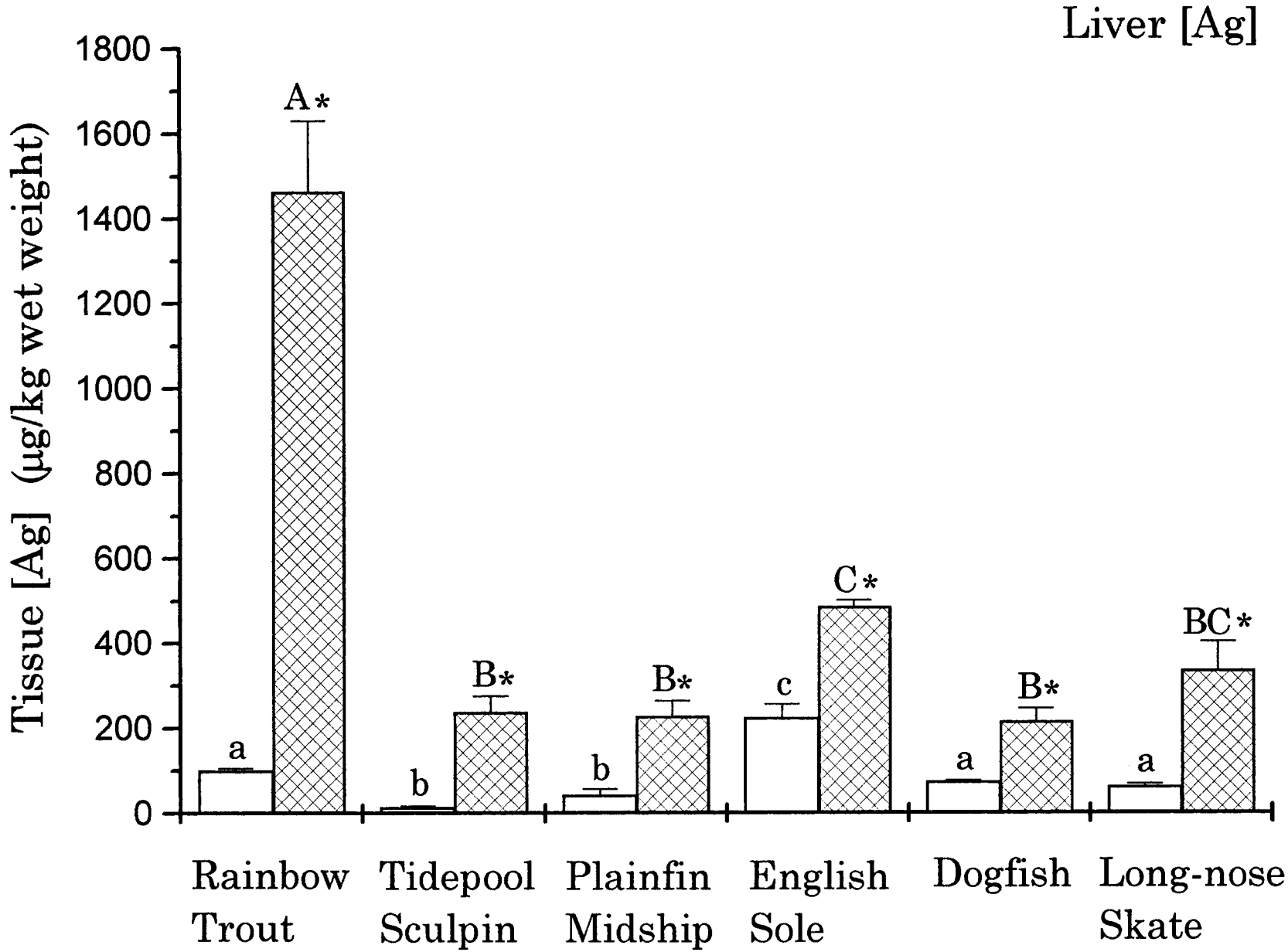

Intestine [Ag]
Tissue [Ag] (µg/kg wet weight)
300
250
200
150
100
50
0
A*
a
A*
a
b
B*
a
A*
a
B
a
B
Rainbow Trout
Tidepool Sculpin
Plainfin Midship
English Sole
Dogfish
Long-nose Skate

Fig. 3.3. Liver silver levels in fish exposed to 14.5 μg/L Ag for 21 d. Other details are as indicated in legend to Figure 3.1.


Liver [Ag]
Tissue [Ag] (µg/kg wet weight)
1800
1600
1400
1200
1000
800
600
400
200
0
A*
a
b
B*
b
B*
c
C*
a
B*
a
BC*
Rainbow Trout
Tidepool Sculpin
Plainfin Midship
English Sole
Dogfish
Long-nose Skate

significantly elevated by more than twofold in all fish. Rainbow trout accumulated the most silver, with levels rising 15-fold to 1464 ± 166 (8) from control levels (100 ± 7 (6)) (Fig. 3.3).

In general, of all tissues, white muscle (Fig. 3.4) in control fish had the lowest background silver levels (20-60 μg Ag/kg wet weight), with midshipmen levels again being significantly lower than the other fish. After 21 d of silver exposure, white muscle silver levels in rainbow trout and midshipmen increased significantly by 3 fold, while levels in sculpin carcasses (mainly white muscle) and sole white muscle did not change. Silver levels in the white muscle of both elasmobranch species increased 2 fold by the end of the exposure (Fig. 3.4).

Overall, in terms of tissue concentration, the liver was the site of greatest silver concentration the teleost fish species, while the gills were the site of highest concentration in the elasmobranchs (Table 3.3).

Salinity comparison. Liver silver levels, in tidepool sculpins (Fig. 3.5) exposed to silver for 21 d, showed a dramatic difference between 30 ppt and 18 ppt seawater, with a greater accumulation in the latter. After 2 d, silver levels in full strength seawater livers at the medium and high silver exposures (14.5 and 50.0 μg/L Ag respectively), had increased slightly but significantly compared to control fish. By the sixth day, all three exposures (1.5, 14.5, and 50.0 μg/L Ag) had significantly more silver in the livers. At the end of 21 d, the increased accumulation of silver was significant and was

Fig. 3.4. White muscle silver levels in fish exposed to 14.5 μg/L Ag for 21 d. The values for tidepool sculpin are a whole carcass measurement. Other details are as indicated in legend to Figure 3.1.

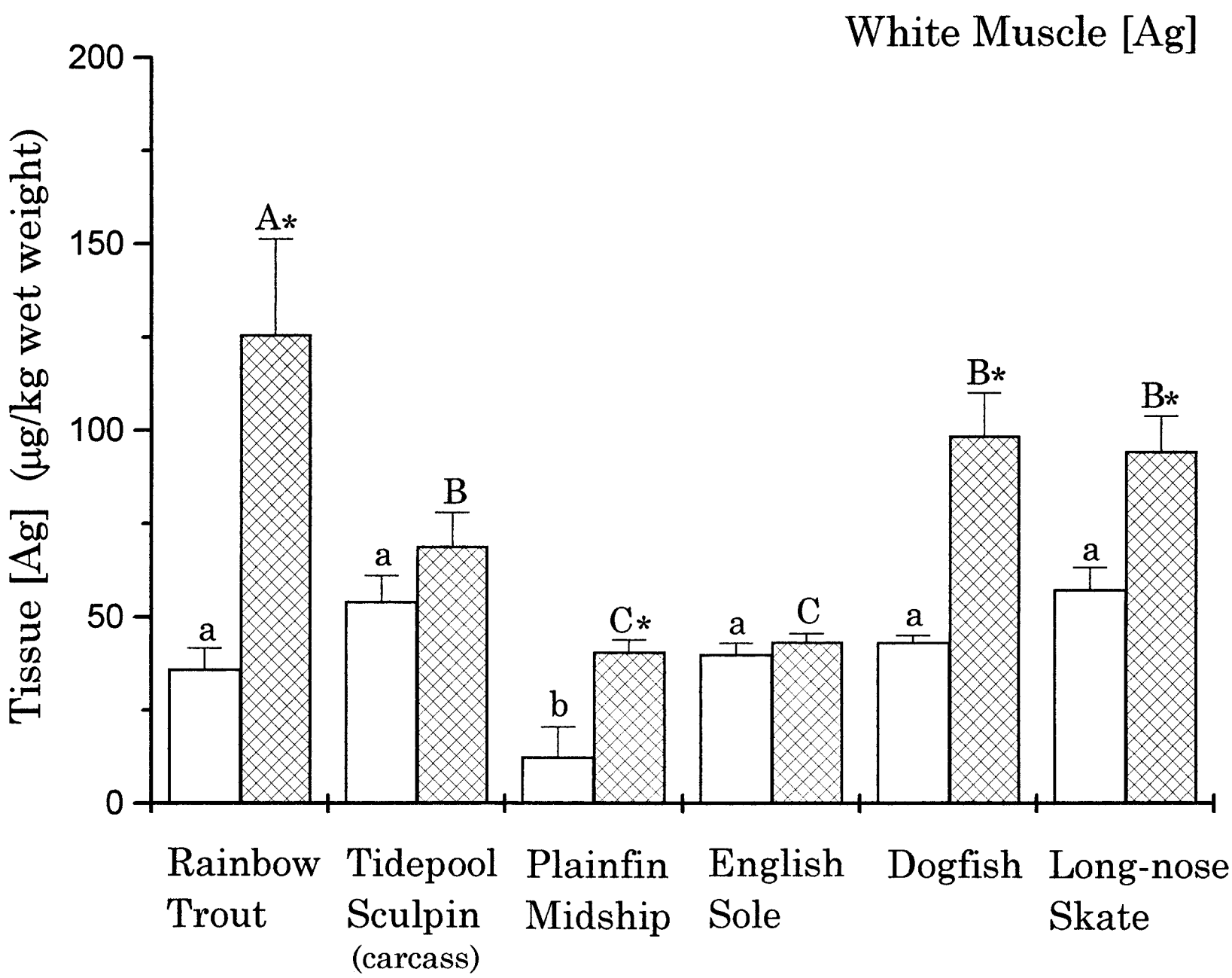
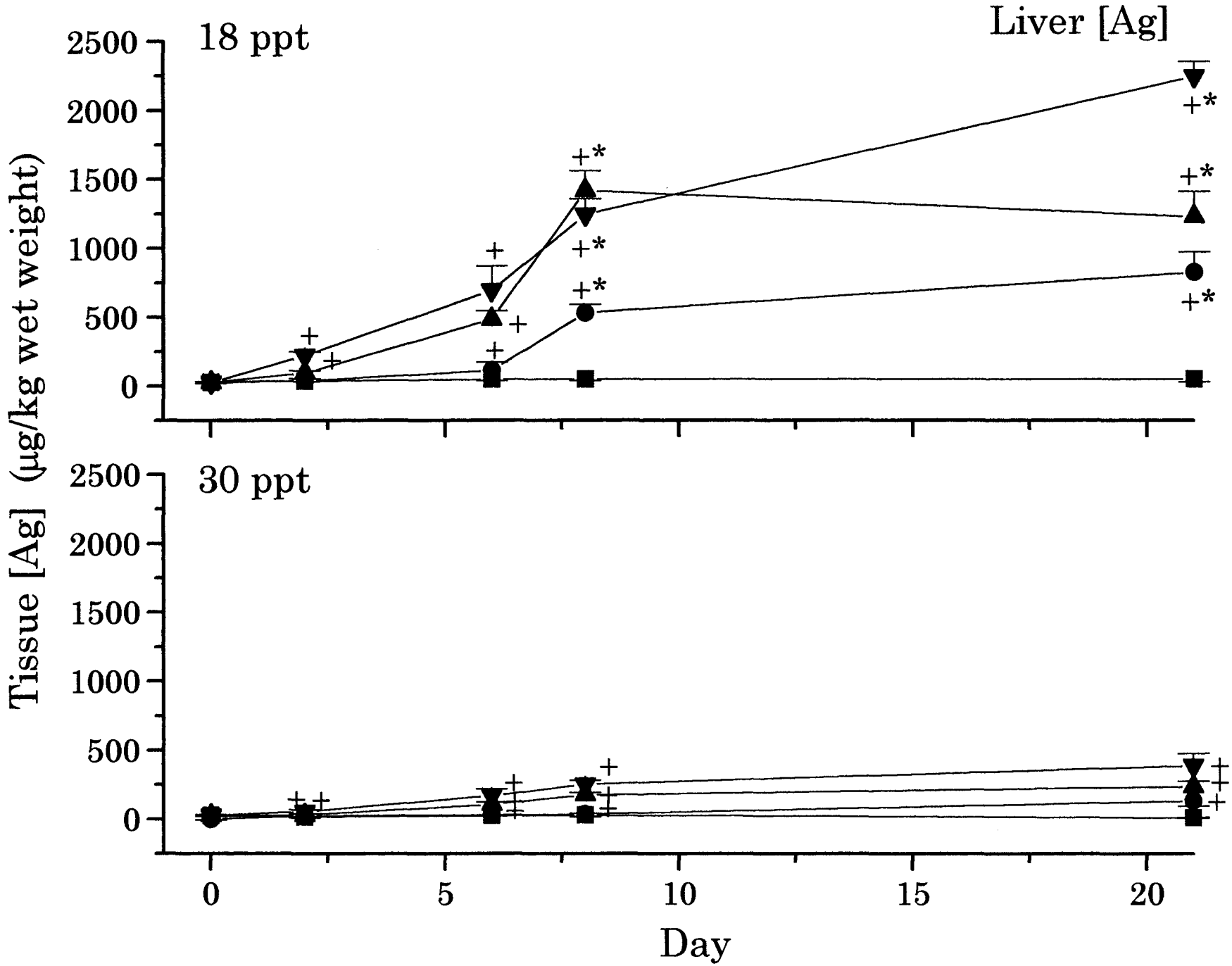

White Muscle [Ag]
Tissue [Ag] (µg/kg wet weight)
200
150
100
50
0
A*
a
B
a
b
C*
a
C
a
B*
a
B*
Rainbow Trout
Tidepool Sculpin (carcass)
Plainfin Midship
English Sole
Dogfish
Long-nose Skate

Table 3.3. Comparison of Ag concentrations in tissues of fish exposed to 14.5 μg/L Ag for 21 d.

Fish Species	**Tissue: ranked by [Ag] concentration**
Rainbow trout	liver >> intestine = gill = white muscle
Tidepool sculpin (30 ppt)	liver = gill > intestine > carcass
Tidepool sculpin (18 ppt)	liver >> gill = intestine > carcass
Plainfin midshipman	liver > gill > intestine > white muscle
English sole	liver > intestine >gill > white muscle
Pacific spiny dogfish	gill > liver >> white muscle > intestine
Long-nose skate	gill > liver >> white muscle > intestine

Fig 3.5. Liver silver levels in control (■) tidepool sculpins and sculpins exposed to 1.5 (●), 14.5 (▲), and 50.0 μg/L Ag (▼) for 21 d at both 18 ppt (top) and 30 ppt (bottom) salinities. Data points are means ± SEM (6). Asterisks (*) indicate a significant difference ($p < 0.05$) between salinity values, while a plus sign (+) indicates a significant difference from control values at the same salinity.


Liver [Ag]
18 ppt
30 ppt
Tissue [Ag] (µg/kg wet weight)
2500
2000
1500
1000
500
0
0
5
10
15
20
Day

concentration dependent (Fig. 3.5). At 18 ppt seawater, it took 6 d for the accumulation of silver to become significant in all exposures. However, by the eighth day of exposure, the accumulation was significantly higher than silver levels in 30 ppt seawater livers at the same silver exposure levels. By the end of the exposure, 18 ppt livers had 5 to 6 fold the levels seen in full strength seawater at the same exposure levels (Fig. 3.5).

A similar salinity dependent difference in Ag accumulation was seen in the intestines (Fig. 3.6). It took 8 d for the accumulation of silver in the intestines of 30 ppt seawater sculpins to become significant for all exposure levels (Fig. 3.6). By day 21, the accumulation was concentration dependent with fish at the high exposure level showing a 10 fold increase of silver. At 18 ppt seawater, there was a significant accumulation of silver in the intestines at all silver levels by the second day. This accumulation was significantly higher than 30 ppt intestines by the eighth day. After 21 d, 18 ppt seawater intestine silver levels were 2 to 3 fold higher than those of 30 ppt seawater intestines at the same exposure concentrations (Fig. 3.6).

Effects of salinity on accumulation of Ag in the gills (Fig. 3.7) were apparent but less clear-cut. The increased accumulation of silver in the gills of 30 ppt seawater sculpins was significant for all exposure concentrations after 6 d of exposure, and remained significant throughout. Silver levels in 18 ppt seawater exposed gills were significantly higher than controls by the second day for all exposures. By the end of the exposure, the increased

Fig 3.6. Intestine silver levels in control (■) tidepool sculpins and sculpins exposed to 1.5 (●), 14.5 (▲), and 50.0 μg/L Ag (▼) for 21 d at both 18 ppt (top) and 30 ppt (bottom) salinities. Other details are as indicated in the legend to Figure 3.5.

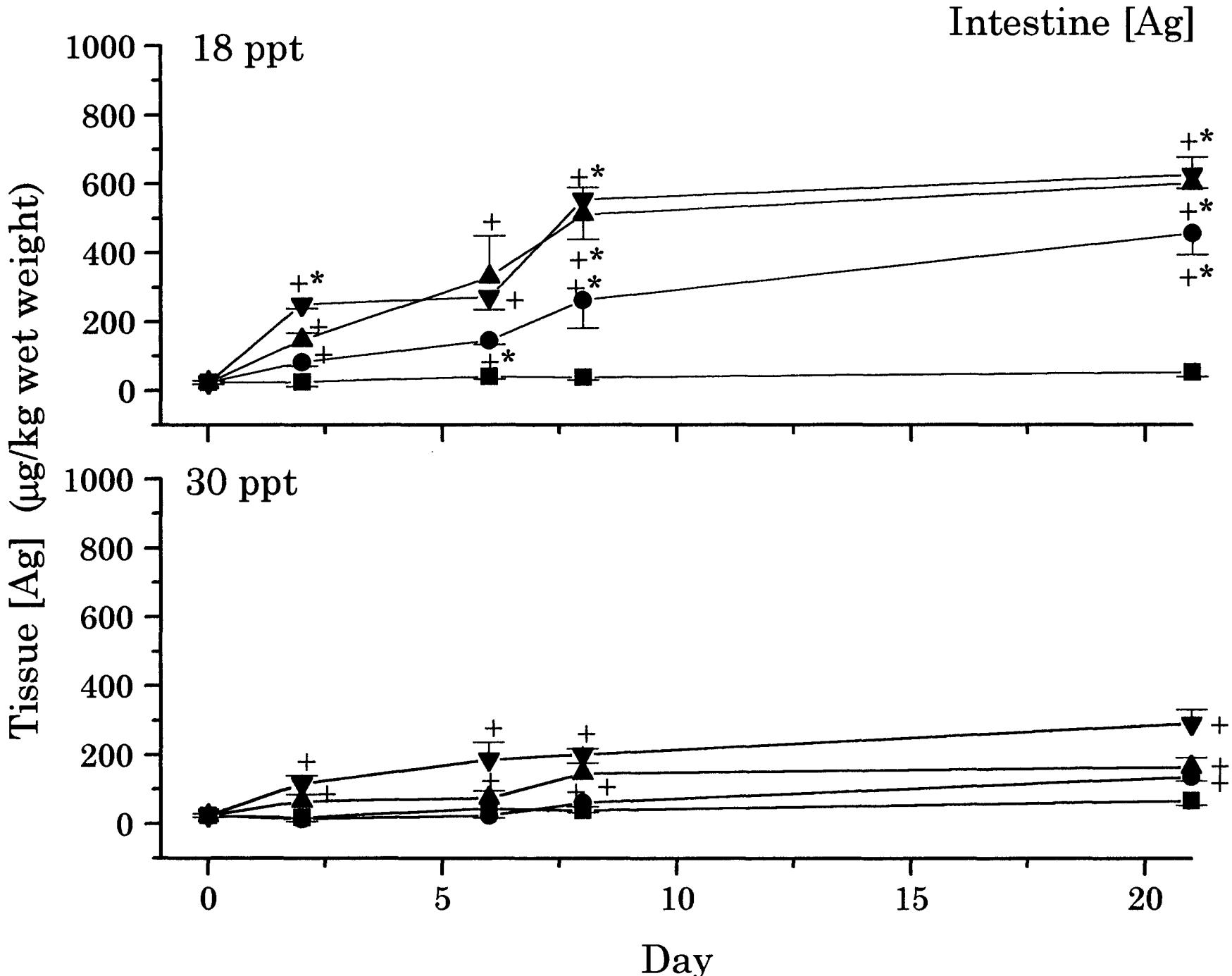
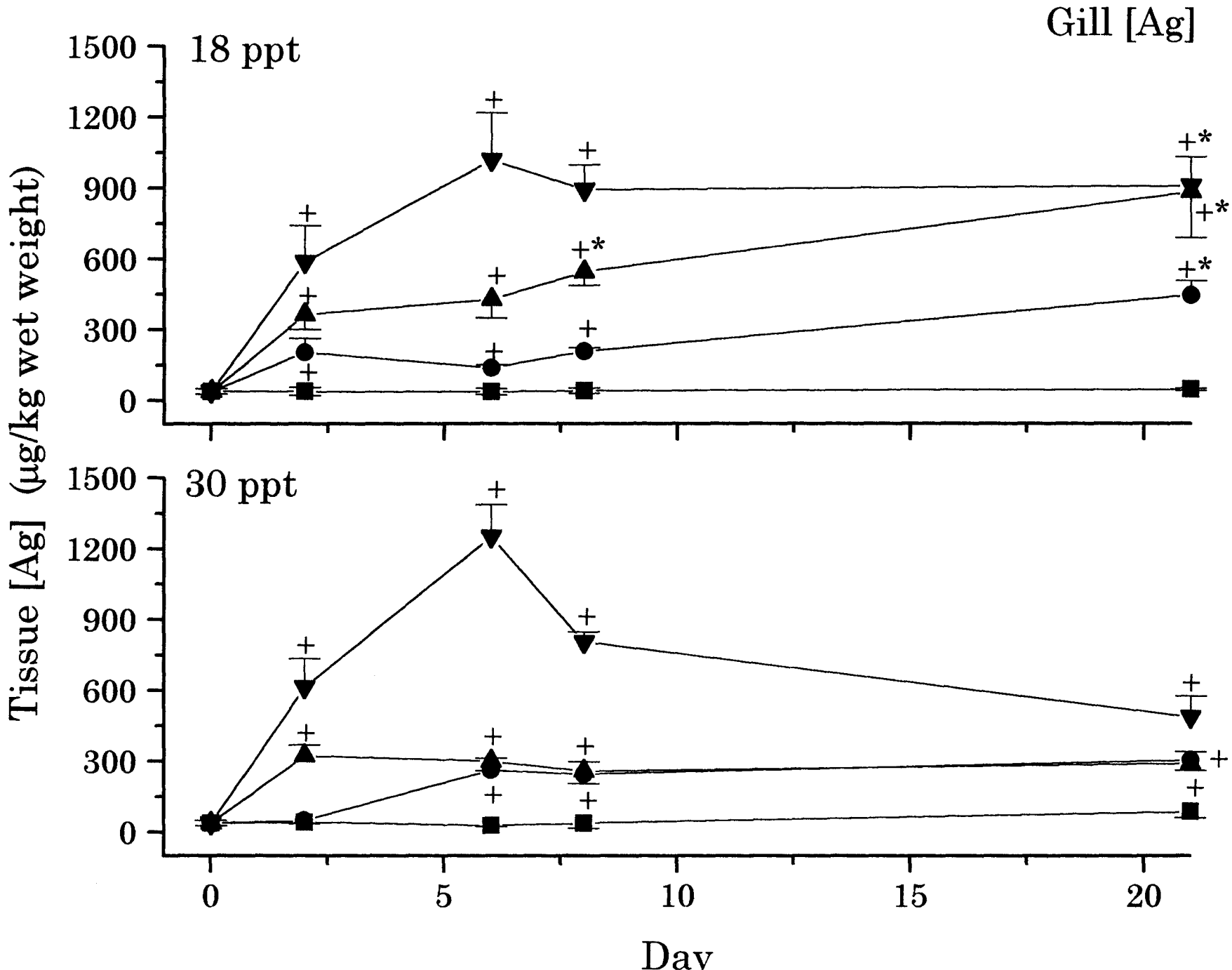

Intestine [Ag]
18 ppt
30 ppt
Tissue [Ag] (µg/kg wet weight)
1000
800
600
400
200
0
0
5
10
15
20
Day

Fig 3.7. Gill silver levels in control (■) tidepool sculpins and sculpins exposed to 1.5 (●), 14.5 (▲), and 50.0 μg/L Ag (▼) for 21 d at both 18 ppt (top) and 30 ppt (bottom) salinities. Other details are as indicated in the legend to Figure 3.5.


Gill [Ag]
18 ppt
30 ppt
Tissue [Ag] (µg/kg wet weight)
1500
1200
900
600
300
0
0
5
10
15
20
Day

accumulation of silver in the 18 ppt gills was also significantly higher than in the 30 ppt seawater gills at the same exposure concentrations (Fig. 3.7).

Exposure concentration comparison. All comparisons were made at Day 21; three species were examined, rainbow trout, tidepool sculpin, and plainfin midshipmen.

Gill silver levels in rainbow trout (Fig. 3.8, top) increased significantly by 2.5 fold (over control levels) in gills of fish exposed to 1.5 and 14.5 μg/L Ag, and 9 fold in fish exposed to 50.0 μg/L Ag. Silver levels in the intestines also increased significantly. The increase was small (~1.5 fold over controls) in fish exposed to 1.5 and 50.0 μg/L Ag, but nearly 4 fold in fish exposed to 14.5 μg/L Ag. Liver silver accumulation in exposed trout was highest of all tissues, at all silver levels. Liver silver increased as the exposure concentration increased, with levels rising 8, 14, and 20 fold (over control levels) in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Accumulation in the white muscle of rainbow trout was significant and uniform (3 to 5 fold over control levels) for all exposure concentrations (Fig. 3.8). Plasma silver concentrations in trout exposed to 14.5 and 50.0 μg/L Ag increased significantly by the end of exposure, to reach values 1.5 and 2 fold higher (respectively) than plasma levels in both control and 1.5 μg/L Ag-exposed fish (Table 3.4).

Silver accumulation in the gills of tidepool sculpins (Fig. 3.8, middle) was significant at all exposure concentrations with the increase being the

Fig. 3.8. Tissue silver levels in rainbow trout (top), tidepool sculpin (middle), and plainfin midshipmen (bottom). Open bars represent control fish, while fish exposed to 1.5, 14.5, and 50.0 μg/L Ag for 21 d are represented by bars with vertical stripes, horizontal stripes, and solid bars respectively. Data points are means ± SEM (8). Bars that share a letter are not significantly different ($p < 0.05$) for each species.

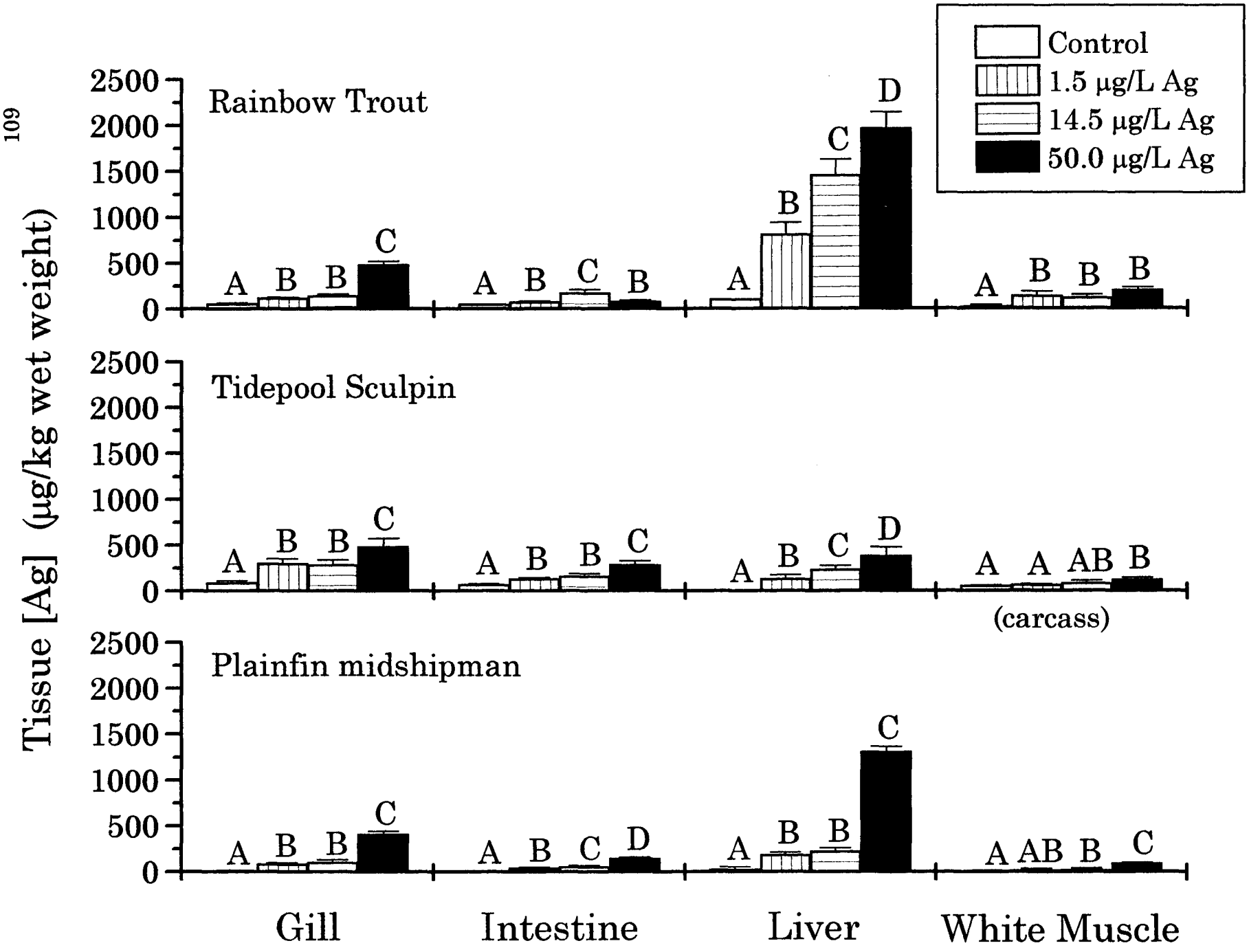

Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag
Rainbow Trout
Tidepool Sculpin
Plainfin midshipman
Tissue [Ag] (µg/kg wet weight)
2500
2000
1500
1000
500
0
A B B C
A B C B
A B C D
A B B B
A B B C
A B C B
A B C D
A A AB B
A B B C
A B C D
A AB B C
Gill
Intestine
Liver
White Muscle
(carcass)

Table 3.4. Plasma Ag levels in rainbow trout exposed to various silver concentrations for 21 d in seawater. [a]

Exposure Concentration (μg/L)	Plasma [Ag] (nmol/L)
Control	18.68 ± 1.24 (8)
1.5	17.51 ± 1.57 (9)
14.5	24.36 ± 1.68 (6)[b]
50.0	36.26 ± 2.71 (6)[b]

[a] Values are means ± SEM (*n*).

[b] $p < 0.05$ from control.

same (3.5 fold over control levels) in gills of fish exposed to 1.5 and 14.5 μg/L Ag, and nearly 6 fold in fish exposed to 50.0 μg/L Ag. Intestine silver levels increased significantly by 2 fold in fish exposed to 1.5 and 14.5 μg/L Ag, and 4 fold in fish exposed to 50.0 μg/L Ag. Silver accumulation in the livers of sculpins was also significant at all exposure concentrations, with liver levels increasing 10, 20, and 30 fold (over control levels) in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Silver accumulation in the carcass of sculpins was only significant in fish exposed to 50.0 μg/L Ag. The level of silver in the carcass of fish exposed to 14.5 μg/L was not significantly different from control fish, but also high enough to be statistically similar to levels in fish exposed to 50.0 μg/L Ag (Fig. 3.8).

Silver accumulation in gills of midshipmen (Fig. 3.8, bottom) was significant in fish at all exposure concentrations with levels increasing by 5 to 6 fold in fish exposed to 1.5 and 14.5 μg/L Ag, and 24 fold in fish exposed to 50.0 μg/L Ag. Accumulation of silver in the intestines of midshipmen was also significant and concentration dependent. The accumulation was 30, 45, and 110 fold in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Liver silver levels were also increased significantly in all exposed fish, with the trend of accumulation being similar to the gills. Silver levels increased 5 fold in fish exposed to 1.5 and 14.5 μg/L, and 32 fold in fish exposed to 50.0 μg/L Ag. Silver accumulation in white muscle of midshipmen was only significant in fish exposed to 14.5 and 50.0 μg/L Ag; rising 3.5 and 8.5 fold

Table 3.5. Net accumulation rates of silver in livers of fish exposed to various silver levels for 21 d. [a]

Fish Species	Net accumulation Rate (nmol/kg/hr)		
	Low [Ag]	**Medium [Ag]**	**High [Ag]**
Tidepool Sculpin (18 ppt)	14.96 ± 2.70 (6)[b]	22.41 ± 3.35 (6)[c]	41.12 ± 2.00 (6)[d]
Tidepool Sculpin (30 ppt)	3.28 ± 0.75 (6)[a]	4.17 ± 0.68 (6)[a]	6.97 ± 1.62 (6)[a]
Rainbow trout (30 ppt)	13.16 ± 2.28 (8)[b]	25.10 ± 3.06 (8)[c]	34.44 ± 3.21 (8)[d]
Midshipman (30 ppt)	2.77± 0.46 (10)[a]	3.45 ± 0.67 (10)[a]	23.42 ± 1.02 (10)[c]
English sole (30 ppt)		4.84 ± 0.03 (5)[a]	
Long-nose skate (30 ppt)		5.06 ± 1.23 (5)[a]	
Dogfish (30 ppt)		2.63 ± 0.56 (8)[a]	

[a] Values are means ± SEM (*n*).

Values that share the same letter (a, b or c) are not significantly different from each other.

respectively. The slight increase of silver in fish exposed to 1.5 μg/L Ag was not significantly different from control fish, nor was it significantly different from silver levels in fish exposed to 14.5 μg/L Ag (Fig. 3.8).

Net accumulation rates, based on terminal liver silver concentrations at the end of 21 d exposure, relative to control fish, were related to exposure concentration (Table 3.5). However, there was no consistent difference between elasmobranchs and teleosts. Rainbow trout and tidepool sculpin (18 ppt salinity) had significantly higher accumulation rates than all other fish at the same exposure concentrations. The only other significant difference was seen in midshipmen exposed to 50 μg/L Ag, where the accumulation rate was also high (Table 3.5).

DISCUSSION

The levels used in this study were chosen based on current and proposed regulatory limits. The current EPA acute criterion for silver levels in seawater is 2.3 μg/L Ag. New acute and chronic criteria for seawater of 14.5 μg/L and 0.92 μg/L Ag respectively, have been proposed, but not yet implemented (Loux, 1993). The province of British Columbia has set criteria for both freshwater (hardness > 100 mg/L) and marine water (open ocean and estuaries) of 1.5 μg/L and 3.0 μg/L for the 30 d average and daily maximum respectively. In water of lower hardness (≤ 100 mg/L), these levels are reduced to 0.05 and 0.1 μg/L respectively (Warrington, 1995). These levels

were used to see if there was any effect on fish, and thus biological significance for these criteria; the 50.0 μg/L Ag exposure was used as a positive control. Silver levels in the open ocean tend to be fairly low (≤0.0025 μg/L Ag; Bryan and Langston, 1992; Eisler, 1996), and thus pose little threat to aquatic organisms. Closer to shore, in the intertidal zone, silver levels tend to be higher (0.06-2.9 μg/L Ag; Fowler and Nordberg, 1986, Eisler, 1996), since these areas are closer to anthropogenic and natural inputs.

It is important to note that there were detectable amounts of silver in the tissues of all fish used in this study, even before exposure to silver. It is not uncommon for fish to accumulate silver throughout their lifetime and similar levels have been seen in many other marine fish (Hall et al., 1978; Eisler, 1996). Accumulation of silver in "non-exposed" fish has also been seen in freshwater fish. Hogstrand et al. (1996) found silver liver levels in unexposed juvenile rainbow trout (1-3 g) to be ~130 μg Ag/kg wet weight, the same range as for the present adult marine fish, while "non-exposed" adults (300-400 g) of the same freshwater strain had liver silver levels of ~9,000 μg Ag/kg wet weight, roughly 70 fold higher than naive seawater fish (Ch. 2; Wood et al., 1996a, b; Galvez and Wood, 1997).

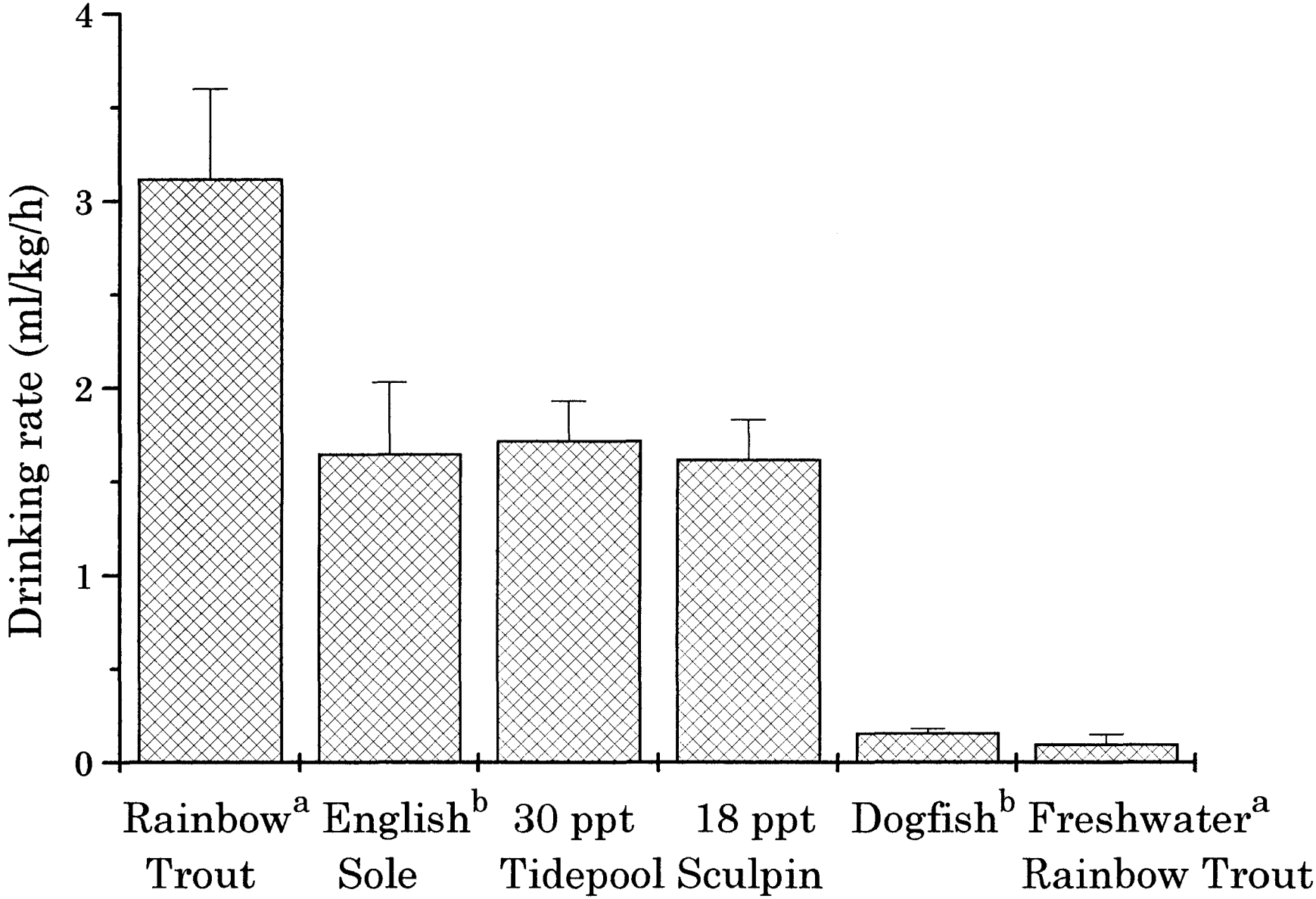
Since the liver silver livers of experimentally unexposed fish in freshwater are 60-70 times higher than seawater fish (9,000 vs. 120 μg Ag/kg wet weight in 250 g rainbow trout), it suggests that silver is much more bioavailable in freshwater than seawater. In fact, even in seawater rainbow

trout exposed to 50.0 μg/L Ag for 21 d, silver levels only reached 1970 ± 175 μg Ag/kg wet weight, still 4-fold less than control rainbow trout in freshwater. This difference in bioavailability is directly due to the change in chloride concentration, and namely the presence of Ag^+ and $AgCl_{aq}$ species in the water (Fig. 1.3, Hogstrand et al., 1996; Galvez and Wood, 1997; Shaw et al., 1998).

A particular focus of this study was to compare the accumulation of silver between marine teleosts and marine elasmobranchs. This comparison is of great interest since the mechanism of silver toxicity, at least in freshwater fish, involves an interference with iono- and osmo-regulation (Wood et al., 1996a, b; Morgan et al,. 1997; Hogstrand and Wood, 1998), and marine teleosts and elasmobranchs have fundamentally different iono- and osmo-regulatory mechanisms (Evans 1979, 1993).

Drinking rates have recently been measured in some, though not all, of the species in the present study (Fig. 3.9). These data confirm a well-known fact. Marine teleosts drink the media to obtain water and excrete divalent ions in a highly concentrated urine, while monovalent ions are actively pumped out of the fish at the gills (Evans 1979, 1993; Demarest, 1984; Marshall, 1995). Marine elasmobranchs do not drink the media (Fig. 3.9), but rather have elevated levels of urea and TMAO in their internal fluids. These help to maintain internal osmotic pressures equal to, or just slightly

Fig. 3.9. Drinking rates in one freshwater teleost, three marine teleosts, and one marine elasmobranch. Data are means ± SEM. Values for freshwater and seawater rainbow trout are taken from Wilson et al. (1996)[a]. Data for English sole and dogfish are taken from M. Grosell, G. De Boeck, and C.M. Wood (unpublished results)[b].


Drinking rate (ml/kg/h)
4
3
2
1
0
Rainbow Trout[a]
English Sole[b]
30 ppt Tidepool Sculpin
18 ppt Sculpin
Dogfish[b]
Freshwater Rainbow Trout[a]

higher, than seawater, thereby avoiding any need for drinking (Evans, 1979, 1993).

The comparison between marine teleosts and elasmobranchs showed a dramatic difference in sites of silver uptake, while no marked differences were seen in the concentrations achieved in the livers, the storage site.

In marine teleosts, both gills and intestines appear to play a significant role in silver uptake from seawater. In fact, the current data suggest that the intestines may be more important than the gills since the increases of silver concentrations (on a weight specific basis), in both tissues were comparable, yet the tissue surface area and water flow through the intestines were undoubtedly less than that of the gills.

The amount of water that passes the gills (i.e., ventilation volume) in marine teleosts is ~20-30 L/kg/hr (Kinkead and Perry, 1990; Kinkead et al., 1993), whereas the amount of water that enters the intestines (i.e., drinking rate) in marine teleosts is roughly 3 ml/kg/hr (Fuentes and Eddy, 1997). Therefore, about 1/10,000th of the water volume accounts for the same amount of silver buildup in the intestines as the gills. This suggests that the intestines either have a greater capacity for Ag uptake, or silver is more bioavailable in the intestines, than at the gills. To our knowledge, the intestinal surface area has not been quantified, but it undoubtedly is less than that of the gills, since the gills account for more than 50% a fish's total

surface area (Hughes 1980, 1982), and are the site of gas exchange with the water.

The greater bioavailability of silver in the gastro-intestinal tract could be directly due to the mechanism of water uptake in the intestines. Na^+ and Cl^- are actively pumped out of the lumen into the epithelial cells, drawing water into the fish osmotically (Loretz, 1995). Current theory suggests that the initial desalination of ingested water occurs rapidly in the esophagus, while the water, together with the remaining NaCl, gets absorbed in the intestines. This lowers the $[Cl^-]$ in the lumen, which theoretically would change the speciation of Ag in the ingested water from being totally complexed as $AgCl_n^{1-n}$ to having a small amount of $AgCl_{aq}$ present. Skadhauge et al. (1980), found $[Cl^-]$ levels in the anterior intestine of tilapia dropped to 136 mMol $[Cl^-]$, while both Shehadeh and Gordon (1969) and Wilson et al. (1996) measured chloride levels throughout the entire intestinal tract and found levels dropped to ~300 mMol $[Cl^-]$ in the stomach and ≤100 mMol $[Cl^-]$ throughout the intestine (relative to >500 mMol in ingested seawater). At these levels of $[Cl^-]$, there is a small portion of the total Ag present in the form of $AgCl_{aq}$ (Fig. 1.3), and small amounts of $AgCl_{aq}$ have been shown to dramatically increase silver uptake from the water (Shaw et al., 1998).

In marine elasmobranchs, only the gills appear to play a significant role in silver uptake from seawater. Elasmobranchs do not drink, so there

was no silver found in the intestines of either the dogfish or skates, but the gills had significant increases, substantially larger than in the teleosts.

Liver silver levels increased uniformly in both teleosts and elasmobranchs, supporting the role of the liver as the primary metal storage site in fish. For the majority of fish in this study, the accumulation rate (based on terminal liver silver concentrations) was fairly low (~4 nmol/kg/h, Table 3.4), suggesting that these fish had a high degree of resistance to silver at the exposure levels. However, rainbow trout, tidepool sculpin at 18 ppt seawater, and midshipmen at 50.0 μg/L Ag had 4 to 10 fold higher silver accumulation rates.

It is important to note that the net accumulation rate in the elasmobranchs was probably ~10 fold higher than the rate determined from terminal liver silver concentrations. Livers in elasmobranchs, on average, account for 20% of the fish's total body weight (Goldstein, 1967; Wendell Burger, 1967), while the teleost liver is only ~2% of the body weight (Smith, 1957; personal observations). This, along with the finding that the terminal liver silver concentrations were the same in elasmobranchs and teleosts, suggests that the elasmobranchs accumulated 10 times the amount of silver. The net accumulation rates from Table 3.4, would therefore be 10 fold higher, giving values of 26.3 ± 5.6 and 50.6 ± 12.3 nmol/kg liver/h for the dogfish and skate respectively.

Although high accumulation rates were found in some of the fish, the rate was only directly proportional to toxicity in the rainbow trout. This suggests that silver accumulation may not have been the sole factor involved in toxicity. It may be, that although silver accumulation is not directly toxic, it reduces the ability of the fish to deal with other stressors such as salinity acclimation. Ferguson and Hogstrand (1998) found an increased toxicity in seawater acclimated rainbow trout relative to those living in brackish water which is isosmotic to the blood plasma. They postulated that the results reflected the additional stress of these freshwater fish having to live in seawater. An additional example of synergistic stress was seen in the study of Shaw et al. (1998), where the presence of silver greatly enhanced the toxicity of ammonia to tidepool sculpins. The levels used here may not be acutely toxic, but they may be able to cause internal problems that can affect the overall health and fitness of an organism. Increased tissue silver levels may lead to interruptions of internal physiological processes that could affect reproduction, swimming ability and/or other physiological factors. Some sublethal physiological impacts were detected in the present study, and are described in Chapter 4. Also, chronic exposure of winter flounder to 10 μg/L Ag for 60 d depressed liver transaminase activity (Calabrese et al., 1977), indicating an interference in ammonia and/or protein metabolism (Lehninger et al., 1993).

The present results do not entirely agree with the Pentreath (1977) study on ^{110m}Ag accumulation in plaice and thornback ray. He found that in fish exposed to 0.04 μg/L Ag for 60 d, both the gills and intestines played a role in silver uptake in marine elasmobranchs, while there was very little bioaccumulation in any tissues of the marine teleost.

Despite the difference in uptake sites, the liver remained as the main site of storage in Pentreath's study. The elasmobranch accumulated 30 times the amount of silver in the liver compared to the teleost. This suggests a greater overall accumulation rate in the elasmobranch. Assuming an initial tissue Ag level of zero (which may not be realistic, see above), we can calculate the liver accumulation rates for the teleost and elasmobranch to be 0.29 nmol/kg/h and 9.0 nmol/kg/h, respectively, in Pentreath's study. Given that his exposure level was 50 times lower than the lowest level used here, the accumulation rate for the teleost agrees quite well with this study (50 * 0.29 = 14.7 nmol/kg/h). The same calculation gives an extremely different accumulation rate in the elasmobranch (450 nmol/kg/h), and even though the elasmobranchs in the present study had higher accumulation rates than most of the teleosts, Pentreath still found an accumulation rate 10 fold higher than the rates determined here. It would be informative to know the actual control tissue silver levels of the fish used in Pentreath's study.

The current study shows that the environmental salinity has a dramatic effect on tissue silver bioaccumulation. In all tissues, at all silver

concentrations, there was significantly more silver in fish held at the lower salinity, 18 ppt than at 30 ppt. Note that differences in drinking rate do not appear to be responsible because, at least in tidepool sculpins, measured drinking rates were identical at the two salinities (Fig. 3.9). Rather, the different bioaccumulation rates are likely due to the difference in silver speciation (and thus bioavailability) between the two types of water. In full strength seawater, all of the silver was complexed as various forms of $AgCl_n^{1-n}$; at the lower salinity, almost 2% of the total silver in the water occurred as $AgCl_{aq}$ (Fig. 1.3). We postulate that this small change leads to a dramatic increase in silver bioaccumulation rates (Hogstrand et al., 1996), and thus increased toxicity, at the lower salinity (Eisler, 1996; Ferguson and Hogstrand, 1998; Shaw et al., 1998).

Since the elasmobranchs, rainbow trout, and midshipmen at 50.0 μg/L Ag where all in 30 ppt seawater, but still had elevated liver accumulation rates, in suggests that these fish were more permeable to silver. Increased gill permeability to ions and water has been seen in fish during stressful situations, and in fact, seawater challenge is used as a stress test to determine the stage of smoltification in salmon parr (Schreck, 1990). The high mortality seen in the rainbow trout, suggests that these fish were under a great deal of stress, and thus it is quite probable that the permeability of their gills was high (Schreck, 1990). Even though no mortality was seen in the midshipmen exposed to 50.0 μg/L Ag, the increased accumulation of

silver suggests that these fish were also stressed, but still able to maintain osmotic and ionic homeostasis. No mortality was also seen in the elasmobranchs, but these fish have gills that are morphologically similar to freshwater gills (Evans, 1993). Thus, increased uptake is most likely due to increased permeability to silver at the gills, especially since the gills are probably the sole site of silver uptake in these fish.

Accumulation of metal in organs involved with ionic and osmotic regulation (namely gills and intestines in marine fish), during sublethal exposures, has been seen with copper. Copper accumulation in gills has been reported for flounder (Stagg and Shuttleworth, 1982a) and *Pseudopleuronectes americanus* (Baker, 1969), interestingly, accumulation in the gills and intestines was found in the freshwater carp (*Cyprinus carpio*) (Yamamoto et al., 1977). The chronic toxic mechanism in seawater appears to be similar to the mechanism if freshwater (Stagg and Shuttleworth, 1982a, b; Wilson and Taylor, 1993a, b), namely a disruption in iono- and osmo-regulation, suggesting that the same may be true for silver. Although little is known about the chronic toxic mechanism of silver in adult fish in either freshwater or seawater (see Hogstrand and Wood, 1998 for review), the acute toxic mechanism in freshwater is very similar to that of copper. This suggests that chronic silver exposure in seawater may have similar results to that of chronic copper exposure. It remains to be seen if silver interferes with regulatory mechanisms in seawater.

CHAPTER 4: Effects of acute and chronic silver exposure on physiological parameters in two marine teleosts

ABSTRACT

The effects of acute and chronic exposure to silver on the physiology of two marine teleosts were examined at different salinities. Tidepool sculpins were either acutely exposed to 250 μg/L Ag at 10, 18, 24, and 30 ppt for 48 h, or chronically exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag at 18 and 30 ppt for 21 d. Plainfin midshipmen were chronically exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag at 30 ppt for 21 days. Sculpins in the first series were monitored for ammonia excretion rate (J_{Amm}) and tissue silver accumulation, while sculpins in the second series were monitored for J_{amm}, oxygen consumption rate (M_{O2}), Na^+/K^+-ATPase activity in gills and intestines, and drinking rate. Midshipmen were monitored for Na^+/K^+-ATPase activity in gills and intestines.

During acute 48 h exposures of tidepool sculpin to high silver levels (250 μg/L Ag), silver accumulated in the gills and intestines, but not in the liver. Ammonia excretion was unaffected by silver exposure, but was altered by salinity, with J_{Amm} increasing as the external salinity decreased.

J_{Amm} was decreased during chronic 21 d exposure to low levels of silver in sculpins at 18 and 30 ppt. M_{O2} also decreased in silver exposed fish, with

a slight but significantly lower M_{O2} in fish at the lower salinity. This depression was gradual over time.

Na^+/K^+-ATPase activity levels in sculpin gills at 18 ppt were lower than at 30 ppt. Activity levels increased in gills of sculpins exposed to 50.0 μg/L Ag at both salinities for the first 6 d of exposure. After 21 d, activity in gills of sculpins at 18 ppt was still elevated, while it was decreased in gills of fish at 30 ppt. Intestinal Na^+/K^+-ATPase levels in sculpins at 18 ppt were generally lower than in fish at 30 ppt. Activity levels in sculpin intestine increased after 21 d exposure to 50 μg/L Ag at 18 ppt, but decreased at 30 ppt. Drinking rates in sculpins showed no difference between salinities, but were inhibited by chronic silver exposure.

Na^+/K^+-ATPase activity in midshipmen gills was inhibited by chronic silver exposure (14.5 and 50.0 μg/L Ag), while no effect of silver was seen on intestinal activities.

These data suggest that silver can enter seawater fish and chronically interfere with ammonia production, metabolic rate, and iono- and osmo-regulation.

Keywords - Silver, Seawater, Teleost, Physiology, Na^+/K^+-ATPase, Ammonia excretion, Metabolic rate

INTRODUCTION

Although silver is one of the most toxic heavy metals to freshwater fish, it is 1 to 2 orders of magnitude less toxic to seawater teleosts (Eisler, 1996; Hogstrand and Wood, 1998). Unfortunately, of the few studies that have been performed on seawater fish, none have studied the physiological effects of low waterborne silver levels (reviewed by Hogstrand and Wood, 1998). In freshwater, the acute toxic mechanism of silver appears to be similar to that of copper (Wood et al., 1996a; Morgan et al., 1997; Ch. 2) and it is likely to be the same in seawater (Hogstrand and Wood, 1996, 1998). The few physiological studies of acute, high level silver exposure in seawater have shown that silver interferes with ammonia metabolism (Hogstrand and Wood, 1996), oxygen consumption (Gould and MacInnes, 1978) and Na^+/K^+-ATPase activity (C. Hogstrand et al., unpublished results). All of these effects have been seen during copper exposure in seawater fish (Lorz and McPherson, 1976; Schreck and Lorz, 1978; Wilson and Taylor, 1993b). The silver studies however, were at extremely high levels of silver (250-1000 μg/L Ag), and the effects of exposure to low silver levels may well be different.

An interference with ammonia metabolism can occur during either production or excretion, with both having similar end results. Inhibited excretion rates would cause plasma ammonia levels to rise, as would increased production rates. However, decreased production would result in lower plasma levels and lower excretion rates. In freshwater rainbow trout,

increased plasma ammonia levels during silver exposure were caused by increased metabolic production since there was no inhibition of ammonia excretion seen throughout the exposure (Ch. 2). In seawater starry flounder, plasma ammonia levels also increased during silver exposure (Hogstrand and Wood, 1996). These studies however, were done at acutely toxic silver levels, and most likely masked any internal disturbances that would occur during chronic exposure to low silver levels. For example, silver could interfere with aspects of ammonia metabolism at the enzymatic level. Calabrese et al. (1977) reported that silver exposure inhibited liver transaminase activity, which would decrease both ammonia production and protein synthesis (Lehninger et al., 1993).

This would also have a direct effect on oxygen consumption, since 20-40% of a fishes metabolic costs is thought to be due to protein synthesis (Hawkins, 1991; Houlihan, 1991). The other main contributors to oxygen consumption (metabolic costs) are Na^+/K^+-ATPase activity involved in iono- and osmo-regulation (~20-40%) and physical activity level (for feeding, reproduction, predator avoidance, etc.) (Jobling, 1994). Since high levels of silver, and low levels of copper, have been seen to inhibit Na^+/K^+-ATPase activity, this suggests that low silver levels may also inhibit enzyme activity, and thus decrease oxygen consumption.

Inhibition of Na^+/K^+-ATPase during low level Ag exposure will also interfere with osmo-regulation. This is because marine teleosts drink

seawater to obtain water, and pump the excess ions out of the plasma at the gills (Eddy, 1979, 1993). Thus, silver could have a direct effect on the rate of drinking in these fish, as well as on branchial and/or intestinal Na^+/K^+-ATPase activity. We are unaware of any data on the effects of copper exposure on drinking rate.

Another aspect to consider when dealing with long term exposure to low levels of a heavy metal, is the possibility of acclimation occurring. Acclimation is the process by which a fish becomes resistant to the toxic effects of a metal. This metal first damages the fish in some way, the fish then repairs the damage and is able to recover and resistant any further damage by the metal (McDonald and Wood, 1993). Since silver toxicity has been shown to be similar to copper toxicity, at least in freshwater (Wood et al., 1996a; Morgan et al., 1997; Hogstrand and Wood, 1998), and since fish are able to acclimate during exposure to low levels of copper (Laurén and McDonald, 1986, 1987), it may be that fish are able to acclimate during exposure to low levels of silver in seawater. This would allow the fish to recover to control levels after the first 1 to 2 weeks of exposure (McDonald and Wood, 1993). Thus, low silver levels may alter oxygen consumption and ammonia production rates during the initial stages of exposure, but no detectable effects of silver exposure would be seen after the fish had acclimated. This could explain the results of Calabrese et al. (1977) who

reported no effect on oxygen consumption after 60 d exposure to 10 μg/L Ag in the winter flounder.

This series of experiments was designed to address some of these issues using silver levels that are of regulatory importance to industry and governments in North America (see Chapter 3 for a summary of regulatory levels). Thus, any results seen in this study may be experienced by fish in natural environments.

The results demonstrate that even at low levels, silver has the ability to diminish oxygen consumption and interfere with ammonia excretion and iono- and osmo-regulation in seawater teleosts. This suggests that low, environmentally realistic levels of silver may have an impact on fish that could decrease fitness and overall health. It is also likely that acclimation to silver occurs in seawater fish, allowing them to recover to control status and become more resistant to further silver exposures.

MATERIALS AND METHODS

Experimental animals

Tidepool sculpins (*Oligocottus maculosus*; 0.5-3.5 g) were collected from local tidepools near Bamfield Marine Station (Bamfield, BC, Canada). Fish were held in a flow through seawater system (pH ~7.9, 29-31 ppt, 12-15°C) for two weeks in 80 L tubs containing rocks for shelter. They were then separated into four groups and acclimated to 10, 18, 24, or 30 ppt

salinity (171, 308, 410, or 513 mMol [Cl^-], respectively) for two weeks. They were fed daily with freshly shucked blue mussels.

Plainfin midshipmen (*Porichthys notatus*; 50-250 g) were obtained as by-catch from local fishermen off the coast of Vancouver Island, BC, Canada. Fish were held at Bamfield Marine Station in fiberglass tanks (450 L), with sandy substrata, in a flow through seawater system (as above). Fish were fed daily with freshly shucked blue muscles.

Acute exposure

Sixteen tidepool sculpins from each salinity were placed into individual amber glass flux chambers (volume = 175 ml), each fitted with an airline for aeration and water mixing, and held in a water table to maintain temperature (13-14°C). At the start of the experiment, the water in each chamber was replaced, and 8 fish from each salinity were exposed to 250 μg/L Ag (2.32 μMol Ag, as $AgNO_3$); the remaining 8 fish were used for control measurements. Water from each flux chamber was replaced every 12 h throughout the 48 h experiment. Water samples were taken before and after each water change to determine the effect of Ag-exposure on ammonia excretion. Fish were sacrificed by cephalic blow and gill, liver, and intestine tissue samples were dissected. Gill and liver tissues were rinsed for 1 min in silver free seawater, while the intestines were flushed with 10 ml of silver

free seawater before rinsing. Samples were then frozen in liquid N_2 and stored at -80°C for future analysis.

Chronic exposure

The exposure set-ups for both sculpin and midshipmen were described in detail in the previous chapter (Ch. 3), and are briefly reiterated here.

Tidepool sculpins from 18 and 30 ppt salinities were separated into 4 groups and exposed statically to 0, 1.5, 14.5, and 50.0 μg/L Ag (0, 13.9, 134.4, and 463.5 nMol Ag, as $AgNO_3$, respectively), for 21 d. Silver was added to the exposure tubs from a stock solution of 1 g/L Ag. Fish were held in 50 L tubs with average initial loading densities of 2.4 g/L wet weight; the tubs were fitted with airlines for aeration and water mixing. The water was replaced every other day with fresh seawater of the appropriate salinity and [Ag]. Each tub was held in a seawater bath to maintain exposure temperatures (11-13°C) and fish were fed fresh shucked blue mussels on a daily basis. Four fish (from both salinities and all Ag levels) were sampled for gill and intestinal Na^+/K^+-ATPase activity on days 0, 2, 6, and 21.

Midshipmen were statically exposed to 0, 1.5, 14.5, and 50.0 μg/L Ag (as $AgNO_3$) for 21 d, in 150 L tanks with initial loading densities of 9.3 g/L wet weight. The water in each tank was changed daily with fresh seawater and [Ag]. Continuous aeration ensured thorough mixing and adequate

oxygen levels. Ten fish from each tank were sampled on days 7 and 21 for gill and intestine Na^+/K^+-ATPase activity.

For all exposures, simultaneous controls in silver free seawater were maintained in a similar manner to the exposed fish.

Oxygen consumption and ammonia excretion

Oxygen consumption (M_{O2}) in tidepool sculpins exposed to all silver levels (0, 1.5, 14.5, and 50.0 μg/L Ag) and both salinities (18 and 30 ppt) was measured on days 0 and 7 in the chronic exposure experiment. In addition, M_{O2} was measured in sculpins exposed to 0 and 50 μg/L Ag in both salinities, from the chronic exposure on days 0, 2, 4, and 6. Ammonia excretion was measured in sculpins exposed to all silver levels (0, 1.5, 14.5, and 50.0 μg/L Ag) in both salinities from the chronic exposure on days 0 and 7.

Eight jars (550 ml, opaque) per treatment, were filled with two to six fish from the same treatment, with each jar being filled with fresh, continuously aerated exposure water. The fish were then allowed to settle for 4 h. The water was then replaced with fresh exposure water, with minimal disturbance to the fish. Two 10 ml water samples were taken and frozen for later analysis of ammonia concentration, and two 1 ml water samples were taken to determine P_{O2}. The jars were then sealed to prevent contact with the air. Throughout the 4 h period, the water was periodically mixed by an internal stir bar. Two more 10 ml and 1 ml water samples were

taken at the end of the exposure. The fish in each jar were weighed and placed back into the exposure tubs. Frozen water samples were later analyzed for ammonia concentrations.

Na^+/K^+-ATPase activity assay

Na^+/K^+-ATPase activity was measured with the method described by Holliday (1985). In brief, this method measures the amount of inorganic phosphate (P_i) produced by the enzyme over a timed period. Homogenized tissue samples were placed in either +K (167 mM NaCl, 50 mM KCl, 33.3 mM imidazole, pH 7.2) or -K (217 mM NaCl, 33.3 imidazole, 3.3 mM ouabain, pH 7.2) media, a start solution of ATP and Mg^{2+} (25 mM Na_2ATP, 50 mM $MgCl_2 \cdot 6H_2O$, pH 7.2) was then added, and the tubes were incubated at 30°C. After 40 min, the reaction was stopped by the addition of a stop solution (an $FeSO_4$-molybdic acid solution that poisons the enzyme and elicits a color change). The absorbance of each tube at 700 nm was measured against a water blank on an LKB Ultraspec 4054 spectrophotometer.

Gills were homogenized in 1:50 (w/v) of homogenization media (0.25 M sucrose, 6.0 mM EDTA) using a small teflon-in-glass homogenizer (2 ml; Wheaton, USA). Tissues were homogenized for 30 s with a drill power setting of 80%. All solutions and homogenizations were done on ice to keep the temperature below 0°C. The homogenate was then transferred to centrifuge tubes and spun at 1000g for 10 min at 5°C. The homogenate was

then frozen at -80°C for future enzyme analysis. Intestine tissues were first disrupted for 20 s with a high speed tissue chopper (Ultra-turrax; Janke and Kunkel, Staufen) before going through the above procedure.

Protein levels were determined by the Bradford (1976) method using Sigma reagents (B6916) and bovine serum albumin as a reference.

Drinking rate

Drinking rates were measured in tidepool sculpins exposed to all silver levels (0, 1.5, 14.5, an 50.0 μg/L Ag) in both salinities from the chronic exposure on days 0, 4, and 8. The inert, non-absorbed marker polyethylene glycol 4000 (Wilson et al., 1996) was used.

Six fish from each chronic exposure treatments were placed as a group into 4 L opaque jars and allowed to settle for 4 h. After this period, the water was changed and the volume was decreased to 750 ml. Fifteen μCi of ^{3}H polyethylene glycol 4000 (PEG 4000, Amersham Canada Ltd., Oakville, ON, Canada; 1.87 mCi/g specific activity) was added to each jar and allowed to mix for 5 min. Water samples (3 x 5 ml) were taken at the start and end of the 6 h exposure. After 6 h, the fish were removed and rinsed in clean water containing MS222 (1 g/L, neutralized with NaOH; Syndel Labs, Vancouver, BC, Canada) for one minute. The fish were then weighed and cut open in the midline; the entire digestive tract was tied off at each end, extracted, and placed into individual pre-weighed scintillation vials and reweighed. Tissues

were digested with tissue solubilizer (TS-2, Research Products International Corp., Mount Prospect, IL, USA) at a ratio of 5 ml/g tissue for 12 h at 80°C. The digests were neutralized by adding 0.05 ml glacial acetic acid per ml of TS-2. Ten ml of organic scintillation fluor (OCS; Amersham Canada Ltd, Oakville, ON, Canada) was then added to each vial. Water samples received 10 ml of aqueous scintillation fluor (ACS; Amersham Canada Ltd, Oakville, ON, Canada). All vials were then counted for β-radioactivity (^3H) on a Rackbeta scintillation counter (LKB Rackbeta 1217, Turku, Finland), using quench curves for tissue digests and seawater constructed by the sample-channels ratio method.

Water parameters

Water pH was measured with Radiometer electrodes (GK 2401C) connected to Radiometer PHM84 meters. Water P_{O2} was monitored with a Radiometer O_2 electrode (E-5046) connected to a Radiometer PHM72 meter. Ammonia levels were determined using the colorimetric assay of Verdouw et al. (1978) which employs the reaction of ammonia with salicylate and hypochlorite. Water [Cl^-] was measured with a Radiometer CMT10 chloride titrator. Water and tissue silver levels were determined by graphite furnace atomic absorption spectrophotometry (AAS; Varian 1275 fitted with a GTA-95 atomizer); see Hogstrand et al. (1996a) and Chapter 3 for an in-depth description of the method.

Calculations

The net flux of ammonia was calculated from changes in water concentrations over the exposure period:

$$J_{Amm} = \frac{([Amm]_i - [Amm]_f) \times V}{W \times t} \quad (4.1)$$

where $[Amm]_i$ and $[Amm]_f$ are the initial and final concentrations of ammonia in the water respectively (μmol/L), V is the volume of the water in the flux chamber (L), W is the weight of the fish (kg), and t is the duration of the flux period (h). Thus net losses from the fish have a negative sign and net gains a positive sign.

Oxygen consumption was calculated from the decrease in the partial pressure of oxygen from the water over the 4 h:

$$M_{O2} = \frac{(P_{O2,I} - P_{O2,F}) \times \alpha O_2 \times V}{W \times t} \quad (4.2)$$

where $P_{O2,I}$ and $P_{O2,F}$ are the initial and final partial pressures of oxygen in the jars, respectively (torr), αO_2 is the solubility of oxygen at the exposure temperature and salinity (μmol/L/torr) from Boutilier et al. (1984), V is the exposure volume (L), W is the weight of the fish (kg), and t is the duration of the exposure (h).

Na^+/K^+-ATPase activity was calculated from the difference in absorption of the +K and -K tubes at 700 nm, and related to the protein

content of the homogenate:

$$Na^+/K^+\text{-ATPase activity} = \frac{[P_i]_{+K} - [P_i]_{-K}}{[\text{Protein}] \times t} \quad (4.3)$$

where $[P_i]_{+K}$ and $[P_i]_{-K}$ are the concentrations of inorganic phosphate liberated in the +K and -K solutions (μmol P_i/ml), [Protein] is the concentration of protein in the sample (mg/ml), and t is the duration of the assay (h).

Drinking rate was calculated from the appearance of radioactivity (^{3}H-PEG 4000) in the gut of the fish over the 6 h period:

$$\text{Drinking rate} = \frac{\text{gut radioactivity}}{MA \times W \times t} \quad (4.4)$$

where gut radioactivity is the total activity counted in the gut (cpm), MA is the mean activity of the exposure water (cpm/ml), W is the weight of the fish (kg), and t is the duration of the flux period (h).

Data have been expressed as means ± SEM (*n*). Differences between control and exposed groups, and between different exposed groups, at the same time, were analyzed by the Student's unpaired two-tailed *t* test. Comparisons of changes over time within treatments were assessed with Student's two-tailed paired *t* test using the Bonferroni procedure to adjust the *t* value for multiple comparisons. Significant differences ($p < 0.05$) between treatments are indicated by asterisks (*) while significant differences within treatments are indicated by a plus sign (+).

RESULTS

Water chemistry and survival

Acute exposure. The mean water Ag levels during the 48 h exposure was 265 ± 8 (128) μg/L (2.26 ± 0.07 μMol Ag), only slightly higher than the nominal value of 250 μg/L. Silver speciation calculated by MINEQL+ (Fig. 4.1) showed that the speciation distribution changed slightly from that determined in Fig. 1.3. The speciation distribution in Fig. 1.3 was determined using a [Ag] of 10 μg/L, while the acute study was performed at 250 μg/L Ag. In Fig. 1.3, $AgCl_{aq}$ was only present at 0, 10, and 18 ppt, while in Fig. 4.1, $AgCl_{aq}$ was present at all salinities. This is due to the increased amount of Ag ions available for chloride complexation.

None of the control or exposed fish died during this exposure.

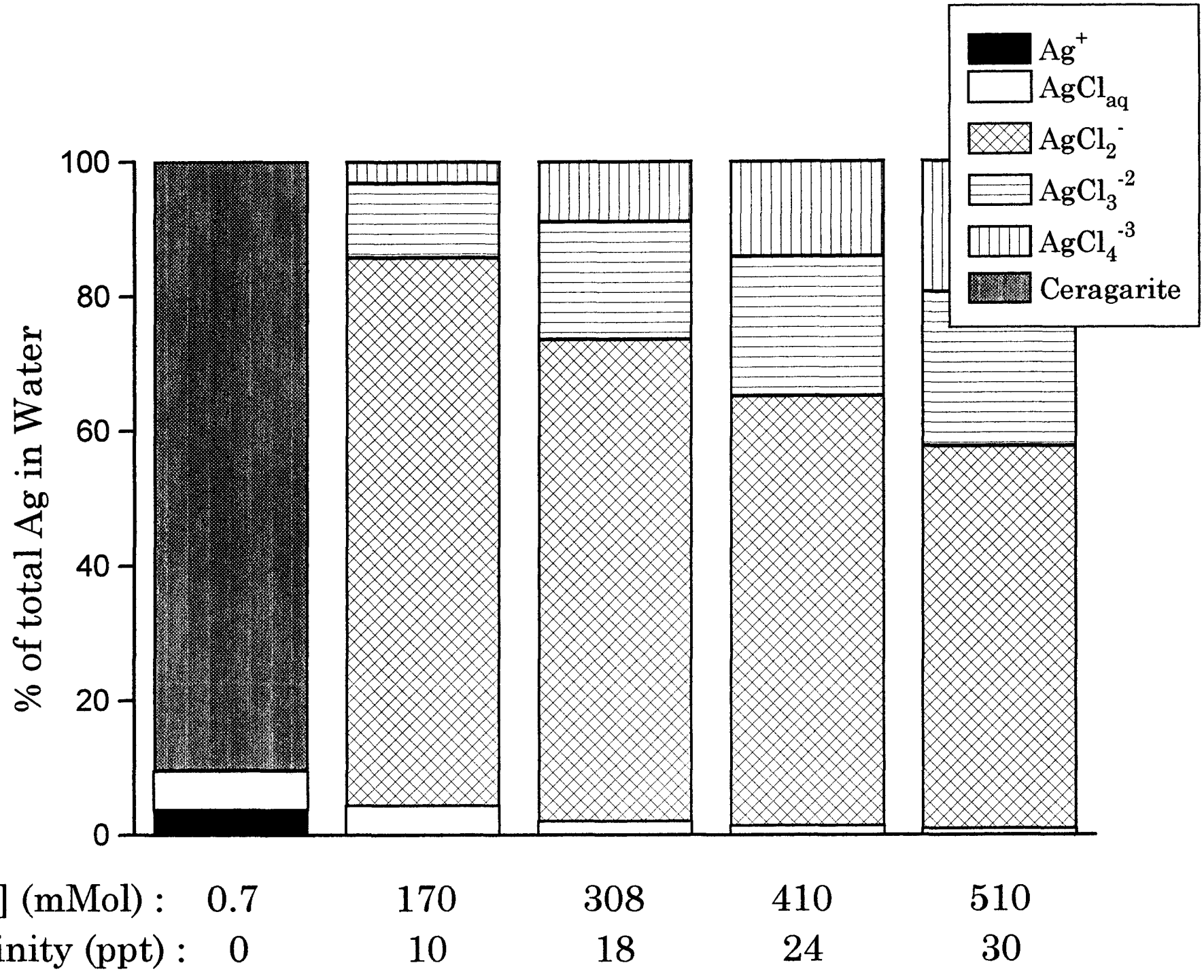
Chronic exposure. The mean water Ag levels were very close to the nominal values and were listed in Table 3.1. Speciation was calculated in the previous chapters and it was seen that all silver ions were complexed with Cl^- ions, with $AgCl_{aq}$ being present only at 18 ppt (Fig. 1.3).

None of the fish at any concentration died during the exposure.

Tissue silver levels

Acute exposure. Both gill and intestines showed significant buildup of silver at all salinities. The silver accumulation in the gills was uniform for

Fig. 4.1. The change of predominant Ag species as [Cl^-] increases from freshwater (0.7 mMol [Cl^-]) to full strength seawater (515 mMol [Cl^-]). In freshwater, nearly 85% of the 250 μg/L of silver exists as ceragarite, with Ag^+ and $AgCl_{aq}$ being present at 10% and 5%, respectively. As the [Cl^-] increases, Ag^+ disappears as more Cl^- binds to silver complexes, however, unlike Fig. 1.3, there is still a small amount (1%) of $AgCl_{aq}$ present at full strength seawater. Charged $AgCl_n^{1-n}$ species make up the remainder of silver species present. It should be noted that this model assumes a DOC concentration of 0, an [S^{2-}] = 0, and a [Ag] = 250 μg/L (as $AgNO_3$).


Ag+
AgClaq
AgCl2-
AgCl3-2
AgCl4-3
Ceragarite
% of total Ag in Water
100
80
60
40
20
0
[Cl-] (mMol) : 0.7 170 308 410 510
Salinity (ppt) : 0 10 18 24 30

the 3 lower salinities, and was significantly greater in the 30 ppt salinity gills (Fig. 4.2). Silver accumulation in the intestines was also significant for all salinities (Fig. 4.3). There was no significant accumulation of silver seen in the livers at 10, 18, and 24 ppt, but a small increase at 30 ppt over the 48 h exposure (Fig. 4.4).

Chronic exposure. Tissue silver levels for these fish have been reported and discussed in Chapter 3 and are shown in Figs. 3.5, 3.6, and 3.7. Briefly, silver accumulated in all tissues during the 21 d exposure. The accumulation was generally higher at the higher silver concentrations. There was also significantly higher accumulation in the tissues of the fish held in the lower salinity (18 ppt) than in full strength seawater (30 ppt).

Ammonia excretion

Acute exposure. There was no effect of 48 h of silver exposure on ammonia excretion (J_{Amm}) seen for any salinity. However, there did appear to be an effect of salinity on J_{Amm}. As the salinity decreased, J_{Amm} increased. This increase was significant in exposed fish at 18 and 30 ppt salinity, while being significant in control fish at 24 and 30 ppt salinity (Fig. 4.5).

Chronic exposure. There was no difference in J_{Amm} seen between 18 and 30 ppt control fish at day 0. On day 7, both the control and 50.0 μg/L Ag

Fig. 4.2. Gill silver levels in control (open bars) and exposed (hatched bars) tidepool sculpins after 48 h at 250 μg/L Ag (as $AgNO_3$). Data points are means ± SEM (8). Asterisks (*) indicate a significant difference ($p < 0.05$) between control values, while bars that share a letter are not significantly different from each other.

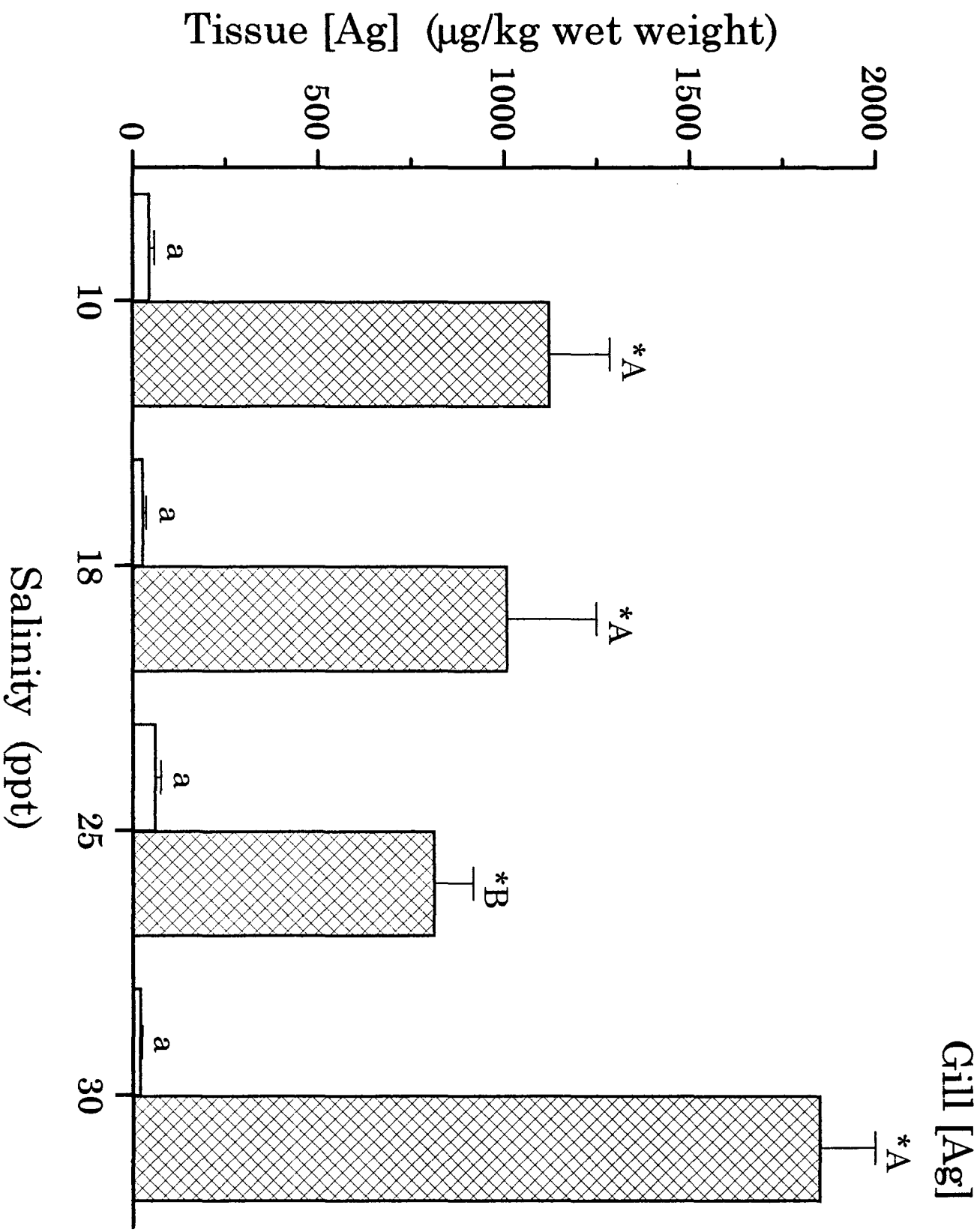

Tissue [Ag] (µg/kg wet weight)
0
500
1000
1500
2000
Salinity (ppt)
10
18
25
30
a
a
a
a
*A
*A
*B
*A
Gill [Ag]

Fig. 4.3. Intestine silver levels in control (open bars) and exposed (hatched bars) tidepool sculpins after 48 h at 250 μg/L Ag (as $AgNO_3$). Other details are as indicated in the legend to Figure 4.2.

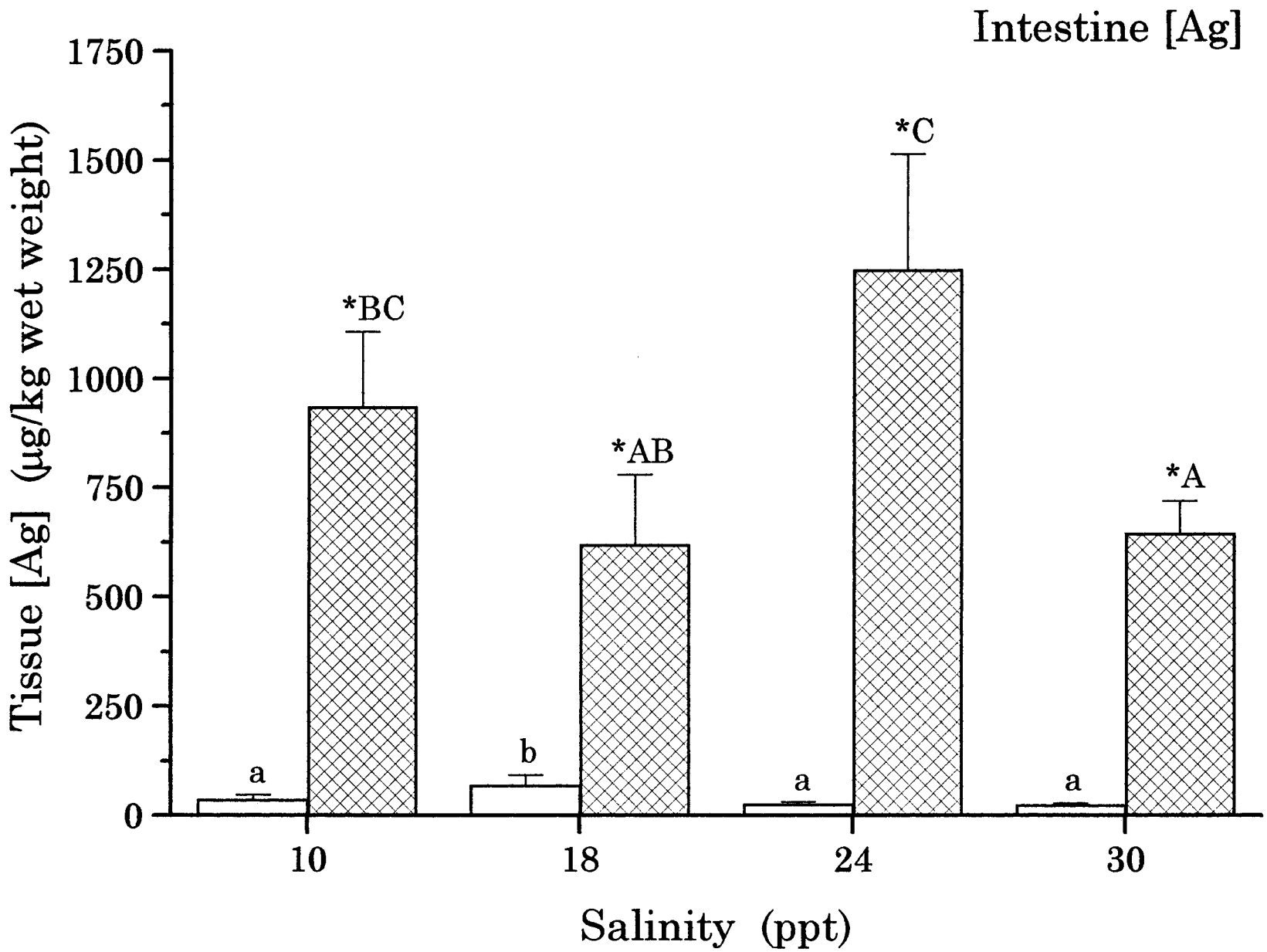

Intestine [Ag]
Tissue [Ag] (µg/kg wet weight)
1750
1500
1250
1000
750
500
250
0
*BC
*AB
*C
*A
a
b
a
a
10
18
24
30
Salinity (ppt)

Fig. 4.4. Liver silver levels in control (open bars) and exposed (hatched bars) tidepool sculpins after 48 h at 250 μg/L Ag (as $AgNO_3$). Other details are as indicated in the legend to Figure 4.2.

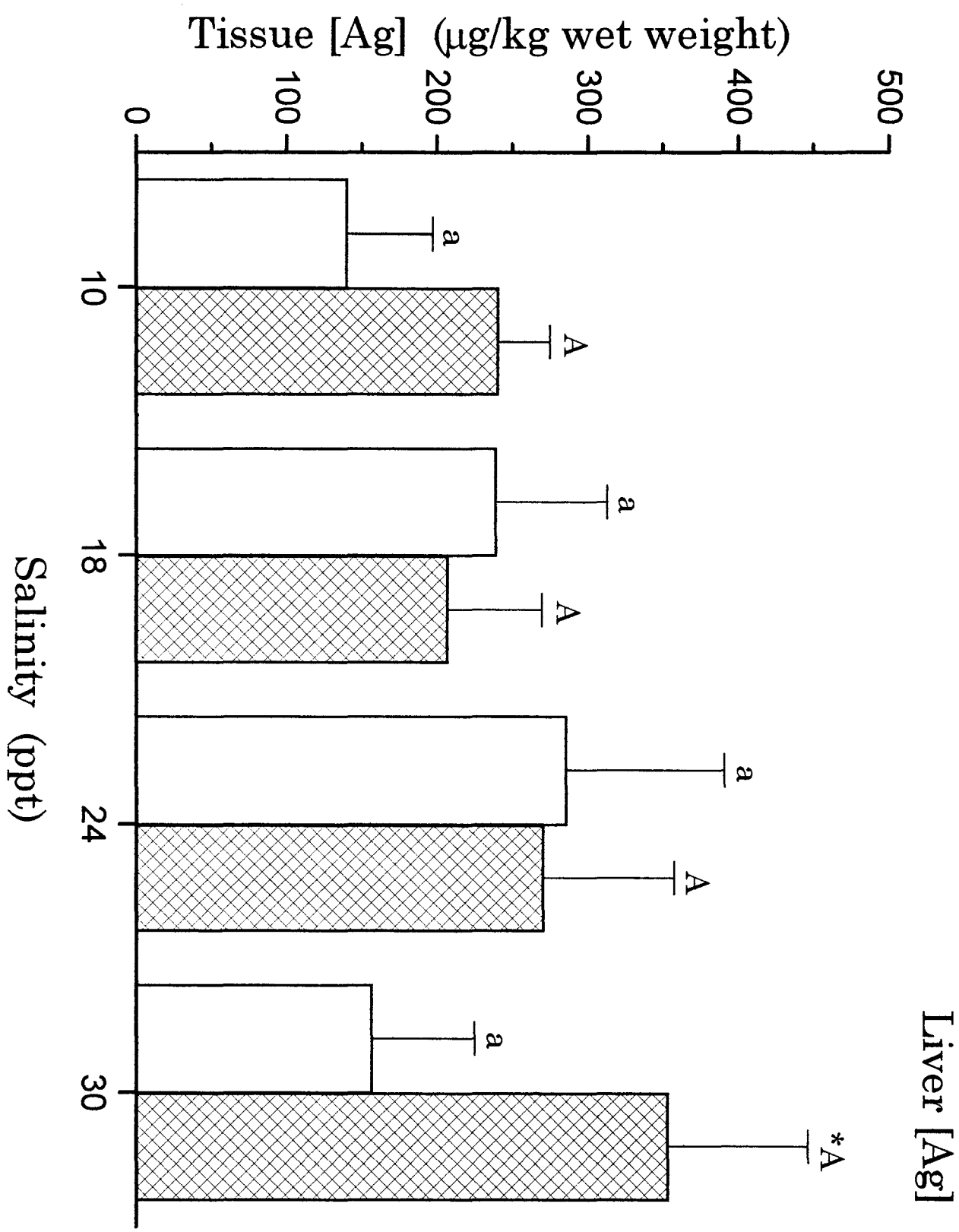
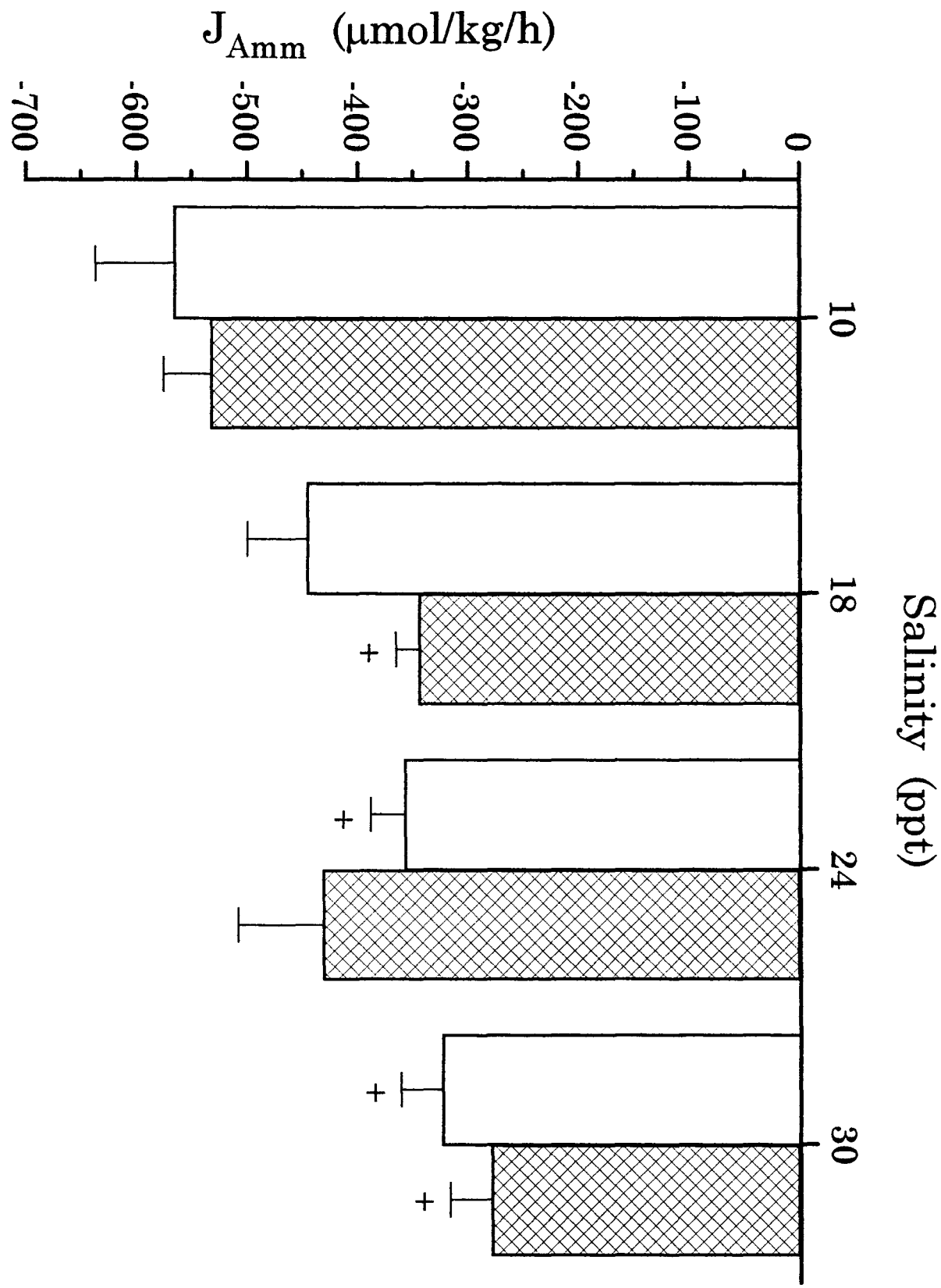

Tissue [Ag] (µg/kg wet weight)
0
100
200
300
400
500
Salinity (ppt)
10
18
24
30
a
A
a
A
a
A
a
*A
Liver [Ag]

Fig. 4.5. Ammonia excretion rates in tidepool sculpins acclimated to four salinities and exposed to 250 μg/L Ag (as $AgNO_3$) for 48 h. Open bars indicate control excretion rates while hatched bars indicate excretion rates of exposed fish. Data are means ± SEM (8). Plus signs (+) indicate a significant difference ($p < 0.05$) from the excretion rates at 10 ppt. There were no significant effects of Ag exposure at any salinity.


J_{Amm} (µmol/kg/h)
-700
-600
-500
-400
-300
-200
-100
0
10
18
24
30
Salinity (ppt)
+
+
+
+

fish at 18 ppt had significantly lower J_{Amm} than day 0 excretion rates (Fig. 4.6).

After 7 d, 30 ppt fish exposed to 14.5 and 50.0 μg/L Ag had significantly lower J_{Amm} compared to day 0 excretion rates. There was no change seen in the control or 1.5 μg/L fish (Fig. 4.6).

Oxygen consumption

The oxygen consumption (M_{O2}) decreased in fish from both salinities as the silver concentration increased. This decrease was only significant at 50.0 μg/L Ag for 18 ppt, but was significant at both 14.5 and 50.0 μg/L Ag for 30 ppt after 7 d (Fig. 4.7). M_{O2} in 18 ppt fish on day 0 and in 18 ppt fish exposed to 50.0 μg/L Ag on day 7 was significantly lower than the corresponding M_{O2} of fish at 30 ppt.

Measurements of M_{O2} throughout the first 6 d of exposure to 50.0 μg/L Ag showed that the depression of M_{O2} occurred gradually, and only became significant (for fish in both salinities) after 4 d of exposure. The only difference between salinities was that the M_{O2} in exposed 18 ppt fish was lower than 30 ppt fish on day 6 (Fig. 4.8).

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity in control midshipmen gills showed a significant decrease from day 0 to day 21. Silver exposure also significantly

Fig. 4.6. Ammonia excretion rates in tidepool sculpins acclimated to 18 and 30 ppt and exposed to Ag (as $AgNO_3$) for 7 d. Open bars indicate control (day 0 and 7) excretion rates, while vertical stripes, horizontal stripes, and solid bars indicate excretion rates in sculpins exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (8). Plus signs (+) indicate a significant difference from the day 0 control value, while asterisks (*) indicate a significant difference between salinities.

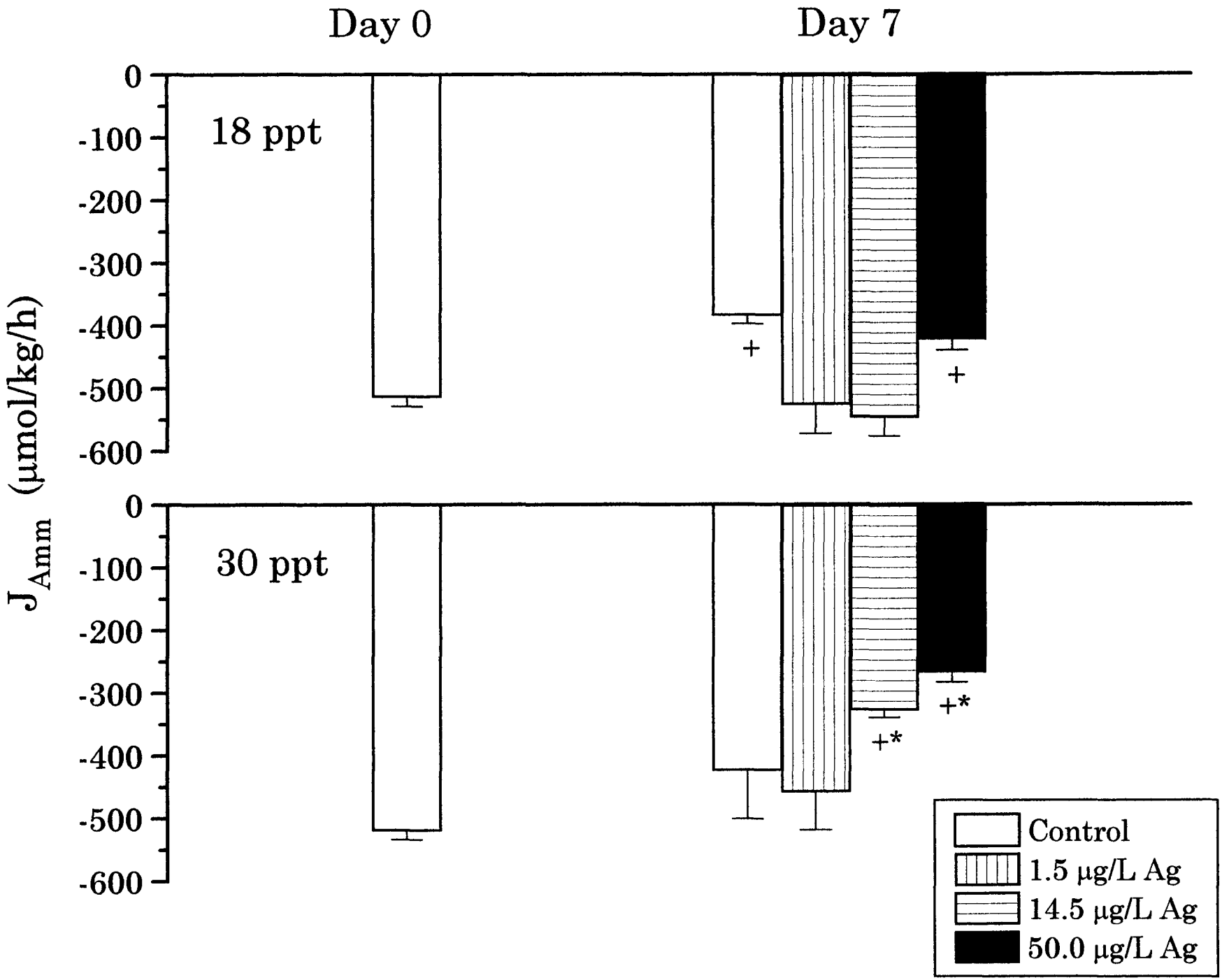

Day 0
Day 7
18 ppt
30 ppt
0
-100
-200
-300
-400
-500
-600
J_{Amm} (µmol/kg/h)
+
+
+*
+*
Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag

Fig. 4.7. Oxygen consumption rates in tidepool sculpins acclimated to 18 and 30 ppt and exposed to Ag (as $AgNO_3$) for 7 d. Open bars indicate control (day 0 and 7) consumption rates, while vertical stripes, horizontal stripes, and solid bars indicate consumption rates in sculpins exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Other details are as indicated in the legend to Figure 4.6.

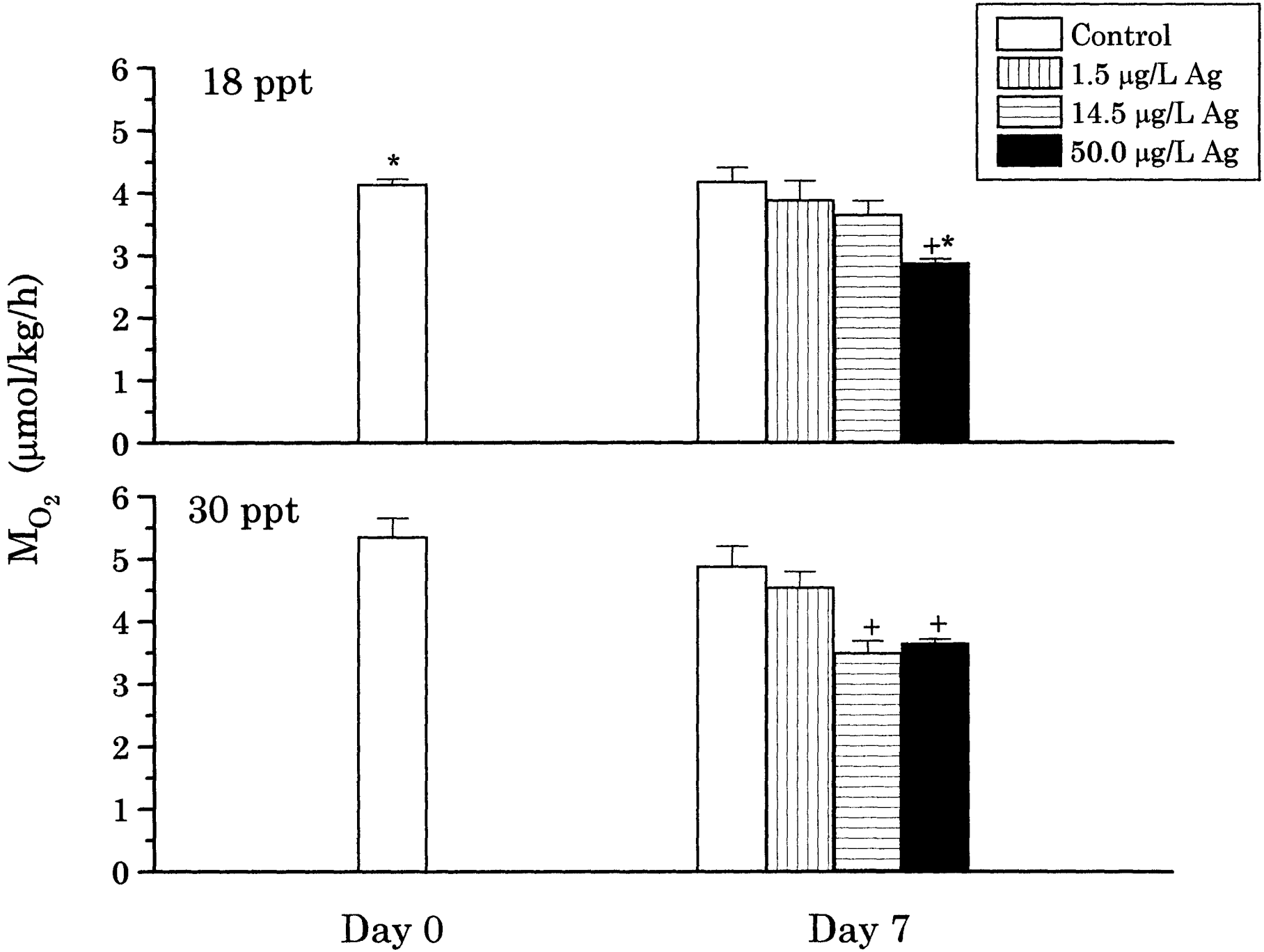
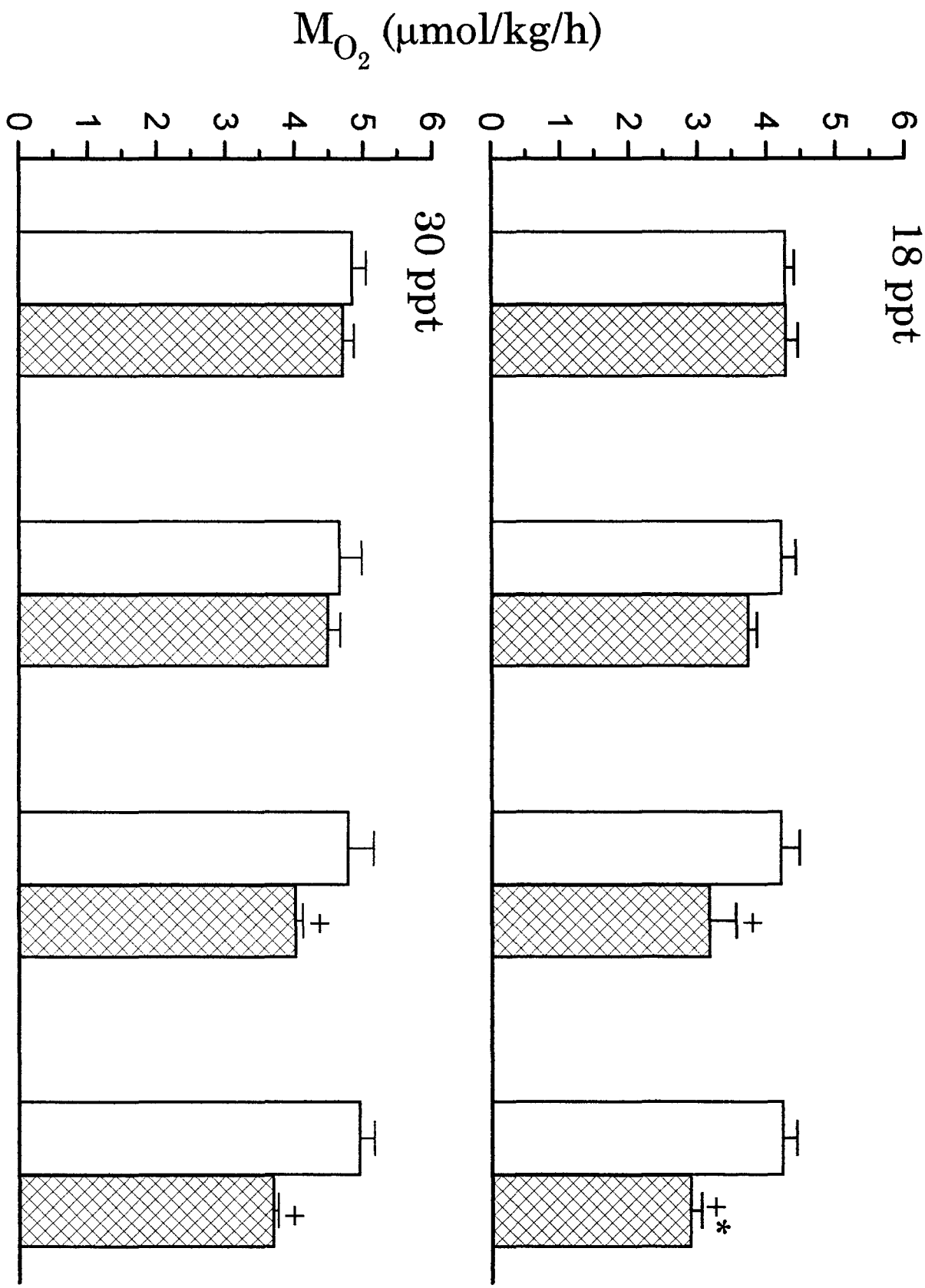

Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag
18 ppt
30 ppt
M_{O_2} (µmol/kg/h)
0
1
2
3
4
5
6
*
+*
+
+
Day 0
Day 7

Fig. 4.8. Oxygen consumption rates in tidepool sculpins acclimated to 18 and 30 ppt and exposed to Ag (as $AgNO_3$) for 6 d. Open bars indicate control consumption rates, while hatched bars indicate consumption rates in sculpins exposed to 50.0 μg/L Ag, respectively. Data are means ± SEM (8). Plus signs (+) indicate a significant difference ($p < 0.05$) from the same day control value. Asterisks (*) indicate a significant difference between salinities.


M_{O_2} (μmol/kg/h)
0
1
2
3
4
5
6
18 ppt
30 ppt
0
2
4
6
Day
*

decreased gill activity in fish exposed to 14.5 and 50.0 μg/L Ag on day 7, and on day 21 in 50.0 μg/L Ag fish (Fig. 4.9). There did not appear to be any effect of time or silver on the activity levels in the intestines of midshipmen (Fig. 4.10).

Activity levels in tidepool sculpins showed a significant difference between 18 and 30 ppt gills. Activity in 18 ppt gills was nearly 50% that of 30 ppt gills throughout the entire exposure (Fig. 4.11).

Activity levels in 18 ppt control gills dropped significantly on day 6, and then recovered to pre-exposure levels by day 21. The only significant effect due to silver exposure was an increase in activity (over same day control levels) in fish exposed to 50.0 μg/L Ag on days 6 and 21 (Fig. 4.11).

Control activity levels in gills of tidepool sculpins at 30 ppt showed the same trend as control gills at 18 ppt. The activity levels dropped significantly by day 6 but recovered to pre-exposure levels by day 21. Compared to the same day control values, the activity in the gills of sculpins exposed to 50.0 μg/L Ag was significantly elevated on days 2 and 6, but was significantly diminished on day 21 (Fig. 4.11).

Intestinal Na^+/K^+-ATPase activity levels in the majority of sculpins at 18 ppt were significantly lower than at 30 ppt. In only four situations was the activity level not significantly lower. In three there was no difference (14.5 μg/L Ag fish on days 6 and 21, and 50.0 μg/L Ag fish on day 21), and

Fig. 4.9. Na^+/K^+-ATPase enzyme activities for gills of plainfin midshipmen exposed to Ag for 21 d. Open bars indicate control values (day 0, 7, and 21), while vertical stripes, horizontal stripes, and solid bars indicate activity levels in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (8). Plus signs (+) indicate a significant difference from the same day control value.

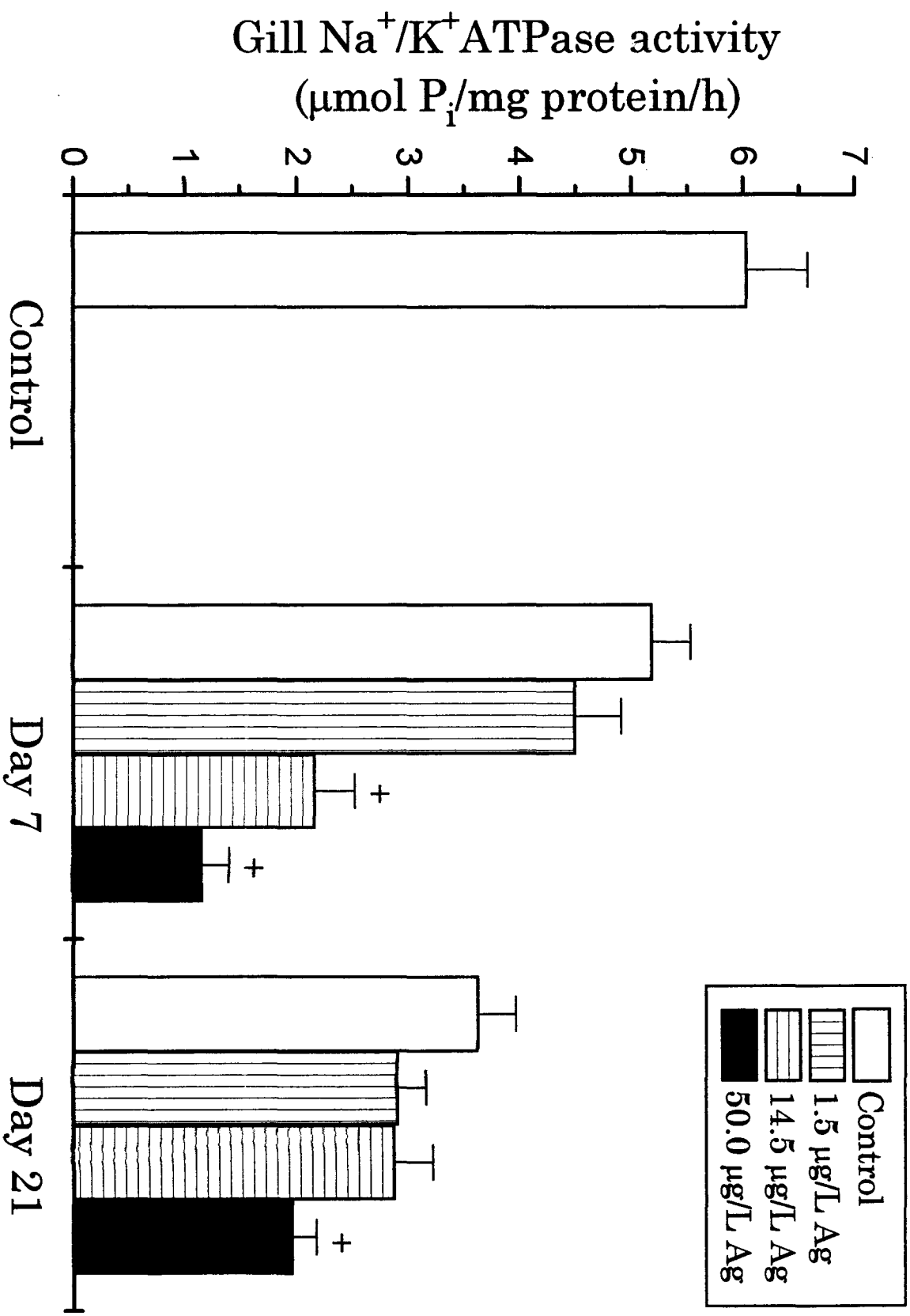

Gill Na+/K+ATPase activity
(µmol Pi/mg protein/h)
0
1
2
3
4
5
6
7
Control
Day 7
Day 21
+
+
+
Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag

Fig. 4.10. Na^+/K^+-ATPase enzyme activities for intestines of plainfin midshipmen exposed to Ag for 21 d. Open bars indicate control values (day 0, 7, and 21), while vertical stripes, horizontal stripes, and solid bars indicate activity levels in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (8). There were no significant differences.

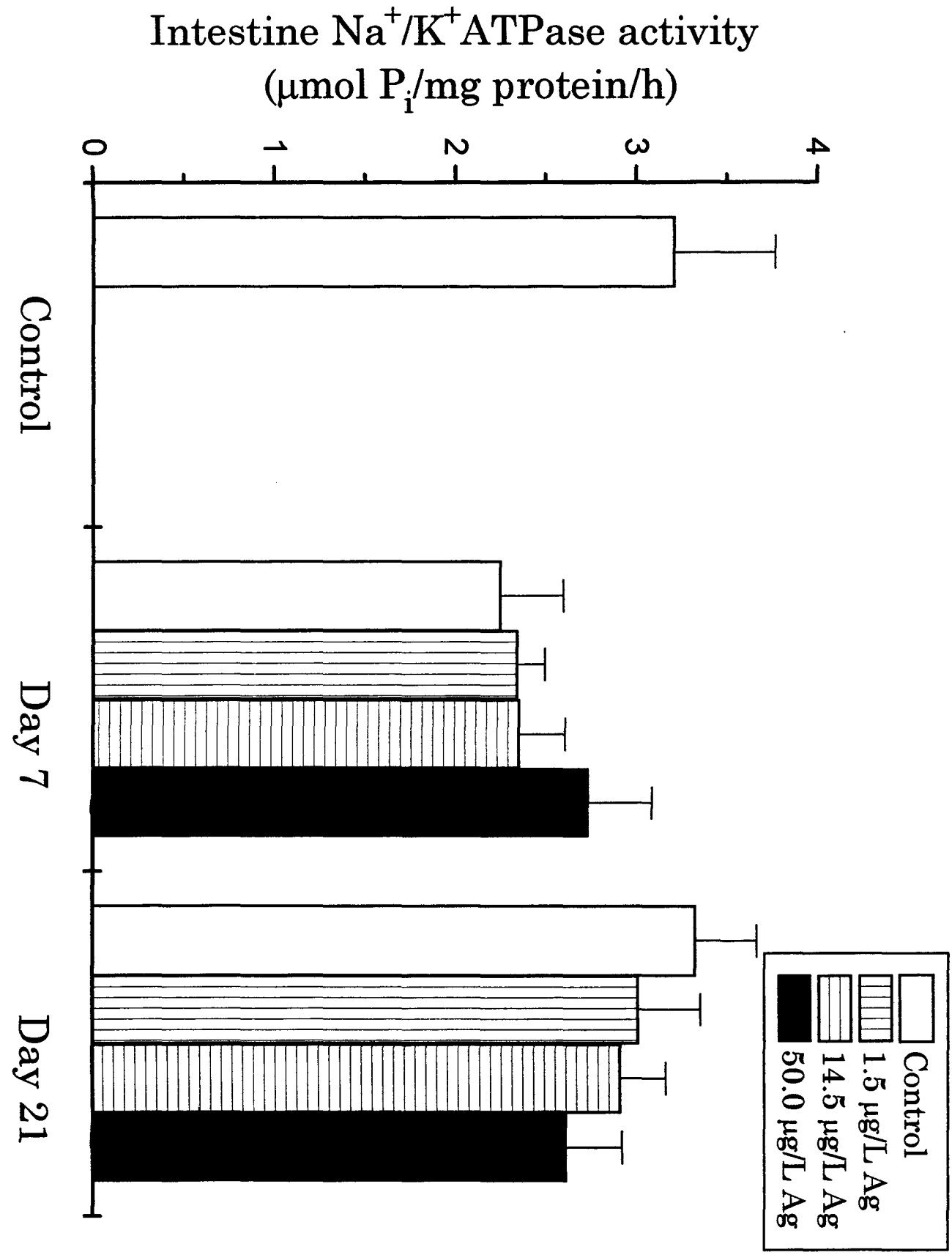
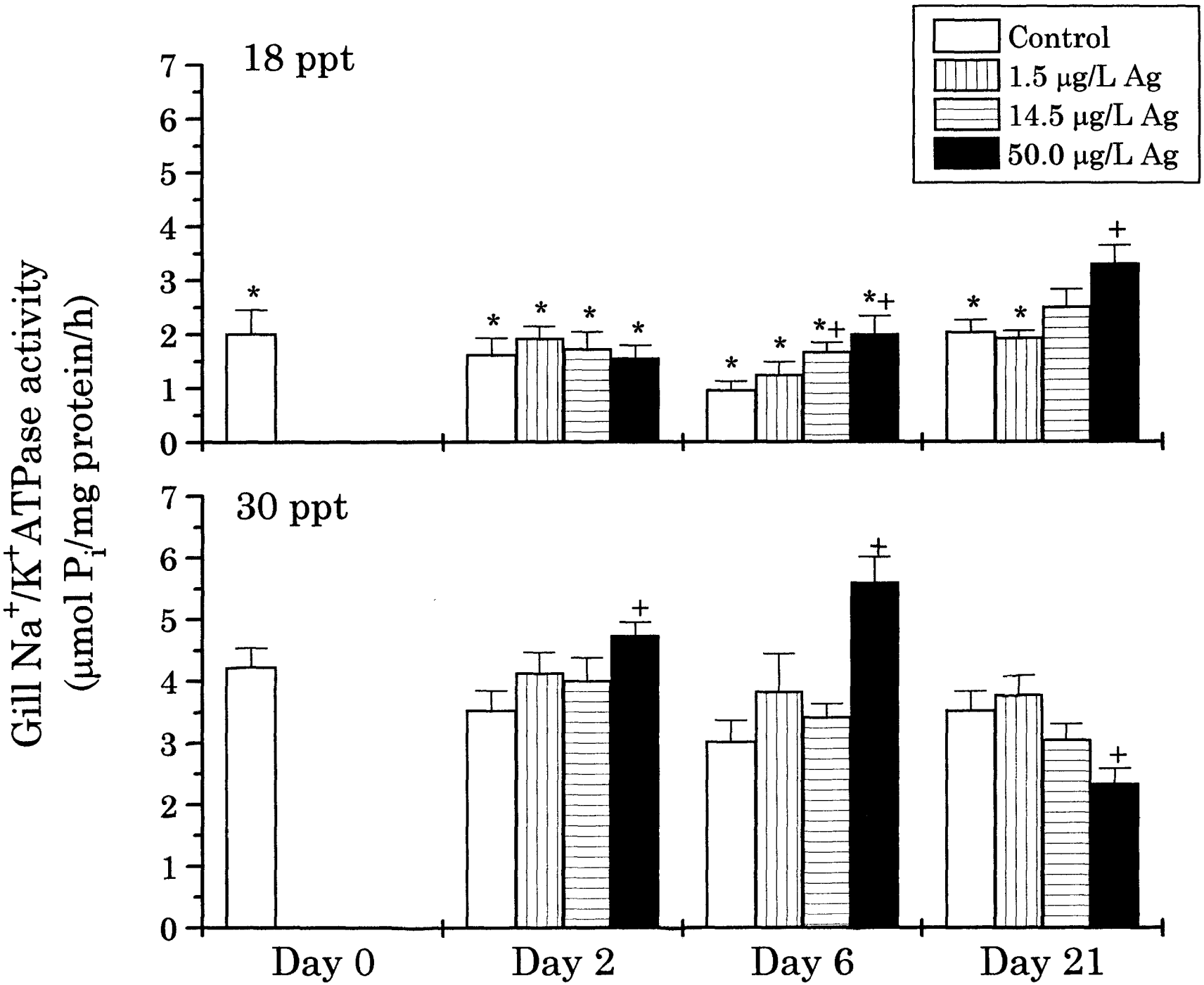

Intestine Na+/K+ATPase activity
(μmol Pi/mg protein/h)
0
1
2
3
4
Control
Day 7
Day 21
Control
1.5 μg/L Ag
14.5 μg/L Ag
50.0 μg/L Ag

Fig. 4.11. Na^+/K^+-ATPase enzyme activities for gills of 18 and 30 ppt tidepool sculpin exposed to Ag for 21 d. Open bars indicate control values (day 0, 7, and 21), while vertical stripes, horizontal stripes, and solid bars indicate activity levels in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (4). Plus signs (+) indicate a significant difference from the same day control value, while asterisks (*) indicate a significant difference between salinities.


Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag
18 ppt
30 ppt
Gill Na+/K+ATPase activity
(µmol Pi/mg protein/h)
0
1
2
3
4
5
6
7
Day 0
Day 2
Day 6
Day 21

one situation where the activity was higher at the lower salinity (50.0 μg/L Ag fish on day 21) (Fig. 4.12).

Activity levels in intestines of control sculpins at 18 ppt dropped significantly by day 6 and then recovered again by day 21. The only significant effect of silver was an enhancement of activity in fish exposed to 50.0 μg/L Ag on day 21 (Fig. 4.12).

Enzyme activity levels in 30 ppt control intestines dropped slightly on day 2, but then increased to be significantly higher than day 0 levels on day 21. Silver significantly decreased the activity (compared to same day control levels) in sculpins exposed to 1.5 μg/L Ag on days 2 and 21, and on days 6 and 21 in sculpins exposed to both 14.5 and 50.0 μg/L Ag (Fig. 4.12).

Drinking rate

There were no significant differences in drinking rate of tidepool sculpins seen between the two salinities. However, there was an effect of silver exposure on drinking rate. At 18 ppt, drinking rate was decreased significantly in fish exposed to all three silver concentrations on day 8. A similar trend was apparent at 30 ppt, but the changes were only significant in fish exposed to 50.0 μg/L on day 4, and 14.5 μg/L on day 8 (Fig. 4.13).

Fig. 4.12. Na^+/K^+-ATPase enzyme activities for intestines of 18 and 30 ppt tidepool sculpin exposed to Ag for 21 d. Open bars indicate control values (day 0, 7, and 21), while vertical stripes, horizontal stripes, and solid bars indicate activity levels in fish exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (4). Plus signs (+) indicate a significant difference from the same day control value, while asterisks (*) indicate a significant difference between salinities.

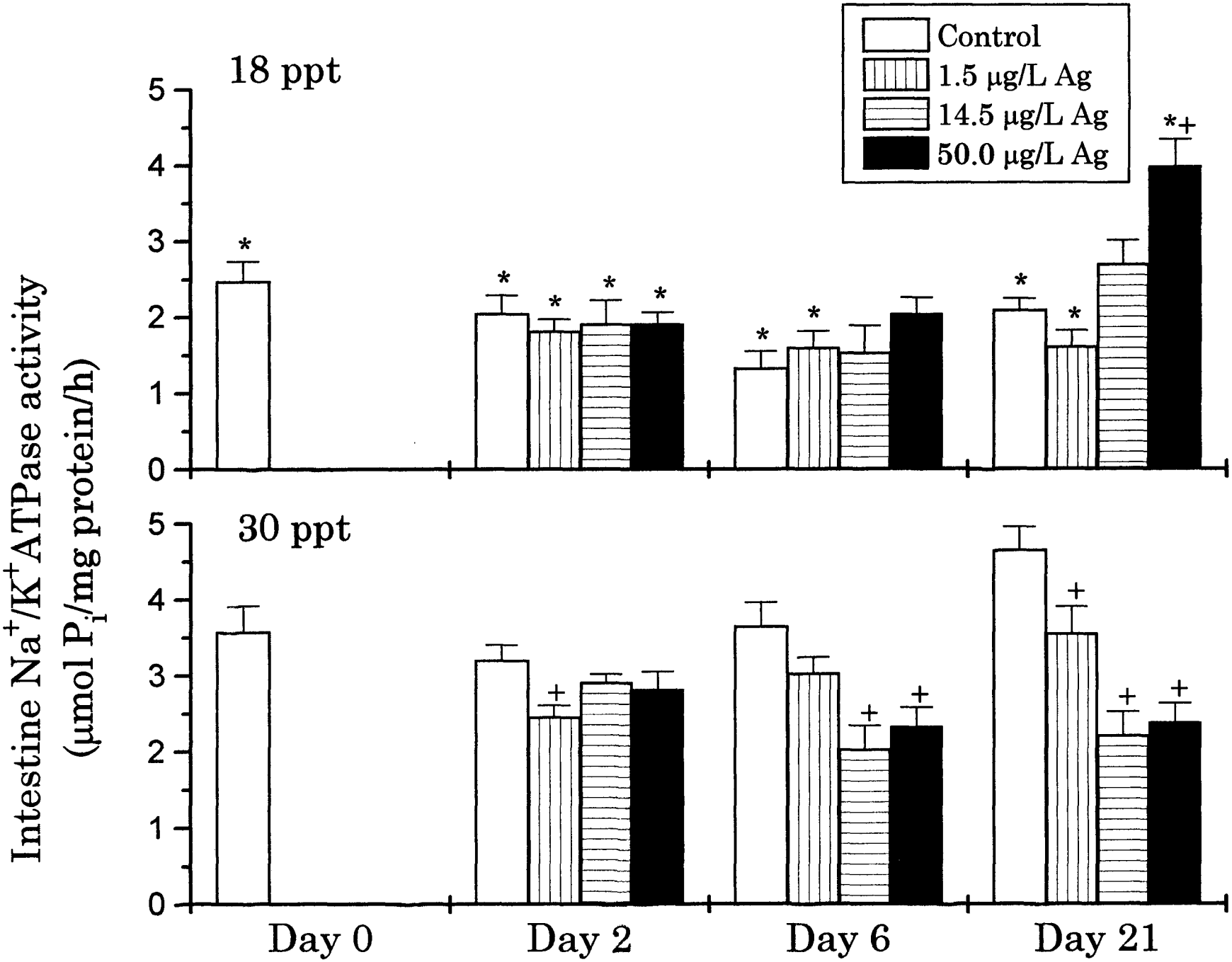
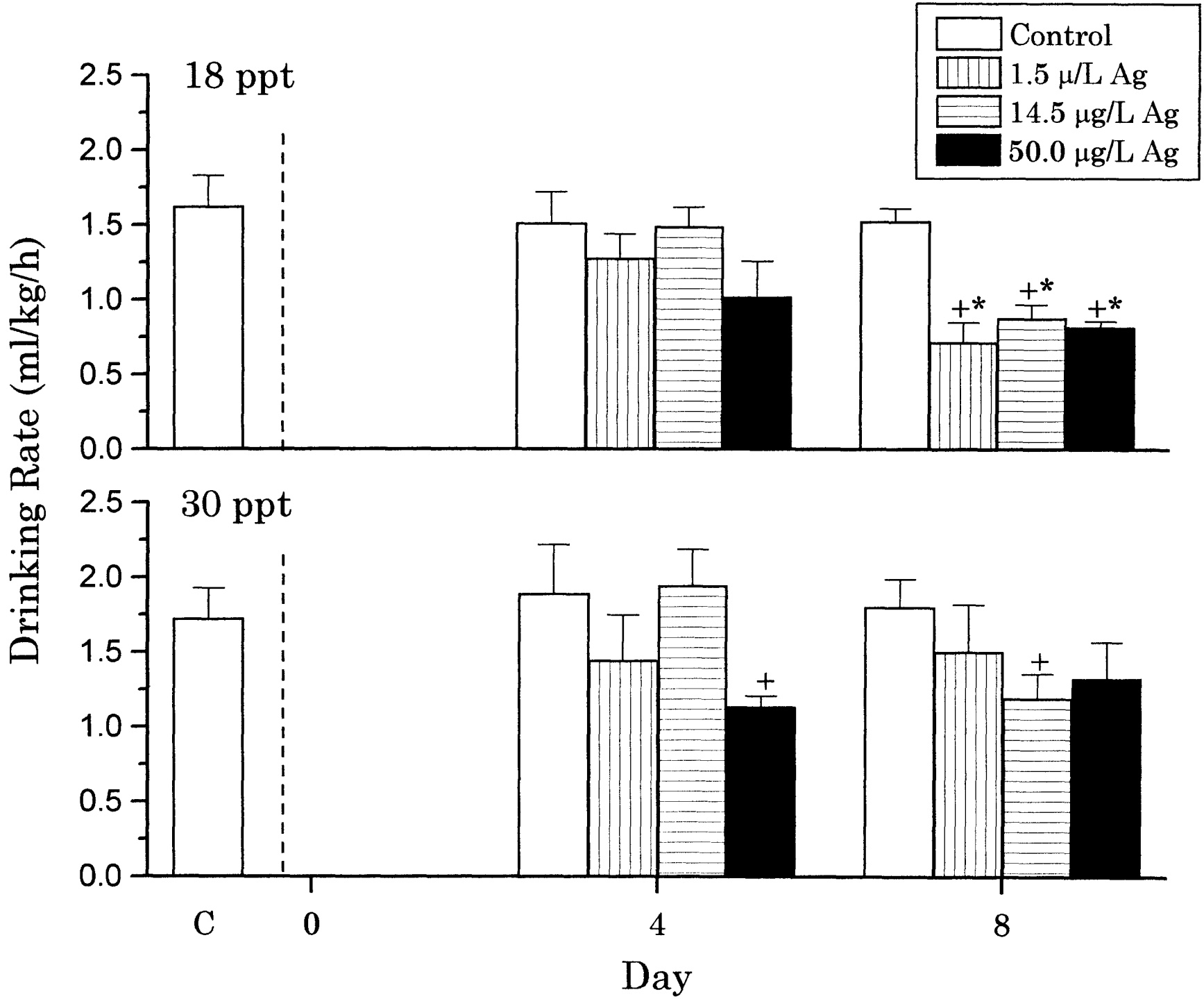

Control
1.5 µg/L Ag
14.5 µg/L Ag
50.0 µg/L Ag
18 ppt
30 ppt
Intestine Na+/K+ATPase activity
(µmol Pi/mg protein/h)
0
1
2
3
4
5
Day 0
Day 2
Day 6
Day 21

Fig. 4.13. Drinking rates in tidepool sculpins acclimated to 18 and 30 ppt and exposed to Ag (as $AgNO_3$) for 8 d. Open bars indicate control drinking rates, while vertical stripes, horizontal stripes, and solid bars indicate drinking rates in sculpins exposed to 1.5, 14.5, and 50.0 μg/L Ag, respectively. Data are means ± SEM (6). Plus signs indicate a significant difference from the same day control value, while asterisks indicate a significant difference between salinities.


Control
1.5 µ/L Ag
14.5 µg/L Ag
50.0 µg/L Ag
18 ppt
30 ppt
Drinking Rate (ml/kg/h)
2.5
2.0
1.5
1.0
0.5
0.0
+*
+*
+*
+
+
C
0
4
8
Day

DISCUSSION

At first glance, the accumulation of silver in the tissues of tidepool sculpins exposed to a high level of Ag (250 μg/L) for 48 h appears to contradict the previous results of Shaw et al. (1998) and chapter 3, since fish in full strength seawater accumulated equal or greater amounts of silver from the water in both the gills and intestines at all salinities. However, MINEQL+ modeling of silver speciation in the water showed that there was a small amount of $AgCl_{aq}$ (1 to 3% of total Ag) present in all salinities because of the higher Ag concentration. It has been seen that this complex is available for uptake by fish in freshwater (Hogstrand et al., 1996; Galvez and Wood, 1997; Hogstrand and Wood, 1998), and is probably the only silver species available for uptake from the water in marine fish (Shaw et al., 1998; Ch. 3). It is important to note that in fish acutely exposed (48 h) to 250 μg/L Ag, only the gills and intestines (the sites of uptake) accumulated substantial amounts of silver. The primary site of storage, the liver, exhibited little or no silver accumulation, suggesting that very little silver entered the fish over the 48 h period. On the other hand, long term exposure (21 d) to low levels (1.5 to 50.0 μg/L Ag) resulted in large amounts of silver accumulating in the gills, intestines, and livers of tidepool sculpins. This suggests that there was a time lag for internalization of silver. Silver must build up in the uptake tissues, and slowly get released into the blood of the fish, which then transports the silver to the liver for storage.

These data suggest that the usefulness of liver silver concentrations as an indicator of environmental exposure depends highly on the time scale of the exposure. It may be more environmentally relevant to monitor accumulation in uptake sites (gills likely respond most quickly in seawater teleosts) after acute high level exposures, while the liver would be more indicative of chronic low level exposures.

Ammonia excretion was only affected after chronic long term silver exposure. The decrease in ammonia excretion could have been due to either an inhibition of excretion or a decreased production of ammonia. Previous studies (Ch. 2; Hogstrand and Wood, 1996) have shown that silver causes an increase in plasma ammonia levels without any inhibition of ammonia excretion. Since silver did not accumulate in the acutely exposed livers, and no effect on J_{Amm} was seen, it suggests that the accumulated silver in the chronically exposed livers, interfered with ammonia production. Calabrese et al. (1977) showed that chronic exposure of winter flounder to 10 μg/L Ag for 60 d depressed liver transaminase activity, an enzyme involved in both ammonia production and protein synthesis. Liver transaminases (also known as aminotransferases) function in a reversible reaction that transfers the α-amino group of L-amino acids to α-ketoglutamate forming α-keto acids and glutamate (Lehninger et al., 1993).

The α-keto acids are than used as fuel in the citric acid cycle to form high energy molecules (ATP and NADPH). Glutamate is then deaminated in

the mitochondria to form NH_4^+ and α-ketoglutamate. When the L-amino acid is alanine, there is a direct effect on energy storage as deamination of alanine yields pyruvate, the starting material for gluconeogenesis (Guyton, 1991; Lehninger et al., 1993).

When there is excess food and energy available, glutamate and citric acid cycle intermediates are shuttled into protein synthesis pathways (Lehninger et al., 1993; Jobling, 1994). Decreased aminotransferase activity would eventually lead to decreased amounts of material available for both the citric acid cycle and protein synthesis pathways, as well as decreased ammonia production. With protein synthesis thought to account for 20 to 40% of the metabolic activity of a fish (Hawkins, 1991; Houlihan, 1991), a depression in these pathways would most likely lead to a depression in M_{O2} as well. Gould and MacInnes (1978) saw a depression in oxygen consumption in the cunner, however, this was an acute response to high levels of waterborne silver (96 h at 120-500 g/L Ag). Calabrese et al. (1977) studied winter flounder and did not see any effect of silver on M_{O2}, however, with such a long exposure (60 d), the possibility of acclimation to silver occurring was high. Acclimation usually occurs during the first 7 to 14 d of exposure and results in the fish recovering from any original damage (McDonald and Wood, 1993).

Another factor that may have affected M_{O2} in the fish, is a change in Na^+/K^+-ATPase enzyme activity. This enzyme accounts for 20 to 40% of the

metabolic rate in mammals, and is thought to be the second most energy demanding process in fish (Jobling, 1994). Silver's toxic mechanism in freshwater fish is via the inhibition of epithelial Na^+/K^+-ATPase enzymes which disrupts iono-regulation and leads to cardiovascular collapse (Wood et al., 1996a; Morgan et al., 1997; Ferguson and Hogstrand, 1998). Thus, if Ag elicited a similar inhibition on Na^+/K^+-ATPase activity in seawater fish, then it should also decrease the oxygen consumption rate. There was a decrease in Na^+/K^+-ATPase activity seen in the gills of midshipmen, while a tendency for increase was seen in gills of tidepool sculpins (except after 21 d at 50.0 μg/L Ag in 30 ppt seawater, where a clear inhibition occurred). This difference may be due to the type of environment in which these two species live. Midshipmen are generally open ocean, bottom dwellers and are not accustomed to changes in salinity, therefore their iono-regulatory mechanism would be at a set rate and slow to change. Tidepool sculpins on the other hand, face changes of salinity on a daily basis and have to be ready to deal with a change in external salinity rapidly, therefore, their iono-regulatory mechanism would be very adaptable and quick to change. Thus, although silver most likely inhibited enzyme activity in both fish, the sculpins were able to counteract the inhibition by producing more Na^+/K^+-ATPase molecules. The decreased activity seen on day 21 in 30 ppt tidepool sculpins exposed to 50.0 μg/L Ag, may have been an effect of attrition. The sculpins

may not have had the energy to maintain such high levels of ATPase activity, and finally succumbed to the inhibitory effect of silver.

The Na^+/K^+-ATPase enzyme is primarily involved in fish iono-regulation, but also plays an important role in water uptake in marine teleosts. The uptake of water in these fish is through osmotic passage into the intestinal epithelium following salt uptake (Evans, 1993; Fuentes and Eddy, 1997). An interference in iono-regulation would lead directly to an interruption in osmo-regulation. Thus, significant build up of silver in the intestines may have a direct inhibitory effect on the uptake of water from the intestinal lumen and thus on the drinking rate. It was seen that although silver did not have an effect on midshipmen intestinal Na^+/K^+-ATPase activity, it did alter tidepool sculpin intestinal activity. ATPase activity was decreased in fish at 30 ppt during silver exposure, while it was increased in fish at 18 ppt. In contrast, the drinking rate was inhibited at both salinities. This makes sense in 30 ppt fish, since lower Na^+/K^+-ATPase activity means less Na^+ and Cl^- ions taken up into the epithelial cells, and thus less water taken up from the lumen, decreasing the drinking rate. At 18 ppt, despite increased intestinal enzyme activity, the drinking rate decreased at all silver concentrations. These fish may have tried to decrease their drinking rate to decrease the amount of silver to which they were exposed. They then increased the Na^+/K^+-ATPase activity to take up as much water as possible from the decreased volume available.

Another way for fish to deal with the adverse effects of a toxicant is to simply decrease energy demand. This would lower the need to breakdown proteins and also lower ammonia production and overall metabolism. The movements of tidepool sculpins during ammonia production experiments did not appear to change, however, no formal measurement of activity was made. Therefore, we cannot be sure as to whether the silver had a direct interference with energy expenditure in the fish, or whether the fish simply became inactive. However, during sublethal copper exposure, a metal with a similar toxic mechanism, sea catfish showed increased activity (Steele, 1983).

It is interesting to note, that despite the ability of copper to inhibit Na^+/K^+-ATPase activity in vitro, no change in total activity was detected during in vivo studies (Stagg and Shuttleworth, 1982b). Stagg and Shuttleworth (1982b) found that this was due to an increase in the number of enzyme units. Thus, even though the individual enzyme unit activity was decreased, the total activity was maintained.

This situation was seen in the current study with silver exposure in tidepool sculpins. Despite the ability of Ag to inhibit Na^+/K^+-ATPase in an in vitro preparation (Morgan et al., 1997), the activity of silver exposed sculpins was either unchanged or increased. The inability of the midshipmen to maintain ATPase activity strengthens the idea that these fish do not respond well to iono-regulatory interference.

This increase in number of enzyme units was thought to be due to increased plasma cortisol levels that was due to the general stress response to metals seen in fish (Schreck and Lorz, 1978; Stagg and Shuttleworth, 1982b). It is not known if the same is responsible for increased enzyme numbers during sublethal silver exposure. Although we are unaware of any cortisol results in seawater fish exposed to silver, cortisol levels did rise in freshwater fish acutely exposed to silver (Ch. 2).

Another aspect for further research is the possibility of acclimation occurring in fish chronically exposed to sublethal silver levels. The inhibition of oxygen consumption here and in cunners (Thurberg and Collier, 1977) was not seen in winter flounder exposed to silver for 60 d (Calabrese et al., 1977), suggesting that fish are able to recover from the initial impairment of basic metabolism. Whether or not acclimation does occur, this study shows that low environmental silver levels can have a negative effect on fish. Not only does silver interfere with iono- and osmo-regulation, but it may also affect the activity of fish, which could lead to decreased feeding rates, as well as decreased ability to avoid predation. It may also affect other energy demanding activities such as smoltification, reproduction, and territory defense.

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