

THE PHYSIOLOGY OF MASSIVE ZINC ACCUMULATION IN THE LIVER OF FEMALE SQUIRRELFISH AND ITS RELATIONSHIP TO REPRODUCTION

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Accepted 26 July 1996

Summary

It is well known that zinc is an essential micronutrient and, as a rule, organisms keep relatively constant low levels of zinc to maintain cellular functions. The squirrelfish family (Holocentridae) is the only known exception from this rule. Squirrelfish accumulate very high concentrations of zinc in the liver. In the present study, we demonstrate that, while female squirrelfish store large amounts of zinc in the liver and ovaries, the males show zinc levels that are typical for vertebrates. The zinc content of the diet is the same in males and females, and zinc is not lost from the liver during starvation. Thus, the difference between genders in zinc storage is not dependent upon the diet. Rather, there are at least two processes that contribute to the accumulation in females. First, females possess high levels of two major zinc-binding proteins: metallothionein (MT) and a novel female-specific zinc-binding protein (FZnBP). In females, but not in males, almost all MT is

present in the hepatocyte nucleus. FZnBP is exclusively found in the hepatocyte cytosol of females. Second, hepatocytes of female squirrelfish have a high capacity to transport zinc across the plasma membrane. In addition to the liver, only the gonads of females showed unusually high concentrations of zinc. Administration of exogenous oestrogen to females decreases the hepatic zinc concentration while there is a matching increase in the zinc content of the ovaries. Thus, oestrogen may trigger a redistribution of zinc from liver to ovaries. Together, our findings suggest that female squirrelfish may be uniquely adapted to detoxify zinc and to utilize it as a macronutrient for processes related to reproduction.

Key words: squirrelfish, *Holocentrus marianus*, zinc accumulation, zinc, metallothionein, FZnBP, physiology, reproduction, fish, pisces.

Introduction

Zinc is an essential micronutrient to all organisms, but it may become toxic if accumulated at higher concentrations (Vallee and Falchuk, 1993; Hogstrand and Wood, 1996). In most vertebrates, the hepatic zinc concentration is within the range 200–600 nmol g⁻¹ wet mass; Underwood, 1977; Hogstrand and Wood, 1996). In contrast, several members of the squirrelfish family (Holocentridae) accumulate extremely high levels of zinc in the liver (up to 70 µmol g⁻¹; Hogstrand and Haux, 1991, 1996). With the exception of the vertebrate retina (Weitzel *et al.* 1954), these are the highest zinc levels reported for any vertebrate tissue. The squirrelfish family is divided into two subfamilies, squirrelfish (Holocentrinae) and soldierfish (Myripristinae), both of which are exclusively associated with coral reefs. The unusual zinc accumulation in this family was first observed in the squirrelfish (*Holocentrus rufus*) from

Bermuda (Hogstrand and Haux, 1991) and was subsequently shown to occur in both squirrelfish and soldierfish species from Queensland, Australia (Hogstrand and Haux, 1996). The fact that several species of this family, from widely separated geographical locations, display this zinc storage makes it reasonable to assume that a high hepatic zinc concentration is normal for squirrelfish and is not related to environmental influence or some inherited disorder in their zinc metabolism.

Although the average hepatic zinc levels of all five squirrelfish species studied to date are high, there seem to be marked differences among species and, more surprising, within each species and population (Hogstrand and Haux, 1996). The most pronounced variation was found in lattice soldierfish (*Myripristis violacea*), which showed a 50-fold variation of hepatic zinc concentration among individuals. Indeed, in each

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population, there appear to be individuals that are quite 'normal' with respect to hepatic zinc content, and these are mixed with individuals that have extremely high concentrations of hepatic zinc. Attempts to link zinc levels to collection sites or fish size were unsuccessful. To date, gender-dependence has not been examined because almost all of the collected specimens were sexually immature.

Zinc accumulation in squirrelfish liver is very closely associated with increased levels of hepatic metallothionein (MT; Hogstrand and Haux, 1990a, 1996). Metallothionein is a low-molecular-mass protein that is believed to play a key role in the intracellular metabolism of zinc and copper (Hogstrand and Haux, 1991; Vallee and Falchuk, 1993). The synthesis of the protein is induced at the transcriptional level by increased intracellular activities of zinc and copper, and also by other metals within groups IB and IIB of the periodic system (Olsson, 1993). In mammals, a broad range of signalling substances and other factors, generally associated with distress and infection, can elicit *de novo* synthesis of MT (Klaassen and Lehman-McKeeman, 1989), but the role of such factors in the regulation of the MT genes in fish is uncertain (Kille *et al.* 1992; Olsson, 1993). However, a recent study shows that the 5'-flanking region of the MT-A gene in the rainbow trout *Oncorhynchus mykiss* has five functional activator protein-1 (AP-1) sites which enhance the transcription of the gene in response to free radicals (Olsson *et al.* 1995). In addition there are putative glucocorticoid-responsive elements (GREs) and nuclear factor interleukin 6 (NF-IL6) sites, but the activities of these sequences have not been confirmed (Olsson *et al.* 1995). Once produced, MT binds metals specifically and avidly (Kille *et al.* 1994). The metals that bind to MT are the same as those that induce the synthesis of the protein.

Although the exact function(s) of MT remains elusive, there is evidence that it may be involved in processes including cell differentiation, proliferation and signalling, as well as enzyme regulation, free-radical scavenging, and metal storage and detoxification (Hogstrand and Haux, 1991; Sato and Bremner, 1993; Cherian, 1994; Hogstrand and Wood, 1996). In squirrelfish, individuals have been found with hepatic MT concentrations of up to $5.4 \mu\text{mol g}^{-1}$ wet mass, and the correlation between MT and zinc concentrations is strong ($0.89 < r < 0.99$; Hogstrand and Haux, 1990a, 1996). Furthermore, in 'high-zinc' individuals, MT bound 60–70% of all the zinc in the liver (Hogstrand and Haux, 1996). From previous research, it is not clear whether MT concentrations in squirrelfish liver are high only because MT synthesis is induced by zinc or whether zinc–MT has some specialized function in the squirrelfish liver. Either way, the squirrelfish is a unique organism for studies of zinc metabolism and the function of MT.

The aim of the present study was to provide a physiological explanation for the elevated concentrations of zinc and MT in squirrelfish liver. The concentration of zinc was measured in the liver and eight other tissues of longjaw squirrelfish (*Holocentrus marianus*) in a search for unusual zinc-dependent activities in other organs. The intracellular partitioning of zinc between organelles and the cytosol of the liver was

investigated to localize any specific subcellular fraction of particular interest. Likewise, the intracellular distribution of MT was investigated, employing western blots of the different subcellular fractions with an antiserum specific for fish MT (Hogstrand and Haux, 1990b). Further, the subcellular fractions were incubated with ^{65}Zn and run on polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions in an attempt to localize zinc-binding proteins other than MT. A food-deprivation experiment and analysis of the zinc content of the intestinal contents were performed to evaluate the mobility of hepatic zinc and the possibility that the high concentrations of zinc are absorbed from a zinc-rich diet. In another experiment, squirrelfish were treated with 17β -oestradiol to investigate possible roles for zinc and MT in the sexual development of females (Olsson *et al.* 1987, 1989). Finally, the kinetics of zinc fluxes within the liver was examined using isolated hepatocytes.

Materials and methods

Animals

Longjaw squirrelfish (*Holocentrus marianus*, Woods; 100–314 g) of both genders were collected by SCUBA-divers using hand-nets off the Florida Keys, Tavernier and Key West, Florida, USA, between the months of January and March. No sedatives were used to capture the fish. The fish were transported to the laboratory, where they were kept in 600 l circular fibreglass tanks, 10 fish in each tank. The tanks were supplied with running, aerated sea water from Biscayne Bay (salinity 29–33‰; temperature 26–28 °C). With the exception of the fish in the food-deprivation experiment, the animals were fed daily to satiation with live shrimp.

Gulf toadfish (*Opsanus beta*, Goode and Bean) were used as a reference fish, with 'normal' hepatic zinc metabolism, for some experiments. Toadfish (84–150 g) were obtained by bottom trawl of the Southern reach of Biscayne Bay, Florida, USA. In the laboratory, the toadfish were kept in glass aquaria under similar conditions to those described for squirrelfish.

Tissue metal concentrations

Fish were killed by an overdose of tricaine methanesulphonate (MS222) and then weighed. A 1 ml blood sample was withdrawn from the caudal vessels using a heparinized syringe. Blood cells and plasma were separated by centrifugation at 14 000 g, at room temperature, for 3 min. Blood cells and plasma were transferred to separate glass tubes for acid digestion. The sex of each fish was determined. Liver, gonads, scales, kidneys, brain, retina and a sample of the dorsal muscle were dissected out and weighed. A sample (0.030–0.75 g) of each tissue, except for muscle, was put into a 16 mm×150 mm borosilicate glass tube for acid digestion. The proximal 10 cm section of the small intestine was cut off and the contents squeezed out. The muscle samples (0.61–1.14 g) and the intestinal contents were transferred to 10 ml polypropylene tubes (Falcon) for acid digestion.

To each of the glass tubes, containing plasma, blood cells,

liver, gonad, scales, kidney, brain or retina, 2 ml of 70 % HNO₃ (trace metal grade) was added. The tubes were heated to 100 °C in a sand bath for 3 h before 300 µl of H₂O₂ was added. The temperature was then gradually increased until all the liquid had evaporated. Finally, 4 ml of 1 % HNO₃ was added and the samples were analyzed for zinc and copper content by atomic absorption spectroscopy (Perkin Elmer, model 2380), using an air/acetylene flame (detection limit for zinc, 0.3 µmol l⁻¹; for copper, 0.6 µmol l⁻¹).

The samples of muscle tissue and intestinal contents were digested in the polypropylene tubes. 4 ml of 35 % HNO₃ was added to each tube, which were sealed and left in an oven at 80 °C overnight. The next day, 1 ml of H₂O₂ was added followed by another 24 h of digestion at the same temperature. The volume of the digest was adjusted to 5 ml with deionized water (Millipore). Finally, the samples were diluted 25-fold with deionized water (Millipore) to reduce the HNO₃ concentration in the matrix and analyzed for zinc and copper content as described above.

Subcellular fractionation of liver

Fish were killed by an overdose of MS222 and then weighed. The liver was dissected out, weighed for calculation of liver-somatic index [LSI; (liver mass/body mass)×100] and placed on ice. The gonads were also removed and weighed for calculation of gonadosomatic index [GSI; (gonad mass/body mass)×100]. Subcellular fractions of the liver were obtained by differential centrifugation of liver homogenate using the protocol of Julshamn *et al.* (1988), for rainbow trout liver, with minor modifications. Liver samples (0.5 g) were individually homogenized in 1.5 ml of isotonic buffer, 35 mmol l⁻¹ Tris-HCl, 0.2 mol l⁻¹ KCl, 0.25 mol l⁻¹ sucrose, pH 7.4 (homogenization buffer), at 0 °C, using a glass-Teflon homogenizer. A 500 µl sample of each homogenate was withdrawn and transferred to glass tubes for preparation for metal analysis as described above. The rest of the homogenate was centrifuged at 370 g, 4 °C, for 5 min. The sediment (nuclear fraction) was saved and immediately placed on ice. The supernatant was centrifuged at 9200 g, 4 °C, for 5 min, and the pellet (mitochondria-lysosome fraction) was saved and immediately placed on ice. Finally, microsomes were separated from the cytosol by centrifugation at 130 000 g, 4 °C, for 60 min. The resulting pellet (microsomal fraction) and the supernatant (cytosolic fraction) were saved and immediately placed on ice.

All pellets from the differential centrifugation were individually resuspended in 500 µl of homogenization buffer using a syringe with a fine-gauge needle. Nine complete sets of liver fractions, from five females and four males, were acid-digested for metal analysis as described above. The subcellular fractions from the livers of 16 longjaw squirrelfish were each subdivided into three portions and frozen in liquid nitrogen for subsequent use in autoradiography of zinc-binding proteins and for western blots for MT (see below).

Western blots

The subcellular distribution of MT in longjaw squirrelfish

liver was examined by western blot of the different fractions obtained from differential centrifugation, using a rabbit anti-perch-MT serum (Hogstrand and Haux, 1990b). Each subcellular fraction was subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous buffer system, according to Laemmli (1970). Samples (1–3 µl) were diluted 1:4 with sample buffer [62 mmol l⁻¹ Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 0.0012 % (w/v) Bromophenol Blue], made up to 20 µl with deionized water (Millipore), and heated at 100 °C for 5 min. The amount of protein loaded into each well was 20 µg. Rainbow Cold Markers RPN 756 (Amersham) and perch (*Perca fluviatilis*) MT (I and II; Olsson and Hogstrand, 1987) were used as molecular mass standards. The electrophoresis was carried out on a 4 % (w/v) stacking gel and a 15 % (w/v) separation gel. Gels were run in a Bio-Rad Mini-Protean II electrophoresis system at 180 V.

Following electrophoresis, the proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting, as described by Towbin *et al.* (1979). The procedure was carried out in a SemiPhor TE 70 semi-dry transfer unit (Hoefer Scientific) at 0.8 mA cm⁻² (60 mA) at room temperature for 60 min. After electroblotting, additional protein binding was blocked by immersing the membrane in 5 % (w/v) dried milk in Tris-buffered saline [20 mmol l⁻¹ Tris, 137 mmol l⁻¹ NaCl, 0.1 % (v/v) Tween-20, pH 7.6; TTBS] for 1 h. The membranes were rinsed in TTBS and washed once for 15 min and twice for 5 min with fresh changes of the same solution. MT bound to the membrane was identified by a rabbit anti-perch-MT serum (Hogstrand and Haux, 1990b), diluted 8000-fold with TTBS, during a 1 h incubation. The incubation in primary antibody was followed by the same washing procedure as before. A horseradish-peroxidase-conjugated donkey anti-rabbit Ig (Amersham), in a 40 000-fold dilution, was used as secondary antibody. The rabbit anti-perch-MT antibodies were labelled with the secondary antibody during a 1 h incubation, which was followed by washing in the same manner as before. All incubations were performed at room temperature with continuous agitation. Immunodetection was carried out with an enhanced chemiluminescence system (ECL, Amersham) according to the manufacturer's recommendations.

Autoradiography of zinc-binding proteins

Subcellular fractions of squirrelfish liver were incubated with ⁶⁵Zn and then run on an SDS-PAGE gel under non-denaturing conditions to identify and localize hepatic zinc-binding proteins. SDS was included in the buffers to mobilize membrane-bound proteins. Each subcellular fraction (50 µg of protein) was incubated with 25 Bq of carrier-free ⁶⁵Zn (NEN; 67.7 Bq ng⁻¹) in 0.3 mol l⁻¹ sucrose at 0 °C for 60 min in a total volume of 10 µl. The sample, 6.5 µl, was mixed with 13 µl of non-reducing sample buffer [62 mmol l⁻¹ Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.0012 % (w/v) Bromophenol Blue], and 15 µl of this mixture (protein 16 mg,

^{65}Zn 12.5 Bq) was loaded, without previous heat denaturation, on a 12% SDS–polyacrylamide gel, with a 4% stacking gel. Purified rainbow trout MT (Olsson and Hogstrand, 1987; 0.5 μg) was incubated with 25 Bq of ^{65}Zn , as described above, and run parallel to the samples on the gel as a standard. One lane was run with 20 Bq of ^{65}Zn only to show the motility of non-protein-bound zinc. The gel was run in a Bio-Rad Mini-Protean II electrophoresis system at 180 V with 25 mmol l^{-1} Tris, 1.4% (w/v) glycine, 1% SDS, pH 8.3, as running buffer. After completion of the electrophoretic separation, the gel was dried and the ^{65}Zn visualized by autoradiography using intensifying screens.

Food-deprivation experiment

A food-deprivation experiment was conducted to assess the mobility of the zinc stored in squirrelfish liver. Nineteen longjaw squirrelfish (172–273 g) were collected off Tavernier, transported to the laboratory the next day, and placed into three tanks. One of the tanks contained five fish (three females, two males) and these animals were fed to satiation with live shrimp, once a day, over 12 days. The fish in the other two tanks were not fed. Nine of the starved fish (five females, four males) were sampled after 4 days in the laboratory and the remaining five fish (three females, two males) were sampled after 12 days. When sampled, the fish were killed by an overdose of MS222 and weighed. The liver and gonads were dissected out and analyzed for zinc and copper as described above.

Oestradiol experiment

Longjaw squirrelfish ($N=11$, six females and four males; 134–301 g) were injected on day 0 and day 5 with 6 mg kg^{-1} 17 β -oestradiol dispersed in peanut oil (1 ml kg^{-1}). A control group ($N=9$, seven females and two males; 175–314 g), which was kept in a separate tank, was injected with vehicle only (1 ml kg^{-1}). The fish were fed live shrimp to satiation once a day. On day 16, the fish were killed by an overdose of MS222 and weighed. The liver and gonads were dissected out, weighed and analyzed for zinc and copper as described above.

Zinc transport in isolated hepatocytes

Hepatocytes from longjaw squirrelfish were isolated according to Walsh (1987) and Kennedy *et al.* (1991). 100 μl of a $^{65}\text{Zn}/\text{ZnSO}_4$ stock solution (223 $\text{Bq } \mu\text{l}^{-1}$, 15 $\mu\text{mol ml}^{-1}$) was added to 10 ml of hepatocytes, containing 24–60 mg cells ml^{-1} in modified Hank's medium, supplemented with 3 mmol l^{-1} glucose and 1 mmol l^{-1} CaCl_2 , pH 7.3, in a plastic vial. Thus, the total concentration of zinc in the transport medium was 148 $\mu\text{mol l}^{-1}$ and the ^{65}Zn activity was 2.21 kBq ml^{-1} , pH 7.2. Cells were agitated during the assay, using a shaking platform. At 1, 2, 4, 6, 10, 15, 30 and 60 min, a 1 ml sample of the cell suspension was separated from the medium by centrifugation through 0.5 ml of 1-bromodecane oil (Sigma). A 800 μl sample of the transport medium was assayed for ^{65}Zn by gamma counting and for zinc by atomic absorption spectroscopy. The oil and remaining medium were discarded and the hepatocytes counted for ^{65}Zn .

For the transport kinetics experiment, seven stock solutions of $^{65}\text{Zn}/\text{ZnSO}_4$ were prepared containing 234, 469, 937, 1870, 3750, 7500 and 15 000 $\text{pmol zinc } \mu\text{l}^{-1}$, respectively. The specific activity of ^{65}Zn was 14.9 Bq nmol^{-1} in all stock solutions. 10 μl of each stock was added to separate Eppendorf tubes. The transport assay was started by the addition of 1 ml of cells (24–60 mg cells ml^{-1}) in modified Hank's medium (described above). The reaction was terminated after 1 min (which gave initial inward transport rates), using the same method as described above for the time-course experiment. The rest of the procedure was the same as that in the time-course experiment.

The unidirectional influx of zinc (J_{in} , $\text{nmol mg}^{-1} \text{min}^{-1}$) was calculated according to the following equation:

$$J_{\text{in}} = CC / (SA \times m \times t), \quad (1)$$

where CC is the counts in the cells (cts min^{-1}), SA is the average specific activity of ^{65}Zn in the transport medium during the assay ($\text{cts min}^{-1} \text{nmol}^{-1}$), m is the mass of the hepatocytes (mg) and t is the time for the flux (min). The time parameter was omitted for the calculation of inward zinc transport (nmol mg^{-1}) in the time-course experiment. Net flux of zinc (J_{net} , $\text{nmol mg}^{-1} \text{min}^{-1}$) was calculated as follows:

$$J_{\text{net}} = ([\text{Zn}]_{t0} - [\text{Zn}]_{t1}) / (m \times t), \quad (2)$$

where $[\text{Zn}]_{t0}$ and $[\text{Zn}]_{t1}$ are the concentrations of total zinc in the transport medium at the beginning and the end, respectively, of the assay (nmol ml^{-1}), m is the mass of the hepatocytes (mg) and t is the time for the flux (min). The time parameter was omitted for the calculation of net zinc transport (nmol mg^{-1}) in the time-course experiment. The unidirectional outward zinc flux (J_{out} , $\text{nmol mg}^{-1} \text{min}^{-1}$) was calculated by subtracting J_{net} from J_{in} . For calculation of the unidirectional outward zinc transport (nmol mg^{-1}), the net zinc transport was subtracted from the inward zinc transport.

Differences between females and males in tissue metal concentrations were assessed using the Mann–Whitney U -test. Analysis of variance (ANOVA), extended with the Tukey HSD test for *post-hoc* comparison of means, was used to detect differences between females and males in zinc transport across the plasma membrane of primary hepatocytes. Groups were considered different at $P < 0.05$.

Results

The longjaw squirrelfish collected were of both sexes and varied considerably in reproductive status. It is not known whether the fish were first-time spawners. Untreated females ($N=12$) had a mean GSI of 0.80 (range 0.16–6.14) and a mean LSI of 1.11 (range 0.48–1.64). The mean GSI and LSI of untreated males ($N=9$) were 0.86 (range 0.07–2.13) and 0.91 (range 0.69–1.28), respectively. Female longjaw squirrelfish accumulated markedly higher concentrations of zinc in the liver than did the males (Fig. 1A). The zinc concentration in the liver of the male squirrelfish did not differ significantly from that of the gulf toadfish ($648 \pm 86 \text{ nmol g}^{-1}$, mean \pm S.E.M.,

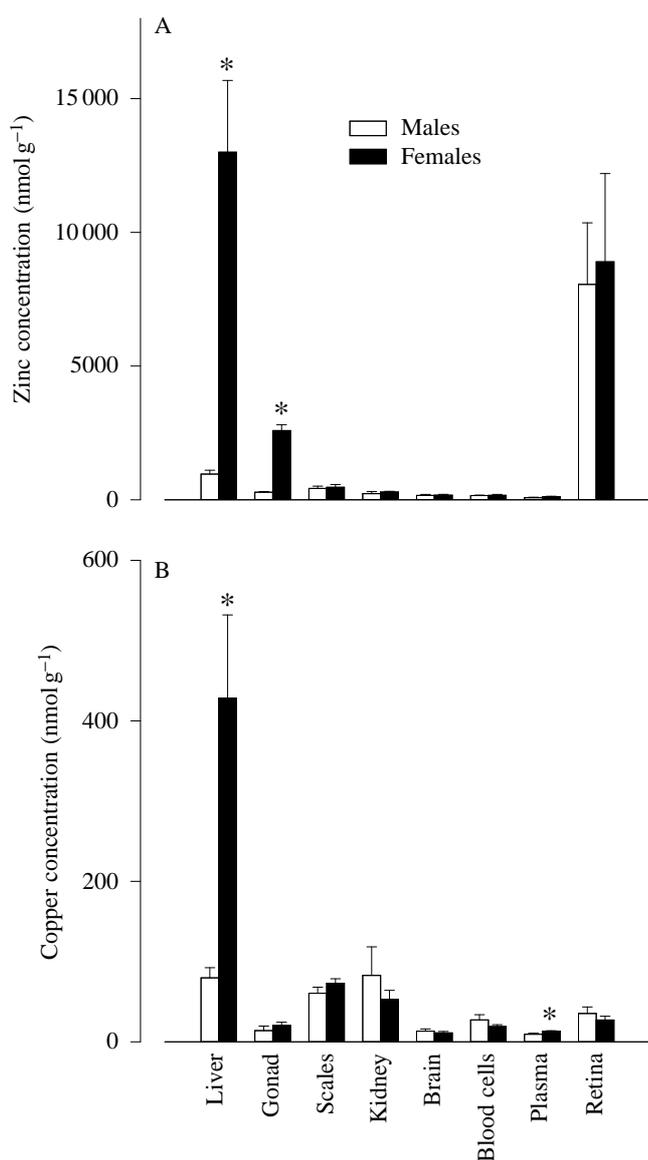


Fig. 1. Concentrations of (A) zinc and (B) copper in various tissues of male (open bars) and female (filled bars) longjaw squirrelfish. Values are expressed as mean + S.E.M. (per gram wet mass). The numbers of observations for males/females were: liver 11/15, gonad 11/5, scales 4/4, kidney 4/5, brain 4/5, blood cells 3/5, plasma 3/5 and retina 4/5. An asterisk indicates that the value for females is statistically different from that for males at $P < 0.05$ (Mann–Whitney U -test).

$N=6$). Of eight additional tissues examined, only the gonads of females showed an elevated concentration of zinc in comparison with those of males (Fig. 1A). The zinc level was high in the retina, but there was no sex-specific difference in the zinc concentration of this tissue. There was no significant correlation between the level of zinc in the liver of individual females and body mass, GSI, LSI, ovarian Zn concentration, or ovarian Zn content (data not shown). The hepatic concentration of copper was significantly higher in females than in males, although the hepatic copper level in female

squirrelfish liver was less than one-thirtieth of the zinc level (Fig. 1B). The copper level was low in all other tissues of both females and males. However, there was a slightly but significantly higher concentration of copper in the blood plasma of females than in that of males (Fig. 1B). The zinc and copper levels of muscle tissue were both below the level of detection (30 and 60 nmol g^{-1} , respectively) in males and females.

The hypothesis that females accumulate more zinc from their food than males was explored in two experiments. In the first of these experiments, we analyzed the intestinal contents of newly captured longjaw squirrelfish for zinc content. The zinc concentration in the intestinal contents of female squirrelfish was $237 \pm 27 \text{ nmol g}^{-1}$ (mean \pm S.E.M., $N=5$), which was not significantly different from the $214 \pm 71 \text{ nmol g}^{-1}$ found in the intestinal contents of males. Thus, female squirrelfish do not seem to eat a more zinc-rich diet than do males. In the second experiment, the fish were starved up to 12 days after capture to determine whether the females need a continuous source of zinc to maintain the high concentration of hepatic zinc. Food deprivation for 4 and 12 days caused a progressive decrease in the liver size of both females and males, as shown by a reduced liver-somatic index (Table 1). There was a trend of increasing zinc concentration in the livers of both genders concomitant with the reduction in liver somatic index, but the total zinc content of the livers remained unchanged during the experiment. There was no evidence for a loss of hepatic zinc during food deprivation, indicating that female squirrelfish are not dependent upon a constant supply of zinc with their food to maintain a high zinc content in the liver. Starvation did not change the zinc concentration in the gonads or the gonadosomatic index in either of the genders (Table 1).

Zinc transport rates across the plasma membrane of isolated hepatocytes were studied to investigate the possibility that differences in transmembrane zinc transport are responsible for the difference in zinc accumulation between female and male livers. Squirrelfish hepatocytes from both genders, incubated at a total extracellular zinc concentration of $148 \mu\text{mol l}^{-1}$, accumulated ^{65}Zn in a biphasic manner (Fig. 2A). An initial fast phase, during the first 1–2 min, was followed by a slower accumulation that continued throughout the 60 min transport period. Inward transport of zinc in male hepatocytes reached the slower second phase more quickly than did the hepatocytes from females (Fig. 2A). This difference resulted in significantly higher amounts of ^{65}Zn accumulated by female hepatocytes at the 10, 15 and 30 min time points, compared with male hepatocytes. In both female and male hepatocytes, the outward zinc transport balanced the inward transport (Fig. 2B), so that there was no net gain or loss of zinc at the end of the transport period (Fig. 2C). Differences in efflux were significant at 2, 10, 15 and 30 min between males and females. Thus, hepatocytes from female squirrelfish have a higher capacity than male hepatocytes to transport zinc in both directions across the plasma membrane.

The concentration-dependence of the initial rate of zinc influx (i.e. 1 min transport assays) indicated that zinc influx

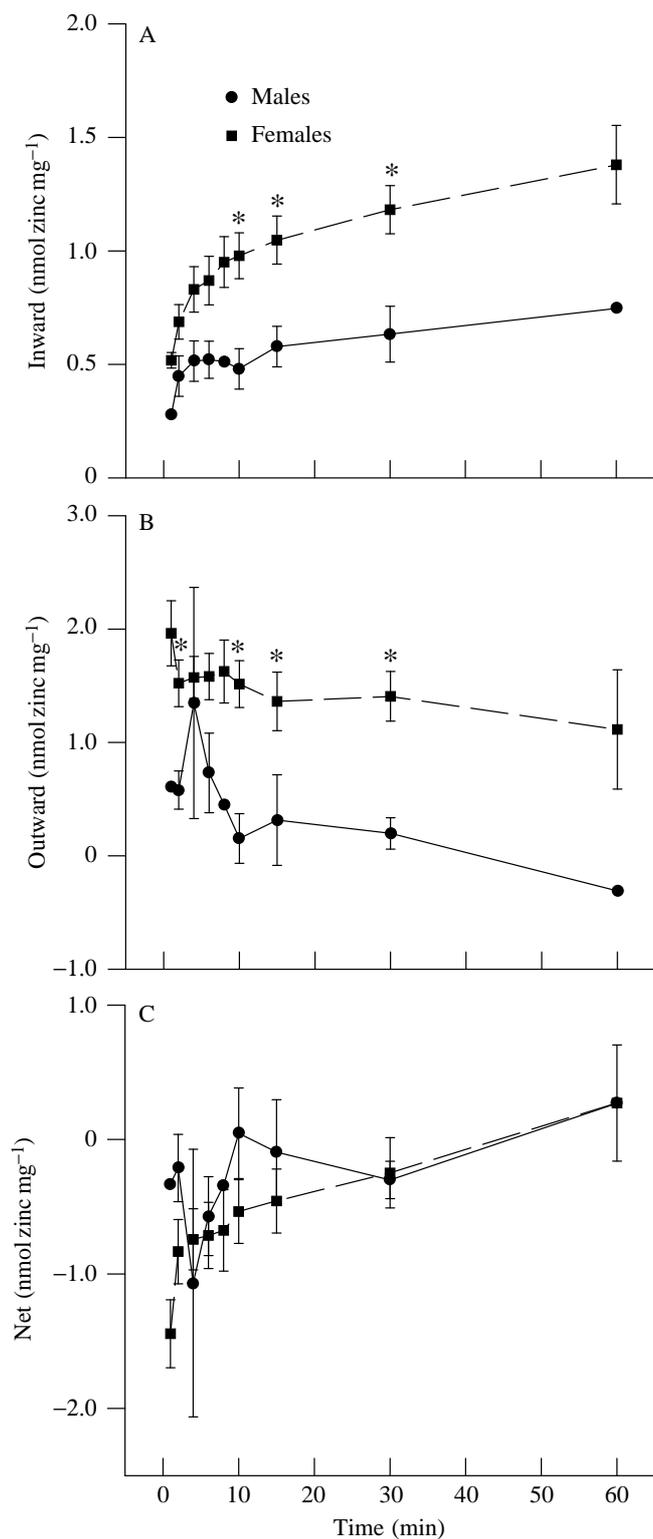


Fig. 2. Time-course kinetics of unidirectional zinc transport across the plasma membrane of primary hepatocytes from longjaw squirrelfish. For females, each point represents the mean of 3–7 fish; for males, the 1 min point is for hepatocytes from a single fish and all other points are the averages from 2–3 fish. The vertical error bars show the S.E.M. An asterisk indicates a significant difference between males and females ($P < 0.05$; ANOVA, Tukey HSD test).

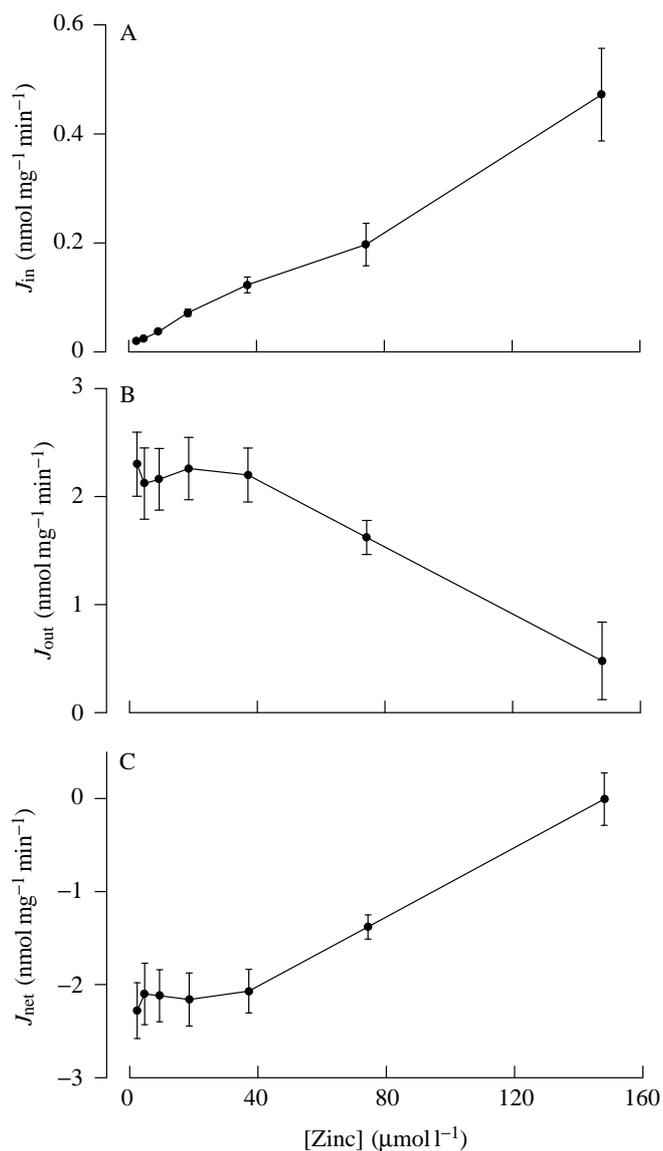


Fig. 3. The effects of zinc concentration on unidirectional zinc fluxes across the plasma membrane of primary hepatocytes from female longjaw squirrelfish. Each point represents the mean of four fish and the vertical error bars show the S.E.M. J_{in} , influx; J_{out} , efflux; J_{net} , net flux.

was a non-saturable process, at least in the concentration range 2.3–148 $\mu\text{mol l}^{-1}$ total zinc (Fig. 3A). Zinc efflux, in contrast, was maintained at a constant level at external total zinc concentrations below 40 $\mu\text{mol l}^{-1}$ (Fig. 3B). Above 40 $\mu\text{mol l}^{-1}$, the efflux rate decreased linearly with increasing concentrations of zinc in the medium. At an external total zinc concentration of 148 $\mu\text{mol l}^{-1}$, which was similar to that in the blood plasma and in the kinetics tests shown in Fig. 2, zinc efflux balanced zinc influx, and there was no net flux across the plasma membrane (Fig. 3C).

Female longjaw squirrelfish were injected with 17β -oestradiol to elucidate whether zinc accumulation in the liver is triggered by oestrogens. Contrary to the prediction, 17β -

Table 1. Effect of food deprivation on liver-somatic index (LSI), gonadosomatic index (GSI) and the concentrations and total contents of zinc in liver and gonads of female and male longjaw squirrelfish

Gender	Time starved (days)	N	LSI	GSI	Zinc			
					Liver ($\mu\text{mol g}^{-1}$)	Liver (μmol)	Gonad ($\mu\text{mol g}^{-1}$)	Gonad (μmol)
Females	0	3	1.43±0.20 ^a	0.78±0.21	12.6±2.5	45.0±22.8	2.66±0.13	4.75±0.32
	4	5	1.05±0.19 ^{b,c}	1.08±1.08	11.8±16.4	28.3±42.2	2.95±0.92	6.20±5.77
	12	3	0.96±0.04 ^c	1.31±0.66	18.0±5.3	45.6±14.9	2.80±0.03	9.72±5.09
Males	0	2	1.16±0.17 ^a	0.76±0.60	0.54±0.01 ^a	1.58±0.14	0.31±0.05	0.54±0.33
	4	4	0.90±0.12 ^{a,c}	1.23±0.86	0.89±0.46 ^{a,b}	1.67±1.14	0.30±0.08	0.78±0.58
	12	2	0.65±0.04 ^c	1.47±1.14	1.73±0.10 ^b	3.46±1.17	0.28±0.05	0.91±0.54

Values are means ± S.D.

Statistically significant differences between groups at $P < 0.05$ are shown by superscript letters. Groups sharing the same superscript letter are not significantly different (ANOVA-Tukey HSD).

Table 2. Effects of 17 β -oestradiol treatment on liver-somatic index (LSI), gonadosomatic index (GSI) and levels of zinc and copper in the liver and ovaries of female longjaw squirrelfish

Treatment	N	LSI	GSI	Zinc ($\mu\text{mol g}^{-1}$)		Copper ($\mu\text{mol g}^{-1}$)	
				Liver	Ovaries	Liver	Ovaries
Sham	6	2.02±0.28	1.62±0.53	5.76±1.34	2.31±0.35	0.58±0.10	BD†
Oestradiol	6	1.93±0.24	1.31±0.33	3.19±1.41*	5.30±1.27*	2.17±0.97	BD†

†BD, below the detection limit, 0.008 $\mu\text{mol g}^{-1}$.

The fish were injected with 17 β -oestradiol (6 mg kg⁻¹) on days 0 and 5, and were then killed on day 16. A control group was sham-injected with vehicle only.

Values are means ± S.E.M.

A statistically significant difference from the control value is shown by an asterisk ($P < 0.05$, Mann-Whitney *U*-test).

Table 3. Concentrations of zinc and copper in subcellular fractions of liver from female and male longjaw squirrelfish

Element	Gender	N	Zinc or copper (nmol g ⁻¹ liver)			
			Nucleus	Mitochondria and lysosomes	Microsomes	Cytosol
Zinc	Females	5	4391±3521*	447±89*	1348±871*	5146±2101*
	Males	4	87±33	69±27	129±13	754±142
Copper	Females	5	331.0±270.1	26.3±4.1	98.6±38.6	88.0±21.3
	Males	4	21.0±1.3	18.7±5.1	40.9±3.8	45.6±16.3

Values are expressed as mean ± S.E.M.

Significant differences from values for male longjaw squirrelfish ($P < 0.05$) are denoted with asterisks.

oestradiol treatment decreased the zinc concentration in the liver by 45 %, compared with that of sham-injected females, while the ovarian zinc concentration increased by 130 % (Table 2). The liver-somatic and gonadosomatic indices were not changed by the treatment. However, it is possible that the liver size increased and then returned to its initial size during the 10 days between the last 17 β -oestradiol injection and sampling.

All subcellular fractions of female squirrelfish liver analyzed contained more zinc than those of male squirrelfish (Table 3). In female livers, zinc was predominantly found in the nucleus and cytosol (39 % and 45 % of the total content, respectively). In males, 73 % of the hepatic zinc was present in the cytosol,

while the nucleus contained only 8 %. The distribution of zinc in males was very similar to that in both females and males of a control species, the gulf toadfish (nucleus 11±2 %; mitochondria and lysosomes 19±3 %; microsomes 13±7 %; cytosol 57±5 %, mean ± S.E.M. expressed as a percentage of total hepatic liver concentration). There were no statistically significant differences between female and male longjaw squirrelfish in copper levels of the various subcellular fractions.

Since it was known that MT is a major zinc-binding ligand in squirrelfish liver (Hogstrand and Haux, 1990a, 1996), we investigated whether MT was co-localized with zinc in the

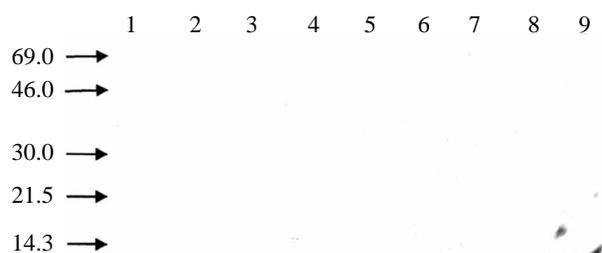


Fig. 4. Western blot for metallothionein (MT) of subcellular fractions from male and female squirrelfish liver. Lanes 1, 3, 5 and 7 are male cytosol, microsomes, mitochondria-lysosomes and nuclei, respectively. Lanes 2, 4, 6 and 8 are female cytosol, microsomes, mitochondria-lysosomes and nuclei, respectively; lane 9 is perch MT. Arrows indicate mobilities of molecular mass standards (given as kDa). The assay was repeated on a total of six females and five males with reproducible results.

nucleus and cytosol of females. MT in subcellular fractions was analyzed using SDS-PAGE, followed by western blotting. The western blot analysis showed that female squirrelfish had a much higher hepatic MT concentration than the males and that virtually all the MT in the females was located to the nucleus (Figs 4, 5). Fig. 4 shows a blot of a gel on which all collected fractions (i.e. nuclei, mitochondria and lysosomes, microsomes, cytosol) were run together to illustrate the vast abundance of MT in the nuclei of females, compared with other fractions from both males and females. Because of the very high MT content in female nuclei, it was not possible to visualize cytosolic MT on the same gel. In Fig. 5, two separate western blots are presented. Fig. 5A shows a blot of nuclear fractions from both females and males; Fig. 5B shows the results from a blot of hepatic cytosol from both sexes. The two gels were run under identical conditions. However, in order to obtain a signal from the MT in cytosolic fractions (Fig. 5B), the exposure time during the ECL detection was longer than that for the blot of the nuclear fractions (Fig. 5A). The western analyses showed that the difference in MT levels between females and males lay in the presence of high quantities of MT

in the hepatic nuclear fraction; there did not seem to be a difference in the cytosolic MT level between females and males. Nevertheless, almost half of the hepatic zinc was found in the cytosol of the females (Table 3), which suggests that the cytosolic zinc was not bound to MT. In an attempt to demonstrate the presence of a major cytosolic non-MT zinc-binding protein, we incubated each subcellular fraction with ^{65}Zn and ran the fractions on 12% SDS-PAGE under non-denaturing conditions (i.e. without addition of mercaptoethanol and boiling). SDS was included in the buffers to mobilize the proteins in the membrane fractions. Autoradiography revealed a single zinc-binding protein and it was only present in the cytosol of females (Fig. 6). This female-specific zinc-binding protein (FZnBP) had a relative mobility of 0.62, which was similar to that of a 28 kDa standard. MT migrated close to the front (relative mobility 0.95). Coomassie Blue staining of the gel confirmed that FZnBP was an abundant protein found exclusively in the cytosol of females (data not shown). To address the potential problem of interference by SDS with the zinc-binding properties of proteins, the same experiment was repeated without SDS in the buffers. This native PAGE provided the same results as the SDS-PAGE (i.e. a single 28 kDa protein band in female liver cytosol).

Discussion

The present study demonstrates that zinc accumulation in the liver of squirrelfish is specific for females, while the males have zinc levels that are typical for that of any vertebrate (Underwood, 1977; Vallee and Falchuk, 1993; Hogstrand and Wood, 1996). Of eight additional tissues examined, only the gonads of females showed an elevated concentration of zinc in comparison with males (Fig. 1). The ovarian zinc level was also much higher than that in other fish species (Hogstrand and Wood, 1996). Furthermore, injection of 17β -oestradiol resulted in a decreased hepatic zinc level while the ovarian zinc content increased, indicating that zinc may have been transferred from the liver to the ovaries (Table 2). The high

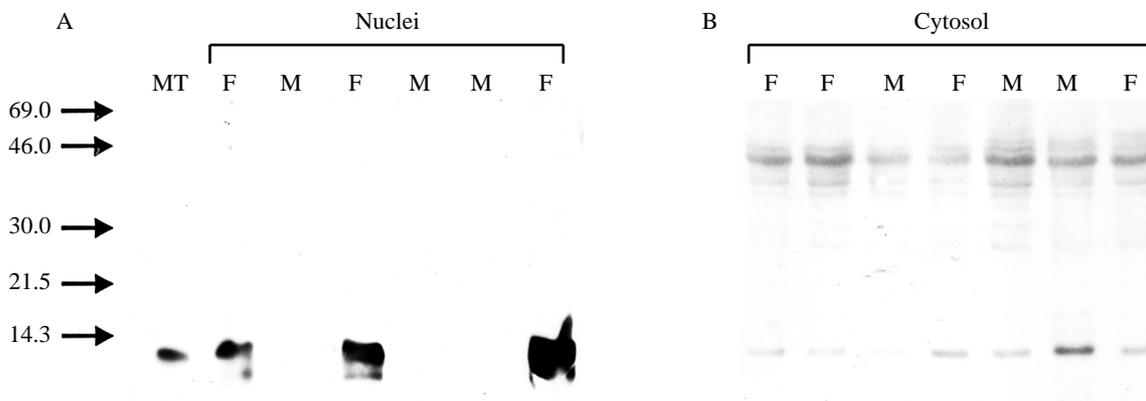


Fig. 5. Western blots for metallothionein in subcellular fractions of female and male squirrelfish livers. (A) A blot of nuclear fractions; (B) a blot of cytosolic fractions. MT, perch metallothionein; M, male squirrelfish; F, female squirrelfish. Arrows indicate mobilities of molecular mass standards (given as kDa).

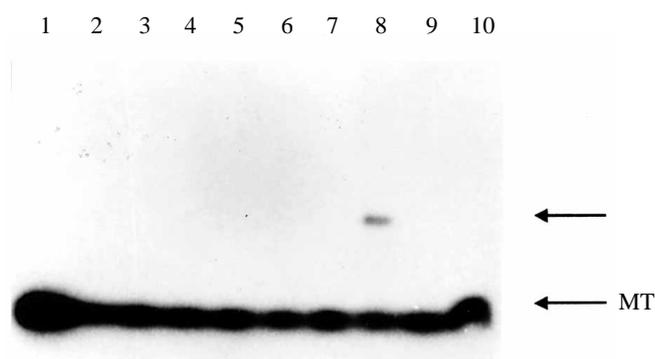


Fig. 6. Autoradiogram of ^{65}Zn -binding proteins in subcellular fractions from longjaw squirrelfish liver, separated on 12% SDS-PAGE under non-denaturing conditions. Lane 1 is ^{65}Zn only; lanes 2, 4, 6 and 8 are female nuclei, mitochondria-lysosomes, microsomes and cytosol, respectively; lanes 3, 5, 7 and 9 are the same subcellular fractions from males; lane 10 is rainbow trout metallothionein (MT). The arrows indicate the location of the female-specific cytosolic ^{65}Zn -binding protein and rainbow trout MT. The front is the dense line at the bottom of the gel. Non-protein-bound ^{65}Zn (lane 1) migrated with the front, presumably because of complexing to components of the running buffer. The assay was repeated on samples from a total of five individuals from each sex, with reproducible results

zinc concentration in the retina, which was not sex-specific, is a general characteristic of vertebrates (Underwood, 1977; Vallee and Falchuk, 1993; Hogstrand and Wood, 1996) and is probably unrelated to zinc accumulation in the liver and ovaries. Thus, female squirrelfish may have evolved a very unusual mechanism to utilize massive amounts of zinc for reproductive purposes.

The higher hepatic zinc levels measured in females than in males are probably not related to differences in food preferences because the zinc concentrations of the intestinal contents of newly collected squirrelfish were equally low ($<240\text{ nmol g}^{-1}$) in both females and males. Furthermore, food deprivation had no effect on the hepatic zinc content. Instead, we found a difference between the genders at the level of the liver cells. Hepatocytes from females transported zinc at a much faster rate than male hepatocytes in both directions across the plasma membrane. This higher zinc-transporting rate of female hepatocytes could explain the ability of females to take up large amounts of zinc in the liver.

The concentration-dependence of zinc influx during the initial fast phase showed a first-order relationship between the concentration of zinc in the medium and the rate of zinc influx. These results suggest that the fast phase of zinc influx in squirrelfish hepatocytes occurs by passive diffusion, possibly through an ion channel. The only previous study on zinc uptake in fish liver is the classic work of Saltman and Boroughs (1960), in which ^{65}Zn and liver slices were used to analyse zinc transport in liver cells of pufferfish (*Tetraodon hispidus*). Using a 40 min incubation period (compared with 1 min in the present study) and a range of zinc concentrations

between 15 and $490\text{ }\mu\text{mol l}^{-1}$ (compared with $2.3\text{--}148\text{ }\mu\text{mol l}^{-1}$ in the present study), it was found that accumulation of ^{65}Zn showed saturation kinetics (Saltman and Boroughs, 1960). Because of the long incubation time, it is possible that this saturation resulted from a limited number of binding sites inside the cells rather than a limitation of the transport rate. In rat hepatocytes, both saturable and non-saturable processes seem to be involved in zinc uptake (Stacey and Klaassen, 1981; Pattison and Cousins, 1986; Taylor and Simons, 1994). The saturable pathway appears to be a high-affinity, energy-dependent carrier with a K_m in the range $2\text{--}13\text{ nmol l}^{-1}$ (Taylor and Simons, 1994). In contrast, the non-saturable pathway has been estimated to make a significant contribution to zinc uptake only at free Zn^{2+} concentrations greater than $1\text{ }\mu\text{mol l}^{-1}$. The total concentration of zinc in the plasma of mammals ranges from 8 to $50\text{ }\mu\text{mol l}^{-1}$ (Underwood, 1977), but the concentration of free Zn^{2+} is probably no more than 0.2 nmol l^{-1} (Magneson *et al.* 1987). Thus, the high-affinity pathway is likely to be the carrier of importance in rat hepatocytes under physiological conditions. If there is a similar high-affinity zinc transporter in squirrelfish, we would not have been able to detect its activity because of the assay conditions used. In rat hepatocytes, saturation kinetics were only observed when a chelator (i.e. albumin or histidine) was used to buffer the concentrations of free Zn^{2+} (Pattison and Cousins, 1986; Taylor and Simons, 1994). No Zn^{2+} chelator was used in the present study. However, squirrelfish hepatocytes may not need a high-affinity zinc transporter. Blood plasma in fish contains about 10 times more zinc than that in mammals (Hogstrand and Wood, 1996). In rainbow trout, 99.8% of the total zinc is bound to plasma proteins, which would mean that the free Zn^{2+} concentration is approximately $0.6\text{ }\mu\text{mol l}^{-1}$ (Bettger *et al.* 1987) or 3000 times higher than that in the rat. Hence, the low-affinity, high-capacity pathway for zinc should be of greater importance for zinc influx in squirrelfish hepatocytes than it is in hepatocytes from the rat.

The outward transport of zinc was also markedly higher in hepatocytes from female squirrelfish than in hepatocytes from males. The outward transport in male hepatocytes was close to zero during the entire experiment (Fig. 2B). At $148\text{ }\mu\text{mol l}^{-1}$ total zinc in the assay medium, the zinc efflux balanced the influx and there was no net transport of zinc. Incidentally, this zinc concentration was close to the total plasma zinc level in female squirrelfish ($115\text{ }\mu\text{mol l}^{-1}$). At lower zinc concentrations, there was a net loss of zinc from female hepatocytes and the efflux increased steadily with decreasing zinc concentration in the medium down to $40\text{ }\mu\text{mol l}^{-1}$. Below this concentration, there was no further change in zinc efflux or net flux with decreasing external zinc concentration. The kinetics of zinc efflux suggests that the outward transport of zinc in female squirrelfish hepatocytes is dependent upon the concentration gradient and that the transport is saturable. Recently, Palmiter and Findley (1995) cloned a zinc transporter (ZnT-1) from rat and mouse that seems to mediate zinc efflux in cultured cells. It would be interesting to probe

for the presence of ZnT-1 in squirrelfish and for differences in its expression in females and males.

The high capacity of female hepatocytes to transport zinc across the plasma membrane may be the mechanism for enhanced zinc uptake, but it cannot account for the dramatically different zinc concentrations in the liver unless females also have a specific mechanism to retain zinc in the liver. Female squirrelfish have at least two zinc-binding polypeptides that could serve to increase the zinc storage capacity. Up to 70% of the zinc in squirrelfish liver is bound to MT, and the level of MT is closely correlated with the hepatic zinc concentration (Hogstrand and Haux, 1996). In the present study, we show that there is not only a marked difference in hepatic MT concentrations between female and male squirrelfish but also that MT has a different subcellular localization in the two genders. MT is considered to be primarily a cytosolic protein (Bremner and Beattie, 1990; Cherian, 1994), and this is where most MT was located in male squirrelfish. In contrast, the females had practically all their MT in the nucleus. Extensive nuclear localization of MT has only been reported under particular conditions (Cherian, 1994). Specifically, MT is found in the nucleus of mammalian hepatocytes during late foetal and early neonatal development (Panemangalore *et al.* 1983; Sato and Bremner, 1983; Nartey *et al.* 1987; Tsujikawa *et al.* 1994). Also, in growing primary cultures of gastric mucosal cells (Tsujikawa *et al.* 1991a) and in primary hepatocytes from partially hepatectomized rats (Tsujikawa *et al.* 1994), MT is predominantly present in the nuclei. In rat hepatocytes stimulated by epidermal growth factor and insulin and in carcinoma cells, MT accumulates in the nucleus during the S phase of the cellular cycle (Tsujikawa *et al.* 1991b; Cherian, 1994). Thus, the presence of nuclear zinc-MT seems to be linked to cell proliferation. At this point, we can only speculate about the significance of nuclear MT in the liver of female squirrelfish. MT could be involved in the expression of female-specific proteins or it may serve as a vehicle to move zinc from the cytosol to the nucleus.

The nuclear localization of MT in female squirrelfish liver can only partially explain the subcellular distribution of zinc. The nucleus contained on average 39% of the zinc contained in the liver. Another 45% of the hepatic zinc was found in the cytosol, where there was little MT. Using non-denaturing SDS-PAGE analysis of ⁶⁵Zn-labelled cytosol, we were able to identify a prominent female-specific cytosolic zinc-binding protein, FZnBP (Fig. 6). This zinc-binding protein had a relative mobility of 0.62 on the gel, similar to a molecular mass standard of 28 kDa. On the same gel, rainbow trout MT had a relative mobility of 0.95, quite different from that of FZnBP. Fish vitellogenin monomers migrate like a 270 kDa molecular mass standard on native PAGE (Silversand and Haux, 1989). Thus, it can be concluded that the FZnBP of female liver cells is most probably neither MT nor vitellogenin. Given that zinc is utilized by some 300 proteins (Vallee and Falchuk, 1993), the presence of a single band after autoradiographic detection of ⁶⁵Zn was surprising. MT migrated very close to the front of this 12% gel, which probably explains why no separate MT

band could be distinguished in the lane corresponding to the nucleus from female fish. Still, there were undoubtedly other zinc-binding proteins in the liver fractions that were not revealed by autoradiography. We considered the possibility that the SDS present in the gel buffer system could have perturbed the zinc-binding properties of proteins other than FZnBP. However, the same results were obtained when SDS was omitted from the buffers, and it was concluded that the results were therefore not an artefact due to the presence of SDS. It is likely that FZnBP was the only protein visualized simply because it bound more ⁶⁵Zn than any other cytosolic zinc-binding protein present. Coomassie Blue staining of the gel confirmed that FZnBP is, indeed, a prominent protein in the female cytosol. Thus, the absence of bands from other zinc-binding proteins was likely to be the result of the limited sensitivity of the method used. At this point, the function of FZnBP is unknown, but it seems to be the major zinc-binding ligand in the hepatic cytosol of female squirrelfish.

In two recent studies, it was shown that vitellogenin from *Xenopus laevis* is a zinc-binding protein that binds 1 g atom of zinc per 220 kDa monomer (Montorzi *et al.* 1994, 1995). Vitellogenin is synthesized in the livers of oviparous animals in response to oestrogens and, in *X. laevis*, the zinc bound to vitellogenin is delivered to the growing oocytes as vitellogenin is transported to the ovaries *via* the blood and is taken up by the oocytes through receptor-mediated endocytosis (Montorzi *et al.* 1995). Thus, redistribution of zinc from the liver to the ovaries in response to oestrogens may be a common theme for oviparous species, but so far it has only been shown to occur in *X. laevis* and is now indicated for longjaw squirrelfish. It is obvious that all animals need zinc for their embryonic development (Vallee and Falchuk, 1993), but what makes squirrelfish unique is the unprecedented quantities of zinc that are stored in the liver and subsequently in the ovaries. It is tempting to speculate that squirrelfish vitellogenin may have a high zinc-binding capacity, but so far we have no data to support this speculation.

The present study shows that female squirrelfish accumulate remarkably high levels of zinc in the liver and ovaries for purposes that are probably related to reproduction. The mechanisms for this unique zinc accumulation include a high capacity of hepatocytes in the female to take up zinc. Influx of zinc into the hepatocytes occurs, at least partially, *via* a low-affinity, high-capacity system, whereas zinc efflux may be mediated by a carrier protein. The female liver contains two prominent polypeptides that seem to be responsible for the accumulation of zinc. One of these polypeptides is MT, which in female squirrelfish liver is found mainly in the nucleus rather than in the cytosol, where it is usually located in other animals. Zinc binding in the cytosol is dominated by a novel female-specific protein, FZnBP. Although zinc is recognized as an essential element, the metal is often regarded as a toxic. The present study demonstrates the excellent ability of squirrelfish hepatocytes to handle zinc; indeed, female squirrelfish seem to utilize zinc as a macronutrient rather than as a micronutrient.

This research was supported by SJFR (98.0702/91 T2), NIEHS (ES-05705), NSF (IBN 95-07239), NSERC and South Florida Water Management District. We thank Dr C. Haux and Dr T. P. Mommsen for their valuable comments on the manuscript.

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