Effects of waterborne silver in a marine teleost, the gulf toadfish (*Opsanus beta*): Effects of feeding and chronic exposure on bioaccumulation and physiological responses

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ABSTRACT

Marine teleosts drink seawater, and the digestive tract is a key organ of osmoregulation. The gastro-intestinal tract therefore offers a second site for the potential uptake and toxicity of waterborne metals, but how these processes might interact with the digestive functions of the tract has not been investigated previously. We therefore compared the responses of adult gulf toadfish (*Opsanus beta*, collected from the wild) to a chronic 22 d exposure to waterborne Ag (nominally $200\,\mu g\,L^{-1} = 1.85\,\mu mol\,L^{-1}$), in the presence or absence of daily satiation feeding (shrimp). Ag exposure did not affect voluntary feeding rate. Feeding reduced the net whole body accumulation of Ag by >50%, with reductions in liver concentrations (high) and white muscle concentrations (relatively low) playing the largest quantitative roles. Feeding also protected against Ag buildup in the esophagus-stomach and kidney, and increased biliary and urinary Ag concentrations. The gill was the only tissue to show the opposite response. Although terminal plasma Na$^+$, Cl$^-$, and Mg^{2+} concentrations were unaffected, there were complex interactive effects on osmoregulatory functions of the gastro-intestinal tract, including drinking rate, gut fluid volumes, and intestinal base secretion rates. At the end of the exposure, the plasma clearance kinetics of an arterially injected tracer dose of ^{110m}Ag were faster in toadfish that had been chronically exposed to waterborne Ag, and ^{110m}Ag accumulation in their red blood cells was reduced. After 32 h, higher amounts of ^{110m}Ag were found in bile and urine, but lower amounts in the intestine of the Ag-exposed toadfish; there were no other differences in tissue-specific distribution. The results suggest that feeding reduces waterborne Ag uptake through the digestive tract and alters physiological responses, while chronic exposure enhances regulatory functions. The time-dependent actions of the liver in Ag scavenging and detoxification are highlighted.

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1. Introduction

Over the past three decades, there has been extensive research on the bioavailability and toxicity of Ag to aquatic organisms, especially fish, motivated by concerns about the potential toxicity of Ag emanating from photographic processing, mining, and other industrial sources (reviewed by Eisler, 1996; Hogstrand and Wood, 1998; Wood et al., 1999; Andren and Bober, 2002; Gorsuch et al., 2003). The major focus has been on freshwater animals, and from this, a fairly clear story has emerged. In fresh water, acute waterborne tox-

icity is mainly due to the free silver ion (Ag$^+$) which interferes with Na$^+$ uptake at the gills, whereas internal bioaccumulation occurs due to uptake of Ag across the gills from both the neutral silver chloride complex (AgCl$_0$) as well as the free Ag$^+$ ion. Marine organisms have received far less study, but from the limited information available, it is very clear that both acute waterborne Ag toxicity and the rate of bioaccumulation are much lower than in freshwater organisms. This likely reflects speciation changes due to the high [Cl$^-$] concentration in full strength seawater which essentially reduces Ag$^+$ and AgCl$_0$ to negligible fractions (see Ward and Kramer, 2002), as well as competitive interactions among ions in seawater.

However, the situation may be far more complex in marine fish, which have two potential target surfaces, the gut as well as the gills. In contrast to freshwater fish which imbibe little water, most seawater fish drink the external medium continuously for the purposes of osmoregulation (reviewed by Grosell, 2006). The relatively high levels of Ag required to cause acute toxicity in sea-

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water appear to exert their toxic effects by interfering with iono- and osmo-regulatory processes at both sites (Grosell et al., 1999; Hogstrand et al., 1999; Grosell and Wood, 2001; Webb et al., 2001). At the gut, this occurs by reduction of the drinking rate and by interference with the mechanisms of transepithelial ion transport and associated water absorption. Indeed by employing continual intestinal perfusion with clean saline to protecting the gut surface from waterborne Ag, Grosell and Wood (2001) were able to reduce physiological symptoms of toxicity by 50–60% in the marine lemon sole.

It remains unclear whether the gut also serves as a site of Ag uptake for bioaccumulation. Grosell and Wood (2001) found no reduction in internal bioaccumulation of Ag in these lemon sole where the gut was protected by perfusion. However, *in vitro* preparations of the teleost intestine certainly transport Ag (Hogstrand et al., 2002; Ojo and Wood, 2007) and several *in vivo* studies have reported circumstantial evidence of gut uptake—i.e. elevated levels of Ag in intestinal tissue, and declining levels of Ag in intestinal fluid as it progresses down the tract (Hogstrand et al., 1999; Grosell et al., 1999; Webb and Wood, 2000; Wood et al., 2004). However during chronic exposures, internal patterns of Ag bioaccumulation were different than during acute exposures, and Ag burdens appeared to reach a plateau in intestinal and internal tissues of several marine species (Webb and Wood, 2000). Indeed in our earlier studies on the gulf toadfish, the elevated intestinal Ag burdens seen during short-term (24 h) exposure (Wood et al., 2004) were not detectable after 6-d exposure (Nichols et al., 2006). These observations suggest that physiological regulation occurs during extended exposures.

An important factor with respect to waterborne Ag uptake and toxicity in marine fish that has been overlooked to date is the other role of the gut—feeding, digestion, and assimilation. We hypothesized that if the gut were a target, then there would be interactive effects of feeding and waterborne Ag exposure on Ag bioaccumulation and physiological indices of toxicity. With this background in mind, we extended our earlier studies on Ag in the toadfish (Wood et al., 2004; Nichols et al., 2006) to examine the impact of chronic exposure (22–23 d) to a high but sublethal concentration of waterborne Ag in animals that were fasted throughout, or fed daily to satiation. Parallel non-exposed fasted and fed controls were also examined. Daily food consumption was monitored, and at the end of the exposure, total and organ-specific Ag burdens were quantified, and physiological indices of toxicity were assessed—plasma ions, drinking rate, and transport of water, ions, and acid–base equivalents in the intestine via *in vitro* gut sac preparations. In addition, the clearance and internal distribution of an arterially injected tracer dose of radioactive ^{110m}Ag were examined in the two fasted treatments, to evaluate the hypothesis that changes in physiological regulation of Ag occur during chronic exposure.

2. Materials and methods

2.1. Experimental animals

Adult gulf toadfish (*Opsanus beta* Goode and Bean, 46.4 ± 4.8 g, $N = 80$, range 23–123 g) were collected in May by commercial shrimp fishermen using a roller trawl. In the laboratory, the fish were held in flowing seawater (salinity = 33–35 ppt, temperature = 24–25 °C, natural photoperiod) in 50-L aquaria at a density of about 25 g L^{-1}. During this time they were treated twice (on days 1 and 3) with malachite green (0.05 mg L^{-1}) plus formalin (15 mg L^{-1}) in seawater to remove any ectoparasites (Wood et al., 1997). They were fed once with shrimp on days 4–6, and not fed thereafter, until the experimental exposures started on day 8 post-collection.

2.2. Exposures

Toadfish with a mean weight of about 40 g were selected and randomly allotted into four treatment groups of 20 fish each. Two of these were weighed in bulk for calculation of ration level. Each group was then placed in a 70-L tank (loading density = approximately 13 g L^{-1}) served with running seawater at 200 mL min^{-1}. Each tank contained PVC pipes for individual shelters and air stones for vigorous aeration. The four treatments were (i) control-fed; (ii) control-fasted; (iii) Ag exposed-fed; and (iv) Ag exposed-fasted. These fish were used for measurements of Ag bioaccumulation and physiological parameters (plasma ions, drinking rates, and *in vitro* gut sac experiments) at the end of 22–23 d exposure.

The two control treatment tanks received clean seawater, and the two Ag-exposure tanks received their flow from a common mixing head tank which received 530 mL min^{-1} of clean seawater by gravity feed and 0.12 mL min^{-1} of a AgNO$_3$ solution (0.88 g Ag L^{-1} as AgNO$_3$, analytical grade, Fisher Scientific, in 0.1 N ultrapure HNO$_3$), the latter delivered from a light-shielded bottle by a peristaltic pump. The resulting nominal Ag concentration in the water flowing to the exposure tanks was 200 μg L^{-1} (1.85 μmol L^{-1}). The AgNO$_3$ solution had no detectable effect on seawater pH (8.05 ± 0.05). Water samples were taken daily (at the end of the feeding period, see below) to monitor total and dissolved Ag concentrations, the latter filtered through 0.45-μm Gelman syringe filters. Samples were immediately acidified (1% ultrapure HNO$_3$, Fisher Scientific) for storage prior to later analysis (see below).

At approximately 15:00 h each day, fish in the two feeding treatments were fed a pre-weighed meal (4% ration) of whole dead shrimp (obtained from a local market) from which the telsons had been removed. Three hours later, remaining pieces of shrimp were retrieved, blotted, and weighed, and the consumed ration calculated from the weight difference. Water samples were taken at this time.

Additional exposures (control-fasted, Ag exposed-fasted) were run in parallel under similar conditions (10 fish per 70-L tank) with larger toadfish of a size (mean weight of about 80 g) sufficient for arterial cannulation. These fish were used for the ^{110m}Ag clearance and tissue distribution experiments.

2.3. Sampling

After 22 d, 6–8 toadfish from each treatment were sacrificed for bioaccumulation and plasma ion measurements, and gut sac experiments, whereas 6–8 toadfish were used for bioaccumulation and drinking rate measurements the following day. There were no significant differences for any tissue, in any treatment, between Ag bioaccumulation data obtained on the two sampling days, so the data were combined. Note that samples of the digestive tract and its contents for Ag analyses could only be taken from the fish on the first day of sampling, because of its removal for drinking rate measurements in the fish on the second day. The Ag content of the anterior intestine was not analyzed, as it was used for gut sac experiments on the first day. In general, several fish were rejected from each experiment because of surface wounds (probably due to aggression), and in some cases urine, bile, plasma, or gut fluid samples could not be obtained.

On the first day of sampling, fish were netted individually into a 10-L bucket containing a lethal concentration of neutralized MS-222 (2.5 g L^{-1}, Syndel Labs) in seawater of the appropriate composition (i.e. with or without 200 μg L^{-1} of Ag as AgNO$_3$). The fish were weighed and blood samples (0.5 mL) were rapidly drawn by caudal puncture into lithium-heparinized syringes and discharged into pre-weighed plastic centrifuge tubes. The plasma was sepa-

rated by rapid centrifugation (13,000 × g for 2 min), then decanted and aliquoted for ionic and Ag analyses, and the tubes re-weighed to determine the mass of the red blood cell pellet. The fish itself was rinsed in clean seawater, and then the coelomic cavity was opened. The gastro-intestinal tract was tied off into segments of esophagus-stomach (a single organ in the toadfish), anterior intestine, mid intestine, and posterior intestine using silk suture. Gut fluids from each segment were then drained into pre-weighed centrifuge tubes, which were re-weighed to determine volumes. The anterior intestine was then used for *in vitro* gut sac experiments (see below), and the other three segments were placed into pre-weighed plastic vials which were re-weighed to determine tissue weights for later digestion. The gall bladder and the larger lobe of the urinary bladder were removed intact and drained into pre-weighed tubes to provide bile and urine samples. Liver, kidney, gill filaments (trimmed from arches), and spleen were removed in their entirety, plus approximately 0.5 g of epaxial white muscle. All tissues were blotted and weighed before being placed into digestion vials for later Ag analysis. These weights were used to determine organ somatic indices, calculated as individual organ weight divided by total body weight. The remaining tissues were taken as a single sample ("carcass") which was weighed, diluted with a known amount of distilled water, and ground to a fine paste in a Waring blender. An aliquot (~7 g) of this paste was preserved for later analysis.

All Ag levels were determined as detailed by Hogstrand et al. (1996). Briefly, acidified water samples were diluted 100-fold with 1% ultrapure HNO_3 (Fisher Scientific), while weighed tissue and body fluid samples were digested overnight at 80 °C in appropriate volumes (generally 3–5 volume:weight) of concentrated ultrapure HNO_3. 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. Ag levels were read using graphite furnace AAS (Varian 1275 fitted with a GTA-95 atomizer) using a certified standard (Aldrich). All Ag concentrations in individual organs were expressed on a wet tissue weight basis. The whole body Ag concentration was calculated as the sum of all the organ-specific contents including the carcass, divided by the total mass of all of the organs plus carcass. On average this accounted for 94% of the original body mass.

2.4. Drinking rate measurements

On the second day of sampling, drinking rates were measured on the remaining 6–8 fish in each treatment, using the inert, non-absorbed drinking rate maker ^{3}[H]polyethylene glycol, M.W. = 4000 (^{3}[H]PEG-4000; specific activity: 2050 mCi g^{-1}; NEN-Dupont) in a protocol similar to that of Wilson et al. (1996). The fish were fed (or not, as appropriate) in the usual fashion. Drinking rate measurements started 1 h after addition of the meal and continued for an 8-h to 10-h period so as to encompass the initial period of digestion. The water flow to the exposure tank was stopped while aeration continued, the water level was gently lowered to 10 L, and 12.5 μCi of ^{3}[H]PEG-4000 was added. Triplicate water samples were taken at the beginning and end to monitor ^{3}[H]PEG-4000 radioactivity which in fact exhibited no detectable change. At the end, the fish were individually netted into appropriate terminal anaesthetic solutions as above. Blood samples were taken by caudal puncture, rectal fluid was sampled from the anus (could not be obtained in all fish), and then the fish were dissected exactly as described earlier, except that the whole digestive tract was first ligated at anterior esophagus and anus, and removed intact for analysis of ^{3}[H]radioactivity. The same organs and the homogenized carcass were preserved for later digestion and analysis of Ag content.

The excised gastro-intestinal sac, together with its fluid contents, was weighed, then homogenized in 3 volumes of 10% $HClO_3$, allowed to incubate for 6 h with constant shaking, then centrifuged at 500 × g for 7 min. The clear protein-free supernatant (0.5 mL aliquots + 2.5 mL seawater), together with the water samples (3 mL) and rectal fluid samples (10–200 μL plus sufficient seawater to make 3 mL) were analyzed for ^{3}[H]radioactivity by scintillation counting, using 10 mL Ecolume fluor (ICN) and a Beckman LS1801 counter. Quench correction was performed using the external standard ratio method and checked by internal standardization. Drinking rate was calculated from the total ^{3}[H] radioactivity of the gastro-intestinal tract and its contents, factored by time, mass of the fish, and mean radioactivity of the external sea water. The rectal fluid samples served as a check that there was no loss of the label through the rectum by the end of the 8–10 h measurement period; in none of the sampled fish (N = 14) was any radioactivity found in these samples.

2.5. In vitro gut sac experiments

Experiments were performed on preparations of the anterior intestine, taken from fish sampled on the first day. The serosal saline (composition in Grosell and Genz, 2006) was representative of toadfish extracellular fluid and contained 3 mmol L^{-1} glucose. The mucosal saline (NaCl = 49, $MgSO_4 \cdot 7H_2O$ = 77.5, $MgCl_2 \cdot 6H_2O$ = 22.5, KCl = 5.0, $CaCl_2 \cdot 2H_2O$ = 5.0, $NaHCO_3$ = 5.0 mmol L^{-1}; pH 7.9) was based on our analyses of toadfish intestinal fluid (Grosell et al., 2004). Mannitol was added to the mucosal saline to raise its osmolality equal to that of the serosal saline (320 mOsm kg^{-1}). Both salines were pre-equilibrated with a mixture of 0.5% CO_2 and 99.5% O_2 provided by a Wosthoff 301 Digamix 2M301/a-F gas-mixing pump (Bochum, Germany).

Methods were based on those of Grosell et al. (1999, 2005). In brief, a short length of heat-flared polyethylene (PE-60) tubing was tied in the anterior end of the intestine through which 20 mL of mucosal saline was flushed to clear the gut contents. The posterior end was then ligated with silk suture, and the sac was moderately overfilled with mucosal saline which had been radio-labeled with ^{22}Na and ^{36}Cl (Amersham, 0.2 and 0.5 μCi mL^{-1}, respectively) to allow for measurements of unidirectional fluxes of these ions. A 300 μL initial mucosal saline sample was obtained by flushing it back and forth several times to ensure complete mixing before the sample was taken. The catheter was then sealed, the preparation was blotted dry, weighed to 0.1 mg accuracy, and placed in 15 mL of serosal saline continually gassed with the 0.5% CO_2 + 99.5% O_2 mixture. A 1-mL sample was taken for measurement of initial serosal radioactivity. At the end of the 3-h flux period, a final serosal sample was taken, and the sac was blotted and weighed again; the change in weight provided a gravimetric measurement of fluid flux. The contents of the sac were then drained into a centrifuge tube, and processed for analyses as the final mucosal saline sample. The sac was then cut open, thoroughly blotted dry, after which the mass of the intestinal tissue (including catheter) was determined. After overnight storage at 4 °C for muscle relaxation, the gross area of the intestinal tissue was determined using graph paper.

All saline samples were assayed for radioactivity on both a Packard Cobra II gamma counter (detecting ^{22}Na only) and a Beckman LS1801 scintillation counter (detecting combined ^{22}Na + ^{36}Cl radioactivity). For scintillation counting, all samples were made up to 3 mL with seawater and added to 10 mL Ecolume fluor (ICN). Tests showed that quench was constant. The dual-label subtraction approach outlined by Grosell et al. (2005) was used to separate the ^{36}Cl cpm from the ^{22}Na cpm. Mucosal samples were assayed for concentrations of Na$^+$ (Varian 1275 flame atomic absorption spectrophotometer), Cl$^-$ (Radiometer-Copenhagen CMT10 coulometric chloridometer), total CO_2 (Corning 965 total CO_2 analyzer), and

pH (Radiometer-Copenhagen GK2401C glass combination electrode connected to a Radiometer PHM 71 blood–gas meter). Total basic equivalent concentrations ($[HCO_3^-] + 2[CO_3^{2-}]$) were calculated from the total CO_2 and pH measurements by means of the Henderson–Hasselbalch equation using the appropriate pK' and pK'' values and CO_2 solubility coefficient at 1/3 strength seawater at 24 °C. The pH dependence of pK' was taken into account using the nomogram of Severinghaus et al. (1956). Grosell et al. (1999) validated this method for intestinal fluid samples, noting it yielded basic equivalent concentrations virtually identical to those determined using the double-end point titration method (Hills, 1973). Unidirectional and net fluxes of Na^+ and Cl^-, and net fluxes of basic equivalents and fluid across the anterior intestine were calculated exactly as described by Grosell et al. (2005), and expressed per unit surface area.

2.6. ^{110m}Ag clearance and tissue distribution experiments

These experiments were performed on the additional, larger toadfish set up in parallel exposures in two of the treatment groups—control-fasting and Ag exposed-fasting. These fish were anaesthetized in appropriate water (i.e. with or without 200 µg L^{-1} of Ag as AgNO$_3$), fitted with indwelling arterial catheters exactly as described by Wood et al. (1997), and then placed in individual 2.0 L containers. The containers were fitted with individual PVC shelters and air stones, and were served with flowing seawater of the appropriate composition.

After 36-h recovery, the water inflow was stopped and the volume was set to 1.5 L in each container. Each fish was injected via the arterial catheter with a tracer dose (10 µCi kg^{-1} in 1 mL kg^{-1} of serosal saline) of ^{110m}AgNO$_3$ (RISØ Nuclear Research Reactor, Risø, Denmark). The injection was washed in with a further 2 mL kg^{-1} of saline. Blood samples (100 µL) were withdrawn from the catheter without disturbance at 10, 20, 40, 80, 160, 320, 600, and 1920 min (32 h) post-injection. Water samples (5 mL) were also taken at each time, and the water in the container was exchanged at 12 h and 24 h. Blood samples were immediately centrifuged in pre-weighed tubes (13,000 × g for 2 min) to separate plasma from red blood cells. The volume of each fraction was measured gravimetrically, and counted, together with the injection stock and the water samples, on a Packard Cobra II gamma counter. The precautions in terms of energy window selection for ^{110m}Ag counting recommended by Hansen et al. (2002) were adopted. After the final sampling at 32 h, the fish was sacrificed by a lethal concentration of neutralized MS-222 (2.5 g L^{-1}, Syndel Labs), and the organs dissected out as in the preceding series, and processed for ^{110m}Ag radioactivity analysis on the same gamma counter.

2.7. Statistical analyses

Data have been expressed as means ± 1 SEM (N) where N refers to number of fish in each treatment. Data were first tested for normality and homogeneity of variances. Where necessary, data were log-transformed prior to further analysis to pass Bartlett's chi square test for homogeneity of variances, and ratio or percentage data were subjected to arcsine transformation. Data were then analyzed by one-way ANOVA, followed by Tukey's honestly significant difference test to detect specific differences (Statistix for Windows). In those few instances where the data still did not pass Bartlett's test, the non-parametric Kruskal–Wallis signed ranks test was used in place of the ANOVA and Tukey's test. Simple comparisons were made using Student's unpaired or paired t-test (two-tailed), as appropriate. A significance level of $P < 0.05$ was used throughout.

The blood ^{110m}Ag time-series data were analyzed by fitting two- or three-phase exponential models (Causton, 1983) to the data for individual fish, so as to calculate rate constants of clearance (frac-

tional loss per hour). Breakpoints were chosen so as to maximize r^2 values for each phase, starting with the shortest one.

3. Results

3.1. Water Ag levels, feeding and mortality

The target level of total water waterborne Ag (1.85 µmol L^{-1} = 200 µg L^{-1}) was achieved in both fed and fasted treatments (2.07 ± 0.14 and 2.05 ± 0.18 µmol L^{-1}, respectively, N = 22) over the 22–23 d exposures. Of this, the dissolved fraction was about 65% (1.35 ± 0.25 and 1.25 ± 0.34 µmol L^{-1}, respectively, N = 22) in both treatments. Waterborne Ag was below detection (<0.009 µmol L^{-1}) in the control exposures. Daily food consumption was unaffected by the presence or absence of chronic Ag exposure (2.89 ± 0.20 and 2.46 ± 0.21% d^{-1}, respectively, N = 22). There were no mortalities in any treatment.

3.2. Bioaccumulation of Ag

There were measurable amounts of background Ag (approximately 500 pmol g^{-1}) in the whole bodies of these wild-collected toadfish, which did not change with feeding or fasting in the control treatments (Fig. 1A). Individual tissues ranged from a low of about 300 pmol g^{-1} in white muscle (Fig. 1B) up to a high of about 1350 pmol g^{-1} (Fig. 2A) in the esophagus-stomach. Again these values did not differ between fed and fasted control animals.

Chronic waterborne Ag exposure for 22–23 d raised the whole body Ag burden significantly by 44% in fasted animals (Fig. 1A). Feeding appeared to be protective, reducing the increase to 17%, a non-significant elevation. Two tissues accounted for most of this difference, the white muscle, which has low [Ag] (Fig. 1B) but comprises about 50% of the body mass, and the liver which has much higher concentrations (Fig. 1C), but accounts for less than 3% of the body mass. Feeding completely eliminated the significant 32% increase in white muscle [Ag] (Fig. 1B), and greatly attenuated the massive 20-fold increase in liver [Ag] (Fig. 1C). Note however, that the liver declined to about one third of its original mass in both fasted treatments (Table 1), so the liver's contribution to the whole body burden was not as great as indicated by the simple ratio of concentrations illustrated in Fig. 1C. Nevertheless, feeding still significantly reduced the total organ Ag burden in the liver by more than 50%. No other tissue differed in mass (expressed as a % of total body mass) as a result of the experimental treatments; all were very close to values reported in an earlier study (Wood et al., 2004) so they have not been tabulated here.

The esophagus-stomach was also completely protected by feeding against additional Ag bioaccumulation, whereas [Ag] in this tissue doubled in fasted animals exposed to waterborne Ag (Fig. 2A). A similar trend was seen in the intestine (mid + posterior combined), but there were no significant differences among treatments (Fig. 2B). Note however, that in all four treatments [Ag], in intestinal tissue was significantly lower than in the esophagus-stomach. Ag concentrations in intestinal fluids were rather variable and N numbers were low (Table 2), as insufficient fluid was available for

Table 1
The influence of feeding or fasting on the percentage contribution of the whole liver and the bile to the total body mass of toadfish exposed to waterborne Ag (nominally 1.85 µmol L^{-1}) or control seawater for 22–23 d. Means ± 1SEM.

	Fasted control	Fasted Ag	Fed control	Fed Ag
Liver	0.76 ± 0.09^A	0.73 ± 0.10^A	2.47 ± 0.31^B	2.22 ± 0.44^B
Bile	0.19 ± .01^{AB}	0.26 ± 0.04^B	0.15 ± 0.02^A	0.14 ± 0.03^A

Notes: (1) N = 10–13 for all means. (2) Within each tissue or fluid, means not sharing the same letter are significantly different ($P < 0.05$).
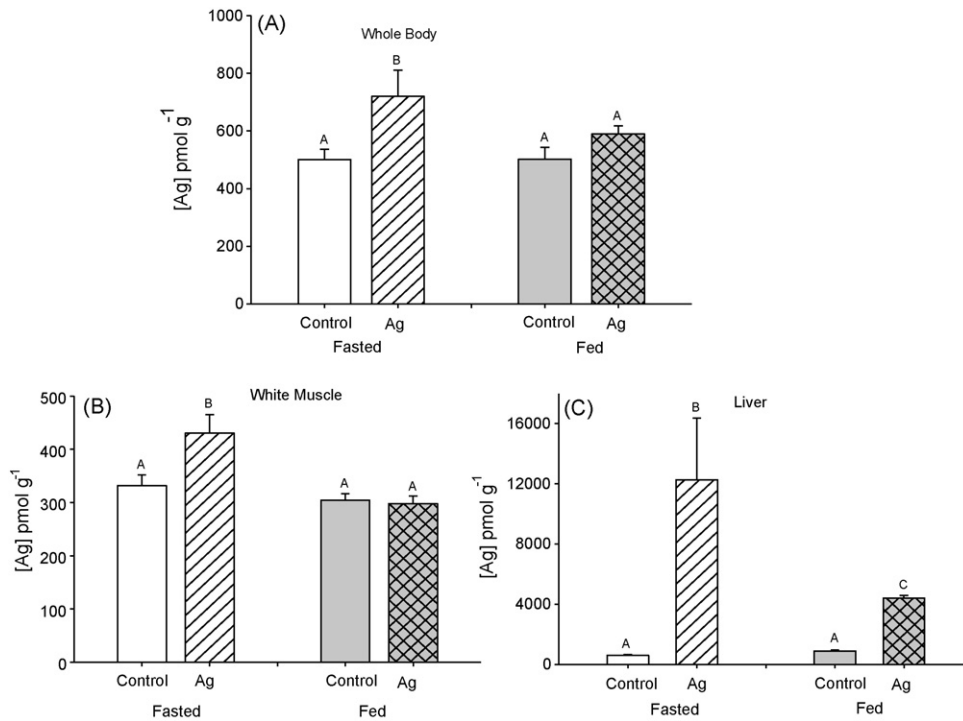
Fig. 1. The influence of feeding or fasting on Ag concentrations (pmol g^{-1} wet weight) in (A) the whole body, (B) the white muscle, and (C) the liver of gulf toadfish exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22–23 d. Note the different scales in the three panels. Means ± 1 SEM (N = 11–13). Means not sharing the same letter are significantly different ($P < 0.05$).

collection in some animals. Waterborne Ag exposure tended to elevate intestinal fluid [Ag] (significant in the mid-intestine only), but all values were below waterborne Ag levels (∼2060 pmol mL^{-1}). There were no substantive differences between fed and fasted animals.

The other two osmoregulatory organs, gills (Fig. 3A) and kidney (Fig. 3B), exhibited contrasting trends. Whereas a significant 40% elevation in kidney Ag burden in fasted animals was again prevented by feeding (Fig. 3B), gill [Ag] increased more than 3-fold in fed animals, but did not change at all in fasted toadfish exposed to waterborne Ag (Fig. 3A).

With regard to possible excretory pathways, qualitatively different responses were seen between bile (Fig. 4A) and urine (Fig. 4B). Biliary Ag concentrations almost doubled in fasted animals in response to waterborne Ag exposure. In fed animals, elevated levels occurred regardless of whether the fish had been exposed to waterborne Ag or control seawater. Not unexpectedly, feeding appeared to influence gall bladder function, with somewhat higher biliary volumes present in fasted animals (Table 1). Urinary Ag concentrations more than tripled in fed toadfish exposed to waterborne Ag, while a much smaller increase was not significant in fasted animals (Fig. 4B). There were no differences among treatments

in urinary volumes present in the bladders of toadfish (data not shown).

The experimental treatments had no significant effects on Ag levels in red blood cells, and there were only minor differences in plasma Ag levels among treatments (Table 2). Notably, absolute red blood cell [Ag] was about 5-fold greater than plasma [Ag], a highly significant difference in all treatments. The spleen, which serves as a storage organ for red blood cells in fish, exhibited elevated levels of Ag in response to waterborne exposure, a response which was significant only in fed toadfish (Table 2).

3.3. Physiological responses

There was clear evidence of disturbances in gut osmoregulatory functions, associated with chronic waterborne Ag exposure, as detailed below. However, there were no significant differences among treatments in the concentrations of plasma ions (overall means: Na$^+$ = 165.0 ± 1.8, Cl$^-$ = 143.4 ± 0.7, Mg^{2+} = 1.16 ± 0.04 mmol L^{-1}, N = 39–41).

In fasted toadfish, drinking rate was unaffected by waterborne Ag exposure, staying at about 0.6 mL kg^{-1} h^{-1} (Fig. 5A). However, in fed animals, drinking was lower at about 0.3 mL kg^{-1} h^{-1}, but

Table 2

The influence of feeding and fasting on Ag concentrations (pmol g^{-1} wet weight) in selected tissues and body fluids in gulf toadfish exposed to waterborne Ag (nominally 185 μmol L^{-1}) or control seawater for 22–23 d. Means ± 1SEM.

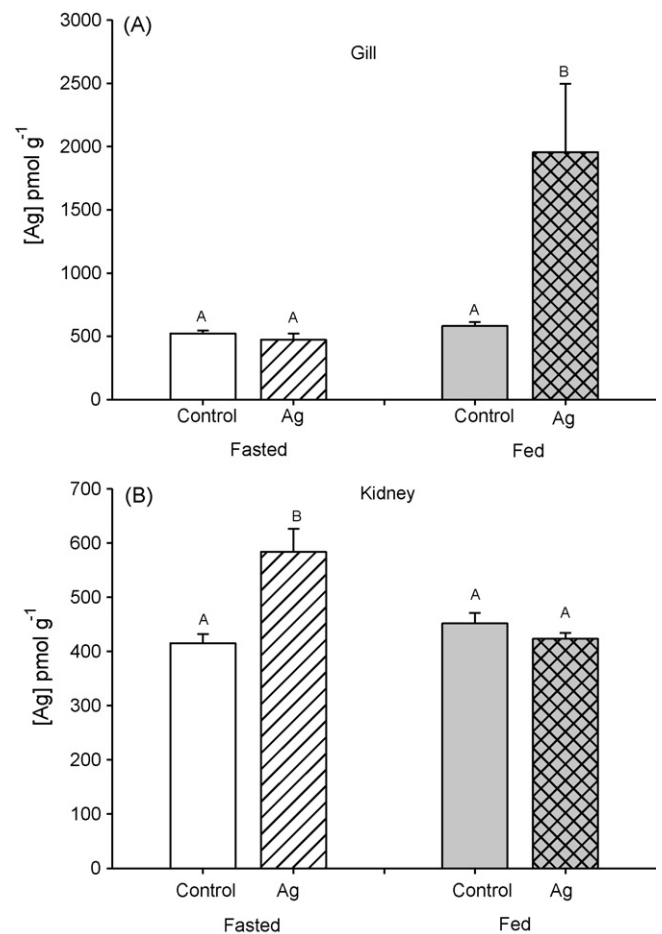|	Fasted control	Fasted Ag	Fed control	Fed Ag
Spleen	538.0 ± 54.9[AB]	702.6 ± 118.4[B]	408.4 ± 30.8[A]	652.4 ± 69.9[B]
RBC's	595.7 ± 70.2[A]	551.7 ± 132.7[A]	862.7 ± 225.5[A]	604.9 ± 75.3[A]
Plasma	89.6 ± 9.1[A]	113.6 ± 23.3[AB]	120.9 ± 13.5[AB]	135.3 ± 17.0[B]
Anterior intestinal fluid	341.0 ± 103.2[AB]	710.0 ± 138.4[B]	217.4 ± 32.4[A]	646.1 ± 248.6[AB]
Mid intestinal fluid	294.9 ± 23.3[A]	1004.9 ± 232.9[B]	201.2 ± 15.4[A]	1428.6 ± 260.2[B]
Posterior intestinal fluid	429.2 ± 154.7[A]	1172.5 ± 426.6[A]	248.7 ± 32.2[A]	762.2 ± 211.6[A]
Carcass	570.9 ± 54.0[A]	709.5 ± 73.1[A]	544.5 ± 27.9[A]	628.7 ± 55.0[A]

Notes: (1) N = 10–13 for all means, except for intestinal fluids, where N = 4–7. (2) Within a particular tissue or fluid, means not sharing the same letter are significantly different ($P < 0.05$).
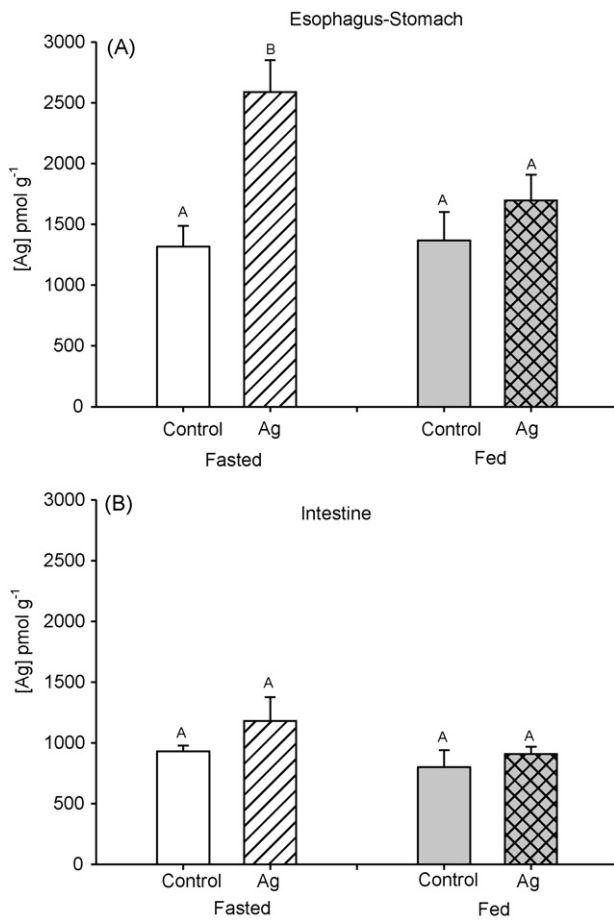
Fig. 2. The influence of feeding or fasting on Ag concentrations (pmol g^{-1} wet weight) in (A) the esophagus-stomach and (B) the intestine (mid and posterior intestine combined) of gulf toadfish exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22–23 d. Means ± 1 SEM (N = 6–7). Means not sharing the same letter are significantly different ($P < 0.05$).

Fig. 3. The influence of feeding or fasting on Ag concentrations (pmol g^{-1} wet weight) in (A) the gills and (B) the kidney of gulf toadfish exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22–23 d. Note the different scales in the two panels. Means ± 1 SEM (N = 12–13). Means not sharing the same letter are significantly different ($P < 0.05$).

was raised back to 0.6 mL kg^{-1} h^{-1} in the presence of chronic Ag exposure. The total fluid volumes collected from the gastrointestinal tract at sacrifice did not correlate with these drinking rates. In fasted animals, chronic waterborne Ag exposure caused a significant 4-fold increase in gut fluid volume (Fig. 5B). However in fed toadfish, gut fluid volume was already high in the control treatment, but was significantly depressed in response to waterborne Ag.

In vitro analysis of gut function using anterior intestinal sacs revealed a significant 55% decrease in the rate of net basic equivalent secretion in fasted toadfish associated with chronic waterborne Ag pre-exposure (Fig. 5C). However, in fed toadfish pre-exposed only to control seawater, intestinal base secretion rates were

already at these lower levels, and did not change significantly with chronic Ag pre-exposure. There were no significant differences in the net water absorption rates *in vitro*, or in the unidirectional or net fluxes of Na$^+$ and Cl$^-$ across the gut sac preparations (Table 3).

3.4. ^{110m}Ag clearance and tissue distribution responses

These experiments were performed on only two groups, both fasted, but exposed to either control seawater or waterborne Ag for 22 d.

After injection of the tracer dose of ^{110m}Ag, whole blood radioactivity declined rapidly in a quasi-exponential fashion (Fig. 6A and

Table 3
The influence of feeding and fasting on rates of water, Na$^+$, and Cl$^-$ fluxes across the anterior intestine, measured *in vitro*, in gulf toadfish pre-exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control conditions for 22 d. Means ± 1 SEM.

	Fasted control	Fasted Ag	Fed control	Fed Ag
H$_2$O flux (μL cm^{-2} h^{-1})	1.89 ± 1.31	2.78 ± 0.72	1.76 ± 0.43	2.82 ± 1.05
Na$^+$ net flux (μmol cm^{-2} h^{-1})	0.29 ± 0.14	0.34 ± 0.16	0.21 ± 0.08	0.21 ± 0.17
Na$^+$ influx (μmol cm^{-2} h^{-1})	8.57 ± 1.84	4.67 ± 0.47	4.64 ± 0.76	7.50 ± 1.21
Na$^+$ efflux (μmol cm^{-2} h^{-1})	−8.27 ± 1.74	−4.33 ± 0.43	−4.43 ± 0.73	−7.29 ± 1.22
Cl$^-$ net flux (μmol cm^{-2} h^{-1})	0.74 ± 0.37	0.82 ± 0.17	0.55 ± 0.07	0.79 ± 0.18
Cl$^-$ influx (μmol cm^{-2} h^{-1})	12.35 ± 3.11	8.85 ± 1.23	6.99 ± 1.25	9.44 ± 1.54
Cl$^-$ efflux (μmol cm^{-2} h^{-1})	−11.61 ± 2.80	−8.03 ± 1.09	−6.44 ± 1.29	−8.05 ± 1.37

Notes: (1) N = 6 in all treatments. (2) There were no significant differences ($P > 0.05$) among treatments for any parameter.
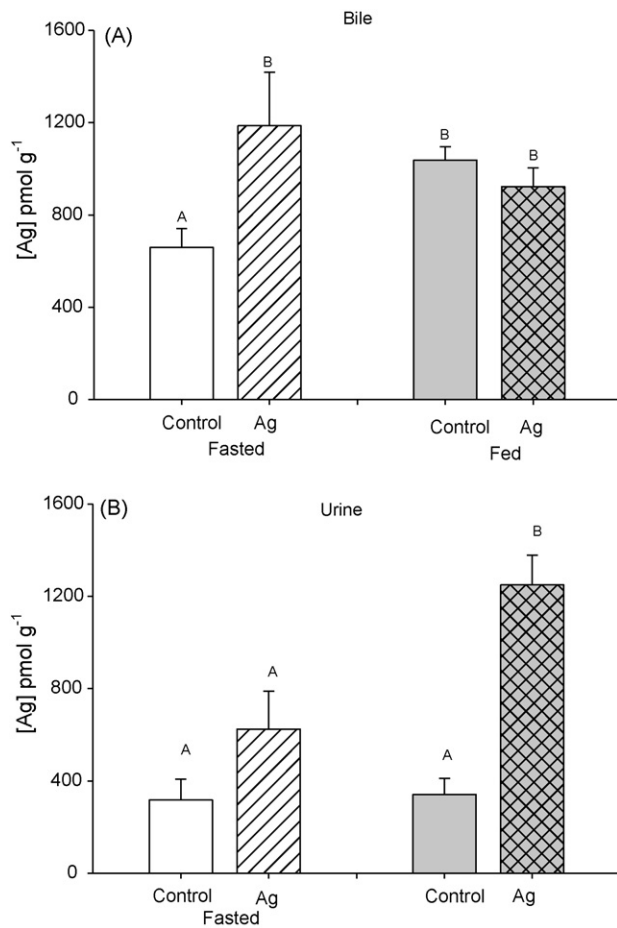
Fig. 4. The influence of feeding or fasting on Ag concentrations (pmol g^{-1} wet weight) in (A) the bile collected from the gall bladder and (B) the urine collected from the urinary bladder of gulf toadfish exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22–23 d. Means ± 1 SEM ($N = 8–13$). Means not sharing the same letter are significantly different ($P < 0.05$).

Table 4). By 320 min post-injection, blood levels were less than 16% of those at 10 min, and were significantly different between the two treatments at this time through until the end of the experiment at 1920 min (32 h), with faster clearance in the group which had been pre-exposed to waterborne Ag.

Table 4
The influence of 22 d pre-exposure to waterborne (nominally 1.85 μmol L^{-1}) or control seawater on the rate constant of clearance (%h^{-1}) of injected ^{110M}Ag from the blood of fasted toadfish. Means ± 1SEM.

	Control	Ag
Whole blood		
K_1	1.093 ± 0.143	1.114 ± 0.103
K_2	0.186 ± 0.025	0.232 ± 0.015
K_3	0.021 ± 0.007	0.027 ± 0.004
Red blood cells		
K_1	–	–
K_2	0.102 ± 0.011	0.136 ± 0.025
K_3	0.029 ± 0.010	0.018 ± 0.005
Plasma		
K_1	0.861 ± 0.059	1.265 ± 0.074[*]
K_2	0.152 ± 0.016	0.212 ± 0.018[*]
K_3	0.018 ± 0.006	0.035 ± 0.009

Notes: (1) $N = 7$ for both treatments. (2) K_1 corresponds to 0–80 min. K_2 to 80–600 min, and K_3 to 600–1920 min post-injection. (3) K_1 could not be calculated for RBC's.

[*] Significant difference ($P < 0.05$) between corresponding means in the two treatments.

There were substantive differences between the two treatments in the partitioning of ^{110m}Ag between red blood cells (RBC's) and plasma. This difference was established as early as 10 min after injection, and remained significant through 160 min (Fig. 6B). Fish which had been previously exposed to only control seawater allowed the mean [RBC ^{110m}Ag]/[plasma ^{110m}Ag] distribution ratio to rise to as high as 3.0 at 40 min post-injection, and the ratio remained high (approximately 2.0) for the remainder of the experiment. In contrast, Ag-pre-exposed toadfish kept the ratio below 1.0 through 160 min, though by 1920 min (by which time blood radioactivity was very low) the ratio had risen to a value similar to that in the naïve fish.

Rate constants (fractional clearance per hour) were determined by fitting two- or three-phase exponential models to the data for individual fish. With only 3 exceptions out of 14 fish (where two-phase models were best), the data were best described by three-phase models covering 10–80 min, 80–600 min, and 600–1920 min, so this analysis was applied to all fish for consistency. The respective rate constants have been designated as K_1, K_2, and K_3 in Table 4.

In whole blood there were no differences between the two treatment groups (Table 4). However, when rate constants were calculated for plasma separately, the rate constants for phase 1 (K_1) and phase 2 (K_2) were significantly higher by about 40% in the fish which had been exposed to waterborne Ag. The difference in K_3 was not significant. Note that K_1 for RBC's could not be calculated because absolute radioactivity in the RBC's sometimes increased over the first 40–80 min, especially in the control group, in accord with the distribution ratio data (Fig. 6B).

The relative distribution of ^{110m}Ag on a whole organ or tissue basis at the end of the experiment (1920 min) was measured. The mass of the white muscle has been estimated as 50% of the body weight for the purposes of this calculation, based on test dissections. A logarithmic scale has been used in Fig. 7 to encompass the large range of variation encountered. The liver (~58%), the white muscle (~10%) and unidentified portions of the carcass ("rest", ~18%; note—does not include white muscle) were the largest contributors, and did not differ between the two treatments. However bile (1.68 ± 0.44% vs. 0.52 ± 0.12%, $N = 7$) and urine (0.023 ± 0.001% vs. 0.003 ± 0.001%, $N = 7$) both made a larger contribution in the Ag-exposed fish, whereas the intestine (1.02 ± 0.16% vs. 3.34 ± 0.63%, $N = 7$) made a larger contribution in the control fish. There were no other significant differences. The appearance of ^{110m}Ag in the external water was undetectable in both treatments.

4. Discussion

4.1. Overview

To our knowledge, this is the first study to examine the consequences of feeding *versus* fasting on the responses to chronic waterborne metal exposure in a seawater teleost. The level of Ag employed was above those of normal environmental occurrence (discussed in Wood et al., 2004), but were chosen so as to be sublethal during chronic exposure, and comparable to previous studies on marine fish; Ag is far less toxic in seawater than in freshwater (see Section 1).

The results provide support for our two main hypotheses. Firstly, we hypothesized there would be interactive effects of feeding and Ag exposure on Ag bioaccumulation and physiological indices of toxicity. With respect to bioaccumulation, this was confirmed by the protective effects of feeding against whole body Ag burden, manifested in the white muscle (Fig. 1B), liver (Fig. 1C), esophagus-stomach (Fig. 2A), and kidney (Fig. 3C), as well as alterations in the excretory pathways of bile (Fig. 4A) and urine (Fig. 4B). Physiologi-
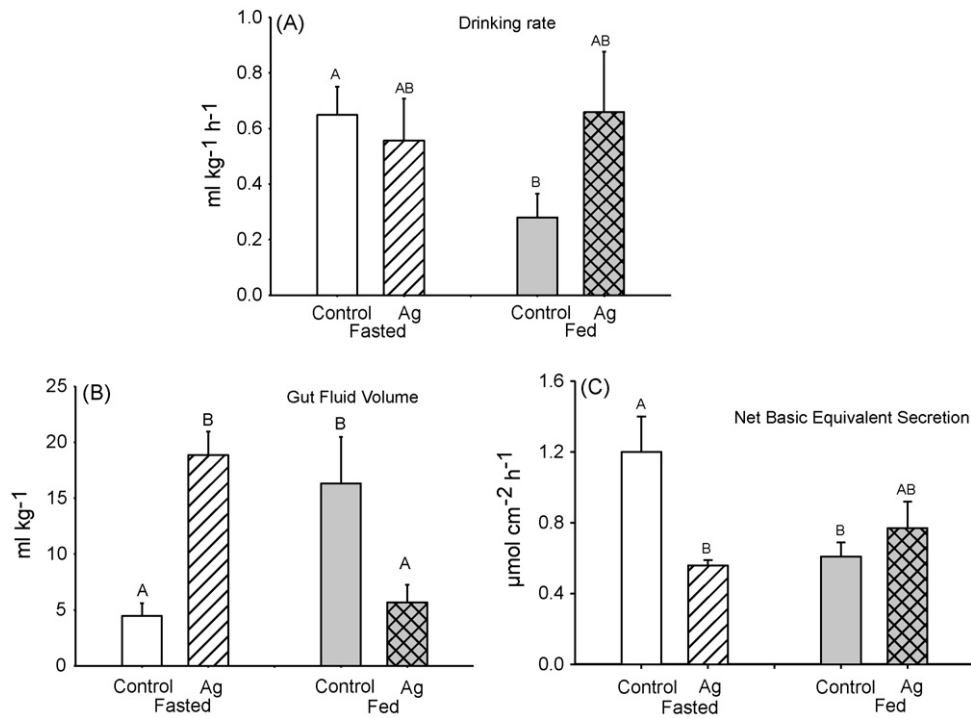
Fig. 5. (A) Drinking rates and (B) total fluid volumes collected from all sections of the gastrointestinal tract at sacrifice in gulf toadfish exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22–23 d. Means ± 1 SEM (N = 5–7). (C) The net rate of basic equivalent secretion by the anterior intestine, measured using an *in vitro* gut sac technique, in gulf toadfish pre-exposed to the same conditions as in (A) and (B). Means ± 1 SEM (N = 6). Means not sharing the same letter are significantly different (P < 0.05). Drinking rates *versus* gut fluid volumes and basic equivalent secretion rates were measured on different fish.
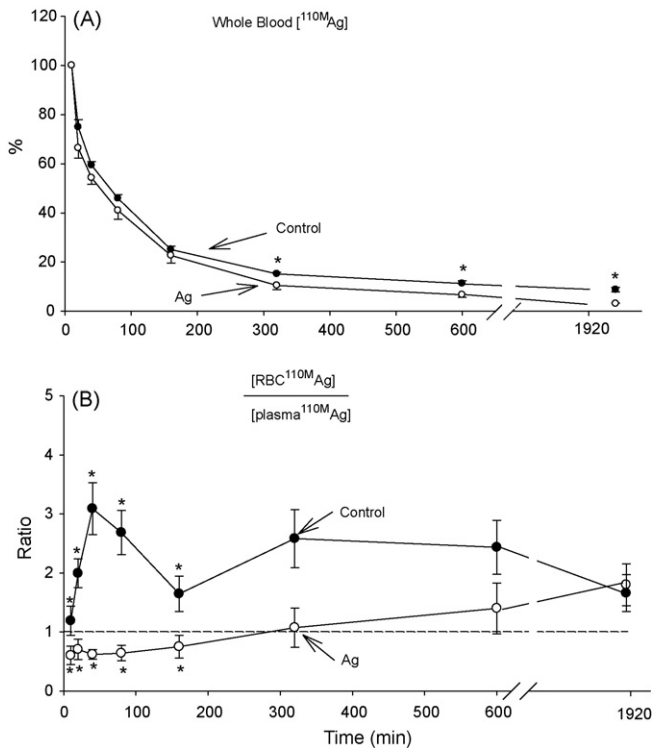
Fig. 6. (A) The time course of clearance of an arterially injected tracer dose of ^{110m}Ag in the whole blood of intact gulf toadfish which had been pre-exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22 d. Both groups had been fasted during the exposures. Concentrations have been expressed as a percentage of the concentration present (100%) at 10 min post-injection in each fish. (B) The partitioning of ^{110m}Ag between red blood cells (RBC's) and plasma in the data of (A), expressed as the [RBC ^{110m}Ag]/[plasma ^{110m}Ag] distribution ratio. Means ± 1 SEM (N = 7). Asterisks indicate significant differences between means at the same time point (P < 0.05).

cal differences associated with feeding or fasting were also seen in the influences of chronic Ag exposure on drinking rates (Fig. 5A), gut fluid volumes (Fig. 5B), and intestinal base secretion rates (Fig. 5C). These results support the view that the gut is a target of water-borne Ag exposure for both Ag uptake and toxic disturbance, and that feeding ameliorates these effects, though admittedly other explanations are possible. Secondly, our hypothesis that changes in the physiological regulation of Ag occur during chronic exposure was supported. There was clear evidence of faster clearance
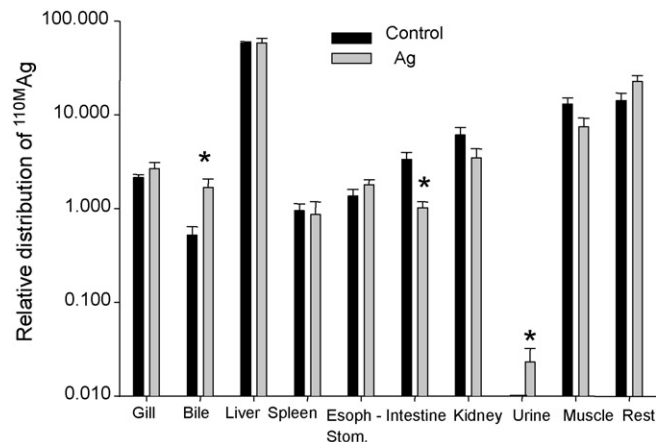
Fig. 7. The relative distribution of an arterially injected tracer dose of ^{110m}Ag on a whole organ or tissue basis after 32 h (1920 min) in gulf toadfish which had been pre-exposed to waterborne Ag (nominally 1.85 μmol L^{-1}) or control seawater for 22 d. Both groups had been fasted during the exposures. Amounts have been expressed as a percentage of the total in the whole body, and a logarithmic scale has been used to encompass the large range of variation among tissues that was observed. White muscle was assumed to contribute 50% of body mass. "Rest" refers to carcass minus white muscle. Means (N = 7). Asterisks indicate significant differences between means for the same tissue or organ (P < 0.05).

of an injected tracer dose of ^{110m}Ag from the whole blood (Fig. 6A) and plasma (Table 4) and protection against Ag accumulation inside RBC's (Fig. 6B), as well as subtle changes in the tissue-specific distribution of the ^{110m}Ag (Fig. 7) in fish exposed to silver for 22 d prior to ^{110m}Ag clearance studies.

4.2. Patterns of Ag bioaccumulation over time

These toadfish collected from Biscayne Bay already had significant background levels of Ag in their tissues. Based on their weight, the animals were 2–3 years old (Malca et al., 2009). Biscayne Bay receives both treated sewage effluent and non-point source runoff from the City of Miami, so it is perhaps not surprising that the fish had accumulated measurable amounts of Ag ($\sim$300 pmol g^{-1} in white muscle, for example; Fig. 1B) over their lifetime. The toadfish is a benthic animal, generally feeding close to the bottom and rummaging in sediments. The elevated background levels in the esophagus-stomach ($\sim$1350 pmol g^{-1}; Fig. 2A) relative to other tissues points to a gastro-intestinal uptake pathway, but whether this burden came from waterborne (i.e. drinking) or dietary exposure is unknown. However, it is noteworthy that these background levels were in fact very comparable to those (e.g. 180–550 pmol g^{-1} in white muscle) measured in other bottom-dwelling species (flounder, sculpins, and the closely related midshipman, *Porichthys notatus*) collected from a so-called pristine site on the west coast of Vancouver Island, B.C., Canada (Hogstrand et al., 1999; Webb and Wood, 2000; Grosell and Wood, 2001). This suggests that this is a general phenomenon for marine benthic fish, and not necessarily indicative of a contaminated ecosystem.

An interesting observation in all of these data sets is that liver levels were not greatly elevated (e.g. $\sim$700 pmol g^{-1} in the present study; Fig. 1C) relative to other tissues as a result of chronic lifetime exposure to background levels of Ag in the environment. This contrasts with the situation during experimental chronic exposures (22–23 d), where liver levels increased 7–20-fold (Fig. 1C) while Ag levels in white muscle and other tissues increased only moderately. Hogstrand et al. (1999), Webb and Wood (2000), and Grosell and Wood (2001) all reported quantitively similar patterns in marine teleosts. The most dramatic accumulation in the liver was seen in the 32-h ^{110m}Ag injection studies (Fig. 7). Fig. 8 illustrates these differences, with the liver accounting for $\sim$1%, 33%, and 58% of the total burden in the lifetime, 22–23 d, and 32 h exposures, respectively. This suggests that the liver, which is known to be a sensitive site for metallothionein induction by Ag (Hogstrand et al., 1996) plays a very important role in short-term scavenging, detoxification, and biliary excretion of Ag (Figs. 4A and 8). In other words, the liver serves as a buffer or surge protector. How-
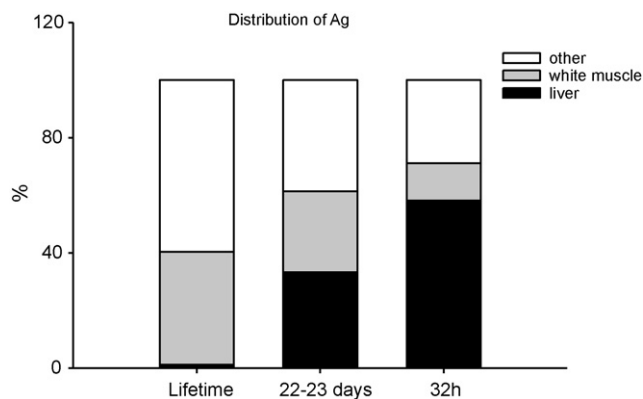
ever, the longer the exposure, the less important is the liver to the total Ag distribution; conversely, the longer the exposure, the more important is the deposition of Ag in white muscle and other tissues. By way of comparison, in another marine benthic teleost, the European plaice, the liver accounted for less than 25% of the total after a 63-d waterborne ^{110m}Ag exposure (Pentreath, 1977), and in the American plaice, the liver accounted for 28% of the total at 42 d after a single dietary exposure to ^{110m}Ag (Rouleau et al., 2000).

This conclusion contrasts with findings on two freshwater teleosts, the rainbow trout and European eel exposed to a 24-h pulse of waterborne ^{110m}Ag, where the contribution of the liver increased from about 25% after 1 d to 70–80% after 67 d of depuration (Hogstrand et al., 2003). Galvez et al. (2002) and Ausseil et al. (2002) reported similar results with longer term waterborne exposures of rainbow trout. Garnier and Baudin (1990) noted a 63% hepatic contribution at the end of a 34-d ^{110m}Ag feeding study with brown trout, and this did not change during a 28-d depuration period. Garnier et al. (1990) reported a 70% liver contribution in this same species at the end of a 57-d waterborne exposure to ^{110m}Ag, and again this remained constant during a 28-d depuration period. Overall, these observations suggest that the differences are salinity-related rather than source-related.

4.3. The influence of feeding on Ag bioaccumulation

The simplest explanation for the clear protective role of feeding against Ag bioaccumulation (Fig. 1) is that the processes of ingestion, digestion, and assimilation reduced the uptake of waterborne Ag through the digestive tract. The fact that feeding prevented any increase in the Ag burden in the esophagus-stomach supports this interpretation; the binding of imbibed Ag to food and chyme in the gastro-intestinal tract would likely reduce its bioavailability for uptake. In this regard, the present findings support the interpretation of those previous studies which have argued that the gut serves as an important site of uptake of waterborne Ag in marine teleosts (Hogstrand et al., 1999, 2002; Grosell et al., 1999; Webb and Wood, 2000; Wood et al., 2004). However, an alternate or additional interpretation is certainly possible, that the energy supplied by feeding allows better homeostatic regulation of Ag. This latter interpretation is supported by the observations of greater elevations of urinary [Ag] in fed toadfish (Fig. 4B), and already higher levels of biliary [Ag], even in non-exposed animals that had been fed (Fig. 4A). Indeed, more frequent biliary discharge would be expected in fed animals.

The only previous feeding *versus* fasting study on Ag bioaccumulation was performed on the freshwater rainbow trout exposed to waterborne Ag, and reached entirely opposite conclusions (Bertram and Playle, 2002). Here uptake and depuration profiles, as well as physiological responses, were identical in the two treatments over 7-d uptake and 15-d depuration periods. Again, the difference may be salinity-related, as drinking-related Ag uptake through the gastro-intestinal tract would be negligible in a freshwater teleost.

The gill was the single tissue to show an opposite trend, where feeding increased Ag accumulation by more than 3-fold, whereas there was no change at all in branchial Ag burden in fasted fish in response to waterborne Ag exposure (Fig. 3A). The likely explanation is the well-known specific dynamic action (SDA) phenomenon, whereby feeding can elevate metabolic rate several fold (reviewed by Secor, 2009). This requirement for greater O_2 consumption is met by both increased blood flow and increased water flow to the gills, as well as the recruitment of a greater gill area. The enhanced delivery of Ag to both internal and external surfaces of the branchial epithelium, as well as the greater available area, could all contribute to the greater gill burden. We might expect that as a result

Fig. 8. A comparison of the tissue-specific distribution of "new" Ag taken up over different time courses in the gulf toadfish. For consistency, data from fasted animals have been used throughout.

of "opening up" their gills, fed fish would lose more water by osmosis, and therefore have to drink more, a point which is addressed below.

4.4. The influence of feeding on physiological responses to waterborne Ag exposure

There were complex interactive effects of feeding *versus* fasting and waterborne Ag exposure on gut function (Figs. 5 and 6), but these did not alter major plasma ions (Na^+, Cl^-, Mg^{2+}), so they were within the range of compensation for the toadfish. In earlier studies on other marine teleosts, waterborne Ag exposure generally inhibited drinking and in some cases raised plasma ions (Grosell et al., 1999; Hogstrand et al., 1999; Grosell and Wood, 2001; Webb et al., 2001), but in none of these studies were the drinking measurements done on long-term fasted fish, or on fish that were actually digesting a recent meal. The present results show that Ag exposure did not alter drinking rate in long-term fasted toadfish, but feeding significantly lowered drinking rate, whereas this effect was eliminated by Ag exposure (Fig. 5A). In two previous studies on marine teleosts, feeding either elevated drinking rate (in seawater Atlantic salmon; Usher et al., 1988) or had no effect on drinking rate (in seawater rainbow trout; Bucking et al., submitted for publication), so results may be species-dependent. Earlier, we have argued that feeding, through its effects in stimulating SDA (Secor, 2009), might be expected to induce a compensatory increase in drinking rate to replace greater osmotic water loss across the gills. The fact that this was not seen may be because the high water content of the food provides the necessary compensation.

Ag exposure caused a marked increase in gut fluid volume in fasted toadfish, suggesting that water absorption was inhibited (Fig. 5B). In turn this would fit with the significant inhibition of intestinal net base secretion rates seen *in vitro* in this treatment (Fig. 5C), because base secretion plays a key role in water absorption through the gut (reviewed by Grosell, 2006; Grosell et al., 2009). Evidence for inhibition by waterborne Ag of intestinal basic equivalent secretion, and Na^+, Cl^-, and water absorption has been reported in several previous *in vivo* studies (Grosell et al., 1999; Grosell and Wood, 2001). However we were unable to detect any associated decrease in water flux or disturbance of Na^+ or Cl^- fluxes in our *in vitro* studies on the gut (Table 3), perhaps because of lack of sensitivity or because luminal Ag was no longer present in these preparations. Base secretion rates *in vitro* in fed toadfish were lower than in fasted toadfish (Fig. 5C), contrary to results of Taylor and Grosell (2006, 2009) on the same species, and Bucking et al. (2009) on seawater rainbow trout. The explanation here may be the much longer fasting period employed in the current study. Feeding appeared to protect base secretion rates against inhibition by waterborne Ag pre-exposure.

Not surprisingly, fed toadfish had much higher gut fluid volumes than fasted toadfish under control conditions (Fig. 5B), and much of this volume may have come from the food and gastrointestinal secretions, since drinking rate was reduced. In contrast to the processing of seawater taken in by drinking, the ingestion of food would necessitate digestive and nutrient-linked absorptive processes. These would likely be slower, keeping more fluid in the gut for a longer period. The reduced gut fluid volume in fed toadfish chronically exposed to Ag suggests more efficient fluid absorption in these fish. The explanation is not clear, though Webb et al. (2001) noted that several marine teleosts which had been both fed and chronically exposed to waterborne Ag tended to have higher intestinal Na^+, K^+-ATPase activities. This was also seen in fed toadfish chronically exposed to waterborne Cu (Grosell et al., 2004). In both cases the response was interpreted as evidence of compensation.

4.5. Evidence for physiological regulation of Ag during chronic waterborne Ag exposure

The ^{110m}Ag injection studies demonstrated faster clearance from the whole blood and plasma (Fig. 6A), protection against Ag accumulation in RBCs (Fig. 6B), and upregulated biliary and urinary excretion pathways (Fig. 7) as a result of chronic waterborne Ag exposure. The lower intestinal burdens in these fish (Fig. 7) suggests that an improved clearance from this tissue also occurs, helping explain why the elevated intestinal Ag burdens seen during short-term (24 h) exposure (Wood et al., 2004) were not detectable after 6 d exposure (Nichols et al., 2006) in our earlier studies on toadfish. They also help explain why there were no significant elevations in intestinal Ag burdens during the chronic exposures of the present study (Fig. 2B). It seems likely that these adaptations would confer acclimation in the toxicological sense, as has been seen in at least one freshwater Ag study with rainbow trout (Galvez and Wood, 2002).

The responses of faster clearance rate from the plasma, protection of the RBCs, and in some cases upregulated biliary and/or renal excretion after chronic exposure appears now to be a common pattern with several other metals, at least in freshwater fish (e.g. Cu—Grosell et al., 1997, 1998, 2001; Zn—Chowdhury et al., 2003; Cd—Chowdhury et al., 2003, 2004; Chowdhury and Wood, 2007; Ni—Pane et al., 2005; Chowdhury et al., 2008). It may represent a co-ordinated systemic response to deal with potentially dangerous metal levels in the bloodstream. In view of the much lower urine flow rates in seawater fish, the renal response is likely to be less important than the biliary response.

In toadfish, RBC Ag levels are already about 5-fold higher than plasma levels and are not allowed to rise during chronic Ag exposure (Table 2), presumably as a result of the same mechanisms responsible for excluding injected ^{110m}Ag from the erythrocytes (Fig. 6B). The protection offered by acclimation against further accumulation of metal in the RBCs must clearly be important for their physiological function, but we are aware of no studies which have examined how erythrocytic metal accumulation specifically affects O_2 or CO_2 transport kinetics in fish. RBC's are rich in carbonic anhydrase which plays a critical role in both O_2 loading and CO_2 unloading at the gills, and *vice versa* at the tissues. Metals such as Ag and Cu are known to inhibit carbonic anhydrase function in fish (Christensen and Tucker, 1976; Morgan et al., 1997, 2004; Lund et al., 2002). This is an important area for future investigation.

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