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# Effects of 17 $\beta$ -estradiol on levels and distribution of metallothionein and zinc in squirrelfish

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**Thompson, E. David, Per-Erik Olsson, Gregory D. Mayer, Carl Haux, Patrick J. Walsh, Erin Burge, and Christer Hogstrand.** Effects of 17 $\beta$ -estradiol on levels and distribution of metallothionein and zinc in squirrelfish. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R527–R535, 2001.—Females of the squirrelfish family (*Holocentridae*) accumulate higher levels of zinc in the liver than any other known animal. This zinc accumulation is made possible by high expression of the zinc-binding protein, metallothionein (MT). In the present study, the squirrelfish (*Holocentrus ascensionis*) MT cDNA was cloned and sequenced. The deduced amino acid sequence was very similar to other teleost MT. The role of estrogens on zinc metabolism was investigated by injecting male and immature female squirrelfish with 17 $\beta$ -estradiol (E<sub>2</sub>). E<sub>2</sub> treatment triggered transient increases in plasma zinc and vitellogenin (VTG) levels, and both of these variables showed very similar time courses. These results suggest that E<sub>2</sub> is responsible for the large hepatoovarian translocation of zinc observed in female squirrelfish and that VTG might be a vehicle for zinc. E<sub>2</sub> did not directly alter the levels of zinc or MT mRNA in the liver. However, the hepatic MT protein concentration increased differentially in the nuclear fraction. Thus E<sub>2</sub> is probably responsible for the association of MT with the nuclear fraction previously observed in untreated mature female squirrelfish.

zinc; metallothionein complimentary deoxyribonucleic acid; reproduction; endocrinology; teleost

ZINC IS AN ESSENTIAL micronutrient that plays a crucial role in many cellular processes involving transcription, enzyme structure and activity, protein interactions, and even cell signaling (45). Zinc is also involved in antioxidant defense (5) and in maintaining membrane integrity (1). As such, minimal levels must be maintained for normal cellular function. However, zinc may become toxic if accumulated at high levels (16), and many proteins are therefore involved in zinc homeosta-

sis. One such protein believed to regulate intercellular zinc concentrations is metallothionein (MT), a low-weight (~6 kDa) protein characterized by high cysteine content (30–35%) and a lack of aromatic amino acids and histidine (21). MT binds elements of groups IB and IIB of the Periodic Table of Elements with a typical stoichiometry of 7 metal atoms of zinc or cadmium per MT molecule or 10–12 atoms of copper, mercury, or silver per MT molecule (22). Although the exact mechanism is uncertain, these metals also induce transcription of the MT gene (28) mediated by metal regulatory elements of the MT promoter (35). MT has been shown to bind zinc with a dissociation constant of  $3.2 \times 10^{-13} \text{ M}^{-1}$  (31). Despite the strong affinity of MT for zinc, recent studies have shown that MT can donate and accept zinc from zinc proteins (18, 25). These characteristics suggest that MT would be well suited for regulating zinc balance within the cell, but this function remains to be demonstrated.

The squirrelfish family (*Holocentridae*) presents an interesting system for studying zinc homeostasis and elucidating the function of MT. Females of this family have been shown to maintain high levels of hepatic zinc, up to 70  $\mu\text{mol/g}$  wet wt, which is unseen in any other studied organism (12, 14, 15). In the liver, females also have unusually high MT levels that are closely correlated to liver zinc concentrations ( $0.89 < r < 0.99$ ) and not to other metals that can bind MT (15). Zinc is believed to be the native metal of squirrelfish MT. Moreover, the only organs displaying conspicuously high zinc levels are the liver and the ovaries (12). There are very high zinc concentrations in the retina as in other vertebrates (47), but this does not seem to be sex specific. Squirrelfish inhabit coral reefs throughout the world, and females in all six species studied contain high zinc and MT levels, suggesting that zinc accumulation is independent of geographical location (12, 14, 15). Also, analysis of intestinal content in wild male

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and female squirrelfish indicated that female-specific zinc accumulation is not a result of dietary differences between the sexes (12). It has been observed previously in rainbow trout (*Oncorhynchus mykiss*) that hepatic MT and zinc levels increase during female sexual maturation (29, 30). These findings suggest that some female-specific factor plays a role in zinc accumulation and that increased zinc and MT levels could be involved in female squirrelfish reproduction. One female-specific factor involved in the reproductive cycle is  $17\beta$ -estradiol ( $E_2$ ).  $E_2$  is the endocrine signal for the hepatic production of vitellogenin (VTG), the precursor to yolk proteins in all oviparous vertebrates, and the vitelline envelope (zona radiata) proteins (17, 46). The purpose of the present study was to evaluate if  $E_2$  plays a role in zinc accumulation and distribution in female squirrelfish and also in the production and distribution of MT. This aim can be relatively easily investigated in fish because immature females and males can express estrogen receptors and are responsive to injections of  $E_2$ . Changes in treated individuals can then be compared with control individuals. Such experiments have been used to study  $E_2$  induction of VTG and vitelline envelope proteins (17, 46) as well as xenoestrogens in the environment (40). In addition, the present study involves the cloning of the squirrelfish MT gene and the examination of the primary amino acid sequence along with comparisons with other known MT sequences to determine if the ability of female squirrelfish to utilize massive amounts of zinc is a result of some unique property of squirrelfish MT.

## MATERIALS AND METHODS

**Animal care and  $E_2$  injections.** Squirrelfish (*Holocentrus ascensionis*) were collected by scuba divers off Tavernier, FL, in May and early June. Fish were transported to the Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, where they were housed in two tanks (4,000 liters) 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 before experimentation.

The squirrelfish in one tank were given interperitoneal injections of 5 mg  $E_2$ /kg body wt (1 ml/kg in peanut oil) on days 0, 2, and 4. A control group housed in the other tank was injected with peanut oil only. Control and treated fish were killed on either day 5, 6, or 10.

**Sampling.** Fish were killed by an overdose of MS-222 (0.5 g/l) and weighed. One milliliter of blood was withdrawn from the caudal vessel with a heparinized syringe, and plasma was separated from blood cells by centrifugation (14,000 g) for 3 min. The plasma was divided into 50- $\mu$ l aliquots and was stored at  $-80^\circ\text{C}$  for subsequent analysis of  $E_2$ , VTG, zinc, and MT as described below. Livers and gonads were dissected out, weighed, and immediately divided into aliquots. A sample of tissue of weighted mass ( $\sim 0.5$  g) was taken from each liver and subjected to subcellular fractionation as described below. Samples of liver and gonad ( $\sim 0.5$  g) were placed into individual 16  $\times$  150-mm borosilicate glass tubes for acid digestion and subsequent zinc analysis. The remaining liver and gonads were wrapped in aluminum foil and frozen in liquid nitrogen. The frozen samples were trans-

ferred to  $-80^\circ\text{C}$ , where they were stored until used for total RNA extraction as described below.

**Tissue zinc content.** Liver and gonad samples were subjected to acid digestion in 2.0 ml of 70%  $\text{HNO}_3$  (trace-metal grade, Fisher). The tubes were heated in a sand bath for 3 h at  $120^\circ\text{C}$  and then cooled to room temperature before 0.50 ml of  $\text{H}_2\text{O}_2$  was added. The temperature was then gradually increased to  $120^\circ\text{C}$  until all liquid had evaporated. The dried residues were reconstituted with 4.0 ml of 0.50%  $\text{HNO}_3$ . These samples were then analyzed for zinc content by air/acetylene flame atomic absorption spectroscopy (model 2380; Perkin-Elmer). Plasma samples taken from these fish were also analyzed for zinc content in the same manner.

**VTG and  $E_2$  analysis.** Plasma samples from each fish were measured for VTG and  $E_2$  content by ELISA (10, 37). The VTG ELISAs were performed according to the procedure of Palmer et al. (32). Each plasma sample was diluted 1:100 in PBS (0.15 M NaCl, 0.0015 M  $\text{KH}_2\text{PO}_4$ , 0.015 M  $\text{Na}_2\text{HPO}_4$ , and 0.002 M KCl, pH 7.4), and 100  $\mu$ l of this dilution were added per well in 96-well microtitre plates (Fisher). Squirrelfish VTG was isolated by ion-exchange chromatography according to the protocol of Silversand and Haux (36), with minor modifications, and this purified VTG was used as a standard. The samples were incubated in duplicate for 2 h at room temperature to allow for optimal adsorption of antigen to the plates and were then washed four times with 300  $\mu$ l PBS per well. After the washes, the plates were treated with 150  $\mu$ l of blocking buffer (5% dried milk in PBS) for 30 min at room temperature. Blocking buffer was then removed, and the wells were subjected to 100  $\mu$ l of primary antibody and incubated overnight at  $4^\circ\text{C}$ . The primary antibody was a rabbit anti-turtle VTG IgG (gift from Dr. Brent Palmer, University of Kentucky) diluted 1:5,000 in blocking buffer. The cross-reactivity of this antibody with squirrelfish VTG was confirmed by Western blot (data not shown). After the primary antibody incubation, the plates were washed six times with PBS, subjected to 100  $\mu$ l of secondary antibody, and incubated for 2 h at room temperature. The secondary antibody was a horseradish peroxidase-linked goat anti-rabbit IgG (Bio-Rad) diluted 1:1,000 in blocking buffer. The plates were then washed four times with PBS, and 100  $\mu$ l of a tetramethylbenzidine peroxidase enzyme immunoassay substrate (Bio-Rad) were added according to the manufacturer's specifications. After a 15-min incubation, 50  $\mu$ l of 0.5 M  $\text{H}_2\text{SO}_4$  were added to the substrate to stop the reaction, and the plates were read on a microplate reader (model 450; Bio-Rad) at 450 nm. Because of limitations in sample volume, these samples were not rerun. Although obtained values from these samples may underestimate the true VTG concentrations, the data were not censored (27). The potential error introduced would not affect any conclusions made.  $E_2$  was analyzed using estradiol ELISA kits (Oxford Biomedical Research) according to the manufacturer's specifications.

**Hepatic subcellular fractionation.** Subcellular fractions of liver were obtained by differential centrifugation of liver homogenates via the procedure for rainbow trout liver as described by Hogstrand et al. (12). Liver samples of known mass ( $\sim 0.5$  g) were homogenized individually in an ice-cold isotonic homogenization buffer (35 mM Tris-HCl, 0.20 M KCl, and 0.25 M sucrose, pH 7.4) using a glass-Teflon homogenizer. The homogenate was centrifuged (370 g) for 5 min at  $4^\circ\text{C}$ , and the supernatant was removed, whereas the pellet (nuclear fraction) was immediately placed on ice. The supernatant was centrifuged (9,200 g) for 5 min at  $4^\circ\text{C}$ , and the pellet (mitochondrial/lysosomal fraction) was saved and placed on ice. This supernatant was then centrifuged (130,000 g) for 60 min at  $4^\circ\text{C}$ , resulting in a small pellet

(microsomal fraction) that was saved and immediately placed on ice. The final supernatant (cytosolic fraction) was divided into 0.50-ml aliquots and immediately placed on ice. All pellets were resuspended in 0.50 ml of fresh homogenization buffer, and subcellular fractions were divided into aliquots and transferred to liquid nitrogen. These samples were then stored at  $-80^{\circ}\text{C}$  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 (23). The protein concentration for each subcellular fraction was determined by Bradford (4) assay, and samples were diluted with distilled water as necessary. These samples were then mixed 1:4 with sample buffer [62 mM Tris·HCl, pH 6.8, 10% glycerol, and 5.0% 2-mercaptoethanol (added just before dilution), 2.0% SDS, and 0.0012% bromophenol blue] and were heated at  $100^{\circ}\text{C}$  for 5 min. A total of 25  $\mu\text{g}$  of protein was loaded into each well. Perch MT was used as a standard (13). 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 (Bio-Rad).

After electrophoresis, proteins were transferred from polyacrylamide gels to nitrocellulose membranes (Schleicher & Schuell) by electroblotting, as described by Towbin et al. (43), in a SemiPhor TE 70 semidry transfer unit (Hoefer Scientific) at  $0.8\text{ mA/cm}^2$  constant current for 60 min at room temperature. Once proteins were transferred, the membranes were blocked with 5.0% dried milk in TBS-T (20 mM Tris·HCl, pH 7.4, 137 mM NaCl, and 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 a rabbit antiperch serum (13) diluted 1:8,000 in TBS-T. After another series of the same washes, the membranes were incubated 1 h in secondary antibody. The secondary antibody was a horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) diluted 1:40,000 in TBS-T. After a final series of washes, immunodetection was performed with an enhanced chemiluminescence system (ECL; Amersham) according to the 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). Each Western blot was exposed for 15, 30, 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 is 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  $-80^{\circ}\text{C}$  were thawed, and 50–100 mg of each sample were homogenized in 1.0 ml of TRIzol Reagent (GIBCO-BRL) using a glass-Teflon homogenizer. After homogenization, samples were centrifuged (12,000  $g$ ) to remove insoluble material. The RNA-containing supernatant was removed and treated with 0.20 ml chloroform. This mixture was shaken for 15 s, incubated for 2–3 min, centrifuged (12,000  $g$ ) for 15 min, and allowed to separate into aqueous and organic phases. The RNA-containing aqueous phase was transferred to fresh tubes and treated with 0.25 ml isopropanol followed by 0.25 ml of a high-salt precipitation solution (1.20 M sodium citrate and 0.80 M NaCl) to remove glycogen, which otherwise made the final RNA pellet difficult to redissolve. The resulting

mixture was shaken and incubated for 10 min before centrifugation (12,000  $g$ ) to pellet the RNA. The supernatant was removed, and 1.0 ml of 75% ethanol was added to the pellet. The sample was vortexed and centrifuged (7,500  $g$ ) for 5 min at  $4^{\circ}\text{C}$  to pellet the RNA. The pellet was allowed to partially air dry before being redissolved in RNase-free water. The extracted RNA was then stored at  $-80^{\circ}\text{C}$  until used as described in Northern analysis.

**Squirrelfish MT cloning and sequencing.** Mature female squirrelfish liver was used as a source of poly(A)<sup>+</sup> RNA for construction of a  $\lambda$  ZAP cDNA library, using the ZAP Express cDNA library kit (Stratagene). After construction, the library, which contained  $\sim 500,000$  individual clones with an average size of 500 base pairs (squirrelfish MT cDNA is  $\sim 200$  base pairs), was amplified and used to screen for MT cDNA. The library was plated out at a density of 20,000 plaques/plate and was screened for MT cDNA using a digoxigenin (Dig)-labeled perch MT cRNA probe. Several positive clones were obtained, and their identity was confirmed after DNA sequencing. A full-length clone of squirrelfish MT cDNA was thereby obtained.

**Northern analysis.** Total RNA extracted from liver samples ( $\sim 10\text{ }\mu\text{g}$ ) was subjected to electrophoresis on a 1.5% agarose gel with formaldehyde as denaturant (34). 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 complete separation, RNA was transferred to a 170-cm<sup>2</sup> Hybond N nylon membrane (Amersham) by capillary/gravity blotting using the Turbo Blotter (Schleicher & Schuell). The RNA was ultravioletly cross-linked to the membrane followed by incubation in 18 ml of the prehybridization buffer [50% formamide,  $5\times$  saline-sodium citrate (SSC), 2.0% blocking reagent (Boehringer Mannheim), 0.10% *N*-laurylsarcosine, and 0.02% SDS] at  $68^{\circ}\text{C}$  for 4 h. The prehybridization buffer was discarded, and the membrane was hybridized for 18 h at  $68^{\circ}\text{C}$  in 18 ml of the same buffer, with 0.30  $\mu\text{l}$  probe/ml added, using a Dig-labeled antisense squirrelfish MT-cRNA probe. The probe was synthesized by in vitro transcription of squirrelfish MT-cDNA cloned downstream of the T7 promoter in the pBK-CMV plasmid (Stratagene). The plasmid was linearized, and the Dig-labeled RNA transcript was made from the DNA template by T7 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  $2\times$  SSC, 0.10% SDS for 5 min at room temperature; 2 washes in  $1\times$  SSC, 0.1% SDS for 5 min at  $68^{\circ}\text{C}$ ), 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, 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, injections were administered to all fish, and males and immature females (operationally defined as gonadosomatic index  $<0.25$ ) were sorted out after death. Statistical analysis, employing the Mann-Whitney *U*-test, showed that immature females were not significantly different from males in all criteria studied in this experiment (Table 1). As such, immature females and males are collectively analyzed throughout the remainder of the text.

Table 1. Comparison of sham injection in males and immature females

	LSI	Liver Zinc, μmol/g	Plasma Zinc, μmol/ml	E <sub>2</sub> , ng/ml	VTG, μg/ml	MT mRNA, arbitrary units	MT C, arbitrary units	MT N, arbitrary units	MT M/L, arbitrary units
Males	1.21 ± 0.10	0.41 ± 0.03	0.036 ± 0.004	2.61 ± 0.46	0.65 ± 0.45	0.45 ± 0.13	0.46 ± 0.09	0.15 ± 0.04	0.17 ± 0.03
Females	1.44 ± 0.11	1.18 ± 0.45	0.041 ± 0.004	1.25 ± 0.87	2.01 ± 1.37	0.41 ± 0.10	0.46 ± 0.10	0.15 ± 0.05	0.10 ± 0.03
P value	0.138	0.382	0.368	0.057	0.254	0.923	0.804	0.999	0.088

Values are means ± SE; no. of observations was 4–9 for immature females and 5–11 for males. Comparison of sham-injected males and immature females in terms of liver somatic index (LSI), plasma zinc, 17β-estradiol (E<sub>2</sub>), vitellogenin (VTG) levels, concentrations of metallothionein (MT) mRNA in liver, and MT protein in cytosolic (C), nuclear (N), and mitochondrial/lysosomal (M/L) hepatic subcellular fractions. Immature females were operationally defined as females with a gonadosomatic index of <0.25. Males were not different from the immature females in any of the variables listed. Statistical comparisons were calculated by the Mann-Whitney U-Test ( $P < 0.05$ ).

Pairwise differences between variables in E<sub>2</sub>-treated fish and the control at each sampling occasion were examined by the Mann-Whitney U-test. Groups were considered significantly different at  $P < 0.05$ . Because squirrelfish samples were divided between the different laboratories involved in the study and further separated into aliquots for different analyses, the number of observations for each treatment group and sampling occasion was not the same for all variables.

The derived primary sequence of squirrelfish MT was submitted to a BLAST search in the SWISS-PROT protein sequence database (41). The squirrelfish MT sequence was compared with existing MT sequences, and similarities were scored.

## RESULTS

Squirrelfish in the experimental group were injected with E<sub>2</sub> to determine any effect this hormone has on

zinc distribution as well as production and intracellular distribution of hepatic MT. Quantification of plasma E<sub>2</sub> indicated that injections were successful in producing increased levels of E<sub>2</sub> in treated fish (Fig. 1A). E<sub>2</sub>-injected squirrelfish sampled 1 day after the final E<sub>2</sub> injection (day 5) had E<sub>2</sub> levels [10.71 ± 3.44 (SE) ng/ml,  $n = 3$ ] fourfold higher than control fish (2.60 ± 1.15 ng/ml,  $n = 6$ ). E<sub>2</sub> levels in the treated group were still markedly elevated on day 6 (E<sub>2</sub> treated: 6.64 ± 1.24 ng/ml,  $n = 6$ ; control: 1.84 ± 1.25 ng/ml,  $n = 3$ ) but were not different from that of the sham-injected control at day 10.

The response to E<sub>2</sub> in treated squirrelfish was evaluated by measuring liver somatic index (LSI = liver body mass<sup>-1</sup> × 100) and plasma VTG levels. There was a significant increase in LSI upon increases in E<sub>2</sub> concentrations (Fig. 1B). By day 5, the LSI of E<sub>2</sub>-

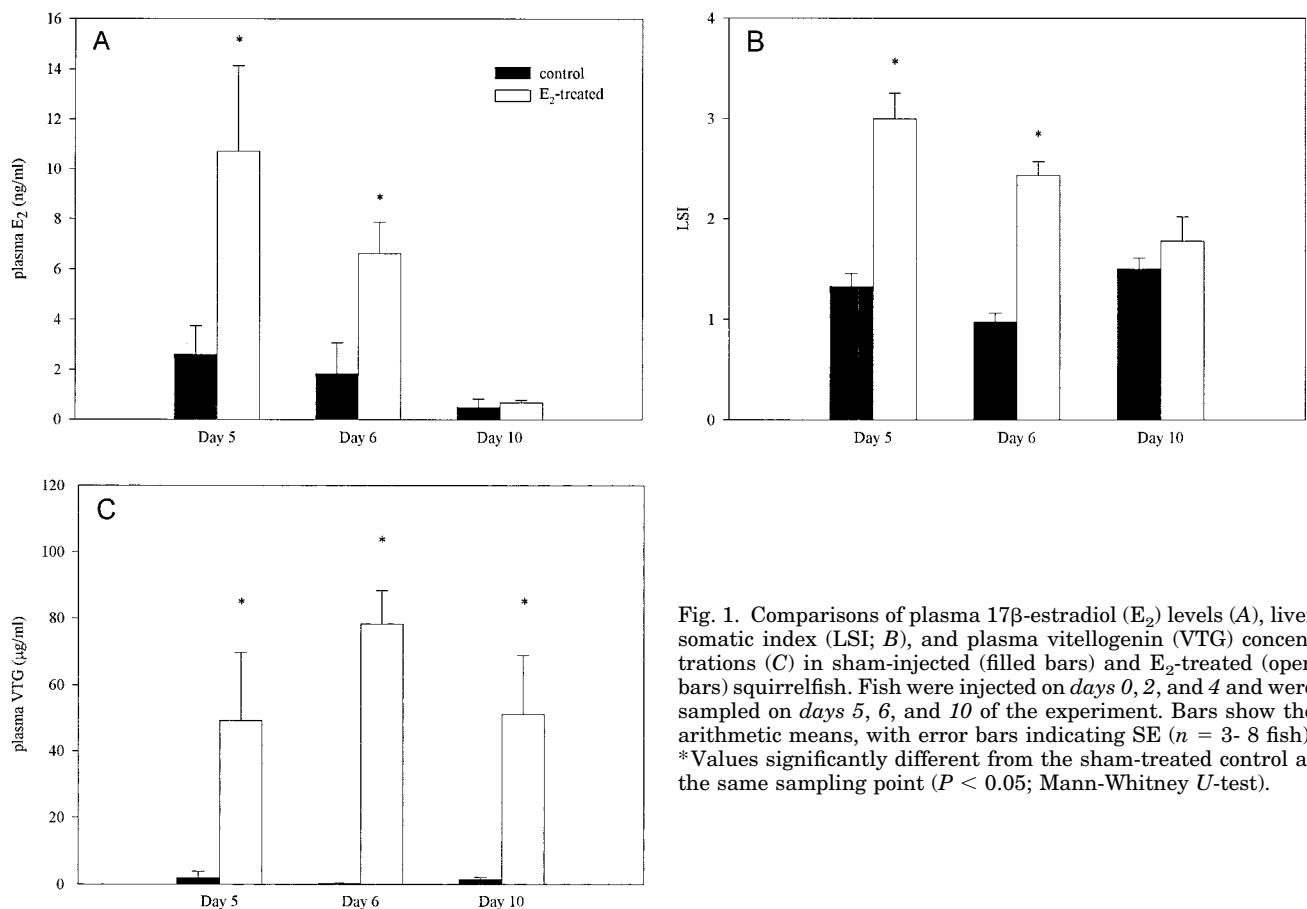


Fig. 1. Comparisons of plasma 17β-estradiol (E<sub>2</sub>) levels (A), liver somatic index (LSI; B), and plasma vitellogenin (VTG) concentrations (C) in sham-injected (filled bars) and E<sub>2</sub>-treated (open bars) squirrelfish. Fish were injected on days 0, 2, and 4 and were sampled on days 5, 6, and 10 of the experiment. Bars show the arithmetic means, with error bars indicating SE ( $n = 3-8$  fish). \* Values significantly different from the sham-treated control at the same sampling point ( $P < 0.05$ ; Mann-Whitney U-test).

treated fish ( $3.00 \pm 0.26$ ,  $n = 5$ ) was 2.3 times higher than the LSI of control fish ( $1.33 \pm 0.13$ ,  $n = 7$ ). At *day 6*,  $E_2$ -treated animals still had a significantly higher LSI ( $2.43 \pm 0.14$ ,  $n = 8$ ) than controls ( $0.98 \pm 0.09$ ,  $n = 5$ ), but this difference was no longer present at *day 10*. Likewise, elevated  $E_2$  levels increased plasma VTG concentrations in the experimental group (Fig. 1C), but the time course was slightly delayed in relation to plasma  $E_2$  concentrations and LSI. Although  $E_2$  levels and LSI both peaked on *day 5*, the highest plasma VTG concentrations were measured in samples from *day 6* ( $78.1 \pm 10.2 \mu\text{g/ml}$ ,  $n = 8$ ). Furthermore, plasma VTG was still markedly elevated on *day 10* ( $E_2$  treated:  $51.1 \pm 17.7 \mu\text{g/ml}$ ,  $n = 5$ ; sham-treated control:  $1.37 \pm 0.64 \mu\text{g/ml}$ ,  $n = 8$ ), although both  $E_2$  and LSI had returned to control levels at this point.

The plasma zinc concentrations increased significantly after  $E_2$  injections compared with control fish and peaked on *day 6* ( $E_2$  treated:  $0.085 \pm 0.013 \mu\text{mol zinc/ml}$ ,  $n = 8$ ; sham-treated control:  $0.041 \pm 0.003 \mu\text{mol zinc/ml}$ ,  $n = 3$ ; Fig. 2A). There was no apparent change in the hepatic zinc concentration, measured as micromoles zinc per gram liver, as a result of  $E_2$  injections (Fig. 2B). In contrast, the hepatic zinc content, as measured by multiplying micromole zinc per gram liver by LSI, increased and followed the pattern of the LSI, thus reflecting the vast increase in liver size (Fig. 2C).

The effect of increased  $E_2$  levels was also investigated in terms of MT induction and distribution. There was no statistically significant change in MT mRNA levels as a result of increased  $E_2$  concentrations at *days 5* and *6* of the sampling period (Fig. 3). There was an apparent increase in MT mRNA at *day 10*, but this was found to be statistically insignificant ( $P = 0.059$ ). However, increased levels of  $E_2$  did have an effect on the MT protein levels and subcellular distribution of MT. Subcellular fractions of control and  $E_2$ -treated squirrelfish liver cells were subjected to Western analysis for MT content (Table 2). There were no significant changes in the absolute cytosolic or mitochondrial/lysosomal MT content at any day of the sample period. MT was not detectable in microsomal fractions. There was, however, an effect on nuclear MT (Fig. 4A). Nuclear MT in  $E_2$ -treated fish at *day 5* ( $0.41 \pm 0.10$ ,  $n = 3$ ) was significantly higher than that in sham-treated control fish ( $0.15 \pm 0.06$ ,  $n = 6$ ). Still at *day 6*, nuclear MT content in treated fish ( $0.58 \pm 0.10$ ,  $n = 8$ ) was significantly higher than that in control fish ( $0.12 \pm 0.07$ ,  $n = 5$ ). This difference was no longer present at *day 10*. Total MT appeared to increase somewhat during the sampling period (Fig. 4B). Although these increases were not significant compared with the simultaneous controls at any single sampling point, the overall effect of  $E_2$  on total MT was significant, as assessed by two-way ANOVA ( $P < 0.05$ ). Thus  $E_2$

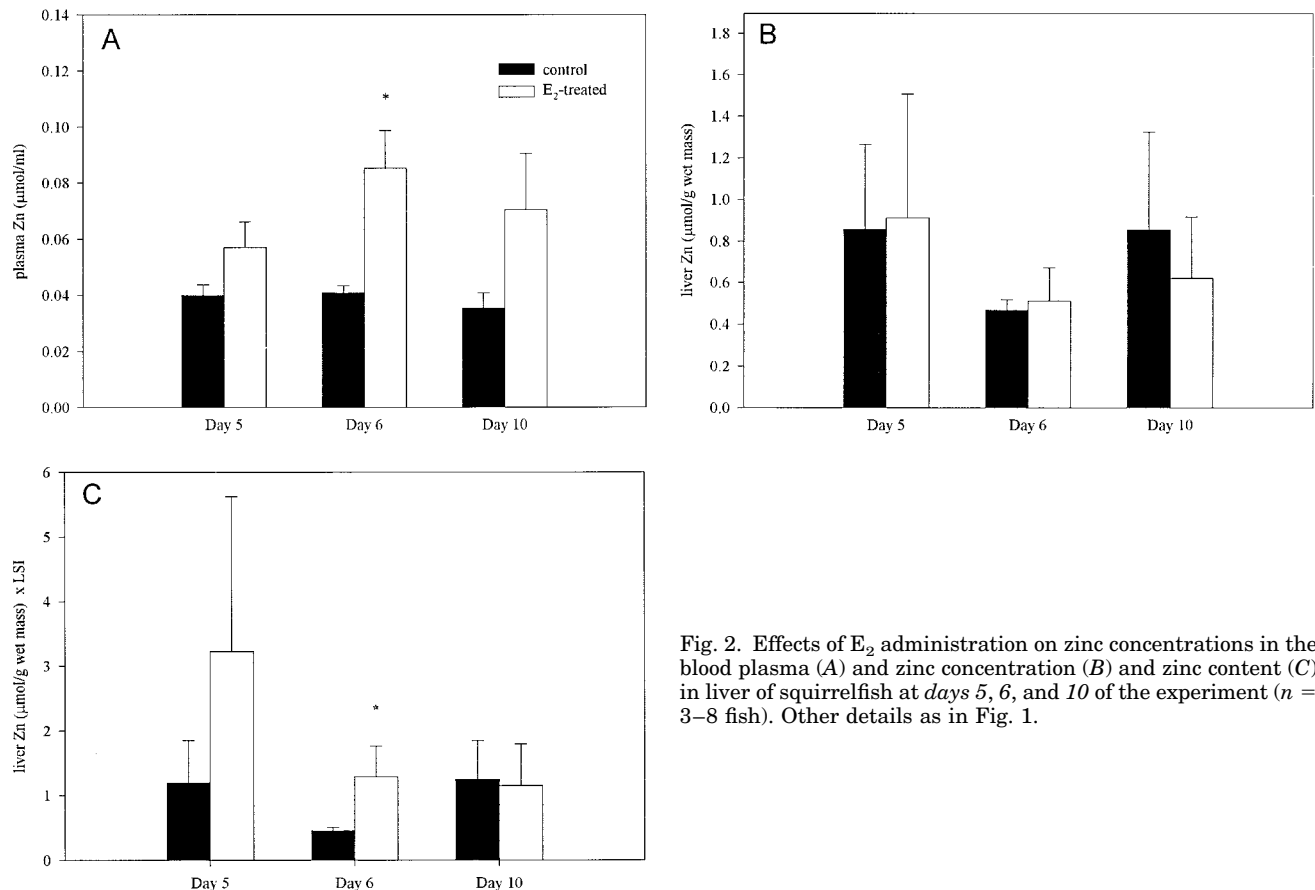


Fig. 2. Effects of  $E_2$  administration on zinc concentrations in the blood plasma (A) and zinc concentration (B) and zinc content (C) in liver of squirrelfish at *days 5, 6, and 10* of the experiment ( $n = 3-8$  fish). Other details as in Fig. 1.

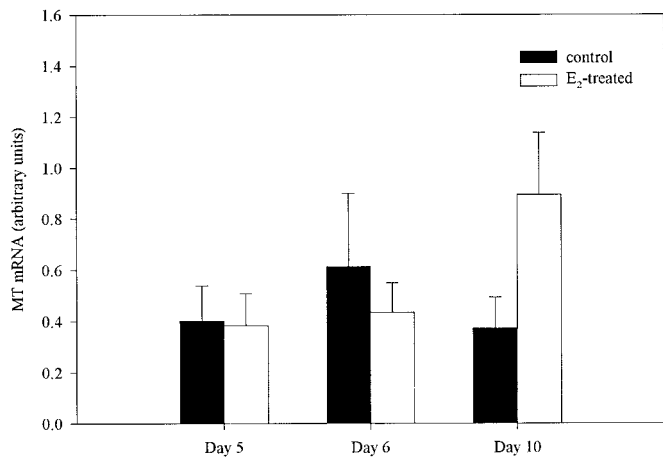


Fig. 3. Effect of  $E_2$  administration on metallothionein (MT) mRNA levels in the liver of squirrelfish at days 5, 6, and 10 of the experiment ( $n = 3-8$  fish). Other details as in Fig. 1.

treatment seems to result in an increase in the concentration of MT in the liver of squirrelfish without a concomitant increase in MT mRNA levels (Fig. 3).

Squirrelfish MT cDNA was cloned and sequenced (Fig. 5). The deduced amino acid sequence was compared with other MT amino acid sequences by BLAST search in the metallo.txt file of the SWISS-PROT protein sequence database (41). The homology of squirrelfish MT to listed MTs from other vertebrates was highly significant ( $P < 3.8e^{-25}$ ), with the closest resemblance found to other fish (*Teleostei*) species ( $>78\%$  identities;  $P < 3.5e^{-38}$ ). The highest score of sequence homology was obtained for viviparous eelpout (*Zoarces viviparus*) MT, with 95% identities to squirrelfish MT and only one "out-of-group" amino acid substitution. Figure 5 shows a comparison of squirrelfish MT with MT from viviparous eelpout (41) and several other species, including mouse (9), human (39), and domestic pigeon (24), and is representative of four other teleost orders (3, 6, 41). All sequenced teleost MTs, including squirrelfish MT, have one less residue than mouse or human MT and two less residues than pigeon MT. In position 13 of the squirrelfish MT sequence, a serine (S) has substituted the asparagine (N) found in almost all other fish MTs. Squirrelfish MT differed from its eelpout counterpart in two additional positions (19 and 41). The uncharged polar threonine (T) at position 19 is the more noteworthy because many fish species have a basic lysine (K) residue located here. The functional

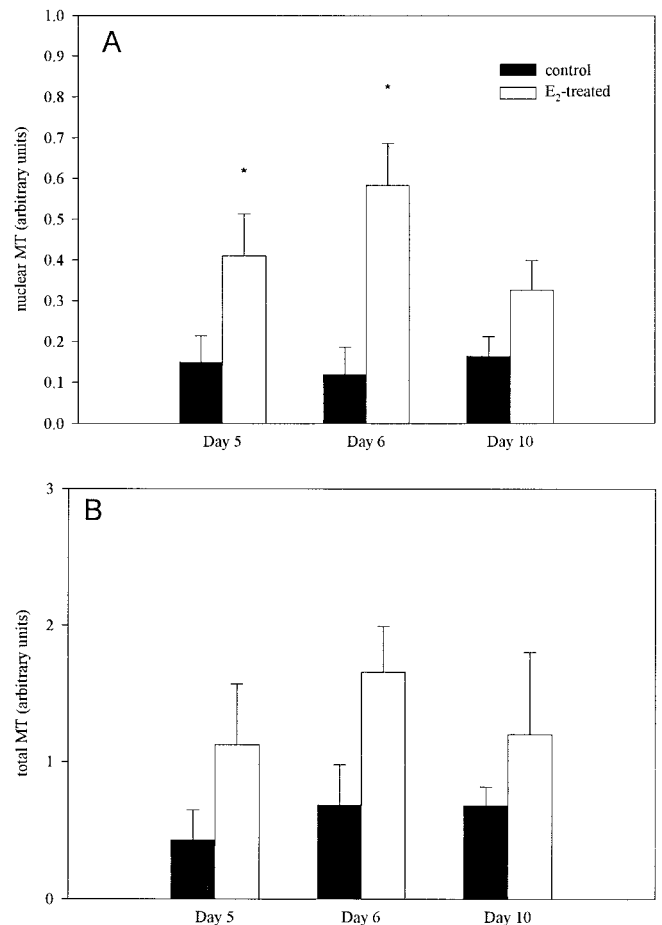


Fig. 4. Effect of  $E_2$  administration on nuclear MT (A) and total MT (B) in subcellular liver fractions obtained by differential centrifugation at days 5, 6, and 10 of the experiment ( $n = 2-8$  fish). Other details as in Fig. 1.

groups, the metal-chelating cysteinyl residues, were perfectly conserved among all sequenced fish MTs, both in terms of numbers and positions.

## DISCUSSION

$E_2$  administration did not change the zinc concentration in the liver of treated squirrelfish. In a previous study in rainbow trout,  $E_2$  was found to mediate the accumulation of zinc in the liver when measured as a function of total liver weight (30). This measurement was obtained by multiplying zinc per gram liver by LSI

Table 2. Subcellular distribution of MT

	C		N		ML	
	Control	$E_2$	Control	$E_2$	Control	$E_2$
Day 5	0.40 ± 0.15	0.84 ± 0.41	0.15 ± 0.06	0.41 ± 0.10*	0.13 ± 0.04	0.29 ± 0.09
Day 6	0.54 ± 0.17	0.78 ± 0.11	0.12 ± 0.07	0.58 ± 0.10*	0.15 ± 0.06	0.30 ± 0.07
Day 10	0.45 ± 0.09	0.74 ± 0.16	0.16 ± 0.05	0.33 ± 0.07	0.14 ± 0.03	0.31 ± 0.18

Values are means ± SE; no. of observations varied from 2 to 8 fish. Subcellular distribution of MT (arbitrary units) in the cytosolic, nuclear, and mitochondrial/lysosomal fractions of control and  $E_2$ -treated squirrelfish livers sampled at days 5, 6, and 10 of the experiment. Microsomal fractions are not shown since no MT was detected. \*Significantly different from the sham-treated control at the same sampling point ( $P < 0.05$ ; Mann-Whitney  $U$ -test).



female squirrelfish interacts with zinc finger proteins to regulate the hepatic transcription of the large amounts of RNA related to the synthesis of VTG (8).

It must also be considered that MT may not actually be present in the nucleus but rather associated with the nuclear envelope. Another possibility is that the MT found in the nuclear fraction is not associated with the nucleus at all but rather in other structures, such as dense vesicles, that would coprecipitate with nuclei during subcellular fractionation via differential centrifugation. The export of VTG from hepatocytes into the plasma involves large Golgi vesicles (46). Recent studies have shown that MT can donate zinc to the apoforms of zinc proteins (18), despite lower affinities of these proteins for zinc than MT, and that this transfer is mediated by the redox activities of glutathione (19). Perhaps MT is localized in Golgi vesicles and is acting to donate zinc to VTG, or other vehicle proteins, for transfer to the ovaries. Such an explanation would be consistent with the increase in plasma zinc concentrations observed in  $E_2$ -injected squirrelfish.

With the use of the suggested criteria for defining the phylogenetic relationships of the MT superfamily (2), squirrelfish MT fall into family 1, vertebrate MTs, and are further divided into subfamily t, teleostean MT. Specifically, squirrelfish MT most closely resembles, with 95% identity, that of the viviparous eelpout (*Z. viviparus*), which belongs to the superorder *Acanthopterygii* and the order *Perciformes*. Squirrelfish sorts under the same superorder as the viviparous eelpout (*Acanthopterygii*) but to a different order, namely *Beryciformes*. Of the teleostean superorders entered into the SWISS-PROT database, the order of peptide sequence homology scores to squirrelfish MT was *Acanthopterygii* > *Procanthopterygii* > *Paracanthopterygii* > *Ostariophysii*. On the basis of the high homology with other teleost MTs and the fact that all cysteinyl residues are perfectly conserved between MT from squirrelfish and other teleosts, we suggest that squirrelfish MT probably does not bind zinc any differently than other teleostean MTs, nor would other functional biochemical properties be different. Therefore, the massive accumulation of zinc in female squirrelfish is most likely not the result of any unusual features of the MT primary sequence.

$E_2$  had no direct effect on the concentrations of zinc or MT mRNA in the liver of treated squirrelfish. Because zinc and MT accumulations observed in squirrelfish are known to be specific to females and because immature females from the present study were not different from males in these respects, it seems likely that some kind of endocrine regulation, linked to the sexual maturation of females, is the impetus for this phenomenon. The identity and characteristics of the signaling system initiating hepatic zinc accumulation and MT transcription are still under investigation in squirrelfish. The role of  $E_2$  in the regulation of zinc in squirrelfish seems to be to stimulate accumulation of MT in the nuclear fraction of liver cells and to promote transfer of zinc from the liver to the ovaries. As expected, an increased  $E_2$  concentration resulted in a

large increase in the hepatic production and secretion of VTG.  $E_2$  was also found to markedly elevate plasma zinc levels. In fact, the peaks in plasma zinc and VTG occurred simultaneously, indicating that VTG could be involved in the transport of zinc from the liver to the ovaries. It will be interesting to isolate and characterize squirrelfish VTG to ascertain whether this protein perhaps has a higher binding capacity for zinc than the VTGs of other species.

### Perspectives

It is fascinating to speculate why squirrelfish accumulate such high levels of zinc. A key to the biological function may be that only liver and gonads of females display higher concentrations of zinc than males or females of other fish species (16). This clue, together with the finding that  $E_2$  treatment triggers redistribution of zinc from liver to ovaries in mature females, suggests that zinc accumulation is related to reproduction (12). Our results have prompted us to further hypothesize that zinc is initially bound to MT in liver and subsequently transferred to a vehicle, which transports the zinc to the ovaries where it is incorporated into the developing oocyte.

Squirrelfish are nocturnal animals with extremely large eyes. There is a connection between eyes and zinc, in that eyes are the organs with the highest zinc concentration in the vertebrate body (16, 47). Furthermore, nocturnal animals tend to have more zinc in their eyes than their day-active relatives (47). Indeed, in squirrelfish of both sexes, the zinc concentration of the retina is almost as high as that of the female liver (12). In the vertebrate retina, zinc is localized to glutamatergic neurons in which it acts as a neuromodulator (38). The nocturnally adapted eyes of squirrelfish, coupled to their physical size, may necessitate large amounts of zinc to be incorporated in the developing retina. Thus the significance of high zinc content in female squirrelfish would be to supply the embryo with sufficient zinc for eye development. This hypothesis remains untested.

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