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Variations in hepatic metallothionen, zinc and copper levels during an annual reproductive cycle in rainbow trout, *Salmo gairdneri*

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Abstract

The normal variations in hepatic levels of metallothionein, zinc and copper were studied during an annual reproductive cycle in rainbow trout of both sexes. In female fish, the total hepatic zinc levels closely followed the estradiol-17β and the LSI levels. Hence, the zinc levels rose in September, peaked in December and dropped in January. No distinct peaks were, however, observed in the whole-liver copper content. The hepatic metallothionein levels in female fish began to increase at the onset of exogenous vitellogenesis. Maximum levels were reached after estradiol-17β and LSI levels had dropped in January. In male fish no distinct peaks in either zinc or copper levels were observed. The metallothionein levels increased somewhat during the time of spermatogenesis. It is suggested that metallothionein may regulate the hepatic zinc distribution during the annual reproductive cycle in female rainbow trout, thereby ensuring the organism of a control mechanism to keep the pool of available zinc at an appropriate level.

Introduction

The biosynthesis of metallothionein (MT) can be induced by a number of factors, such as heavy metals (Durnam and Palmiter 1981), glucocorticoids (Karin *et al.* 1980; Yangle and Palmiter 1985), infection (Sobocinski *et al.* 1978; Friedman 1984), stress (Oh *et al.* 1978) and endotoxin treatment (DiSilvestro and Cousins 1984; Durnam *et al.* 1984), and involves increased levels of MT mRNA in the cytosol (Karin *et al.* 1985). The biological role of MT has been subject to numerous investigations but the physiological function is still unclear. MT appears, however, to be involved in the metabolism of zinc and copper, and may participate in the gastrointestinal uptake and in the storage of these metals in the liver and the kidney (Cousins 1983).

Several investigations have focused on the normal variations in MT content of the liver during foetal and neonatal development in mammals (Oulette 1982; Andrews *et al.* 1984). In rats, it has been shown that the translation products of foetal hepatic MT mRNA increase relative to other mRNA translation products from day 18 of gestation to birth (Andersen *et al.* 1983). An increased demand for zinc and copper has been observed during foetal and neonatal development (Bell 1979; Kern *et al.* 1981; Brady *et al.* 1982) and the MT levels have been observed to rise in association with the increased zinc levels (Charles-Shannon *et al.* 1981; Panemangalore *et al.* 1983). Copper is a constituent of cytochrome oxidase together with iron

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and may influence growth by affecting the energy supply (Kirchgessner *et al.* 1977). Zinc is an essential cofactor to many of the critical enzymes in DNA, RNA and protein synthesis (Taylor *et al.* 1982) and it has been suggested that MT serves as a temporary reservoir or as an effective storage protein for zinc and copper during early development in mammalian neonates (Pangemangalore *et al.* 1983). The role of MT in zinc regulation has further been implicated in a study of the variations of zinc and MT during periods of thymic growth in mice (Olafson 1985). Together these studies imply a role for MT in zinc and copper regulation during periods of growth and development.

The effects of zinc exposure on reproduction in fish has received some attention (Holcombe *et al.* 1979; Pierson 1981), but the normal variations in zinc during the annual reproductive cycle has not previously been studied. Further, little is known of the normal variations in copper during the annual reproductive cycle. In fish, no data are available on the variations of MT during the annual reproductive cycle.

During the period of exogenous vitellogenesis and spawning, the female fish undergoes large metabolic changes. There is a marked increase in plasma levels of steroids and this is accompanied by hypertrophy of the liver and the gonads (Bohemen *et al.* 1981). Enhanced levels of RNA are observed in the liver and large quantities of vitellogenin are produced when the plasma levels of estradiol-17β increase during the onset of the exogenous vitellogenesis (DeVlaming *et al.* 1977; Haux and Norberg 1984). After being synthesised in the liver, vitellogenin is released to the blood and transported to the ovaries, where it is incorporated into the oocytes (Wallace 1978).

In a previous paper (Olsson and Haux 1985a), the presence of hepatic MT in rainbow trout with characteristics similar to mammalian MT was established. The zinc inducibility of this protein has been shown by Ley *et al.* (1983), and a relationship between hepatic MT levels and zinc concentrations in the water has been demonstrated (Roch and McCarter 1984). Although little is known of the normal function of MT, it is conceivable that control of zinc levels could be mediated by MT.

The present study was initiated to investigate the changes in MT, zinc and copper levels occurring in fish liver during an annual reproductive cycle, and to provide evidence for a role for MT in zinc and copper regulation. During the time of exogenous vitellogenesis, the metabolic activity of the liver is increased, which implicates an increased need for zinc during this period. We propose that MT is involved in the regulation of the hepatic zinc distribution during the annual reproductive cycle in female rainbow trout.

Material and methods

About 250 male and female rainbow trout, *Salmo gairdneri*, of a spring spawning strain were obtained in late winter from a local hatchery, Antens Laxodling AB. The fish were two years old and weighed around 600 g. Before the rainbow trout were transported to the laboratory, a total of 50 sexually mature males showing typical secondary sex characteristics were sorted out and kept separately during the course of the experiment. No sexually mature females were identified.

In the laboratory, the rainbow trout were kept in two basins, each containing 5 m^3, supplied with filtered, aerated and recirculating tap water at a temperature of 10°C. The daily light and dark cycle was adjusted to follow the natural photoperiod occurring in Göteborg. The fish were fed dry pelleted food (Ewos AB) in ratios calculated to maintain the fish at a constant weight. An acclimation period of six weeks to these laboratory conditions was allowed before sampling of the immature fish started in March, and sampling of the mature males started in June. Mortality was negligible among the immature fish, while some initial losses of mature males probably were due to factors related to spawning.

Sampling was performed during the first week of every month during twelve months. On each sampling occasion, the fish were stunned by a blow to the head, weighed, and blood was withdrawn from the caudal vessels using a heparinized syringe. The liver was rapidly excised, weighed, cut into suitable pieces, wrapped in aluminium foil and immediately frozen on dry ice. The liver somatic index (LSI) and

gonadal somatic index (GSI) were calculated according to the formula: organ weight × body weight^{-1} × 100.

A 200 mg sample of each liver was homogenized in 1.0 ml ice-cold 10 mM Tris-HCl, pH 8.1, and divided into four 0.25 ml aliquots of which two were used for MT determination and the remaining two were prepared for heavy metal determinations.

Two aliquots of 0.25 ml were rehomogenized and made up to 2.5 ml with 10 mM Tris-HCl, pH 8.1, and prepared for MT determination by centrifugation at 10.000 × g for 20 minutes at 4°C. The supernatants were heat denatured at 95°C for 5 min. Denatured samples were recentrifuged at 1.000 × g for 10 min and the supernatant was filtered through a 0.45 μm filter. MT in the filtrate was determined by differential pulse polarography at 20°C (PARC Model 174 analyser, PARC/EG & G Model 303 SMDE) using a modification of the Brdicka (1933) procedure as described by Olafson and Sim (1979) and Thompson and Cosson (1984). The sensitivity of the electrode was 6.42 μA/nmole MT at 20°C. The linear working range was found to be 1.0 to 20.0 nmoles MT/litre, based on an average MW of 6500 dalton. The detection limit was approximately 0.5 nmoles MT/litre. Rabbit MT 1 (Sigma) was used as a standard. The modified Brdicka supporting electrolyte contained 1.0 M NH_4Cl, 1.0 M NH_4OH and 1.2 mM $[Co(NH_3)_6]Cl_3$. A 100 μl aliquot of Triton X-100, from a working solution of 1.25 × 10^{-2}% (v/v), was added to 10 ml of electrolyte and the sample was mixed by passing nitrogen through the solution. The purge time was set to 2 min. The electrolyte was prepared weekly and was refrigerated (4°C) when not required.

The two remaining 0.25 ml aliquots of liver homogenate were digested in 1.0 ml nitric acid (70%) at 120°C for 3 hours followed by further digestion with 0.15 ml hydrogen peroxide (50%) at 120°C for 1 hour. When the solution was colourless, it was made up to 5.0 ml and analysed for copper and zinc using air/acetylene flame atomic absorption spectrophotometry. The detection limits were 20 ppb for zinc and cadmium and 50 ppb for copper. Both the precision and the accuracy of the measurements were routinely better than ± 5%.

To determine cytosolic levels of heavy metals, 200 mg samples of individual livers were homogenized in 1 ml ice-cold 10 mM Tris-HCl, pH 8.1, using a glass-teflon homogenizer. The homogenates were centrifuged at 10.000 × g for 20 min at 4°C and recentrifuged at 105.000 × g for 60 min

Table 1. The gonadal somatic index (GSI) and liver somatic index (LSI) of male and female fish of the present study

Month	GSI		LSI	
	Males[5]	Females[5]	Males	Females[5]
Mar	0.15 ± 0.10[1]	0.11 ± 0.01	0.93 ± 0.04	0.91 ± 0.05
Apr	0.06 ± 0.01	0.19 ± 0.02	0.92 ± 0.04	0.86 ± 0.02
May	0.24 ± 0.09	0.35 ± 0.02	0.99 ± 0.01	1.00 ± 0.04
Jun	1.78 ± 0.64	0.26 ± 0.04	1.06 ± 0.08	0.91 ± 0.06
Jul	1.11 ± 0.31	0.33 ± 0.09	0.99 ± 0.04	0.95 ± 0.04
Aug	1.48 ± 0.33	1.05 ± 0.34	0.87 ± 0.05	1.10 ± 0.07
Sep	2.42 ± 0.42	1.02 ± 0.11	0.98 ± 0.04	1.05 ± 0.04
Oct	3.71 ± 0.43	2.17 ± 0.43	0.91 ± 0.04	1.27 ± 0.08
Nov	3.49 ± 0.43	5.81 ± 1.02	0.95 ± 0.02	1.51 ± 0.04
Dec	3.31 ± 0.32	8.73 ± 0.66[2]	1.01 ± 0.04	1.71 ± 0.08
Jan	2.01 ± 0.13	15.30 ± 0.53[3]	1.09 ± 0.07	1.44 ± 0.15
Feb	2.00 ± 0.16	0.87 ± 0.07[4]	1.19 ± 0.09	0.77 ± 0.04

[1] Mean ± SE; the number of individuals in each group is given in Fig. 3. [2] No females ovulated. [3] Five out of nine females ovulated. [4] All females ovulated. [5] Statistically significant variance ($p < 0.01$).

at 4°C. Aliquots of 0.25 ml of the resulting ultrasupernatants were treated as described above for heavy metal determination.

The sex steroids were determined by radioimmunoassays as described by Scott *et al.* (1982) and Scott and Sumpter (1983).

The significance of variance was determined by statistical analysis of the data. This was performed as a special case of a general linear model according to the formula $E\{x_k\} = a_0 + a_1\sin(2\pi_m^k) + a_2\cos(2\pi_m^k)$, where a_0, a_1 and a_2 are parameters in the model (Scheffé 1959).

Results

Growth of the livers and the gonads was followed by recording their somatic indices (Table 1). The LSI showed no apparent seasonal variation in the males, while the LSI in the females increased from October to reach a maximum in December. The GSI values increased in an annual fashion in both sexes. The enlargement of the gonads was much more pronounced in the females than in the males.

The development of sexual maturity was followed by monitoring plasma levels of the sex steroids in both sexes. The plasma levels of testosterone in male fish are presented in Fig. 1. In female fish estradiol-17β and testosterone were followed throughout the cycle, while 17α-20β-dihydroxyprogesterone was monitored the months around the time of ovulation (Fig. 2).

In female rainbow trout, the plasma estradiol-17β and testosterone levels showed a small progressive increase from March to August. Estradiol-17β increased from 1 ng/ml to 3 ng/ml, while testosterone increased from 1 ng/ml to 5 ng/ml. In September, at the onset of exogenous vitellogenesis, the levels of both steroids increased more rapidly and reached maximum levels in December (estradiol-17β, 40 ng/ml) and in January (testosterone, 180 ng/ml). The 17α-20β-dihydroxyprogesterone levels peaked in January at the time of ovulation. In male fish, the testosterone levels were rather constant at about 5 ng/ml plasma from March to August. In September at the beginning of spermatogenesis the levels started to rise

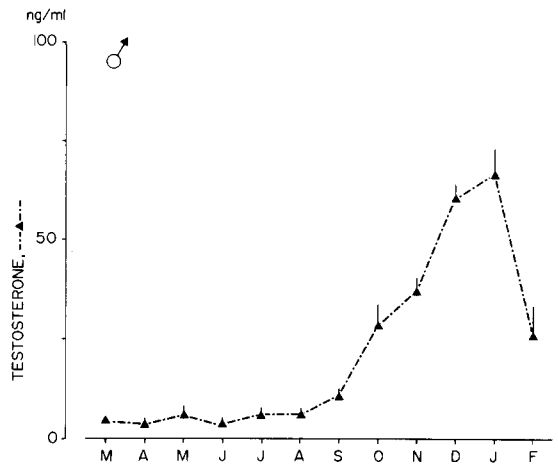

Fig. 1. Plasma levels (ng/ml) of testosterone in male rainbow trout. The number of individuals in each group is indicated in Fig. 3. The vertical bars indicate SE. Statistically significant variation ($p < 0.01$).

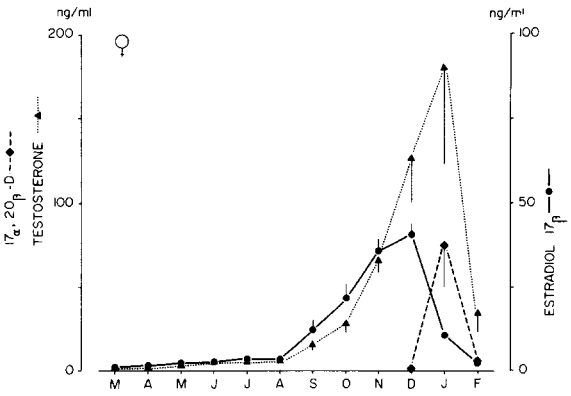

Fig. 2. Plasma levels (ng/ml) of testosterone (· · · · · ·), estradiol-17β (———) and 17α-20β-dihydroxyprogesterone (– – –). The number of individuals in each group is indicated in Fig. 3. The vertical bars indicate SE. All parameters showed statistically significant variations ($p < 0.01$).

and reached a maximum in December-January.

The total zinc levels rose in female fish during the period of exogenous vitellogenesis (Fig. 3). From March to August the total hepatic zinc levels were maintained at a fairly low and constant level in the females. The zinc levels began to increase in September and peaked in December at 241 μg Zn/liver (w.w.). In January the levels dropped to 145 μg/liver. The total cytosolic zinc levels were relatively stable from March to September in female

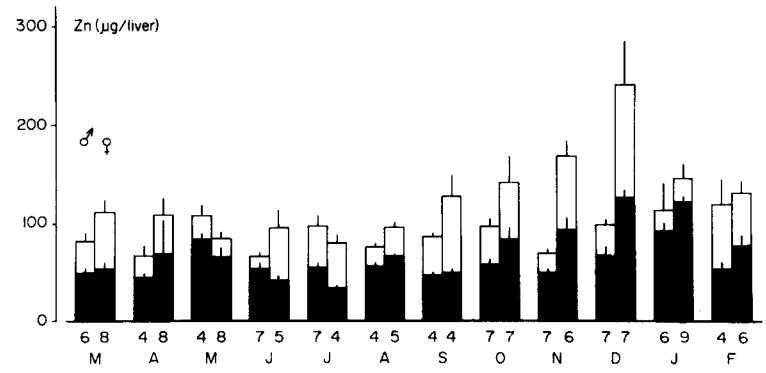

Fig. 3. Hepatic levels of zinc (µg/liver, w.w.) in male (left) and female (right) rainbow trout. The number of animals in each group is given beneath the columns. Vertical bars indicate SE. The total zinc content in whole liver is given in open column and the zinc content in liver cytosol is given in shaded column. Statistically significant variation at the $p < 0.01$ level was found in total zinc content in females and in cytosolic zinc content in fish of both sexes.

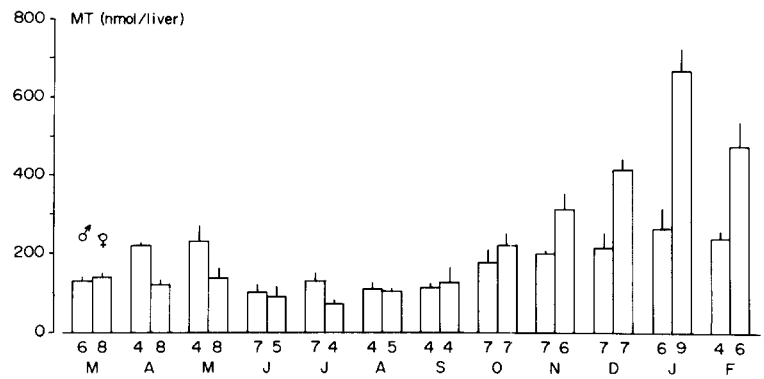

Fig. 4. Metallothionein levels (nmoles/liver) in male (left) and female (right) rainbow trout. The number of individuals in each group is indicated beneath the columns. Vertical bars indicate SE. Both groups showed statistically significant variations ($p < 0.01$).

fish, but increased in October to peak in December-January at a level of 125 µg/liver. This peak was thus reached one month later than the peak in whole liver zinc content. The zinc levels in male fish showed some fluctuations during the experimental period but no clear peaks, related to maturation or spawning, could be observed (Fig. 3).

During the period of low zinc content in the liver of female fish, from March to August, the MT levels were stable at a low level. Maximum MT levels were reached in January (646 nmoles/liver) when the hepatic MT content had increased five-fold over the basal levels. In male fish, the MT levels rose slightly toward the end of spermatogenesis and reached a maximum in January at a level of 262 nmoles/liver (Fig. 4). No major increase in total zinc (Fig. 3) or total copper (Table 2) content could be observed. A small increase in cytosolic zinc could, however, be seen in January.

The hepatic copper levels and the cytosolic copper levels in female fish were relatively stable throughout the period (Table 2). In male fish, the total hepatic copper levels were higher than those observed in the females and the copper levels also showed larger fluctuations (Table 2). The cytosolic levels fluctuated in the males in association with the fluctuations in whole liver levels.

When calculated per gram liver, the increase in MT was very pronounced in January and February in female rainbow trout (Fig. 5). The amount of zinc/g liver increased from September and reached maximum first in February. The cytosolic zinc

Table 2. The total copper content (μg/liver, w.w.) of liver and liver cytosol

Month	Whole liver[1]		Liver cytosol[2]	
	Males	Females	Males	Females
Mar	864 ± 179	723 ± 88	412 ± 26	313 ± 27
Apr	927 ± 93	742 ± 103	438 ± 28	340 ± 20
May	1186 ± 207	649 ± 84	510 ± 68	310 ± 34
Jun	1131 ± 126	740 ± 79	470 ± 41	294 ± 30
Jul	1005 ± 277	839 ± 111	407 ± 59	344 ± 13
Aug	746 ± 135	880 ± 115	335 ± 40	353 ± 25
Sep	1241 ± 358	862 ± 266	478 ± 103	327 ± 12
Oct	1004 ± 36	815 ± 165	402 ± 50	293 ± 19
Nov	1191 ± 242	724 ± 88	513 ± 56	288 ± 34
Dec	1022 ± 184	774 ± 79	578 ± 101	409 ± 42
Jan	907 ± 84	766 ± 80	508 ± 70	386 ± 31
Feb	886 ± 271	733 ± 53	474 ± 38	300 ± 29

[1]Mean ± SE; the number of individuals in each group is given in Fig. 3. [2]Mean ± SE; n = 2. [3]Statistically significant variance ($p < 0.01$).

Table 3. Copper content in liver and liver cytosol (μg/g liver, w.w.)

Month	Liver[1]		Liver cytosol[1]	
	Males	Females[2]	Males	Females[2]
Mar	160 ± 26	148 ± 17	77 ± 12	64 ± 6
Apr	211 ± 12	155 ± 16	99 ± 6	71 ± 4
May	164 ± 37	128 ± 18	70 ± 9	61 ± 7
Jun	218 ± 32	128 ± 18	91 ± 7	51 ± 2
Jul	191 ± 29	173 ± 18	77 ± 11	71 ± 3
Aug	158 ± 30	136 ± 25	71 ± 10	55 ± 4
Sep	189 ± 54	138 ± 32	73 ± 16	51 ± 2
Oct	176 ± 6	125 ± 33	70 ± 9	45 ± 3
Nov	231 ± 44	86 ± 11	99 ± 11	34 ± 1
Dec	146 ± 36	70 ± 8	83 ± 12	37 ± 1
Jan	144 ± 25	114 ± 12	81 ± 10	57 ± 8
Feb	141 ± 32	150 ± 8	75 ± 13	61 ± 5

[1]Mean ± SE; the number of individuals in each group is given in Fig. 3. [2]Statistically significant variation ($p < 0.01$).

levels showed a marked increase in January, which coincided with the elevation of MT. The copper content/g liver showed a distinct drop during the time of increased LSI and returned to a higher level in January and February (Table 3). In male fish no major variations could be observed per gram liver (Table 3).

Discussion

The results from the present study provide evidence for a possible role of MT in zinc regulation in female rainbow trout liver during exogenous vitellogenesis. Characteristic of the period of exogenous vitellogenesis are increased levels of estradiol-17β in

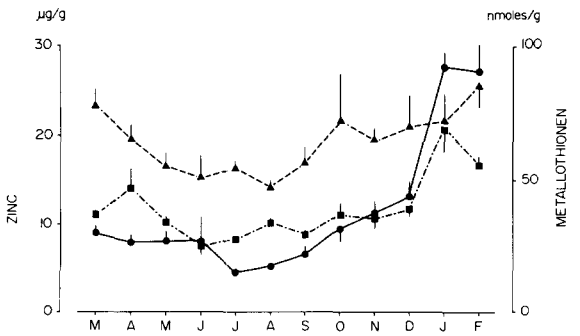

Fig. 5. Levels of total zinc (– – – –), cytosolic zinc (–.–.–.) and metallothionein (———) in the livers of female rainbow trout. Zinc and copper are shown as µg/g liver (w.w.) while metallothionein is shown as nmoles/g liver. The vertical bars indicate SE. The number of individuals in each group is given in Fig. 3. All parameters showed statistically significant variations ($p < 0.01$).

the plasma, as well as increases in both LSI and GSI (Bohemen *et al.* 1981). Increased levels of hepatic mRNA have been demonstrated in juvenile rainbow trout treated with estradiol-17β, indicated an increased ability of the liver to synthezise new protein (Haux and Norberg 1985). During the period of exogenous vitellogenesis, the amount of RNA is enhanced in the liver of female rainbow trout (Haux and Norberg 1984). In males, the testosterone levels increase during the period of spermatogenesis (Baynes and Scott 1985), and a similar profile for plasma testosterone was found in the present study. However, no increase in LSI could be seen in the males. In mammals, zinc has been implicated as an essential element in DNA, RNA and protein synthesis (Sandstead 1975; Taylor *et al.* 1982). In view of this, the observed increase in total hepatic zinc levels in female fish most probably reflects the enhanced metabolic activity of the liver, due to the synthesis of large amounts of vitellogenin.

In the rat, it appears that zinc may be involved in the regulation of foetal changes in MT synthesis (Charles-Shannon *et al.* 1981). In the present study, the hepatic MT levels were observed to increase during exogenous vitellogenesis. The zinc levels increased in association with the elevations in MT and LSI in the females. The peak in hepatic zinc content corresponded to the time of maximum liver size, when the metabolic activity of the liver was high. When the hepatic zinc content returned to a lower level there was a redistribution of zinc from the microsomal and mitochondrial fractions to the cytosolic fractions. This corresponds to the time of maximum levels of hepatic MT in the females and suggests that zinc binds to MT. Thus, MT may have a zinc regulatory role in rainbow trout. A further indication of such a role was obtained when expressing the content of zinc, cytosolic zinc and MT on a per gram liver basis. In this case a 3 fold increase of MT was observed in January at the time of zinc redistribution in the liver. Thus, the cytosolic MT levels increase concomitantly with the increase in zinc.

In experiments on rats it has been observed that both foetal and maternal MT levels undergo changes during the period of gestation (Kern *et al.* 1981; Piletz *et al.* 1983). The zinc levels in foetal rats have been shown to increase together with the increase in MT during late gestation (Charles-Shannon *et al.* 1981; Kern *et al.* 1981). The maternal MT levels have been observed to drop between days 17 and 20 of gestation (Kern *et al.* 1981; Piletz *et al.* 1983), and this drop corresponds to the increase of foetal hepatic MT. It has been shown that it is during this period that the mobilization of zinc from maternal serum to the foetus takes place (Vojnik and Hurley 1977). A role of MT in zinc regulation has further been implicated in a study of the variations of zinc and MT during periods of thymic growth in mice (Olafson 1985). Together these studies imply a role for MT in zinc regulation during periods of growth and development.

While there were very distinct changes in total zinc, cytosolic zinc and MT levels in the females, the copper levels were unaffected by the process of sexual maturation. In rainbow trout, the hepatic copper levels are high in comparison to other teleosts (Ley *et al.* 1983; Olsson and Haux 1985a). This is also the case in the present study and the possible redistribution or changes in copper content can therefore be difficult to distinguish due to the high variations in the different groups. It can be observed that, although the liver size doubles towards the end of exogenous vitellogenesis, the total copper content is maintained at a constant

level. Studies of the subcellular cytosolic distribution of copper might, however, reveal shifts in the copper content of different subfractions during the period of sexual maturation in rainbow trout.

Much attention has been focused on the role of MT in adaptation to heavy metals and its possible involvement in heavy metal detoxification in fish (Roch and McCarter 1984; Olsson and Haux 1985b). Possibly, cadmium binds to MT in heavy metal-exposed fish, thereby reducing the toxic effects of this metal (Olsson and Haux 1985b). MT can function as a valuable indicator of metal pollution in fish, as recently demonstrated for copper (Roch *et al.* 1982; Roch and McCarter 1984). Sublethal effects obtained in field studies must, however, be viewed with caution as several of the biochemical and physiological response parameters are influenced by abiotic and biotic factors, such as sex, stage of sexual development, nutritional state, temperature and season (Förlin *et al.* 1986). This has also been demonstrated in the present paper where the MT and zinc content of rainbow trout liver vary as a function of sex as well as state of sexual maturation.

The function of MT during and after exogenous vitellogenesis could be to regulate the levels of available intracellular zinc and perhaps also copper. This can be achieved by binding the metals to MT during periods of low metabolic activity and by releasing the metals during times of high demand. The present study indicates that MT may be involved in the regulation of zinc during the annual reproductive cycle in rainbow trout, thereby ensuring the organism of a control mechanism to keep the pool of available zinc at an appropriate level.

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