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Sexual maturation and reproductive zinc physiology in the female squirrelfish

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Summary

Female squirrelfish (Holocentridae) accumulate higher concentrations of hepatic zinc than any other known organism. In the liver cells, up to 70% of zinc is bound to metallothionein (MT), which is expressed at extremely high levels. These attributes are related to reproduction in ways that have not been fully characterized. In the present study, we have demonstrated that female-specific zinc and MT accumulation and distribution are strongly correlated to the onset of sexual maturity in *Holocentrus adsenscionis*. Sexual maturation not only resulted in increased concentrations of zinc in the liver and plasma, but also increased levels of hepatic MT mRNA. Furthermore, mature female squirrelfish exhibited greater proportions of MT protein in the nuclear liver cell fractions. To characterize the physiology further, we have examined the influence of the female sex hormone 17 β-estradiol (E_2). E_2 was not sufficient to elicit an increase in hepatic zinc concentrations or MT mRNA levels. E_2 administration did, however, result in increased levels of MT in the nuclear fraction as well as overall hepatic MT protein. E_2 also increased concentrations of zinc in the plasma. The changes in zinc concentration in the bloodstream followed the same time course as vitellogenin (VTG) transport from the liver. However, the high ratio of molar concentrations of zinc to VTG in the bloodstream suggest that VTG may not be the primary vehicle for hepato-ovarian zinc transport in squirrelfish.

Key words: zinc, metallothionein, vitellogenin, estradiol, sexual maturity, reproductive physiology, female, squirrelfish.

Introduction

The squirrelfish family (Holocentridae) is very unusual in that its members have been found to accumulate extremely high concentrations of zinc (Hogstrand and Haux, 1991, 1996; Hogstrand et al., 1996). This phenomenon is both gender and organ specific, with only female squirrelfish accumulating large amounts of zinc in the liver and ovaries, while males maintain normal zinc concentrations (Hogstrand et al., 1996). This difference between the sexes is independent of geographic location or dietary preference (Hogstrand et al., 1996). As in other vertebrates, the retinae of both males and females contain high concentrations of zinc (Weitzel et al., 1954). However, the eyes of the nocturnal squirrelfish are conspicuously large, so the total amount of zinc in the retina is especially high when compared with total body mass (Hogstrand et al., 1996; Thompson et al., 1999). Recent studies suggest that zinc may play a protective role for photoreceptors during night vision (Wu et al., 1993; Busselberg et al., 1994), thereby raising the possibility of increased zinc accumulation serving as a means for proper visual function for the nocturnal squirrelfish.

Zinc is an essential micronutrient that is required for normal cellular function (for a general review, see Vallee and Falchuk, 1993). The absorption of zinc in marine fish occurs by two major pathways. Aqueous Zn^{2+} is taken in through the gills while the majority of zinc uptake occurs via intestinal absorption of dietary zinc and waterborne zinc (Hogstrand and Wood, 1996). Upon entering the bloodstream, zinc is bound to proteins such as albumin, which act in its transport (Fletcher and Fletcher, 1980; Dyke et al., 1987). Although the exact mechanism remains unclear, the recent discovery of the ZIP family of membrane-associated proteins seems to indicate a means for zinc to pass into the cell (Guerinot, 2000). The cellular accumulation of zinc is likely to be the result of interplay between the relative activities of this ZIP-mediated zinc import, and export by other membrane proteins such as ZnT-1 (Palmiter and Findley, 1995; Cousins and McMahon, 2000). Once within the cell, zinc is almost instantaneously chelated to a variety of zinc-binding proteins (Vallee and Falchuk, 1993). One such zinc-binding protein is metallothionein (MT). MT is capable of binding not only zinc but also other elements of groups IB and IIB of the periodic table (Kagi and Schaffer, 1988). One function of MT is to sequester potentially toxic metal species within the cell, which

is probably an extension of its role in the regulation of labile intracellular zinc. It has been shown previously that zinc can be toxic if accumulated in large amounts (Hogstrand and Wood, 1996). Thus, it is remarkable that the concentrations of zinc accumulated in the liver of female squirrelfish (up to 70 μmol g^{-1} wet weight) are higher than any other studied organism (Hogstrand and Haux, 1991, 1996; Hogstrand et al., 1996). In addition, it has also been discovered that female squirrelfish have high hepatic concentrations of MT, and MT levels are closely correlated to zinc concentrations ($0.89<r<0.99$) but not other metals that can bind MT (Hogstrand and Haux, 1996), further suggesting a role for MT in protecting female squirrelfish against potentially toxic zinc hyperaccumulation.

A seasonal shift in the subcellular localization of MT in female squirrelfish liver also exists, with MT located primarily in the liver cell cytosol in late spring and co-precipitating with the nuclear fraction in winter months (Thompson et al., 1999). Conversely, MT in male squirrelfish liver cells is maintained in the cytosol throughout the year (Hogstrand et al., 1996; Thompson et al., 1999). Overall, this gender specificity suggests that the dynamics of zinc and MT accumulation and distribution observed in female squirrelfish are in some way related to the reproductive cycle. However, the point of development at which females become segregated from males in terms of zinc metabolism remains unclear. In addition, the mechanism by which this occurs has not been identified. Because squirrelfish are not bred in captivity and are collected from their reef environment, it is logistically difficult to characterize their reproductive biology, physiology and endocrinology. Thus, we are still faced with the questions of when, as well as how, this unique phenomenon is initiated.

The purpose of the present study was therefore twofold. The first phase was to investigate the role of sexual maturation in female squirrelfish as pertaining to the accumulation and localization of zinc and MT. To do so, we have measured zinc, MT, MT mRNA and sex steroids in female squirrelfish as a function of ovary size. In a previous study involving mature females, administration of 17β-estradiol (E_2) elicited a decrease in hepatic zinc and at the same time moderated an increase in ovarian zinc (Hogstrand et al., 1996). Therefore, to further characterize the effects of this female sex hormone, the second aspect of this study was to inject immature and mature female squirrelfish with E_2, followed by analyses of the same variables as described above, to determine if E_2 is responsible for any previously observed physiological differences in zinc regulation brought on by the onset of sexual maturity.

Materials and methods

Animal care and E_2 injections

Squirrelfish [*Holocentrus adscensionis* (Osbeck, 1765)] were collected by SCUBA divers off Tavernier, FL, USA in May and June, without the use of tranquilizers. Fish were transported to the Rosenstiel School of Marine and Atmospheric Science (RSMAS), University of Miami, where they were housed in two tanks (4000 l) supplied with a continuous flow of aerated seawater (28°C) from Biscayne Bay. Fish were fed daily to satiation with live shrimp and were allowed to acclimate to laboratory conditions for a total of 4 days prior to experimentation.

The squirrelfish in one tank were given interperitoneal injections of 5 mg E_2 kg body $weight^{-1}$ (1 ml kg^{-1} in peanut oil) on days 0, 2 and 4, to maintain circulating E_2 levels. A control group housed in the other tank was injected with peanut oil only. Control and treated fish were sacrificed on either day 5, 6 or 10.

Sampling

Fish were euthanized by overdose of MS222 (0.5 g l^{-1}) and weighed. Approximately 2 ml of blood was withdrawn from the caudal vessel with a heparinized syringe, and plasma was separated from blood cells by centrifugation (14000 ***g***) for 3 min. The plasma was divided into 50 μl samples and stored at –80°C for subsequent analysis of E_2, testosterone (T), progesterone (P), vitellogenin (VTG), zinc and MT as described below. Livers and gonads were removed, weighed and immediately divided into aliquot samples. A tissue sample (approximately 0.5 g) was taken from each liver and subjected to subcellular fractionation as described below. Samples of liver (approximately 0.5 g) were placed into individual 16 mm × 150 mm borosilicate glass tubes for acid digestion and subsequent zinc analysis as described below. The remaining liver samples were wrapped in aluminum foil and frozen in liquid nitrogen. The frozen samples were transferred to –80°C, where they were stored until used for total RNA extraction as described below.

Tissue zinc content

Liver samples were subjected to acid digestion in 2.0 ml of 70% HNO_3 (trace-metal grade, Fisher, Pittsburgh, PA, USA). The tubes were heated in a sand bath for 3 h at 120°C and then cooled to room temperature before 0.5 ml of H_2O_2 was added. The temperature was then gradually increased to 120°C until all liquid had evaporated. The dried residues were reconstituted with 5.0 ml of 0.5% HNO_3. These samples were then analyzed for zinc content by air/acetylene flame atomic absorption spectroscopy (Perkin Elmer, model 2380, Shelton, CT, USA). Plasma samples (0.05 ml) were analyzed for zinc content in the same manner.

VTG and steroid analysis

Plasma samples from each fish were measured for VTG and steroid content by enzyme-linked immunosorbent assay (ELISA) (Specker and Anderson, 1994). The VTG ELISAs were performed according to the procedure of Thompson et al. (2001). Each plasma sample was diluted 1:100 in phosphate-buffered saline [PBS (0.15 mol l^{-1} NaCl, 0.0015 mol l^{-1} KH_2PO_4, 0.015 mol l^{-1} Na_2HPO_4, 0.002 mol l^{-1} KCl, pH 7.4)] and 100 μl of this dilution was added per well in 96-well Nunc-Immuno™ MaxiSorp™ microtiter plates (Nalge Nunc International, Rochester, NY, USA). Squirrelfish VTG was

isolated by ion-exchange chromatography according to the protocol of Silversand and Haux (1989), with minor modifications. This purified squirrelfish VTG was used to produce standard curves. The samples were incubated in duplicate for 1 h at 4°C to allow for optimal adsorption of antigen to the plates and were then washed four times with 300 µl PBS per well. Following the washes, the plates were treated with 150 µl of blocking buffer (5% dehydrated nonfat milk in PBS) for 30 min at room temperature. Blocking buffer was then removed and the wells were subjected to 100 µl of primary antibody and incubated for 1 h at room temperature on an orbital shaker. The primary antibody (gift from Dr Ram Abuknesha, King's College London) was a sheep immunoglobulin G (IgG) reared against a conserved VTG peptide sequence diluted 1:30000 in PBS. The crossreactivity and specificity of this antibody with squirrelfish VTG was confirmed by western blot (data not shown). After the primary antibody incubation, the plates were washed four times with PBS, subjected to 100 µl of secondary antibody, and incubated for 1 h at 37°C. The secondary antibody was a horseradish peroxidase-linked donkey anti-sheep IgG (BioRad, Hercules, CA, USA) diluted 1:2000 in PBS. The plates were then washed four times with PBS, and 100 µl of a tetramethylbenzidine (TMB) peroxidase enzyme immunoassay substrate (BioRad) was added according to manufacturer's specifications. After a 15 min incubation, 50 µl of 0.5 mol l^{-1} H_2SO_4 was added to the substrate to stop the reaction, and the plates were read on a microplate reader (BioRad, model 450) at 450 nm. E_2, T and P were analyzed using the respective steroid ELISA kits (Oxford Biomedical Research, Oxford, MI, USA) according to manufacturer's specifications.

Hepatic subcellular fractionation

Subcellular fractions of liver were obtained by differential centrifugation of liver homogenates immediately following dissection via the procedure for rainbow trout liver described by Julshamn et al. (1988), modified for squirrelfish by Hogstrand et al. (1996). Liver samples (approximately 0.5 g) were individually homogenized in an ice-cold homogenization buffer (35 mmol l^{-1} Tris-HCl, 0.20 mol l^{-1} KCl, 0.25 mol l^{-1} sucrose, pH 7.4) using a glass–Teflon homogenizer. The homogenate was centrifuged (370 ***g***) for 5 min at 4°C and the supernatant was removed, while the pellet (nuclear fraction) was immediately placed on ice. The supernatant was centrifuged (9200 ***g***) for 5 min at 4°C, and the pellet (mitochondrial/lysosomal fraction) was saved and placed on ice. This supernatant was then centrifuged (13,0000 ***g***) for 60 min at 4°C, resulting in a small pellet (microsomal fraction), which was saved and immediately placed on ice. The final supernatant (cytosolic fraction) was divided into 0.5 ml aliquot samples and immediately placed on ice. All pellets were resuspended in 0.5 ml of fresh homogenization buffer and subcellular fractions were divided into aliquots and transferred into liquid nitrogen. These samples were then stored at –80°C until used in western analysis for MT.

Western analysis

Each subcellular fraction was subjected to SDS-PAGE with a discontinuous buffer system according to the protocol of Laemmli (1970). The protein concentration for each subcellular fraction was determined by Bradford assay (Bradford, 1976), and samples were diluted with distilled water as necessary. These samples were then diluted 1:4 with sample buffer [62 mmol l^{-1} Tris-HCl, pH 6.8, 10% glycerol, 5.0% 2-mercaptoethanol (added just before dilution), 2.0% SDS, 0.0012% Bromophenol Blue] and heated at 100°C for 5 min. A total of 25 µg of protein was loaded into each well. Perch (*Perca fluviatilis*) MT was used as a standard (Hogstrand and Haux, 1990). Electrophoresis was carried out on a 4% stacking gel and a 12.5% separating gel at 100 V for 2 h in a Mini-Protean II electrophoresis system (BioRad).

After electrophoresis, proteins were transferred from polyacrylamide gels onto 54 cm^2 nitrocellulose membranes (Schleicher & Schuell, 0.2 µm pore diameter) by electroblotting, as described by Towbin et al. (1979), in a SemiPhor TE 70 semi-dry transfer unit (Hoefer Scientific, Moorestown, NJ, USA) at 0.8 mA cm^{-2} constant current for 60 min at room temperature. Once proteins were transferred, the membranes were blocked with 5% dehydrated nonfat milk in TBS-T (20 mmol l^{-1} Tris-HCl, pH 7.4, 137 mmol l^{-1} NaCl, 0.10% Tween-20) for 60 min to prohibit further nonspecific protein binding. All incubations were carried out at room temperature. The membranes were then subjected to a series of washes in fresh TBS-T (one 15 min and two 5 min) followed by a 1 h incubation in primary antibody. The primary antibody was rabbit anti-perch MT (Hogstrand and Haux, 1990) diluted 1:8000 in TBS-T. After another series of the same washes, the membranes were incubated for 1 h in secondary antibody. The secondary antibody was a horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Piscataway, NJ, USA) diluted 1:40000 in TBS-T. Following a final series of washes, immunodetection was performed with an enhanced chemiluminescence system (ECL, Amersham) according to manufacturer's specifications. Chemiluminescence was captured on photographic film (Kodak), and the optical density (OD) of each band was quantified using Sigma Gel software (Jandel Scientific, Chicago, IL, USA). Each western blot was exposed for 15 s, 30 s and 60 s to ensure the OD linearity of the film. The OD of each unknown was compared with the OD of the internal standard of each gel. Although a known amount of the perch MT standard was added to each gel, the results are presented as arbitrary units because it has not been determined if the immunoreactivity of perch and squirrelfish MT are the same. The relative amount of MT in each fraction was thus calculated as the ratio of sample OD to standard OD.

Total RNA extraction

Liver and gonad tissues previously stored at –80°C were thawed, and 50–100 mg of each sample was homogenized in 1 ml of TRIzol Reagent (Gibco BRL, Rockville, MD, USA) using a glass–Teflon homogenizer. Total RNA was then extracted from the homogenates according to manufacturer's

Table 1. *Comparison of untreated immature and mature female squirrelfish*

	GSI	LSI	VTG (μg/ml)	T (ng/ml)	E_2 (ng/ml)	P (ng/ml)	MT mRNA (relative units)	N (relative units)
Immature	0.16±0.03	1.44±0.11	0.26±0.11	3.07±0.10	1.29±0.05	34.2±0.38	0.33±0.10	0.15±0.05
Mature	1.28±0.20*	1.64±0.10	0.32±0.10	2.95±0.11	1.82±0.33	33.3±2.26	1.18±0.16*	0.49±0.10*
	P<0.001	P=0.290	P=0.553	P=0.596	P=0.536	P=0.930	P=0.001	P=0.020

Comparison of untreated immature and mature female squirrelfish in terms of gonadosomatic index (GSI); liver-somatic index (LSI); plasma vitellogenin (VTG), testosterone (T), 17β-estradiol (E_2) and progesterone (P) levels; hepatic metallothionein mRNA levels (MT mRNA); and MT protein levels in nuclear (N) liver fractions. Values are mean±S.E.M. Mann–Whitney values are included for each variable, N=5–14. Values were considered significant at P<0.05.

specifications, with minor modifications as suggested by the manufacturer. Specifically, the homogenates were centrifuged (12,000 ***g***) to remove insoluble material, and treated with a high salt precipitant solution (1.20 mol l^{-1} sodium citrate, 0.80 mol l^{-1} NaCl) to remove glycogen.

Northern analysis

Total RNA extracted from liver samples (approximately 10 μg) was subjected to electrophoresis on a 1.5% agarose gel with formaldehyde as denaturant (Sambrook et al., 1989). To standardize results, a reference sample with a constant amount of MT mRNA was loaded onto each gel. In addition, some experimental samples overlapped between gels as a control for interassay variability. After completed separation, RNA was transferred onto a 170 cm^2 Hybond N nylon membrane (Amersham) by capillary/gravity blotting using the Turbo Blotter (Schleicher & Schuell, Moorestown, NJ, USA). The RNA was UV crosslinked to the membrane followed by incubation in 18 ml of the prehybridization buffer [50% formamide, 5×SSC, 2.0% Blocking reagent (Boehringer Mannheim, Indianapolis, IN, USA), 0.10% N-laurosarcosine, 0.02% SDS] at 68°C for 4 h. The prehybridization buffer was discarded and the membrane was hybridized for 18 h at 68°C in 18 ml of the same buffer, with 0.30 μl probe ml^{-1} added, using a digoxigenin (DIG)-labeled antisense squirrelfish MT-cRNA probe (Thompson et al., 2001). The plasmid was linearized and the DIG-labeled RNA riboprobe was made from the DNA template by T7 RNA polymerase in the presence of a nucleotide mix containing DIG-labeled dUTP (Boehringer Mannheim). After hybridization, the membrane was subjected to stringency washes (2 washes in 2×SSC, 0.10% SDS for 5 min at room temperature; two washes in 0.1×SSC, 0.1% SDS for 5 min at 68°C), followed by immunochemiluminescent detection of DIG-labeled probe using a Fab fragment of sheep anti-DIG-AP conjugate (Boehringer Mannheim) and CSPD (Boehringer Mannheim) as chemiluminescent substrate. Chemiluminescence was captured on X-ray film (Kodak), and MT mRNA bands were quantified using Sigma Gel software (Jandel Scientific). Each northern blot was exposed for at least four exposure times to ensure OD linearity of the film. Arbitrary units were derived by the ratio of sample OD to reference OD.

Statistics

Because gonadal examination is the only known way to distinguish gender and sexual maturity in squirrelfish, immature females [operationally defined as gonadosomatic index (GSI) <0.25] and mature females were sorted into separate groups after sacrifice. To further characterize that selected females were indeed immature and not merely adults in a regressed ovarian stage, the mean body mass of immature and mature females was compared (immature, 157.5±5.54 g, N=9; mature, 245.8±10.8 g, N=14). E_2-injected fish were also sorted into groups of immature and mature females following sacrifice (immature, 153.9±11.6 g, N=9; mature, 240.8±11.5 g, N=13).

Squirrelfish samples were divided between the different laboratories involved in the study and further divided for different analyses. Therefore, the number of observations for each treatment group and sampling occasion was not the same for all variables, and the resulting distributions were not normal and did not exhibit equal variances. As a result, nonparametric statistical analyses have been used. Pair-wise comparisons between immature and mature female squirrelfish were determined by the Mann–Whitney U-test. For the E_2-injection experiment, trend analyses were performed by Kruskal–Wallis one-way analysis of variance (ANOVA), and any significant findings were further subjected to *post-hoc* pair-wise comparisons performed by Dunn's Test. Data are presented as means ± S.E.M. Results were considered significant at P<0.05.

Results

Immature female squirrelfish were compared with mature females to examine differences in a variety of reproductive parameters brought on by sexual maturity (Table 1). As expected according to the definition of sexual maturity, there was a significant difference in gonadosomatic index (GSI), with mature females having a mean GSI approximately eight times higher than immature females. There was no significant difference in the liver-somatic index (LSI) of immature and mature females. There was, however, a significant difference in the hepatic zinc accumulation between the two groups (Fig. 1). Mature females were found to maintain hepatic zinc

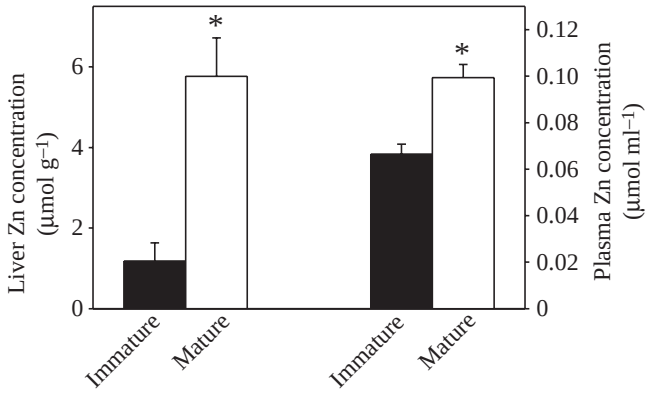

Fig. 1. Comparisons of zinc concentrations in the liver and blood plasma of untreated immature and mature female squirrelfish. Results are arithmetic means ± S.E.M. (N=11–15 fish). Asterisks indicate values that are significantly different from the sham-treated control at the same sampling point (P<0.05; Mann–Whitney U-test).

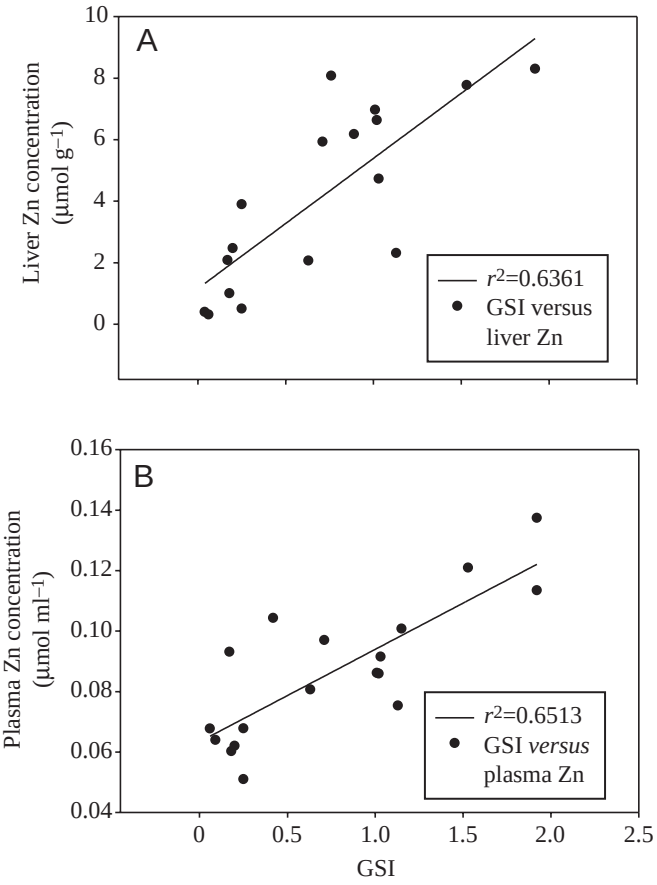

Fig. 2. Relationship of gonadosomatic index (GSI) and (A) hepatic and (B) plasma zinc levels in female squirrelfish (N=21 fish).

concentrations nearly five times greater than those observed in immature individuals. Furthermore, there was a significant linear correlation between hepatic zinc concentration and GSI (Fig. 2A, P<0.001, N=21). Plasma zinc concentrations (Fig. 1)

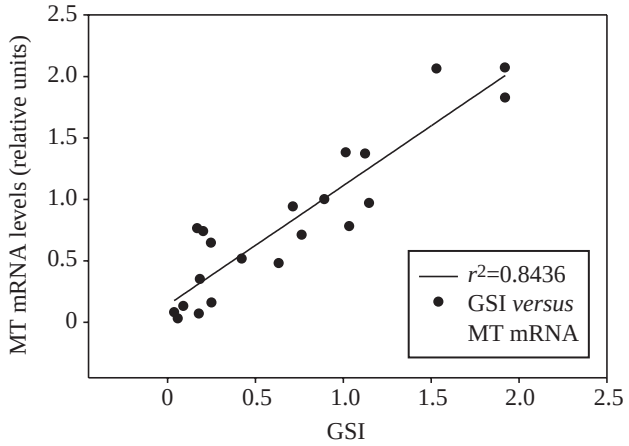

Fig. 3. Relationship of gonadosomatic index (GSI) and hepatic metallothionein (MT) mRNA levels in female squirrelfish (N=21 fish).

were significantly higher in mature females than in immature females. As with liver zinc, plasma zinc concentrations were significantly correlated with increasing GSI (Fig. 2B, P<0.001, N=21). There was no significant difference in the circulating levels of VTG between mature and immature female squirrelfish (Table 1). Plasma concentrations of E_2, T and P were also not significantly different between the two groups.

Hepatic MT mRNA levels (Table 1) observed in mature female squirrelfish were nearly four times that of their immature counterparts. Furthermore, the levels of hepatic MT mRNA were significantly correlated with increasing GSI (Fig. 3, P<0.001, N=21). On a subcellular basis, MT protein in the hepatic nuclear fraction (Table 1) was significantly greater in mature females than in immature females. There was no significant difference in the amount of MT protein observed in the cytosolic or mitochondrial/lysosomal fractions, and MT in the microsomal fraction was undetectable.

The effects of E_2 treatment were determined for both immature and mature female squirrelfish. E_2 treatment resulted in a significant increase in LSI of immature females during the sampling period as compared with control individuals (Fig. 4A), with the highest measurements occurring at day 5 and remaining elevated in E_2-treated fish at day 6 before returning to control levels by day 10 (Kruskal–Wallis ANOVA; P=0.009, N=18). The trend was similar for mature female squirrelfish (Fig. 4B), with a peak in the LSI of E_2-treated individuals at day 5 before falling back to levels similar to control individuals by day 10 (P=0.008, N=13). E_2 treatment had no effect on the GSI of immature female squirrelfish (Fig. 5A). The overall effect of E_2 on GSI of mature females was not found to be statistically significant (P=0.101, N=13). Interestingly, pair-wise comparison of means indicates that GSI of E_2-treated individuals was significantly greater than control measurements both at days 5 and 6 of the sampling period, while significantly less than control GSI measurements at day 10 (Fig. 5B). E_2 treatment had no effect on the hepatic accumulation of zinc in either immature or mature females (Tables 2, 3). However, there was an effect

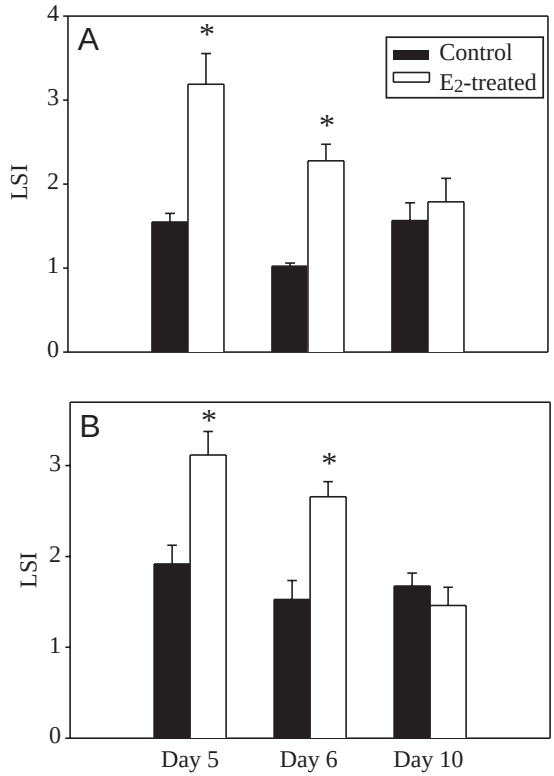

Fig. 4. Comparisons of liver-somatic index (LSI) in sham-injected (black bars) and E_2-treated (white bars) immature (A) and mature (B) female squirrelfish. Fish were injected on days 0, 2 and 4, and sampled on days 5, 6 and 10 of the experiment. Results are arithmetic means ± S.E.M. (*N*=2–7 fish). Asterisks indicate values that are significantly different from the sham-treated control at the same sampling point (*P*<0.05; Dunn's test).

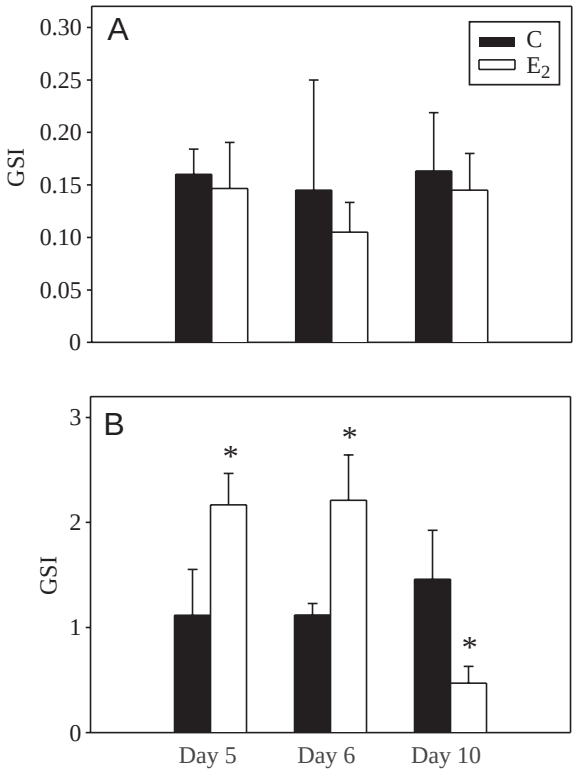

Fig. 5. Effects of E_2 administration on gonadosomatic index (GSI) in immature (A) and mature (B) female squirrelfish at days 5, 6, and 10 of the experiment (*N*=2–7 fish). Results are arithmetic means ± S.E.M. Asterisks indicate values that are significantly different from the sham-treated control at the same sampling point (*P*<0.05; Dunn's test).

on zinc accumulation in the blood plasma of immature females (Fig. 6A) with plasma zinc remaining significantly elevated throughout the sampling period (*P*=0.002, *N*=6). The same effect was observed in mature female squirrelfish (Fig. 6B; *P*<0.001, *N*=12), although levels at day 10 were approximately 50% less than plasma zinc concentrations at days 5 and 6 in E_2-treated individuals. Likewise, plasma VTG concentrations were significantly increased in E_2-treated fish compared with simultaneous controls in both immature (Table 2, *P*=0.006, *N*=8) and mature (Table 3, *P*=0.001, *N*=11) female squirrelfish, with VTG concentrations in both E_2-treated groups reaching the highest levels at day 5 of the sampling period.

Table 2. *Effects of 17β-estradiol (E_2) on immature female squirrelfish*

	Day 5		Day 6		Day 10	
	Control	E_2 treated	Control	E_2 treated	Control	E_2 treated
Liver zinc	1.14±0.76	1.34±1.04	0.45±0.08	0.43±0.17	1.74±1.34	6.10±3.63
VTG	0.31±0.18	36.2±1.32*	0.04±0.05	28.9±7.02*	0.27±0.38	12.1±2.12*
E_2	1.29±0.01	28.0±14.1*	1.31±0.04	6.95±3.71	1.28±0.15	0.88±1.17
T	3.03±0.11	0.10±0.07*	3.08±0.04	0.04±0.04*	3.10±0.31	0.15±0.00*
P	34.2±0.02	3.84±0.08*	33.4±1.10	2.80±0.64*	34.7±0.88	2.09±1.13*
MT mRNA	0.48±0.23	0.56±0.33	0.29±0.20	0.28±0.14	0.37±0.21	0.60±0.00
C MT	0.58±0.24	1.17±0.37	0.26±0.01	0.79±0.21	0.47±0.23	0.68±0.04

Effects of 17β-estradiol (E_2) on immature female squirrelfish in terms of liver zinc, plasma vitellogenin (VTG), testosterone (T), 17β-estradiol (E_2) and progesterone (P) levels; hepatic metallothionein mRNA levels (MT mRNA); and MT protein levels in cytosolic (C) liver fractions. Values are mean±S.E.M. Each variable was analyzed by Kruskal–Wallis ANOVA and pair-wise differences in means were statistically evaluated by Dunn's post-hoc test, *N*=2–7 fish. Values were considered significant at *P*<0.05.

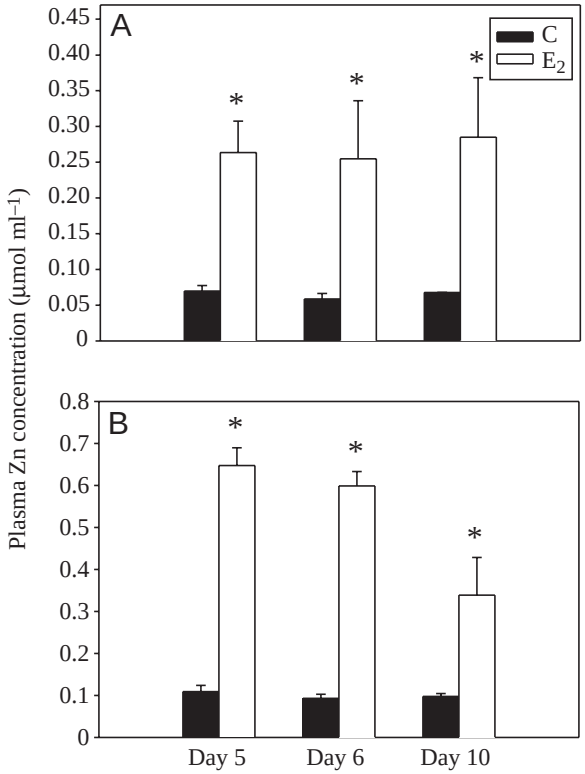

Fig. 6. Effects of E_2 administration on plasma zinc concentrations in immature (A) and mature (B) female squirrelfish at days 5, 6, and 10 of the experiment (N=2–6 fish). Results are arithmetic means ± S.E.M. Asterisks indicate values that are significantly different from the sham-treated control at the same sampling point (P<0.05; Dunn's test).

E_2 treatment of mature female squirrelfish did result in increased plasma E_2 levels during the sampling period when compared with controls (Table 3; P= 0.024, N=10). Conversely, the overall effect of E_2 concentration in plasma of immature females was not found to be statistically significant (Table 2; P=0.142, N=7), even though there was a pair-wise significance at day 5 of the sampling period. This probably resulted from low sample numbers plus the lower-than-control average E_2 level of treated fish on day 10. Plasma E_2 levels peaked in mature females at day 6 of the sampling period before dropping to levels at day 10 that were approximately 10 times less than the levels of simultaneous controls (Table 3). E_2 treatment resulted in a significant decrease (up to 30 times less) in plasma T levels of immature (Table 2; P=0.002, N=7) and mature (Table 3; P=0.001, N=10) females throughout the sampling period when compared with control female squirrelfish. Likewise, plasma P levels were significantly decreased (approximately 10 times less) in E_2-treated immature females (Table 2; P=0.002, N=7) and mature females (Table 3; P=0.002, N=9) when compared with controls. These drastic reductions in the levels of T and P in both immature and mature female squirrelfish are likely to be in response to increased concentrations of E_2. Thus, the addition of large doses of exogenous E_2 to female squirrelfish seems to indicate a negative feedback loop mechanism within the hypophyseal–gonadal sex steroid production axis.

E_2 treatment did not result in increased hepatic MT mRNA levels in either immature or mature female squirrelfish (Tables 2, 3) during the sampling period. However, E_2 treatment did result in an increase in the levels of MT protein in the nuclear fraction of immature female squirrelfish liver cells (Fig. 7A) when compared with control individuals (P=0.002, N=8), with levels peaking at day 6 of the sampling period. There was no significant trend in the levels of MT in the nuclear fraction of E_2-treated mature females (Fig. 7B). Furthermore, E_2 treatment resulted in increased levels of cytosolic MT protein in immature females (Table 2; P=0.046, N=8), while, again, a significant effect did not occur in mature females (Table 3). In fact, there was a significant increase in the ratio of nuclear MT to cytosolic MT in E_2-treated immature females (P=0.046, N=8). This trend did not occur in E_2-injected mature female squirrelfish.

Discussion

Female squirrelfish maintain higher concentrations of zinc and MT in the liver than any other studied species. The developmental timeframe of this zinc uptake into the female

Table 3. *Effects of 17β-estradiol (E_2) on mature female squirrelfish*

	Day 5		Day 6		Day 10	
	Control	E_2 treated	Control	E_2 treated	Control	E_2 treated
Liver zinc	2.32±2.84	3.36±0.69	6.12±1.06	3.61±1.01	7.13±1.83	14.2±6.35
VTG	0.43±0.27	33.0±2.82*	0.55±0.27	24.9±8.66*	0.13±0.06	12.1±2.12*
E_2	1.17±0.08	11.4±5.10*	2.53±0.95	18.2±8.95*	1.61±0.21	0.13±0.04*
T	2.92±0.28	0.10±0.03*	3.06±0.13	0.09±0.05*	2.88±0.29	0.25±0.14*
P	31.2±8.51	3.95±0.52*	32.0±3.31	3.67±0.83*	36.1±3.65	3.47±0.77*
MT mRNA	1.03±0.18	0.82±0.10	1.01±0.13	0.72±0.11	0.98±0.24	0.93±0.14
C MT	1.29±0.60	0.48±0.05*	0.63±0.20	1.01±0.08	0.64±0.09	0.81±0.04

Effects of 17β-estradiol (E_2) on mature female squirrelfish in terms of liver zinc, plasma vitellogenin (VTG), testosterone (T), 17β-estradiol (E_2) and progesterone (P) levels; hepatic metallothionein mRNA levels (MT mRNA); and MT protein levels in cytosolic (C) liver fractions. Other details as in Table 2.

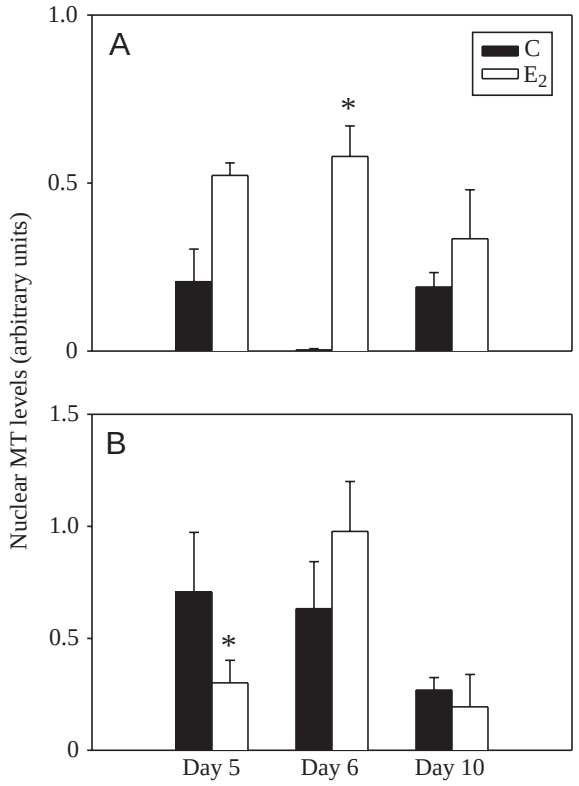

Fig. 7. Effects of E_2 administration on MT levels in the nuclear fraction of liver cells of immature (A) and mature (B) female squirrelfish at days 5, 6, and 10 of the experiment (N=2–6 fish). Results are arithmetic means ± S.E.M. Asterisks indicate values that are significantly different from the sham-treated control at the same sampling point (P<0.05; Dunn's test).

liver, as well as the increased production and variable distribution of MT, have to this point been unclear. This study presents evidence that the massive accumulation of hepatic zinc in female squirrelfish occurs as a result of the onset of sexual maturation, as hepatic zinc uptake is significantly correlated to increasing GSI. One factor that plays a crucial role in the sexual development of female vertebrates is E_2. Immature and mature female squirrelfish were injected with E_2 to determine if this sex hormone was responsible for increases in hepatic zinc concentration. In this experiment, E_2 administration did not result in the increase in hepatic zinc accumulation indicative of the onset of sexual maturity. We have previously shown that E_2 injection of male squirrelfish also produced no such effects (Thompson et al., 2001). Functional E_2 receptors are probably present in the liver because VTG was produced and LSI doubled in response to E_2 treatment. Thus, E_2 alone is not sufficient to cause accumulation of zinc in squirrelfish liver.

In addition to hepatic zinc, circulating levels of zinc in the bloodstream also increased as a result of sexual maturity. This corroborates evidence from previous studies in squirrelfish, which indicates that zinc is accumulated in the liver and passed on to the ovaries via the bloodstream in mature females, under the influence of E_2 (Hogstrand et al., 1996; Thompson et al., 2001). Furthermore, E_2 administration in this study resulted in an increase in plasma zinc levels. As stated previously, hepatic zinc concentrations were not altered by increased E_2 levels. If zinc in the plasma originates from the liver, then both import and export of zinc in the liver must be proportionately increased by E_2. Interestingly, the hepatocytes of female squirrelfish have indeed been shown to have higher zinc efflux as well as higher zinc influx than the hepatocytes of males (Hogstrand et al., 1996). It remains to be investigated if this upregulation of bilateral zinc fluxes is under the control of E_2 or some other endocrine factor.

Zinc transport from the liver followed the same time course as the hepatic production and transport of VTG. However, the molar ratio of plasma zinc to VTG was consistently high (approximately 11:1), suggesting that either squirrelfish VTG has the capacity to bind much more zinc than do VTGs of other species or that VTG is not the primary vehicle of hepato–ovarian zinc transport in squirrelfish. In the present study, immature and mature females were not significantly different in terms of LSI, an indication of hepatic VTG production. Furthermore, there was no difference in the plasma VTG levels between immature and mature female squirrelfish from the respective control groups. This was in spite of 35% higher plasma zinc levels in mature females in comparison with immature females. E_2 administration of immature and mature females did result in increased plasma concentrations of VTG and zinc in all females irrespective of sexual development stage. However, whereas plasma VTG levels decrease by day 10 of the sampling period in both immature and mature females, plasma zinc levels only decreased in mature females. Furthermore, plasma zinc levels were twice as high in mature females than immature females after E_2 administration, while VTG concentrations were similar in both mature and immature females. These findings seem to suggest that plasma zinc is not primarily bound to VTG in the plasma of female squirrelfish. In *Xenopus*, VTG-bound zinc appears to be the only form of zinc taken up by the developing oocyte (Falchuk et al., 1995). Thus, it is unclear if the zinc in the plasma of squirrelfish that is not bound to VTG is a direct indication of zinc that is available for uptake into the ovaries. Certainly, the zinc concentration in squirrelfish ovaries is unusually high, so zinc is likely to be taken up by the follicle in one form or another.

The onset of sexual maturation also resulted in increased levels of hepatic MT mRNA. However, administration of E_2 in immature and mature female squirrelfish did not enhance MT mRNA levels. This result is similar to results obtained previously with male squirrelfish (Thompson et al., 2001). Similarly, in *Oncorhynchus mykiss*, E_2 treatment did not directly result in the production of MT mRNA (Olsson et al., 1989). Furthermore, promoter analyses of various fish MT loci have revealed no elements that would be expected to respond to sex steroids (Olsson, 1993). Thus, there is at present little experimental evidence to suggest that E_2 directly triggers MT gene transcription in fish in general. Likewise, E_2 treatment

alone does not seem to be sufficient to induce MT transcription in squirrelfish. It seems more likely that accumulation of zinc itself, or in combination with a sex hormone (e.g. E_2), activates transcription of MT. This would mean that zinc absorption and its uptake in liver are upregulated during female maturation. Indeed, recent results suggest the female squirrelfish have an unusually high intestinal zinc uptake rate and that absorbed zinc is rapidly translocated to the liver (Glover et al., 2002). Furthermore, a study on isolated squirrelfish hepatocytes demonstrated that hepatocytes from females take up zinc more rapidly than those of males (Hogstrand et al., 1996). Whatever the signal for MT synthesis, the fact remains that the very potent production of MT allows zinc to be accumulated at very high levels in the liver of mature female squirrelfish.

Not only are the levels of MT mRNA and MT protein increased during maturation of the female squirrelfish, but the subcellular distribution of MT protein is also altered. Mature female squirrelfish liver has a greater proportion of MT in the cell nuclear fraction than immature females. This distribution could be the result of increased levels of E_2, as immature females treated with E_2 do indeed show increased proportions of MT in the nuclear fraction of liver cells. However, this is not the case in E_2-treated mature females. This result was unexpected, and is difficult to explain, but could mean that the female liver has to be in a responsive state for the occurrence of MT movement into the nuclear fraction. Interestingly, the increase of MT in the nuclear fraction of immature females did not occur at the expense of MT in the cytosolic fractions of these fish, indicating a possible preferential degradation or other post-transcriptional mechanism by which E_2 increases total liver MT. Similar results have been presented for male squirrelfish (Thompson et al., 2001).

E_2 treatment also resulted in an increase in the GSI of mature female squirrelfish, but this effect is not observed in E_2-treated immature females. The pre-spawning development of the female teleost oocyte occurs in two phases, a period of bulk growth and a period of final maturation (Wallace, 1985). Oocyte growth is primarily the result of the uptake of VTG (Wallace, 1985). VTG is produced in the liver in response to E_2, and is transported via the bloodstream to the ovary where it is incorporated into the developing oocyte (Wallace, 1985). Indeed, plasma VTG concentrations were increased in E_2-treated mature and immature females. The uptake of VTG by the oocyte is a receptor-mediated endocytotic process (Wallace, 1985) via a membrane receptor specific for VTG (Tyler and Lancaster, 1993). One possibility for the lack of gonadal growth stimulation by E_2 in immature female squirrelfish could be that sexually immature oocytes lack functional VTG receptors and therefore do not respond to circulating VTG in the same manner as in mature females.

We have now established the developmental stage for the female-specific zinc and MT accumulation and distribution in squirrelfish to be concurrent with sexual maturation. As in most vertebrates, E_2 is a very important regulator of sexual maturation in fish. Yet E_2 does not seem to be sufficient for enhancing hepatic uptake of zinc or transcription of MT. Thus, the factors that initiate these physiological differences indicative of sexual maturity in female squirrelfish remain unclear. Studies of sex hormone profiles during the female squirrelfish reproductive cycle are currently under way. E_2 does seem to stimulate an increase in plasma zinc concentration and we hypothesize that this is to supply zinc to the oocytes. The purpose of such a hepato–ovarian zinc transfer is not known, but Thompson et al. (2001) speculated that it might be to supply the nocturnal squirrelfish larvae with sufficient zinc to ensure proper night vision.

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References

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Busselberg, D., Platt, B., Michael, D., Carpenter, D. O. and Haas, H. L. (1994). Mammalian voltage-activated calcium channel currents are blocked by Pb^{2+}, Zn^{2+}, and Al^{3+}. *J. Neurophysiol.* **71**, 1491-1497.

Cousins, R. J. and McMahon, R. J. (2000). Integrative aspects of zinc transporters. *J. Nutr.* **130**, 1384S-1387S.

Dyke, B., Hegenauer, J. and Saltman, P. (1987). Isolation and characterization of a new zinc-binding protein from albacore tuna plasma. *Biochemistry* **26**, 3228-3234.

Falchuk, K. H., Montorzi, M. and Vallee, B. L. (1995). Zinc uptake and distribution in *Xenopus laevis* oocytes and embryos. *Biochemistry* **34**, 16524-16531.

Fletcher, P. E. and Fletcher, G. L. (1980). Zinc- and copper-binding proteins in the plasma of winter flounder (*Pseudopleuronectus americanus*). *Can. J. Zool.* **58**, 609-613.

Glover, C. N., Balesaria, S., Mayer, G. D., Thompson, E. D., Walsh, P. J. and Hogstrand, C. (2002). Intestinal zinc uptake in the marine teleosts, squirrelfish (*Holocentrus adscensionis*) and Gulf toadfish (*Opsanus beta*) *Physiol. Biochem. Zool.* (in press).

Guerinot, M. L. (2000). The ZIP family of metal transporters. *Biochim. Biophys. Acta* **1465**, 190-198.

Hogstrand, C. and Haux, C. (1990). A radioimmunoassay for perch (*Perca fluviatilis*) metallothionein. *Toxicol. Appl. Pharmacol.* **103**, 56-65.

Hogstrand, C. and Haux, C. (1991). Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comp. Biochem. Physiol.* **100C**, 137-141.

Hogstrand, C. and Haux, C. (1996). Naturally high levels of zinc and metallothionein in liver of several species of the squirrelfish family from Queensland, Australia. *Mar. Biol.* **125**, 23-31.

Hogstrand, C. and Wood, C. M. (1996). The physiology and toxicology of zinc in fish. In *Aquatic Toxicology*, vol. 1 (ed. E.W. Taylor), pp. 61-84. Cambridge: Cambridge University Press.

Hogstrand, C., Gassman, N. J., Popova, B., Wood, C. M. and Walsh, P. J. (1996). The physiology of massive zinc accumulation in the liver of female squirrelfish and its relationship to reproduction. *J. Exp. Biol.* **199**, 2543-2554.

Julshamn, K., Anderson, K.-J., Ringdal, O. and Brenna, J. (1988). Effect of dietary copper on the hepatic concentration and subcellular distribution of copper and zinc in the rainbow trout (*Salmo gairdneri*). *Aquaculture* **73**, 143-155.

Kagi, J. H. R. and Schaffer, A. (1988). Biochemistry of metallothionein. *Perspect. Biochem.* **27**, 8509-8515.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Olsson, P.-E., Zafarullah, M. and Gedamu, L. (1989). A role for metallothionein in zinc regulation after oestradiol induction of vitellogenin synthesis in rainbow trout, *Salmo gairdneri*. *Biochem. J.* **257**, 555-559.

Olsson, P.-E. (1993). Metallothionein gene expression and regulation in fish. In *Molecular Biology Frontiers*, vol. 2 (ed. P.W. Hochachka and T. P. Mommsen), pp. 259-278. Amsterdam: Elsevier.

Palmiter, R. D. and Findley, S. D. (1995). Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J.* **14**, 639-649.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Silversand, C. and Haux, C. (1989). Isolation of turbot (*Scophthalmus maximus*) vitellogenin by high-performance anion-exchange chromatography. *J. Chromatogr.* **478**, 387-397.

Specker, J. L. and Anderson, T. R. (1994). Developing an ELISA for a model protein – vitellogenin. In *Biochemistry and Molecular Biology of Fishes*, vol. 3 (ed. P. W. Hochachka and T. P. Mommsen), pp. 567-578. Amsterdam: Elsevier.

Thompson, E. D., Olsson, P.-E., Mayer, G. D., Haux, C., Walsh, P. J. and Hogstrand, C. (1999). Involvement of metallothionein in female squirrelfish reproduction. In *Metallothionein IV* (ed. C. D. Klaassen), pp. 157-165. Basel: Birkhäuser Verlag.

Thompson, E. D., Olsson, P.-E., Mayer, G. D., Walsh, P. J., Haux, C., Burge, E. and Hogstrand, C. (2001). Effects of 17β-estradiol on levels and distribution of metallothionein and zinc in squirrelfish. *Am. J. Phys. Reg. Int. Comp. Phys.* **280**, R527-R535.

Towbin, H., Stachelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Tyler, C. R. and Lancaster, P. (1993). Isolation and characterization of the receptor for vitellogenin from follicles of the rainbow trout, *Oncorhynchus mykiss*. *J. Comp. Physiol. B* **163**, 225-233.

Vallee, B. L. and Falchuk, K. H. (1993). The biochemical basis of zinc physiology. *Physiol. Rev.* **73**, 79-118.

Wallace, R. A. (1985). Vitellogenesis and oocyte growth in nonmamallian vertebrates. In *Developmental Biology*, vol. 1 (ed. L. W. Browder), pp. 127-177. New York: Plenum.

Weitzel, G., Strecker, F., Roester, U., Buddecke, E. and Fretzdorff, A. (1954). Zink im Tapetum lucidum. *Hoppe–Seyler's Z. Physiol. Chem.* **296**, 19-30.

Wu, S. M., Qiao, X., Noebels, J. L. and Yang, X. L. (1993). Localization and modulatory actions of zinc in vertebrate retina. *Vision Res.* **33**, 2611-2616.