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PHYSIOLOGICAL EFFECTS OF SUBLETHAL COPPER EXPOSURE ON THE ENERGY METABOLISM OF THE COMMON CARP, *CYPRINUS CARPIO.*

FYSIOLOGISCHE EFFECTEN VAN SUBLETHALE KOPERBLOOTSTELLING OP HET ENERGIEMETABOLISME VAN DE GEWONE KARPER, *CYPRINUS CARPIO*.

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen aan de Universitaire Instelling Antwerpen te verdedigen door Gudrun De Boeck.

Promotor: Dr. Ronny Blust

Wilrijk, 1996.

TO ALL THE PEOPLE

WHO BELIEVED IN ME

ALL THE WAY

AND SUPPORTED ME:

THANK YOU!

LOVE,

GUDRUN

What if we live to be fifty
And the optimists win by a mile
Supposing we stop the starvation
And the century ends with a smile
Maybe recycling paper
Will bring back the forests again
And maybe five year old psychos with knives
Will grow up to be happy and sane

So give up your cigarettes
Work out and study
And carry a packet of three
We'll live to be rich
and one hundred and seven
Unless you know better than me

What if we live to be fifty
And help all the weak and oppressed
We'll cancel their debts & no-one will expect us
To work any harder for less
We'll spend our way out of recession
The West will invest in the East
So hordes of poor never swarm out door
Demanding a share of the feast

So give up your cigarettes
Work out and study
And carry a packet of three
We'll live to be rich
and one hundred and seven
Unless you know better than me

Science will beat every fatal disease
Plutonium's perfectly save
They'll find a solution to all the pollution
It's only a matter of faith

What if we live to be fifty
And the bomb doesn't drop after all
And we never lie destitute, freezing and sick
As the mortar shells batter our walls
We'll cheer as our glorious leaders
Develop new weapons for peace
They'll base a new military laser in space
And the ozone will heal in a week...

So give up your cigarettes
Work out and study
And carry a packet of three
We'll live to be rich
and one hundred and seven
Unless you know better than me

Tom Robinson

GENERAL INTRODUCTION

Through human activities, continuous changes have occurred in aquatic ecosystems throughout the world. For example levels of metals have increased in aquatic ecosystems in a number of industrialised areas (Nriagu and Pacyna, 1988). They increase directly, as a result of atmospheric deposition, waste water discharge and runoff (e.g. Hg, Cd, Cu and Zn), or indirectly, through increased solubilisation and mobilisation from sediments owing acidification (e.g. Al and Fe). While both marine and freshwater ecosystems are threatened, soft fresh waters that are poorly buffered are particularly sensitive because they are the only aquatic habitats prone to acidification. Metals which are of the greatest concern are Hg, Cd, Cu, Pb, Zn, Al, Mn and Cr, approximately in order of decreasing toxicity.

Severe changes in an aquatic environment, such as toxic spills, may result in sudden mortality of inhabiting species. These episodic events are relatively easily detected, and the presence of acute toxicity in the environment can be measured using acute toxicity tests. Much more difficult to detect is the biological impact of more subtle environmental changes, which might not lead to immediate mortality, but could affect growth and reproduction at longer terms.

If individual organisms are exposed to environmental changes, they will try to cope with these changes, and may do so by altering physiological and biochemical processes. These alterations, even when relatively small, may not only lead to modifications in the energy status and fitness of the organism, but can also affect populations and entire ecosystems. For example, the appearance of a pollutant in the aquatic environment may increase the metabolic demand of an organism to maintain homeostasis. As a consequence, its nutritional requirements may increase, potentially above food availability. The oxygen consumption may increase, making the organisms more susceptible to oxygen stress. Growth rates may change, potentially altering predator-prey relations. The adaptation processes may require energy, thereby

decreasing the fitness of the organism, and rendering it more susceptible to other stressors. The pollutant may also trigger a migration towards other regions. All these effects may eventually result in local disappearance of species and profound changes in the structure and functioning of ecosystems. Therefore, it could be of great importance to recognise changes in the physiology and biochemistry of organisms exposed to environmental changes at an early stage, and to acknowledge their importance at longer terms. Early detection of such events, may allow sufficient time to interfere with the cause of pollution before irreversible changes in the ecosystem have occurred.

Aquatic toxicologists have long sought for a single all purpose method to assess environmental health or condition. As Cairns and Van der Schalie (1980) so appropriately have put it: 'This is the contemporary version of the search for the Holy Grail and almost certainly will be no more successful'. In other words, no single method will ever serve for all situations. It seems safe to state that the same conclusion applies to the use of physiological and biochemical measures to monitor the health of fishes. There will always be a need for a variety of methods in order to be responsive to all types of potential forms of degradation of water quality. In order to detect possible effects of pollutants on the fitness of fish, fish physiology has become an integral part of aquatic toxicology during the last decades. From approximately the mid 70s, aquatic toxicology has increasingly used the tools of physiologists. This is partly to understand why a fish or invertebrate is debilitated, but it is also because of a realisation that many sublethal effects may occur without necessarily resulting in death of the individual organism. While bioassays extending over several generations are useful and certainly the more sensitive tools to be used, they are also extremely time consuming and expensive to carry out. Due to this disadvantage, there has been a considerable interest in developing physiological and biochemical tests, commonly known as biomarkers , to assess the fitness of aquatic animals.

From purely physiological standpoint, the presence of pollutants in the environment at sublethal concentrations can be considered to be an important

environmental variable to which fish respond physiologically and thereby reveal basic mechanisms of fish physiology. Because physical, chemical and biological conditions of ecosystems are continuously changing due to mankind, the curiosity to understand the results of those perturbations at several levels of biological organisation is a goal in itself. In the long run, it might lead to a broader theoretical construct, which might allow better predictions of effects of environmental perturbations on organisms in the future.

The different levels of biological organisation in an organism should always be kept in mind when investigating the effects of pollution. When environmental changes affect an organism at the cellular level, this will also reflect on organ functioning, which on its turn can cause changes at the hormonal or neural level and alter the total physiology of the organism. Or, if a pollutant affects sensory organs, this may change the release of neurotransmitters, which might reflect in a different hormonal control of organ and cellular functioning. Looking at the different levels of organisation, it is important to realise that no level is more important than another. Each level offers its unique problems and insights; each level finds its explanation of mechanism in the lower levels, and its significance in the higher levels. As a rule, the higher the level, the more generalised the response. So, if one wishes to assess the general "health" of an organism, higher levels are more appropriate. However, if one is interested in studying more specific actions of various processes and wishes to understand mechanisms, lower levels should be investigated.

When considering the biochemical functioning of organisms, copper is an essential element in several processes. It is an essential part in different enzymes and it is important in the formation of bone and brain tissue. Thus, low levels of copper intake are necessary for vertebrate function. When environmental copper levels increase beyond the normal levels in an aquatic ecosystem, the metal becomes toxic to the different species inhabiting that ecosystem, and the disruptive effects of the heavy metal become apparent. Indeed, copper appears to be a very puissant toxicant when present in excessive amounts.

The aim of the presented research is twofold: 1) to study the effects of sublethal concentrations of copper on the physiology of the common carp, *Cyprinus carpio*, at different levels of biological organisation, and 2) to evaluate the possibility to employ the changes in these different physiological processes as sensitive biomarkers for sublethal stress. Four series of experimental studies were made, studying the following processes during copper exposure: growth, capacity for protein synthesis, use of energy stores, total aerobic metabolism, relative protein catabolism, use of phosphorous compounds during rest and after an additional mild exercise, and changes in neurotransmitters involved in hormonal control.

CHAPTER I: LITERATURE STUDY

1.1. General aspects of metal toxicity

1.1.1. Fish gills, the main site of water-borne metal uptake: structure and function

There is an important difference between the routes by which a foreign chemical is taken into a terrestrial animal or into an aquatic animal. This is because the aquatic environment imposes more constraints on the animals in it. For example, to obtain sufficient oxygen, a fish must breathe roughly 20 times more of the respiratory medium (i.e. water) than a terrestrial animal. Therefore, the gill tissue will be exposed to a far greater amount of disolved pollutants than an air breathing animal, even taking into account the lower oxygen consumption of a water dwelling poikilotherm. The gill comprises over half the body surface area; here only a few microns of delicate epithelium separate the internal environment from a continually flowing external environment of which the chemical and physical characteristics may vary greatly over time and space. The organ is both morphologically and physiologically complex, performing multiple functions simultaneously such as gas and solute exchange, acid-base regulation, nitrogenous waste excretion, etc... As such, it is the primary receptor surface for all changes in the external environment, and its response, whether adaptive or pathological, invariably determines the extent of homeostatic regulation of the internal environment.

The gills of fish constitute a sieve like structure placed in the path of the respiratory water flow (Hughes, 1984; Laurent, 1984). The sieve is formed by the gill arches, filaments and lamellae (Fig. 1.1.). The predominant cell type are the pavement cells which generally cover more than 90% of the filament and lamellar epithelium and are joined to each other by tight junctions (Perry and Laurent, 1993). The filament

epithelium is typically multi-layered while the lamellar epithelium is normally composed of a double layer of cells separated by extracellular spaces. Whereas the lamellae and its pillar capillary network are considered to be the gill exchange area, the filaments contain the major sites of vascular perfusion control and musculature for orientation of the exchange surface (Laurent and Dunel, 1980). Blood flows through the lamellae in a direction countercurrent to that of the water flowing over the lamellae, and gasses and ions are transferred between water and blood across the lamellar wall.

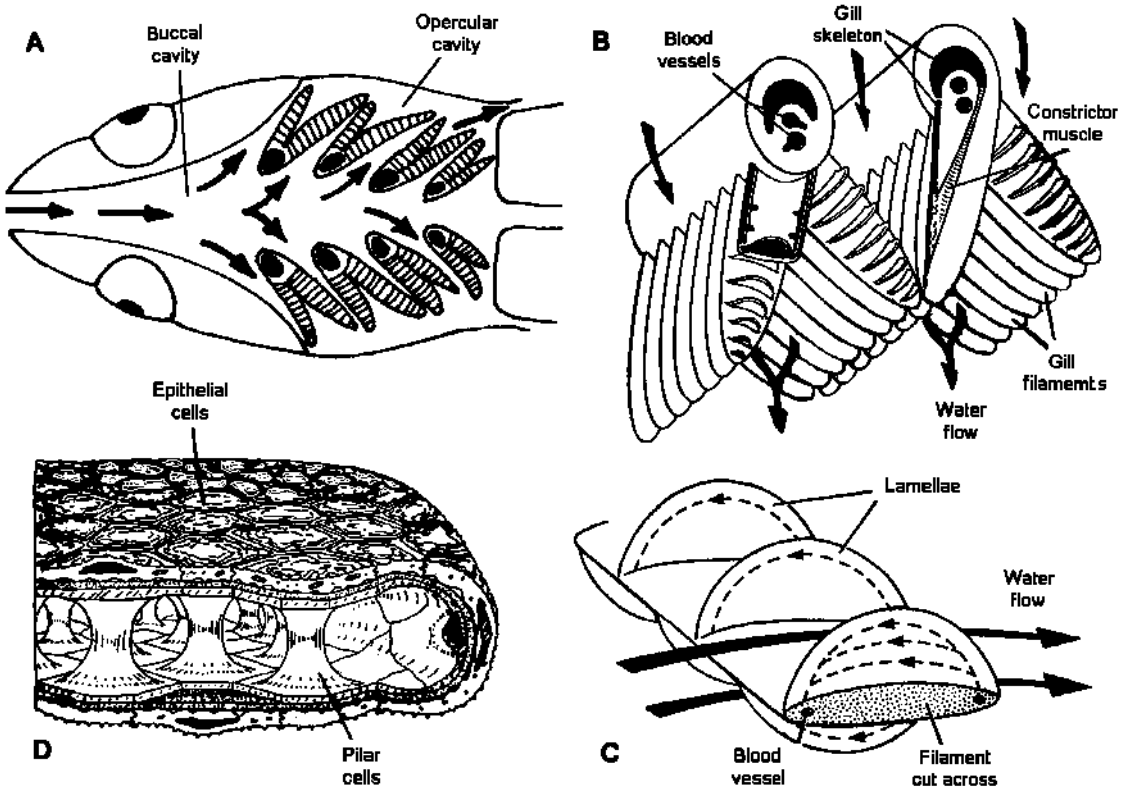

Figure 1.1. General structure of a teleost gill: a) horizontal section through the head of a generalised teleost showing four pairs of gill arches; b) a part of two gill arches with two rows of gill filaments; c) a transverse section of a filament showing lamella in side view; d) a transverse section of a lamella. Arrows indicate directions of water and blood flow (redrawn from Perry and McDonald, 1993; Eckert and Randall, 1978; Olson, 1991).

Most important for ion exchange are the chloride cells, which comprise less than 10% of the gill surface area. The ultrastructural features of a chloride cell include an abundance of mitochondria, an amplification of the basolateral membrane to form an extensive tubular network within the cell and a well developed vesicular system in the apical regions of the cell, all characteristics of cells which are involved in ion transport. Chloride cells are most abundant in the interlamellar regions and on the afferent side (trailing edge) of the filament (Laurent and Dunel, 1980).

A third type of cells are the mucous cells, which are most abundant on the efferent side (leading edge) of the filament (Laurent and Hebibi, 1989). The mucus secreted by these cells is a polyanionic glycoprotein and its suggested functions include defence against pathogens, prevention of turbulent water flow during swimming and ionic regulation. It has been shown that mucus not only binds metals but also retards their diffusion rate (Pärt and Lock, 1983). The thickness of the mucus layer is known to vary, but has been estimated to be about three µm in resting, normoxic fish (Randall *et al.*, 1991). When gills are ventilated with water of a neutral pH, the interaction between CO_2 and ammonia excretion in this mucus layer results in a net excretion of acid (Lin, 1989). The overall effect of this acidification in the gill microenvironment has important consequences for metal binding and uptake (see below: part 1.1.2.).

1.1.2. Mechanisms of acclimation to metals in fish gills

As stated above, the main, or at least the initial target of metals in fish are the gills. The disturbances caused by metal exposure, are such that an impairment of both gill transport and gill barrier functions are suggested. At low exposure concentrations, or short periods of exposure, the normal homeostatic mechanisms of the gill are able to cope, and no damage is sustained (Fig. 1.2.). Increasing concentrations or longer exposure however, may initially cause reversible physiological changes and

subsequently some impairment of gill function. Typically, the disturbances caused by metal exposure are characterised by an initial ‘shock’ phase of fairly short duration during which the disturbances develop fairly rapidly, and a longer-term ‘recovery’ phase during which the disturbances diminish gradually. The organism may, in some instances, fully recover in the continued presence of the metal, but more usually it moves into a new physiological steady state (McDonald and Wood, 1993).

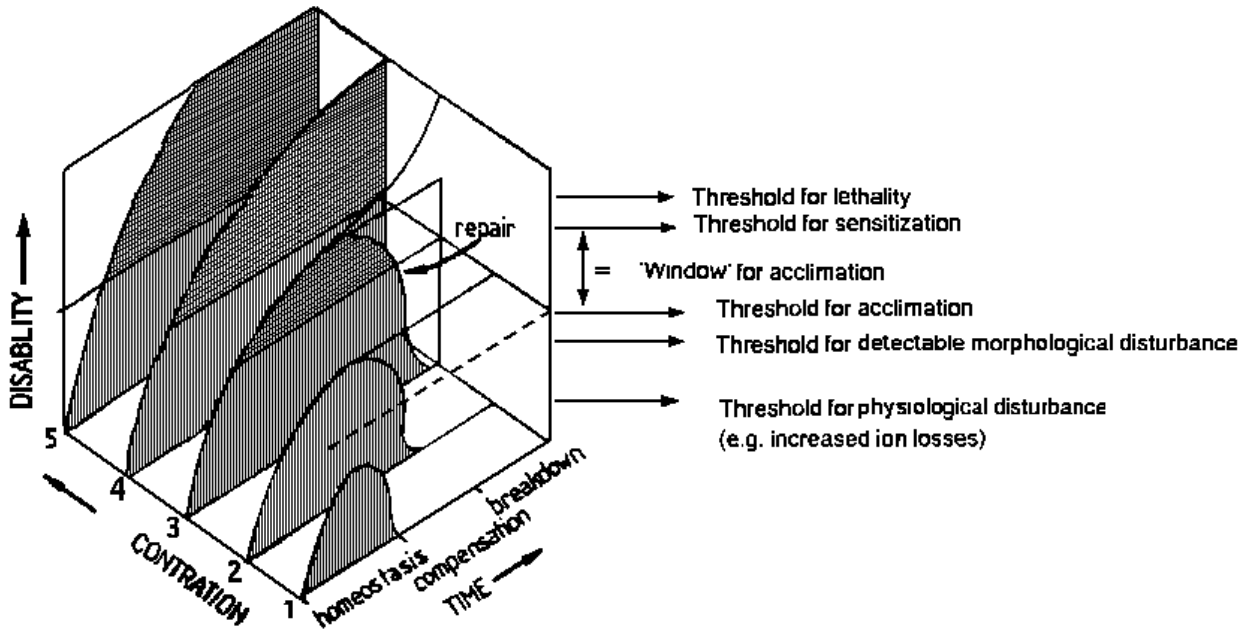

Figure 1.2. Effects of an increasing dose of a toxicant on the physiological processes of an organism (redrawn from Hellawell, 1986; McDonald and Wood, 1993).

1.1.2.1. The shock phase

In the initial shock phase morphological gill damage occurs. At moderate levels of exposure, the damage consists of separation of epithelial layers, tissue oedema and clubbing of lamellae, while at more severe levels, tissue necrosis and rupture and fusion of lamellae become more prominent (Mallat, 1985). The morphological damage phase roughly corresponds to the physiological ‘shock’ phase. The latter is

characterised by inhibition of ion uptake at low metal levels, with stimulation of ion efflux at higher metal levels, thereby leading to net ion losses (Spry and Wood, 1985; Laurén and McDonald, 1987a; Wood and McDonald, 1987; Reid and McDonald, 1988). A direct poisoning of branchial ion-transport ATPases (Stagg and Shuttleworth, 1982a; Staurnes *et al.*, 1984; Laurén and McDonald, 1987b; Verbost *et al.* 1988) is implied in the inhibition of ion uptake, whereas disruption and widening of tight junction are implicated in the increased efflux (Freda *et al.*, 1991). The effects of Cd, Zn and Mn are directed towards disrupting Ca^{2+} balance, whereas the effects of Cu, Al, and Cr are directed towards disrupting Na^{+} and Cl^{-} balance. These initial responses are followed later by compensatory responses, including hypertrophy of mucous and chloride cells, and a general thickening of the filamental and lamellar epithelia.

1.1.2.2. The recovery phase

There is a general agreement that the secretion of mucus, as one of the first lines of defence, is an important mechanism for protecting the gill tissues from toxic metals. Mucus release has been shown to be a more sensitive indicator of metal exposure than mucus content, which on its turn is a better indicator than the amount of mucus producing cells (Lock and Van Overbeeke, 1981). Indeed, Playle and Wood (1989, 1991) showed that for Al-exposed rainbow trout the binding of aluminium by the gills could only account for 10 to 15% of the aluminium extracted from the water passing over the gills. It is therefore likely that the bulk of aluminium extracted from the water is bound by mucus produced by the gills which afterwards is quickly washed away. Furthermore, it has been shown that mucus not only binds metals, but also retards their diffusion rate (Pärt and Lock, 1983). The way mucus retards electrolyte loss is less clear. Mucus certainly inhibits O_2 diffusion (Ultsch and Gros, 1979), but there is no evidence that it also retards diffusion rates of Na^{+}, Cl^{-} or Ca^{2+}. It is more likely that the presence of mucus creates an unstirred layer with much higher concentrations of electrolytes in the matrix next to the epithelial surface than the water (Shepard, 1982;

Handy, 1989). In this way, both ion loss is retarded and active ion uptake across the epithelium is facilitated.

A second line of defence is the reduction in ionic permeability of the gills, i.e. a rapid response which probably already occurs while physical damage is still increasing (Laurén and McDonald, 1986). The exact mechanism of this reduced permeability is unknown, but possible mechanisms include: the lamellar fusion and clubbing which reduces the surface area, the swelling of epithelial cells which increases the blood to water diffusion distance, and finally hormonal actions are suggested to stabilise the tight junctions and the binding of protective divalent ions (Ca^{2+}, Mg^{2+}) on the surface. Also mucus secretion appears to be under hormonal control (Mattheij and Sprangers, 1969; Mattheij and Stroband, 1971; Marshall, 1976, 1979).

After these first lines of defence a phase of compensation and repair follows, even in the continuous presence of the metal depending on the metal concentration. The gills return to a more normal appearance meaning that rupture of epithelial layers, tissue necrosis, and clubbing and fussion of lamellae disappear. Hypertrophy of the mucous and chloride cells can remain, together with a thickening of the lamellar epithelium. Ionic uptake rates recover and ion loss rates are reduced, and the internal physiology returns to normal. Moreover, the fish even become more resistant when challenged with a further increase of the metal level. At least three mechanisms can account for this phenomenon (McDonald and Wood, 1993): 1) alterations in the barrier properties of the tissues which act to decrease the net uptake rate of the metal (continuous higher rate of mucus secretion by the hypertrophic mucous cells and a thicker lamellar epithelium), 2) an increase in storage and detoxification of the metal once it has entered the gill tissue (enhanced synthesis of metallothioneins), and 3) an increase in resistance of metal sensitive branchial processes (increased synthesis of Na^+/K^+ ATPase and Ca^{2+} ATPase in hypertrophic chloride cells, higher binding affinity of the cell surface ligands for Ca^{2+}).

1.2. Copper

Copper (Cu) has an atomic number of 29 and molecular weight of 63.546. It occupies a position between nickel and zinc in the subgroup IB of the periodic table. Copper comprises about 0.007% of the earth's crust and exists as sulphide ores such as chalcocite (Cu_2S), covellite (CuS), chalcopyrite ($CuFeS_2$) and chalcanthite ($CuSO_4.5H_2O$). Copper's valencies are 1^+, 2^+ and 3^+.

1.2.1. The physiological role of copper

Copper is an essential element and part of about thirty enzymes and glycoproteins (e.g. amine oxidase, catalase, cytochrome oxidase, feroxidases, peroxidase, superoxide dismutase...). Since oxidative enzymes (e.g. catalase, cytochrome oxidase and peroxidase) require Cu, the metal is involved in hydrogen peroxide organic substance destruction and energy production. Copper promotes iron absorption from the gastrointestinal system, is involved in the transport of Fe from tissue into plasma, helps to maintain myelin in the nervous system, is important in formation of bone and brain tissue, necessary for haemoglobin synthesis, and other important functions (Abdel-Mageed and Oehme, 1989). Copper is bound to α-globulin (cerukoplasmin) in the blood and transported to the liver, kidney, heart, central nervous system, bone, and muscle for storage.

Because Cu is essential to so many functions in the vertebrate body, homeostatic mechanisms are involved in regulation of internal levels. Under normal circumstances, uptake of trace levels of copper are sufficient to maintain internal homeostasis. When copper levels exceed certain values however, regulatory capacity is overcome, defence mechanisms to protect against excess copper are insufficient, and toxic effects appear.

1.2.2. Copper in the aquatic environment

When metals enter a natural aquatic system, their bioavailability may be significantly decreased. If there is a considerable amount of organic material or suspended solids, the actual amount of dissolved metal available for uptake by the fish is greatly reduced. This tendency to form complexes with organic and inorganic ligands (primarily carbonate and hydroxide) varies with the metal. For example, copper binds to organics more readily than does either cadmium or zinc (Engel *et al.*, 1981). As a result it is important that only dissolved metal is measured rather than its total concentration in the water, in order to assess the amount of metal actually available to the fish. Most natural water systems contain a vast array of organic complexing agents which can substantially reduce free Cu^{2+} concentrations in aqueous systems. This list includes humic and fulvic acids derived from rotting vegetation (Zitko *et al*., 1973; Brown *et al*., 1974; Sylva, 1976), amino acids (Brown *et al*., 1974), suspended solids (Brown *et al*., 1974) and nitrogenous waste products (Sylva, 1976). Moreover, absorption of free Cu^{2+} by sand, gravel and suspended clay is important in these systems (Sylva, 1976). Because many complexing agents are normally present in water, synthetic water solutions are best suited for laboratory testing (Sörensen, 1991). Therefore, synthetic 'standard moderately hard water' was used in our studies which was prepared according to the 'Standard methods for the examination of water and waste water.' (American Public Health Association, 1989).

Even when only the dissolved copper fraction is considered, identification of specific, toxic copper species in the aquatic environment is a controversial issue (Sörensen, 1991). Speciation of copper is expected to be highly variable from system to system and from one time to another within the same system. The list of toxic copper species often includes $CuOH^{+}$ (Pagenkopf *et al*., 1974; Howarth and Sprague, 1978), and $Cu_2(OH)_2^{2+}$ (Howarth and Sprague, 1978). However, without question, Cu^{2+} is considered to be the most toxic (Stiff, 1971a,b; Pagenkopf *et al*., 1974; Sylva, 1976; Howarth and Sprague, 1978). With the water composition as in Table 1.1., the

speciation of the different copper species was calculated by the SOLUTION speciation program and is given in figure 1.3.

$CaSO_4.2H_2O$	0.348 mM
$MgSO_4.2H_2O$	0.500 mM
$NaHCO_3$	1.143 mM
KCl	0.054 mM
pH	7.4-7.8

Table 1.1: Composition of standard moderately hard water according to the 'Standard methods for the examination of water and waste water.' (American Public Health Association, 1989).

It is clear from figure 1.3. that pH plays an important role in copper specation. In fact, with a pH of 7.5 in the water, pH of the water in the narrow spaces between the gill lamellae would have been about 7.0 (according to the results of Lin, 1989 for rainbow trout, see above: part 1.1.2), and therefore, copper at this main site of water-borne metal uptake would occur as: 77.3% Cu^{2+} and 6.6% $CuOH^{+}$, i.e. two of the major toxic forms of copper.

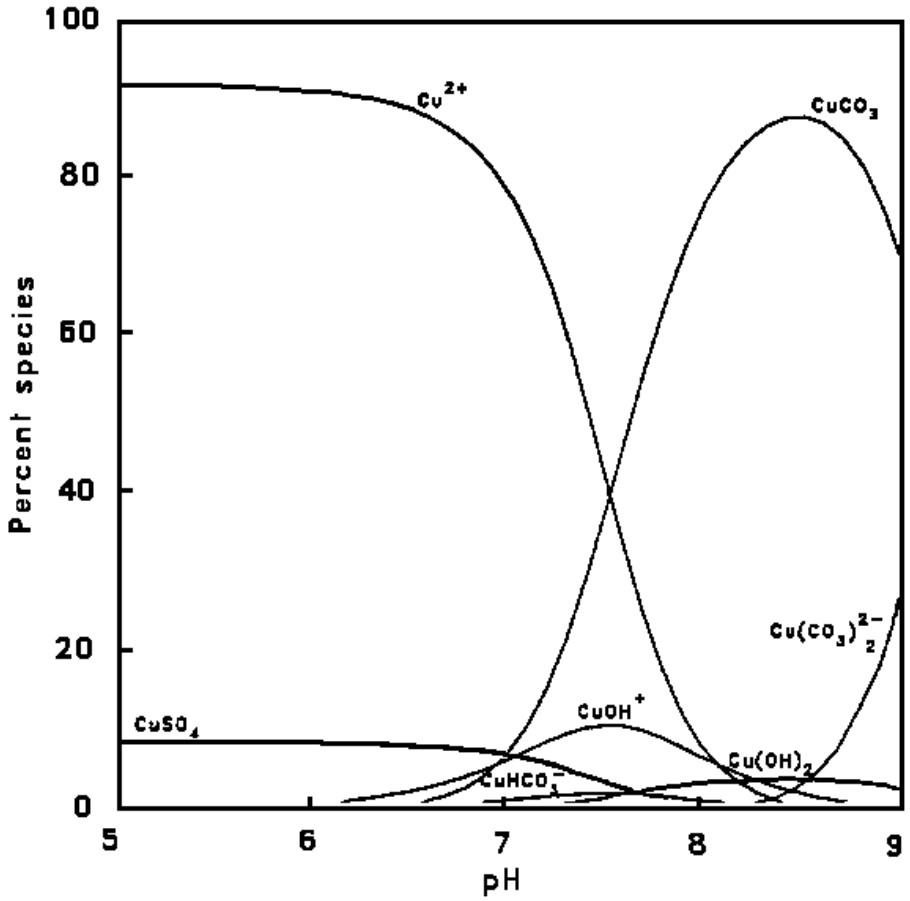

Figure 1.3. Copper speciation in synthetic standard moderately hard water at 20°C as a function of pH calculated with the SOLUTION speciation program.

During the 70's and 80's, research was completely directed towards explaining copper toxicity as a function of copper speciation. One of the most debated issues was whether total hardness or alkalinity of water is the major modulating factor controlling the toxicity of copper in fish. Stiff (1971a) measured Cu^{2+} concentration and pH in a series of three bicarbonate systems (0.001, 0.002 and 0.005 M) and four concentrations of total copper (0.79 µM, 1.57 µM, 7.87µM and 15.74 µM) to simulate the water composition of most natural waters. If Cu^{2+} were the toxic form of copper and $CuCO_3$ was relatively non toxic, this would account for the difference in copper toxicity in water of different hardness. Toxicity would not be related, to hardness *per se*, but to the covarying alkalinity. Other researchers also considered alkalinity of more

importance than hardness in protecting fish from copper toxicity (Pagenkopf *et al*., 1974; Laurén and McDonald, 1986).

In contrast, other scientists believed that total hardness is more important than carbonate-bicarbonate alkalinity in protecting fish from copper poisoning (Miller and MacKay, 1980). Fingerling rainbow trout (*Salmo gairdneri*) were used to determine effects of water hardness and alkalinity on the incipient lethal concentration which caused 50% lethality (ILC50) for copper. At low water hardness (12 ppm), the ILC50 of copper did not change despite a five-fold change in alkalinity (10 to 50 ppm). However, at 10 ppm alkalinity, a ten-fold increase in hardness (12-120 ppm) caused a five-fold increase in ILC50. Moreover, at a hardness of 100 ppm, increasing the alkalinity significantly reduced copper toxicity. Therefore, although increasing alkalinity reduces copper toxicity in fish exposed in hard water, Ca levels (or total hardness) were thought to be more important than the carbonate-bicarbonate alkalinity in protecting fish from copper toxicity.

More recently, research has been extended towards membrane biology to incorporate the characteristics of membrane protein ligands as well as the speciation of the metals in the water (Simkiss and Taylor, 1989, 1995). Indeed, some observations could not be explained by metal speciation only. For example, even at high pH values of the surrounding water, copper was observed to be very toxic, although Cu^{2+} concentrations were very low (Howarth and Sprague, 1978, Miller and Mackay, 1980). Recalculation of the data from a number of sources to standardise it to a constant hardness led Borgmann (1983) to conclude that the toxicity of copper ions increased as the water pH increased. This implies either that some inorganic complexes are extremely toxic, or that pH has a direct effect on the uptake of copper. The latter suggestion is being favoured now. If copper is transported across cell membranes by binding to carrier ligands, then pH will directly affect the ionisation of these groups, and protons may actually compete with the ligands that were involved in copper transport. This is supported by the fact that at low pH biological uptake of copper decreased although the metal availability increased (Campbell and Stokes, 1985).

Furthermore, a gene encoding for a Cu^{2+}ATPase has been isolated from human patients suffering Menkes disease recently, a condition associated with copper deficiency. This finding of the Menkes gene is the first characterised example of a eucariotic trace metal binding membrane protein (Hamer, 1993).

1.2.3. Toxic effects of copper on fish

A vast number of papers have been published concerning the effects of copper on fish, displaying a wide variety in responses and toxic levels. The differences in copper toxicity depend on water composition (see above: part 1.2.2.), temperature (Lydy and Wissing, 1988), size of the fish (Laurén and McDonald, 1986) and the species used. Also previous exposure to copper can alter the toxicity of copper in fish (Dixon and Sprague, 1981).

The large number of copper containing enzymes and glycoproteins in fish probably accounts for the diversity of biological effects; effects which appear in nearly every system evaluated in teleosts. Haematology is altered, as is respiratory and cardiac physiology. Copper induced histological alterations are found in the gill, kidney, hematopoietic tissue, mechanoreceptors, chemoreceptors, and other tissues. Reproductive effects are noted at low levels of copper inducing blockage of spawning, reduced egg production per female, abnormalities in newly hatched fry, reduced survival of young, and other effects.

1.2.3.1. Behaviour/Spawning/Reproduction

Fish are sensitive to low levels of copper in the water. Giattina and co-workers (1982) show that rainbow trout (*Salmo gairdneri*) are capable of detecting as little as 0.02-0.04 µM of copper (12°C, pH 7.3, total hardness 28 ppm as $CaCO_3$). These values are based on declines in residence times in shallow concentration-gradient tests. In steep concentration-gradient tests, trout avoid about 0.07-0.10 µM of copper. Trout spend over 30% less time in 0.09 µM of copper than in control solutions. Residence times and activity of trout in copper contaminated water are inversely related to copper at low concentrations. However, rainbow trout apparently are attracted to higher copper levels in the water (5.26-6.07 µM). This might be due to narcotic effects, changes in chemoreceptor sensitivity, or damage to chemoreceptors (Gardner and LaRoche, 1973).

Once fish have detected the presence of copper, they usually attempt to escape the source of copper pollution as fast as possible with increased swimming speed and skipping of exploratory behaviour (fish swim longer distances between turns and make fewer directional changes). For Atlantic silverside (*Medidia*) a typical migration schooling behaviour occurred (Koltes, 1985). In a study on hardhead sea catfish (*Arius felis)* Steele (1983) showed that this tendency of hyperactivity is dependent on copper concentration: catfish exposed to 1.57 or 3.15 µM of copper showed the typical hyperactive response, while catfish exposed to 0.08 to 0.79 µM of copper were less active than fish in the control group. Also Ellgaard and Guillot (1988) found a decreased level of activity in bluegill (*Lepomis macrochirus*), which was dependent both upon concentration and duration of exposure. Marked impairment of migratory ability in yearling coho salmon (*Oncorhynchus kisutch*) after exposure to 0.08 µM of copper, affirms the potential for copper induced behavioural impairment at levels previously considered to be no-effect levels (Lorz and McPherson, 1976).

The social rank of a fish may also alter the response to copper intoxication. When exposing bluegill to copper, the behaviour of the most dominant and the most

subordinate fish were affected by the metal to the greatest extent (Henry and Atchinson, 1986). When a variety of body movements is evaluated at the same time, copper concentrations as low as 0.54 µM were detectable. Also feeding behaviour can be altered by copper exposure. Copper has shown to depress feeding rate (Drummond *et al.*, 1973; Waiwood and Beamish, 1978) and increase prey handling time (interval between the moment a prey is taken and the moment that the fish starts to hunt another prey) (Sandheinrich and Atchinson, 1989).

Copper affects fish in most life stages, but the reproductive and embryonic stages can be considered 'bottlenecks' in the life cycle, since reproductive adults and embryos are susceptible to considerably lower levels of copper than other life stages. It is already long known that copper exposure retards sexual development and growth (Mount and Stephan, 1969), egg production per female can be decreased (McKim and Benoit, 1971; Pickering *et al.*, 1977), spawning can be blocked partially or entirely (Mount, 1968; Mount and Stephan, 1969; Pickering *et al.*, 1977) and migration can be impended (Lorz and McPherson, 1976). At the embryonic stage, copper can alter hatchability as well as hatching time (McKim and Benoit, 1971; Gardner and LaRoche, 1973, Scudder *et al.*, 1988) without changing developmental rate (Scudder *et al.*, 1988), which can lead to premature hatching. Also embryonic deformities are reported, especially when fish are exposed during day 6-9 of their development, when chorion permeability is high (Scudder *et al.*, 1988, Stouthart *et al.*, 1996).

1.2.3.2. Physiology/Energetics/Osmoregulation

Introduction of copper into an aqueous environment has usually been reported to increase coughing frequencies (Drummond *et al.*, 1973), ventilation rates (Spoor *et al.*, 1971; Sellers *et al.*, 1975) and oxygen consumption (Jones, 1947; O'Hara, 1971). But when routine oxygen consumption of bluegill (*Lepomis macrochirus*) was measured, while they were exposed to copper for 7 days, the initial stimulation was followed by an inhibition (O'Hara, 1971). Both the stimulation and inhibition were

concentration dependent. The initial stimulatory effect is most likely due to simple irritation which causes the fish to greater activity. The depression in the metabolism following this initial stimulation can be delayed several days (Felts and Heath, 1984), and can not be explained by depressed oxygen consumption in different tissues (liver, brain, gill) as was tested *in vitro* in this study. Most probably, this depressed metabolism is caused by a reduced spontaneous activity, as has been observed in bluegill (Ellegaard and Guillot, 1988).

When fish are forced to swim at controlled speeds, copper causes the oxygen consumption to increase (Waiwood and Beamish, 1978). Thus, although overall metabolism can be depressed because of the reduced locomotor activity, the metabolic cost for maintenance metabolism seems to be elevated. This hypothesis is supported by results obtained by Collvin (1985) on perch. In copper exposed perch, growth was suppressed at very low levels of copper even though food consumption remained unchanged. Copper presumably does not affect assimilation efficiency in fish (Lett *et al.*, 1976), so the explanation for the reduced growth must be a greater utilisation of energy by the fish, leaving less to be used for growth. Also in young trout, copper has been shown to reduce growth, this time accompanied by reduced appetite (Lett *et al.*, 1976). As with the perch, reduction in growth disappeared after several days (Collvin, 1985). Since appetite returned to normal more rapidly than growth rate, it appears that the lack of eating was not the sole cause of the reduced growth rate.

Copper causes a comparatively large upset in osmoregulation in freshwater fish. Exposed fish exhibit a rather rapid decrease in plasma electrolytes and/or osmolality. The cause of death from acute exposure in freshwater fish appears to be a sequence of events which start with electrolyte loss. If this occurs fast enough, as it would with acute exposures, a massive hemoconcentration follows which in turn causes a big increase (ca. 2x) in arterial blood pressure. Finally, the heart apparently fails from having to deal with the viscous blood and excessive pressure head (Wilson and Taylor, 1993). This sequence of physiological dysfunctions is very similar to that seen in fish dying from acid exposure (Milligan and Wood, 1982). The mechanism of the copper

effect on osmoregulation in freshwater appears to be an inhibition of sodium and chloride uptake by the gills, although at fairly high copper concentrations also a stimulation of passive electrolyte efflux occurs (McDonald *et al.*, 1988). The inhibition of electrolyte uptake is probably due to an inhibition by copper *in vivo* of the enzyme Na^+/K^+ATPase in gill tissue (Lorz and McPherson, 1976). The decreased plasma electrolytes in fish exposed to copper also causes a movement of water from the blood to the tissues which would facilitate the hemoconcentration observed (Heath, 1984).

Little is known regarding to endocrine responses to copper. Generalised endocrine responses to stress involve activation of the hypothalamo-pituitary-interrenal axis resulting in release of corticosteroids, and in the activation of the adrenergic system, resulting in the release of catacholamines (Mazeaud *et al.*, 1977; Donaldson, 1981; Mazeaud and Mazeaud, 1981). The primary release of hormones will inevitably lead to secondary physiological and biochemical events. Some of these, such as alterations in the energy metabolism, may be immediately beneficial to the fish, but it is equally clear that this is not always the case, and maladaptive effects such as disturbed osmoregulation, immunosuppression, and in the longer term, depressed growth and reproductive function, have become apparent. One of the first reactions to stress is the immediate release of the catacholamines adrenaline and noradrenaline. These catacholamines have several beneficial effects on oxygen transport, and have been suggested to make an important contribution to acid-base regulation (Brown, 1993), but since they also increase branchial permeability and blood flow, they are unlikely to have any beneficial effects on osmotic and ionic regulation. Few minutes later (5-10 minutes) plasma cortisol concentrations start to rise. Elevated levels of cortisol have been measured in copper exposed fish (Donaldson and Dye, 1975; Schreck and Lorz, 1978), as well as elevated levels of plasma glucose (Laurén and McDonald, 1985) which can be caused both by corticosteroid and catacholinergic stimulation. In contrast to catacholamines, cortisol has a beneficial effect on osmoregulation, it stimulates proliferation of chloride cells (Fu *et al.*, 1990; Laurent and Perry, 1990) and hence increases Na^+/K^+ATPase and Ca^{2+}ATPase activity (Flik

and Perry, 1989; Madsen, 1990). In longer terms, cortisol reduces growth and suppresses the immune system. The possible role of prolactin in copper intoxication is not yet investigated. Prolactin reduces the gill permeability to ions and osmotic permeability to water (Flik *et al*., 1989) and can stimulate proliferation of epidermal mucocytes.

1.2.3.3. Hematology/Immunology

McKim *et al.* (1970) observed that copper increased the hematocrit, hemoglobin, and red blood cell count in trout when exposed for 6 days. Waiwood (1980) also reported increased hematocrits in trout exposed to copper but he was able to account for the entire change as being due to a shift of water from the plasma to the muscle cells, thereby producing hemoconcentration, which was also observed by Wilson and Taylor (1993) in trout exposed to lethal copper concentrations. The McKim *et al.* (1970) chronic study found just the opposite; a slight increase in plasma water. Because copper is required for hemoglobin synthesis, a mild exess may be stimulatory, particularly if the fish were marginally deficient in copper at the start of the experiment. Furthermore, if copper causes a slight hypoxic condition for the fish, increases in the oxygen capacity of the blood can be seen as an adaptation to an altered respiratory homeostasis caused by the pollutant and not a toxic or direct stimulatory action of the chemical on the blood cell-forming tissues. A similar thing happens during acclimation to environmental hypoxia, and, in any situation where acute stress is imposed on an animal, adrenergic stimulation of the spleen can cause it to contract and release stored erythrocytes into the circulation (Nilsson and Grove, 1974). On the contrary, O'Neill (1981) found decreased hematocrits after copper exposure. Possibly these fish were more sensitive to a rise in plasma cortisol, which is frequently elevated in fish under a variety of stressors (physically, chemically or physiologically), and has been seen to cause anaemia (Bollard *et al.*, 1993).

Although copper sulphate is frequently used to control external columnaris infections of pond fishes, the general effect of copper on immune systems of fish is suppressive. The positive effects of copper on infections with external unicellular parasites is probably based on the lower resistance of the parasites against the copper toxicity compared to the fish. O`Neill (1981) shows that copper exposure reduces antibody levels in brown trout (*Salmo trutta*) and common carp (*Cyprinus carpio*). Also in an *in vitro* study with antibody-producing cells, copper works immunosuppresive (Anderson *et al*., 1989). In air-breathing catfish (*Saccobranchus fossilis*), a 28 day exposure to copper resulted in a dose dependent depression of antibody production, depression of phagocytic activity of spleen and kidney macrophages, and a suppression of T-cell activity (Khangarot and Tripathi, 1991). When zebrafish were exposed to copper for 7 days, a dose dependent suppression of kidney lymphocyte number was observed, and macrophage activity was lowered *in vivo* as well as *in vitro* (Rougier *et al*., 1994). When striped bass were exposed during 96 hours to copper prior to a challenge with two naturally occurring bacterial species (*Vibrio anguillarum* and *Pasteurella piscicida*) susceptibility of the fish to these infections was increased (Hetrick *et al*., 1982). So, while copper may be protective for some external diseases, defence against internal infections is compromised.

1.2.3.4. Accumulation/Metallothioneins

Deposition of copper in tissues and organs is very variable. In liver, the organ with the highest concentration factor for copper (Brungs *et al.*, 1973; Buckley *et al.*, 1982; Felts and Heath, 1984; Stagg and Shuttleworth, 1982b), one-thousand-fold differences can exist between related species collected from uncontaminated areas (Frazier, 1984). White perch (*Morone americana*) can accumulate hepatic levels of 43.98 mM of copper, compared to the normal level of about 0.05 mM found in striped bass (*Morone saxatilis*). In white perch, 70 % more copper is found in the nuclei and plasma membrane fraction compared to striped bass. Copper containing granules are observed at optical and ultrastructural levels. Cytosolyc copper in both species associates with a protein similar in weight to metallothionein. Mechanisms to explain elevated hepatic levels of copper in white perch have not been elucidated, however, possibilities include accelerated metallothionein synthesis with enhanced storage capacity (e.g. cytosolic or lysosomal). In contrast to renal uptake, hepatic accumulation is both time- and concentration-dependent. Neither the brain, spleen, gonad or muscle seem to be involved in copper accumulation since copper levels in exposed fish are similar to those of control fish (Benoit, 1975).

In a study by Felts and Heath (1984), three days of exposure of bluegill sunfish to sublethal levels of copper only resulted in a significant rise of copper in the gills. Only after 7 days of exposure, the liver exhibited large increases in copper concentrations. At least some of this elevated copper concentration in the gills could be accounted for by the mucus on the gills, which cannot be removed completely during gill preparation for analysis. In coho salmon, plasma copper rose on day one of copper exposure, but after two weeks or more of exposure, this elevated level had dissapeared (Buckley *et al.*, 1982). During this time, the copper concentration in the liver steadily increased. Thus, it appears that copper was rapidly removed from the plasma by the liver and/or there was a reduction in copper uptake rate by the gills during the first few days of copper exposure. Accumulation of copper by liver can be

greatly influenced by the nutritional level of the experimental fish. When fed and starved yearling roach (*Rutilus rutilus*) were exposed to waterborne copper for 7 days, only the starved fish accumulated copper in the liver (Segner, 1987). Because one of the major functions of bile is digestion, it is suggested that starved fish were unable to dump copper from the liver into the bile due to reduced bile production.

Metallothioneins have been found in at least 34 species of elasmobranchs and teleosts (Roesijadi, 1992). They are low molecular weight polypeptides with many sulfhydryl groups due to the large amount of cysteine in the molecule. Because of these sulfhydryl groups, metallothioneins bind a variety of metals, both essential and non-essential. For essential elements (such as copper and zinc), the metallothioneins sequester or donate the metals depending on demand for and concentration of the metal in the cell. Non-essential metals are sequestered by the metallothioneins, thereby reducing toxic interactions with other cellular proteins. In fish, they have been found in gills, intestine, kidney and liver. Metallothioneins generally exist in metal-saturated form (Hodson, 1988). Thus, in order for them to detoxify additional metal, there must be synthesis of additional metallothioneins (or other metal binding proteins) or displacement of one metal by another. Each of these mechanisms comes into play at certain times.

It is well established that a variety of metals, including copper, induce the synthesis of additional metallothioneins (Hamer, 1986). It has also been found that acclimation to low levels of copper can result in lower toxicity (Buckley *et al.*, 1982; Dixon and Sprague, 1981). This increased tolerance towards a metal has been associated with the elevated levels of metallothioneins in one or more tissues. Elevated levels of metallothioneins in gills or intestine may reduce the rate of metal uptake in the blood (Petering *et al.*, 1990), although the major mechanism of increased tolerance is presumed to be the enhanced ability to sequester the metal. The ability of fish to acclimate to a metal varies with the particular metal. Chapman (1985) reviewed the literature and ranked Zn, Cu and Cd in decreasing order: Zn > Cu > Cd while Eaton (1985) found the reverse for their binding affinities to the metallothioneins which is:

Cd > Cu > Zn so the acclimation may not be as obvious as it appears. The kinetics of metallothionein formation after copper exposure has been investigated by McCarter and Roch (1984). With continuous copper exposure, hepatic metallothioneins rose steadily and stabilised after 4 weeks at a level dependent on the copper exposure concentration in the water. Thus the rate of metallothionein synthesis was controlled by the rate of copper uptake. Transfer of the acclimated fish to water not contaminated with copper did not result in a significant loss of metallothioneins after 4 weeks, so once they have been formed, metallothioneins appear to be rather stable.

1.2.4. Official copper guidelines

Official water quality criteria for copper differ in different states and countries, but in all cases copper is concluded in the list of priority pollutants. The most recent water quality standards of the United States Environmental Protection Agency lists under freshwater aquatic life criteria for copper a value of 6.54 µg/l (0.10 µM). The Dutch normation for copper is in the same range: 3 µg/l (0.05 µM). The Belgian normation (Animal, Vlarem) is considerably higher: 30 µg/l (0.47 µM).

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CHAPTER II: INTRODUCTION TO THE EXPERIMENTAL WORK

2.1. The test organism: the common carp *Cyprinus carpio* Linneaus

Class Osteichthyes
Subclass Actinopterygii
Order Cypriniformes
Suborder Cyprinoidei
Family Cyprinidae
Species *Cyprinus carpio*

Cyprinids have been cultured by man since ages. They have many natural advantages for cultivation. Species in this family have colonised a very wide variety of biotopes, posses the ability to withstand a wide range of temperatures and oxygen levels (e.g. common carp, *Cyprinus carpio*, are especially tolerant to low dissolved oxygen concentrations), feed at all levels of the trophic chain (phytoplankton, micro- and macrozooplankton, benthos, macrophytes and fish), and display variable modes of reproduction. Cyprinids are naturally found in very diverse habitats (e.g. streams, rivers, lakes, ponds) and can therefore be raised in a variety of diverse culture conditions. Cyprinids have a wide geographic distribution, thanks in part to human intervention, and are now found and cultured on most continents (yearly production: 1.8 million tonnes). They are characterised by: a protrusile mouth, toothless jaws, toothless palate, and enlarged toothbearing pharyngeal bones which grind the food against a keratinised pad at the opposite side of the throat. The pharyngeal bones bear three rows of teeth (4.1.1) important for determining the species.

Carp culture in China extends back to at least 475 BC when Fan Li wrote a treatise on the subject. In fact, carp were the primary object of culture until the Tang

dynasty came to power in AD 68. The name of the Royal Family and the Chinese word for carp, ‘Li’ were similar and the carp became a royal fish which could not be caught or eaten. This stimulated than cultivation of grass carp and other species. In Japan, the carp became the emblem of the samurai, because it ‘withstands opposition and swims against the current’ and as the symbol of courage , energy and firmness (Bănărescu and Coad, 1991).

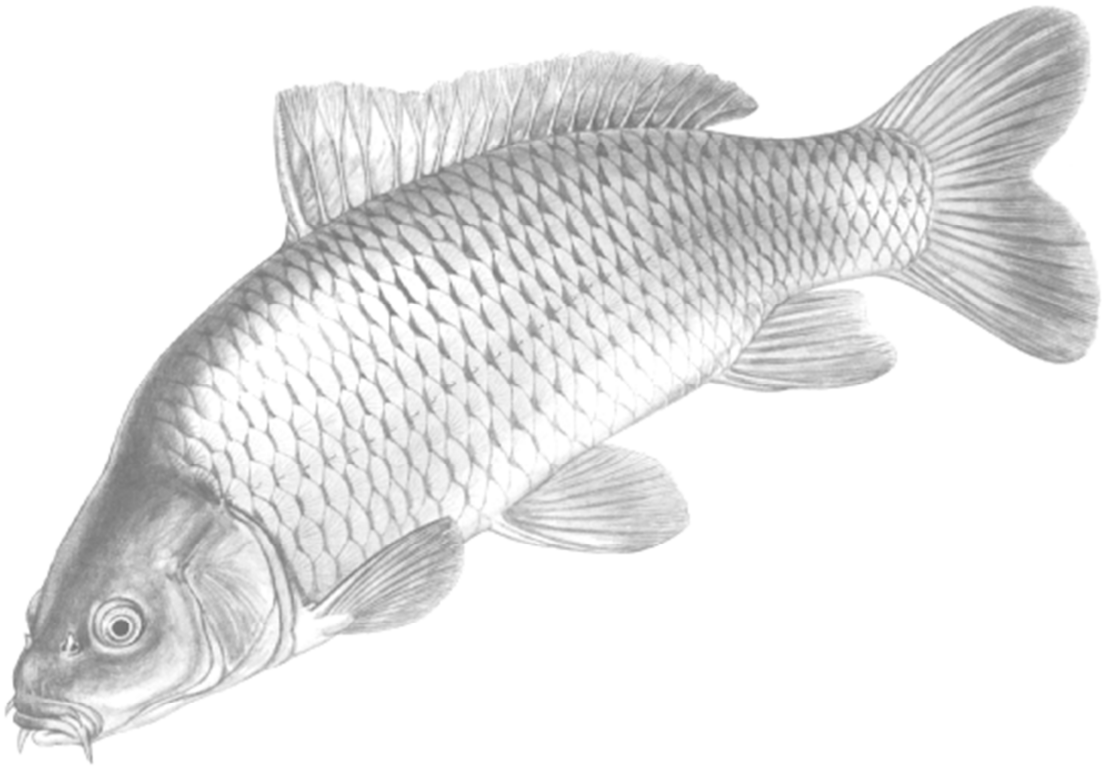

Figure 2.1. Wild type of the common carp, Cyprinus carpio (from Pivnicka and Cerny, 1990).

Common carp are abundant in streams, ponds and lakes of Europe and Asia. Originating from the region of the Kaspic Sea, they were distributed over Europe by monks who cultivated carp in ponds during the Middle Ages. Besides in Asia, common carp are still of major economic importance in Eastern Europe (yearly production: ± 141.000 tonnes) and Israel. The wild form of common carp (Fig. 2.1.) is characterised by two long and two short barbels on the upper lip and 33-40 large cycloid scales on the lateral line, total length usually 25-75 cm. Other forms have been

bred (Fig. 2.2.) featuring higher backs and less scales. Breeding season is June-July, when they mate in shallow water putting the eggs between water plants (93.000 to 1.664.000 eggs per female). Carp larvae emerge after 3-6 days and grow up to be adults after 3-5 years. Under cultured conditions, reproduction is initiated artificially.

Cyprinus carpio has been chosen as test organism because of its abundance in Europe and its economic importance. In water of low hardness (50 mg l^{-1} as $CaCO_3$), sensitivities of *Cyprinus carpio* and *Salmo gairdneri* to copper are similar (Peres and Pihan, 1991). Furthermore, *Cyprinus carpio* has been approved as test organism by OECD and EPA. We obtained our carp from the Agricultural University of Wageningen (The Netherlands) where they are bred under controlled conditions.

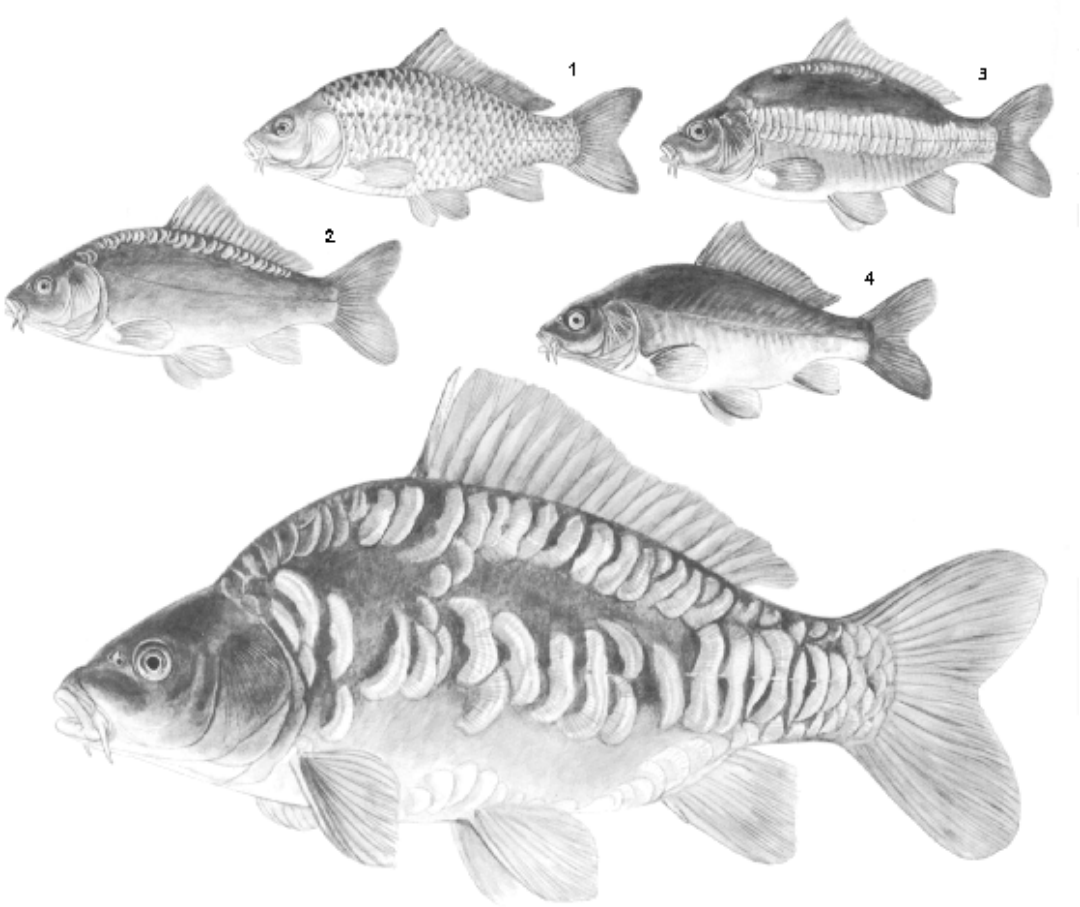

Figure 2.2. Different cultivated types of common carp, Cyprinus carpio (from Pivnicka and Cerny, 1990).

2.2. The different methods used for monitoring physiological processes

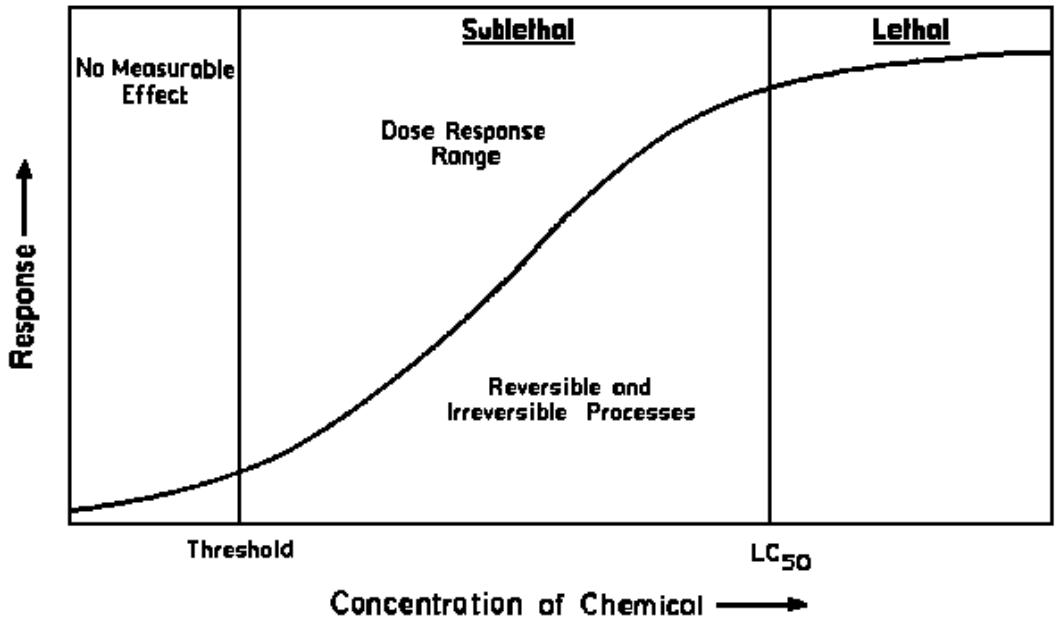

Figure 2.3. Idealised diagram of the effect of the concentration of a pollutant on the physiological response of an organism (from Heath, 1995).

Figure 2.3. illustrates the general effect of an environmental concentration of a chemical on an organism. At very low concentrations, the stress responses caused by the chemical are minimal, and can therefore not be measured. The sublethal threshold for observing a physiological effect when an organism is being exposed to a pollutant will vary with the response that is being measured. Monitoring different physiological processes at the same time therefore not only makes understanding of the different effects of a pollutant on an organism possible, it also enhances the chances of finding a biomarker with a threshold of physiological relevance. Within the sublethal range, a wide variety of reversible and irreversible processes take place. Prolonged exposure within the upper end of the sublethal range may cause death through general weakening of the animal so it becomes more susceptible to disease and/or predation. When physiological processes are to be used as a biomarker, the aim of the researcher

has to be a development of methods that allow to measure a threshold below the concentration where irreversible effects occur, and which detect weakening of the organisms at an early stage.

In the presented research four different methods have been used to determine effects of sublethal copper exposure on the metabolism of the common carp. The results obtained by these methods will be discussed in the next four chapters. Here, the different methods are briefly introduced.

2.2.1. Growth, food consumption, energy stores and nucleid acid content.

Protein is a major component of an organisms body mass, and RNA is necessary for the synthesis of protein. Consequently, a positive relationship between the concentration of RNA and the rate of protein synthesis has been demonstrated in many organisms. Typically, maximum RNA/DNA ratios occur during peaks of protein production. Based on these observations, protein, RNA and DNA relationships have been suggested as a promising biomarker of reduced growth (Heath, 1995). The RNA/DNA ratio has been shown useful to detect starvation as well as toxic effects (Buckley,1979; Barron and Adelman, 1984; Cleveland et al, 1986).

The use of the RNA/DNA ratio is also strongly related to the use of the AQ ratio (see below, part 2.2.2.) as a bioindicator. As a healthy, growing organism has a high protein synthesis rate and thus a high RNA/DNA ratio, an other consequence is a low nitrogen excretion rate (a decrease in the rate of protein catabolism and nitrogen retention in favour of protein synthesis). Besides protein, RNA and DNA also lipid and glycogen are quantified to determine the endogenous energy stores of the organism.

Together with the determination of these biochemical components, food consumption and growth were evaluated over a 28 day period. Growth is a classic biomarker, which obviously reflects the fitness of an organism, and it has been used in

these studies as a standard to evaluate the usefulness of the other determinations as biomarkers.

The results of these studies are discussed in chapter III which is based on the manuscript by De Boeck, G., Vlaeminck, A. and Blust, R., 1996. Effects of sublethal copper exposure on copper accumulation, growth, food consumption, energy stores and nucleic acid content in common carp. *Comp. Biochem. Physiol.*, submitted.

2.2.2. Oxygen consumption and ammonia excretion: the ammonia quotient (AQ)

Determination of the oxygen consumption and ammonia excretion rates gives information on two critical physiological processes. The use of the ratio between the ammonia excreted and the oxygen consumed (AQ: mole to mole ratio) is based on two principles: 1) the oxygen consumption is a measure of the total amount of energy that has been used, and 2) the excretion of nitrogen, in carp excreted mainly as ammonium, is a measure for the amount of proteins that has been used to provide this energy. The ratio between these two processes should therefore represent the relative use of proteins compared to lipids and carbohydrates. Stroganov (1956) uses the relationship between oxygen consumption and ammonium excretion for the first time as the ammonia quotient (AQ). Later on, the O:N ratio in atomic equivalents has also been used, mostly on invertebrates (Widdows, 1978; Carr, 1985; McKenney et al, 1991), but in fish, the AQ remains the most commonly used ratio. In fish, stored glycogen and especially lipids are the preferred substrate, but when these are depleted due to exercise or stress, muscle protein becomes the main energy source. The major pathway for the production of ammonia is through the transamination of various amino acids. The primary site for this ammonia production is the liver, but the necessary enzymes have also been allocated in the kidneys, gills, and skeletal muscle.

The results of these studies are discussed in chapter IV which is based on the manuscripts by De Boeck, G., De Smet, H. and Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. *Aquat. Toxicol.* **32**: 127-146 and by Blust, R., G. De Boeck, R. Borger and W. Decleir, 1993. Effects of changing environmental conditions on the energy metabolism of aquatic organisms. *In: Proceedings of the Global Change Symposium*, Belgian Science Policy Office: 141-168.

2.2.3. Phosphorous compounds of the energy metabolism

In vivo Phosphor Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMRS) allows simultaneous and continuous measurement of the phosphorous compounds involved in the energy metabolism of a living organism. In fish, the main phosphorous energy reserve is phosphocreatine. When ATP gets depleted, phosphocreatine is converted by creatine kinase into creatine in order to restore the ATP content. During this process, inorganic phosphate increases in the fish muscle (due to ATP breakdown), while the phosphocreatine content slowly decreases. Using the ^{31}P-NMRS technique, concentrations of inorganic phosphate (P_i), phosphocreatine (P_{Cr}) and ATP can be monitored in time in a non invasive way and intracellular pH (pH_i) can be determined. Although ^{31}P-NMRS has been used before in fish to examine the energy metabolism in fish muscle under normoxic, hypoxic and anoxic conditions (Van den Thillart *et al.*, 1989, Van Waarde *et al.*, 1991), this promising technique has not yet been used to study the effects of pollutants on the energy metabolism of fish.

The results of these studies are discussed in chapter V which is based on the manuscript by De Boeck, G., Borger, R., Van der Linden, A. and Blust, R., 1996. Effects of sublethal copper exposure on the energy metabolism of common carp, measured by ^{31}P-NMRS. *Environ. Toxicol. Chem.*, submitted.

2.2.4. Monoamine neurotransmitters

Monoaminergic neurons compose a very small fraction of the neurons in the vertebrate brain. In fact, monoaminergic neurons number in the thousands whereas the total quantity of neurons in the vertebrate brain numbers in the hundreds of millions or more. However, the influence of monoaminergic neurons on their target sites appears to go far beyond their numbers (Winberg, 1993). In the mammalian brain, where the monoaminergic systems have been extensively studied, monoaminergic neurotransmitters are believed to be involved in the control of several behavioural patterns, notably aggression (Mason, 1984), mating (Meyerson and Malmnäs, 1978) and feeding (Leibowitz, 1992). Monoaminergic systems in brain have also been connected to stress reactions (Dunn, 1989) as well as to the central regulation of autonomic and neuroendocrine functions (Tuomisto and Männistö, 1985). Moreover, human diseases, including schizophrenia, depression and Parkinsons disease, seem to be more or less directly related to unbalance or malfunction of brain monoaminergic systems (Mason, 1984). In comparison to the wealth of information, although sometimes inconclusive, on brain monoaminergic functions in mammals, very little is known about the function of monoamine neurotransmitters in non-mammalian vertebrates. In fish, excellent work has been done by Winberg (1993) who found relationships between the monoamine brain neurotransmitters and social rank, aggression, food intake, locomotor activity, and stress in salmonids. In our research, the effects of copper exposure on the content and activity of the catacholamine dopamine and the indoleamine serotonin were assessed.

The results of these studies are discussed in chapter VI which is based on the manuscript by De Boeck, G., Nilsson G.E., Elofsson, U., Vlaeminck, A. and Blust, R., 1995. Brain monoamine levels and energy status in common carp (*Cyprinus carpio*) after exposure to sublethal levels of copper. *Aquat. Toxicol.* **33**: 265-277.

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CHAPTER III: EFFECTS OF SUBLETHAL COPPER EXPOSURE ON COPPER ACCUMULATION, GROWTH, FOOD CONSUMPTION, ENERGY STORES AND NUCLEIC ACID CONTENT IN COMMON CARP*

3.1. Summary

Juvenile common carp were exposed for 28 days to four different sublethal copper concentrations (0.00 µM, 0.20 µM, 0.55 µM and 0.80 µM). Food consumption was monitored on a daily basis during the exposure period while growth was assessed weekly. Every week 8 fish from each group were sacrificed for determination of copper accumulation, energy stores and nucleic acid contents. Copper exposure to 0.80 µM affected both growth and feeding behaviour in common carp. At 0.55 µM, growth was affected despite normal food consumption. Even at the lowest copper concentration (0.20 µM), metabolic demand for the fish increased, challenging the carp with an increased demand for food. Copper accumulation mainly occurred in the liver, reaching an equilibrium between uptake and excretion after one month of exposure. Substantial biochemical changes were observed at the two highest copper exposure concentrations and are discussed. The correlation between growth rate and RNA/DNA ratio was poor considering the substantial differences in growth rate.

* Based on the manuscript by De Boeck, G., Vlaeminck, A. and Blust, R., 1996. Effects of sublethal copper exposure on copper accumulation, growth, food consumption, energy stores and nucleic acid content in common carp. *Comp. Biochem. Physiol.*, submitted.

3.2. Introduction

Growth of an organism is generally used as a sensitive and reliable endpoint in chronic toxicological investigations. Sublethal levels of a wide variety of toxicants have been found to slow the growth of fish larvae or juveniles (see Woltering, 1984 for review). This can be due to a reduced food intake, but also due to increased metabolic expenditure for detoxification and maintenance of the normal body functions. Sublethal copper exposure has proven to reduce as well appetite as growth in different fish species (Benoit, 1975; Drummond *et al*., 1973; Buckley *et al*., 1982; Lett *et al*., 1976; McKim and Benoit, 1971; Mount, 1968). Even at copper concentrations where food consumption is not affected, copper exposure is known to slow growth (Collvin, 1985).

Before changes in growth occur, changes in biochemical composition should become apparent. The use of energy stores (glycogen, fat) might be initiated, and protein synthesis might decrease. Protein is a major component of an organisms body mass, and RNA is necessary for the synthesis of protein. Consequently, a positive relationship between the concentration of RNA and the rate of protein synthesis has been suggested. Since cells contain a fixed amount of DNA, the DNA content in the tissues is used as a reference to calculate a RNA/DNA ratio. Typically, maximum RNA/DNA ratios should occur during peaks of protein production. Based on these observations, protein, RNA and DNA relationships have been implied as a promising biomarker of reduced growth (Heath, 1995; Jobling, 1994). The RNA/DNA ratio has been shown useful in some cases to detect starvation as well as toxic effects (Buckley, 1979; Barron and Adelman, 1984; Cleveland *et al*, 1986; Haines, 1973). Other studies do not confirm the RNA/DNA ratio as being a such a sensitive indicator of growth (Jürss *et al*., 1987; McKee *et al*., 1989; Pinkney *et al*., 1990).

The aim of the present study was to evaluate the effects of three sublethal copper concentrations on growth and feeding of common carp, *Cyprinus carpio*, and to compare these effects with some changes in biochemical composition of muscle and

liver tissue. Also copper accumulation in brain, muscle and liver tissues was followed. Determination of the biochemical composition included measurement of the energy stores of glycogen and fat, as well as determination of the levels of protein and nucleic acids (RNA and DNA) in white muscle and liver tissue.

3.3. Materials and methods

3.3.1. Animal holding and copper exposure

Juvenile (1 month old) common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Agricultural University of Wageningen, The Netherlands. They were grown at the University of Antwerp at the optimal temperature of 25 °C (Elliott, 1981) in softened Antwerp city tap water (0.875 mM Ca, 0.145 mM Mg, pH 7.0-8.0). Water was filtered with a trickling filter and water quality was checked weekly for ammonia, nitrite and nitrate with Visicolor Test Kits (Macherey-Nagel, Düren).

Two weeks before starting the experiments, carp weighing 18±2 g (means±S.D.) were transferred into 150 l aquaria filled with standard moderately hard fresh water (FW) according to Standard Methods (American Public Health Association, 1989: 0.348 mM $CaSO_4$.$2H_2O$; 0.5 mM $MgSO_4$.$2H_2O$; 1.143 mM $NaHCO_3$; 0.054 mM KCl; pH 7.8-8.0). The FW was well aerated during at least 24 hour before use. The photoperiod was set at 14 hour light, 10 hour dark period and temperature remained at 20 °C. Carp were fed once a day, with 'Pond Sticks' (Tetrapond, Henckel). After 15 min, the remaining food was removed, dried overnight at 60°C and weighed to calculate mean food consumption. Water quality was checked daily for pH, ammonia, nitrite and nitrate and 50 % of the water was renewed twice a week.

Copper exposure was started by adding 250, 125 or 62.5 µg copper l^{-1} FW. Copper was added by means of copper nitrate solution ($Cu(NO_3)_2.2H_2O$ 1 $g.l^{-1}$)

(Merck, Darmstadt). Water was well aerated and mixed during at least 24 hour. In the aquaria, water was filtered with Eheim filter, filled with Rivalon synthetic filter cotton wool. Twice a week 75 % of the water was renewed with standard water containing copper. Five times a week the exact amount of copper levels in the aquaria was determined using an atomic absorption spectrophotometer and nominal copper levels were 0.80±0.38 µM, 0.55±0.15µM and 0.20±0.08 µM respectively. When extrapolating the results of a study by Peres and Pihan (1991) while considering the hardness of the water used in this study (85 mg l^{-1} as $CaCO_3$), a 48h-LC50 value for common carp of 3.41 µM can be calculated. Marek *et al*. (1991) found a 100% survival for carp with a mean weight of 19 g at copper concentrations up to 1.57 µM during a 10 day exposure period and 90% survival at a copper concentration of 7.87 µM under the same circumstances. Therefore, the copper concentrations used in this study are considered as sublethal concentrations.

3.3.2. Sampling of tissue

Liver, white muscle and brain tissue were sampled before, after one week, two weeks, three weeks and four weeks of exposure to copper for all groups. At the day the tissues were sampled, fish were not fed in the morning, because sampling started at their normal feeding time. Each fish was quickly anaesthetised with ethyl 3-aminobenzoate, methanesulfonic acid salt (MS222), weighed and decapitated. The liver and white muscle tissues were dissected for biochemical and copper determinations, brain tissue was dissected into three parts (telencephalon, hypothalamus and brain stem) for copper determination. Tissues were frozen in liquid nitrogen within 5 min of decapitation and stored in an eppendorf tube at -80 °C. Remaining fish were weighed and fed after samples were taken. Weighing of the fish was performed by transferring them in a small container filled with water on the balance. Mean weight obtained in this way was 3.5±1% (means±S.D.) higher than

when fish were dried carefully before weighing after sedation but the wet method was preferred for the lower impact it had on the fish.

3.3.3. Isolation and determination of biomolecules

The procedure for isolation has been described by McKee and Knowles (1986). After being weighed the frozen liver and white muscle samples were homogenised in the eppendorf tube at 0 °C with ice cold 0.2 M perchloric acid (PCA) (200 µl 1 M PCA + 800 µl H_2O) using an Ultra-Turrax T8.01 homogeniser. The homogenised samples were kept on ice throughout subsequent extraction steps because nucleic acids are susceptible to enzymatic and heat degradation (Hutchinson et. al.,1962). A 50 µl aliquot was taken from the tube for protein determination. The remaining homogenate was centrifuged for 10 min at 10.000g and 4 °C (Sorval RC-5). After centrifugation a 200 µl aliquot was taken from the supernatant and kept at -80 °C until further determination for glycogen. The rest of the supernatant was discarded and the pellet was rinsed twice with 0.2 M PCA and centrifuged. The pellet was resuspended in 0.3 M NaOH and incubated for 1 hour at 37 °C to hydrolyse the RNA. After hydrolysation the sample pH was adjusted (pH 4.0) by adding 10 M PCA. The sample was centrifuged (10 min, 10.000 g, 4°C) and 900 µl supernatant was transferred into a small tube and kept at 0 °C (first RNA fraction). The pellet was rinsed twice with 0.2 M PCA and centrifuged, yielding the second and third RNA fraction. The pellet was treated with ethanol-ether 3:1 (v/v) at -80 °C overnight, to extract lipid material. The sample was centrifuged, supernatant discarded, pellet rinsed with ethanol-ether 3:1 (v/v), centrifuged, and again supernatant was discarded. The pellet was resuspended in 5 mM NaOH - 1 M PCA 1:1 (v/v) and kept at -80 °C until determination of DNA.

3.3.4. Protein determination

The 50 µl aliquot for the determination of protein was diluted with 950 µl 0.01 M NaOH and incubated for 24 hour at 37 °C in order to hydrolyse the sample. The next day the protein samples were analysed for protein content by the method of Bradford (Bradford, 1976). Protein content was quantified by VIS spectrophotometry at a wavelength of 595 nm. Bovine serum albumin (Serva, Heidelberg) was used as a standard (Lowry *et al.*, 1951). The calibration curve was non-linear over the concentration range of the samples (rectangular hyperbola).

3.3.5. Glycogen determination

The supernatant (200 µl) which was stored at -80 °C was used for the determination of glycogen. Liver samples were diluted up to 8x with 0.2 M PCA and an aliquot of 200 µl was taken. White muscle samples were used as such. Stock solution was prepared by dissolving glycogen (Merck, Darmstadt) in 0.2 M PCA. Anthrone reagent (0.5 g anthrone in 72 % H_2SO_4 containing thiourea 30g.l^{-1}) (Roe et. al., 1966) was added to the aliquots and the diluted stock solutions, then incubated for 30 min at 100 °C. The reaction was stopped by cooling in an ice bath. The glycogen concentrations were quantified by VIS spectrophotometry at a wavelength of 620 nm. The calibration curve was linear over the concentration range of the samples.

3.3.6. RNA determination

The pooled white muscle RNA fractions were used as such, the pooled liver RNA fractions were diluted up to 4x and then used as such. The samples were transferred into a quartz cuvette. RNA was quantified by UV absorption spectrophotometry at a wavelength of 260 nm. Calf liver RNA (Sigma Chemicals, St-Louis) was used as a standard (Dagg and Littlepage, 1972). The calibration curve was linear over the concentration range of the samples.

3.3.7. DNA determination

The defrosted DNA samples were analysed as described by Vytasek (1982). Stock solutions of DNA were prepared by dissolving calf thymus DNA (Sigma Chemicals, St-Louis) in 5 mM NaOH. Reagent 1 consisted of 10 mM Na_2CO_3 in 1 M NaOH. Reagent 2 was a freshly prepared 20 % solution of 3.5-diaminobenzoic acid (DABA.2HCl). The samples and the diluted stock solutions were incubated at 90 °C for 30 min. At the same time reagent 1 and 2 were mixed together 3:1 (v/v) (reagent 3) and let to react for exactly 60 min at room temperature. After 30 min the DNA stock solutions and the samples were removed from the incubator and were kept at room temperature for the next 30 min. After exactly 1 hour 200 µl of reagent 3 was added to all samples and stock solutions. The fluorescence-yielding reaction was initiated by incubating the samples and the stock solutions at 37 °C for 1 hour. The reaction was stopped by cooling in an ice bath. The samples were centrifuged and aliquots of 350 µl were taken. The stock solutions and the aliquots were diluted with 2.5 ml 1 M HCl. The fluorescence was measured on a fluorescence spectrophotometer at an emission wavelength of 520 nm. The calibration curve was linear over the concentration range of the samples.

3.3.8. Lipid determination

Lipid content of liver and white muscle was determined gravimeticaly using a Soxtec System. In order to obtain a sufficient amount of tissue for the extraction procedure, samples from each exposure group were pooled per day. First, samples were submitted to a acid hydrolysis in order to get all lipids into extractable forms by boiling them for one hour in 4 M HCl in a Soxtec System 1047 Hydrolysation Unit. Thereupon samples were dried for 3 h at 100°C. Then lipid extraction was accomplished with petroleum ether using the Soxtec System 1043 Extraction Unit (30 min boiling time, 45 min rinsing time).

3.3.9. Statistics

All values are given as means ± S.D. Statistics were performed with GraphPad InStat, using one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test if significant differences were found. If conditions for ANOVA were not fulfilled, The Kruskal-Walis and Dunn's test were used. For statistical analysis of food consumption, two-way analysis of variance was used, followed by a Tukey HSD comparisons test.

3.4. Results

3.4.1. Food consumption and growth

From the first day of copper exposure on, changes in food consumption could be seen (Figure 3.1.). Fish exposed to the highest copper concentration (0.80 µM) became apathic and slow and consumed significantly less food ($P<0.001$) during the first two weeks of the exposure period. The effect was most striking during the first week, when food consumption dropped to 36% of control values. Food consumption then slowly recovered to control values: 46% of control values during the second week of exposure, 73% during the third week ($P<0.01$) and 101% during the last week ($P<0.001$). Food consumption in the lowest exposure group (0.20 µM) appeared to be stimulated, and was significantly higher compared to food consumption in the control group ($P<0.01$ during the first week, $P<0.05$ during the second week). No changes in food consumption were seen in the group exposed to 0.55 µM of copper.

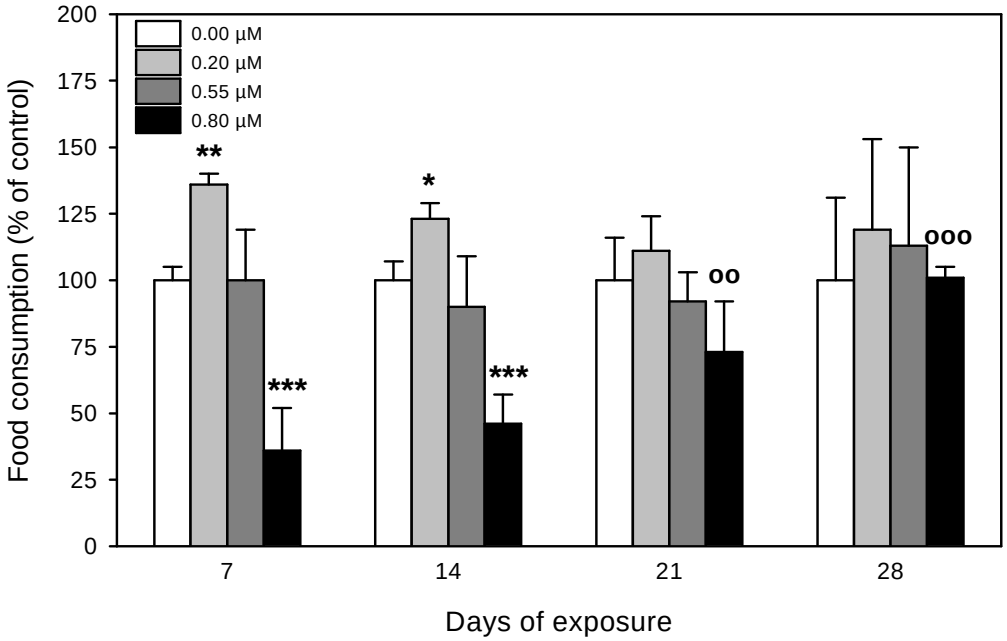

*Figure 3.1. Food consumption as % of control in common carp during copper exposure to different copper concentrations. Each bar represents the mean value ± S.D. of 4-6 samples of food consumed on the days of the preceding week by the different exposure groups. Significant differences compared to control value from the same week are indicated with * (*: P<0.05; **: P<0.01; ***: P<0.001), significant differences compared to the first week from the same copper concentration are indicated with o (oo: P<0.01; ooo: P<0.001).*

Whereas food consumption in the lowest exposure group was elevated compared to the control group, growth remained very similar during the entire exposure period (Figure 3.2.). In the group exposed to 0.55 µM of copper, growth was reduced, and fish even lost weight during the first three weeks of exposure despite the normal food intake. In the highest exposure group, weight loss of the fish was considerable, certainly during the first week of exposure. This effect slowly diminishes, and in the fourth week of exposure fish were again gaining weight, although less than the fish in the control group.

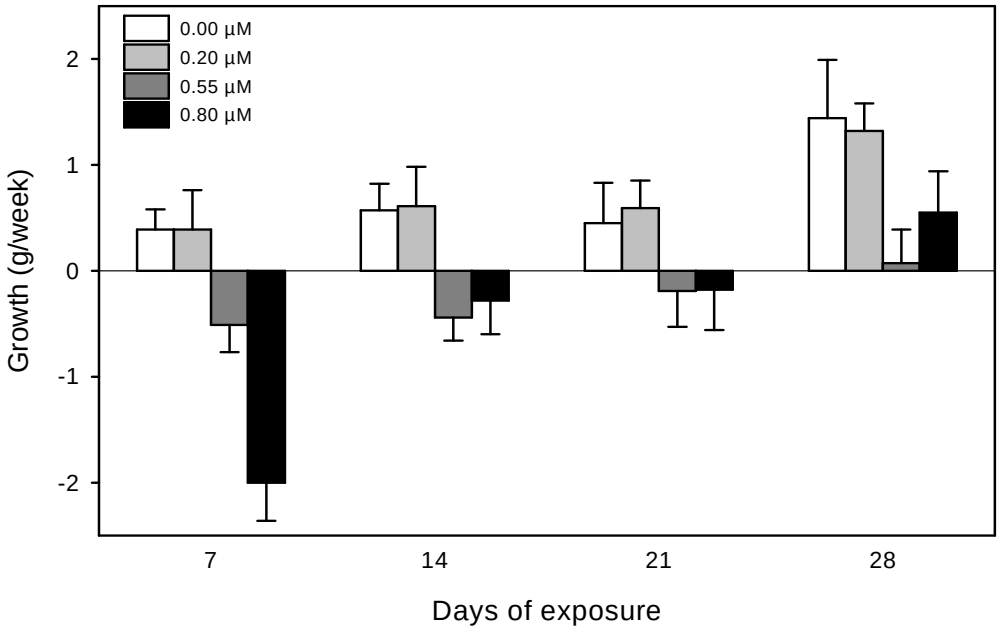

Figure 3.2. Mean growth in g/week of common carp exposed to different copper concentrations. Each bar represents mean values ± S.D. from fish remaining at that time (n=32 at day 7, n=24 at day 14, n=16 at day 21, n=8 at day 28).

3.4.2. Copper accumulation in different tissues

Copper accumulation was followed during the 28 day exposure period in three different brain parts and in muscle and liver tissue (Table 3.1.). The most pronounced copper accumulation was found in the liver. After one week of exposure to 0.80 µM of copper, the level of accumulated copper had increased from 20 µg per gram of wet liver tissue to 39 µg ($P<0.001$). This increase continued, and after 28 days of copper exposure, the copper level in liver tissue had augmented to 65 µg per gram wet tissue

Table 3.1. Copper accumulation in brain, muscle and liver tissues of common carp exposed to 0.00, 0.020, 0.55 and 0.80 µM of copper. Values are means ± S.D. from eight fish, significant differences were indicated when accumulation of the copper was significantly higher than controls of the same day (: p< 0.05, **: p<0.01, ***: p<0.001).*

	Copper in water	Copper concentrations in different tissues (µg/g) Day 0	Day 7	Day 14	Day 21	Day 28
TELENCEPHALON	0.00µM	1.11±0.12	1.24±0.26	1.18±0.14	1.24±0.20	1.10±0.20
	0.20 µM	1.12±0.13	1.33±0.23	1.32±0.27	1.13±0.11	1.06±0.16
	0.55 µM	1.24±0.28	1.39±0.12	1.39±0.28	1.19±0.16	1.18±0.15
	0.80 µM	1.29±0.16	1.14±0.27	1.44±0.24	1.30±0.22	1.12±0.16
HYPOTHALAMUS	0.00µM	2.11±0.31	1.84±0.23	1.85±0.24	1.60±0.25	1.64±0.31
	0.20 µM	1.89±0.11	1.88±0.19	1.98±0.29	1.58±0.31	1.64±0.09
	0.55 µM	1.85±0.20	1.96±0.23	1.79±0.36	1.70±0.31	1.61±0.27
	0.80 µM	1.94±0.25	2.12±0.18	1.96±0.38	1.76±0.30	1.74±0.44
BRAIN STEM	0.00µM	1.73±0.33	1.63±0.24	1.70±0.14	1.62±0.15	1.80±0.10
	0.20 µM	1.76±0.14	1.71±0.18	1.74±0.14	1.60±0.10	1.88±0.16
	0.55 µM	1.68±0.15	1.71±0.12	1.80±0.13	1.80±0.21	1.71±0.12
	0.80 µM	1.60±0.04	1.80±0.03	1.89±0.11 *	1.99±0.08 ***	1.89±0.10
MUSCLE	0.00µM	0.44±0.14	0.48±0.11	0.40±0.19	0.42±0.06	0.46±0.15
	0.20 µM	0.33±0.07	0.39±0.10	0.44±0.08	0.41±0.05	0.46±0.07
	0.55 µM	0.35±0.06	0.42±0.08	0.41±0.04	0.38±0.08	0.49±0.10
	0.80 µM	0.39±0.06	0.38±0.10	0.41±0.06	0.39±0.06	0.36±0.06
LIVER	0.00µM	19.91±4.78	18.94±5.38	21.93±6.76	25.04±4.96	19.18±3.94
	0.20 µM	20.46±4.10	25.42±6.65	29.29±8.68	29.29±5.58	25.65±4.76
	0.55 µM	19.03±4.76	24.06±3.99	37.66±11.25 **	45.09±13.46 ***	50.26±10.08 ***
	0.80 µM	19.66±2.95	38.85±8.05 ***	51.92±8.83 ***	62.66±7.21 ***	65.16±11.94 ***

($P<0.001$). At a copper concentration of 0.55 µM, a significant copper accumulation in the liver appeared after two weeks of exposure; copper levels increased from 19 µg per gram of wet tissue to 38 µg ($P<0.01$). The accumulation continued, and after four weeks of continuous exposure to 0.55 µM of copper, levels had increased to 50 µg per gram of wet liver tissue.

No significant copper accumulation occurred in muscle tissue, and also in telencephalon or hypothalamus a significant rise in copper levels was absent. In brain stem, a significant rise in copper concentration was observed at the highest copper exposure concentration after two ($P<0.05$) and three ($P<0.001$) weeks only, not after four weeks of exposure.

3.4.3. Effect of copper on protein content

Protein content of white muscle remained stable during the first three weeks of exposure (Figure 3.3.), but after four weeks of copper exposure, protein content dropped in the exposure group of 0.55 µM ($P<0.05$) as well as in the exposure group of 0.80 µM ($P<0.001$). In liver tissue, a drop in protein levels only occurred at the highest exposure concentration were levels were significantly reduced after two ($P<0.01$) and three ($P<0.05$) weeks of copper exposure. After four weeks of copper exposure, liver protein levels had returned to a normal level.

3.4.4. Effect of copper on glycogen content

In muscle tissue, copper exposure had a pronounced effect on glycogen levels (Figure 3.4.). At all three exposure copper concentrations glycogen levels started to drop after the first week of exposure, the decrease being significant after two weeks of exposure at the highest copper concentration ($P<0.01$) and after three weeks of

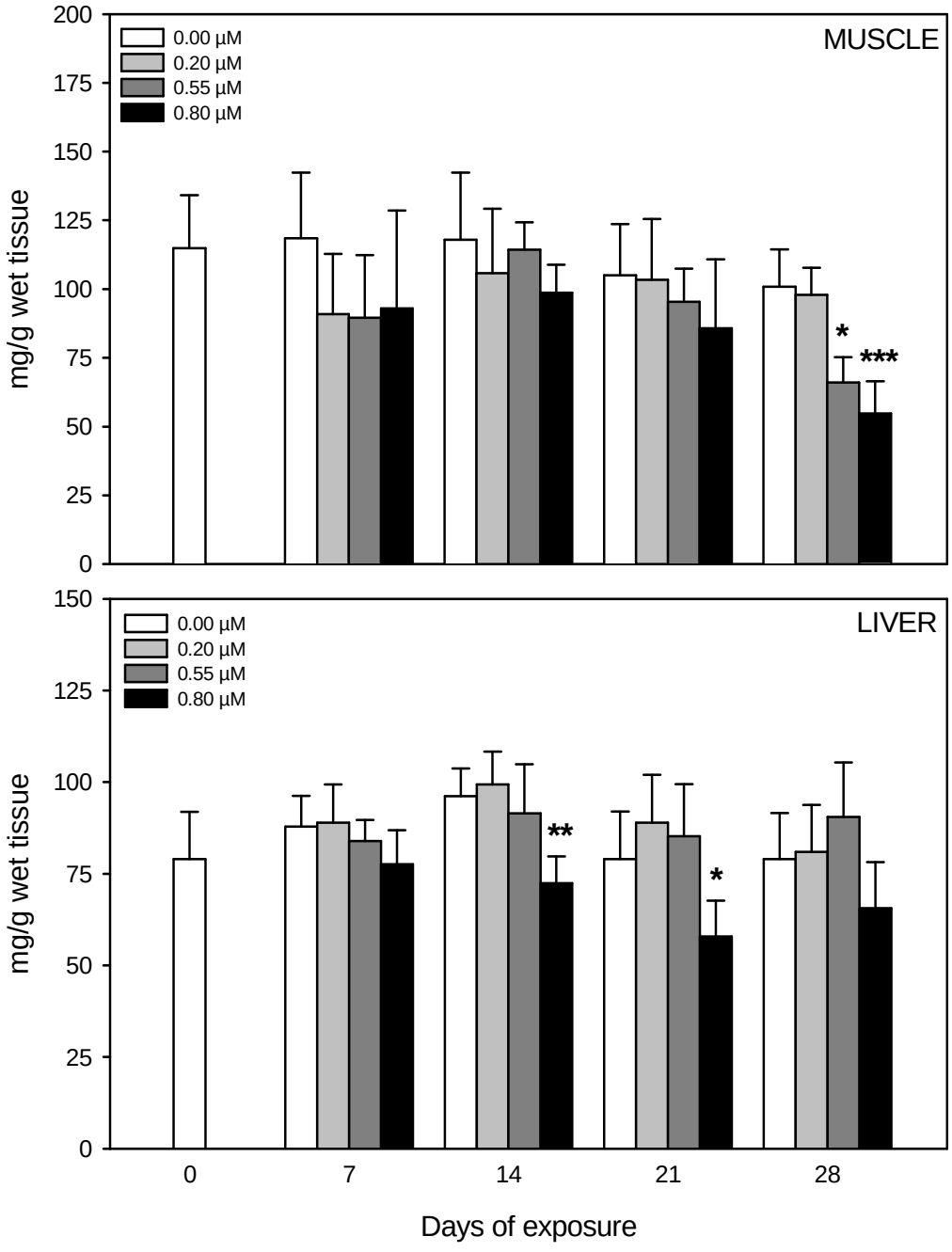

Figure 3.3. Protein content of muscle and liver tissue from copper exposed carp. Values are means ± S.D. (n=8), significant differences compared to control value at the same day are indicated (: P<0.05; **: P<0.01; ***: P<0.001).*

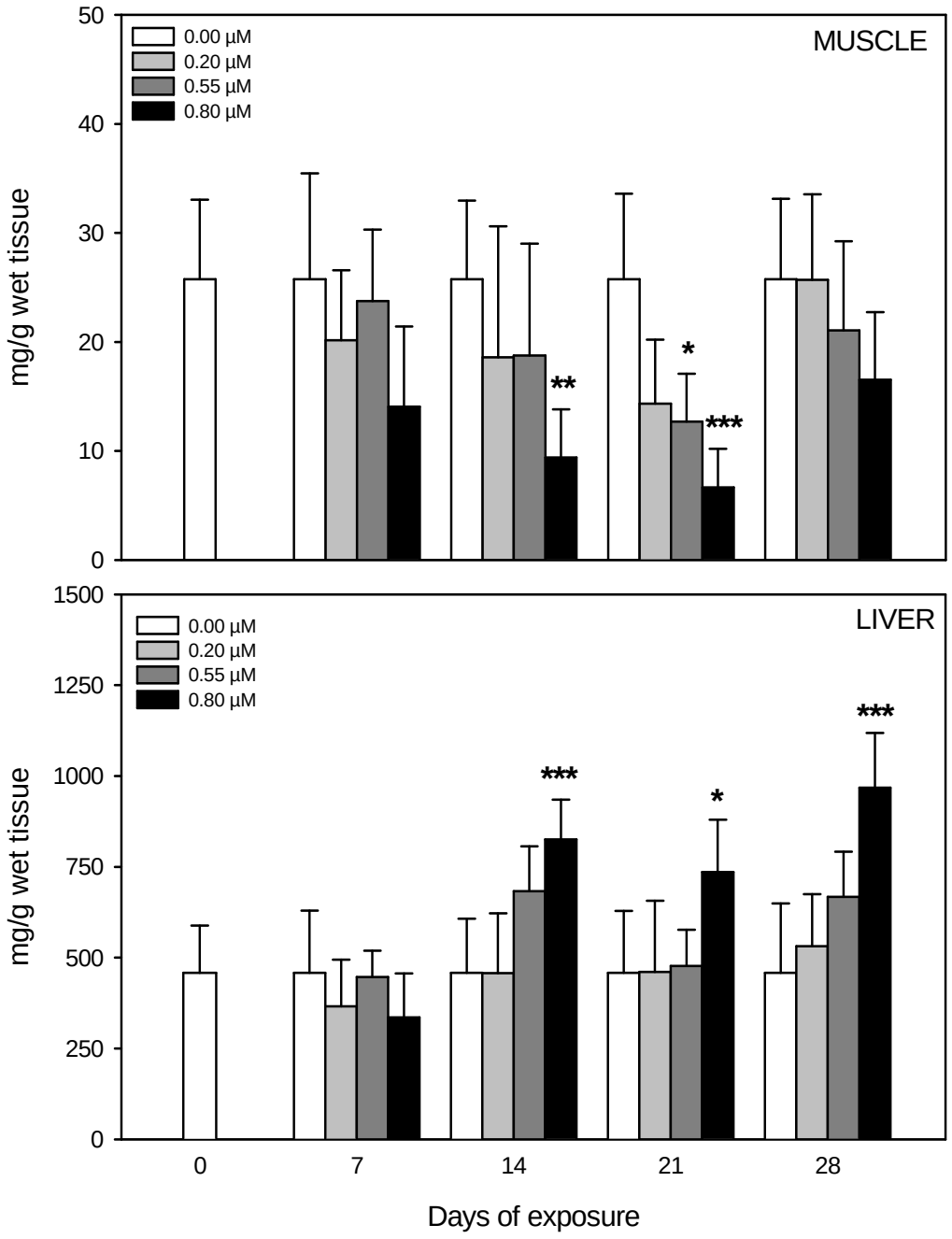

Figure 3.4. Glycogen content of muscle and liver tissue from copper exposed carp. Values are means ± S.D. (n=8), significant differences compared to control value at the same day are indicated (: P<0.05; **: P<0.01; ***: P<0.001).*

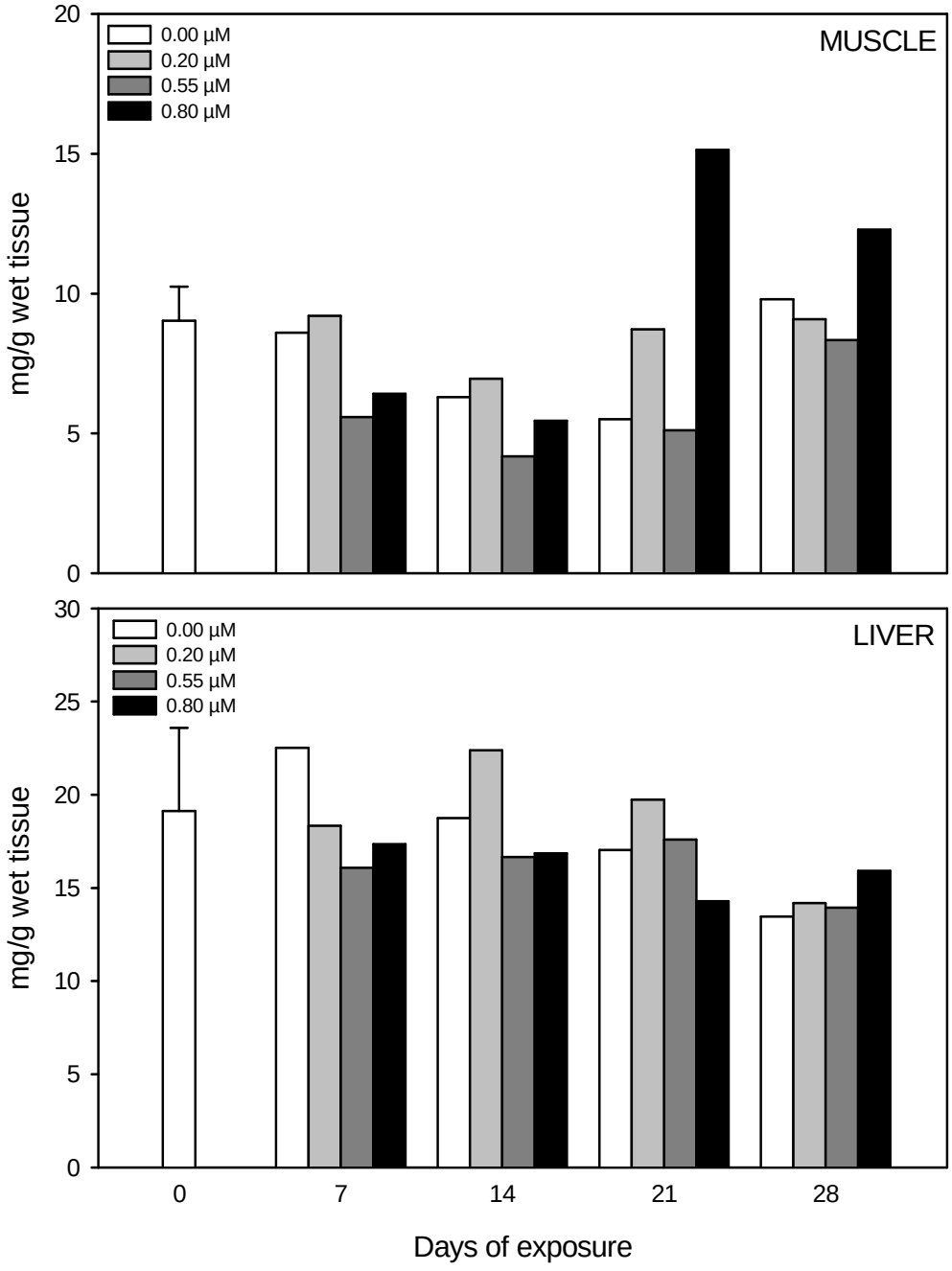

Figure 3.5. Lipid content of muscle and liver tissue from copper exposed carp. Values are means from pooled samples of eight fish for each exposure group.

exposure at exposure copper concentrations of 0.55 µM ($P<0.05$) and 0.80 µM ($P<0.001$). During the fourth week of exposure, glycogen levels in muscle tissue returned to normal.

In the liver, on the contrary, glycogen concentrations tended to rise after two weeks of exposure, the effect being significant at the highest copper concentration after two weeks ($P<0.001$), three weeks ($P<0.05$) and four weeks of exposure ($P<0.001$).

3.4.5. Effect of copper on lipid content

In both in white muscle tissue and in liver tissue (Figure 3.5.), lipid content remained rather stable. In the liver, lipid levels remain roughly between 1.5 and 2% of the wet tissue weight, whereas in white muscle levels vary between 0.4 and 1% except at the highest copper concentration where lipid levels appear to be elevated during the second half of the exposure time.

3.4.6. Effect of copper on nucleic acid content

Both in white muscle and in liver tissue (Figure 3.6.), RNA levels were stable during the entire exposure period. DNA levels (Figure 3.7.) differed only in white muscle after four weeks of copper exposure. DNA levels were significantly increased at all three copper exposure concentrations ($P<0.05$ at 0.20 µM, $P<0.001$ at 0.55 and 0.80 µM). Therefore, RNA/DNA ratios were quite stable and no good correlation was obtained between growth rate and RNA/DNA ratio, either in muscle (not significant) or liver tissue ($P<0.05$) (Figure 3.8.). Also protein/RNA or protein/RNA/DNA ratios failed to give a significant indication of growth rates (results not shown).

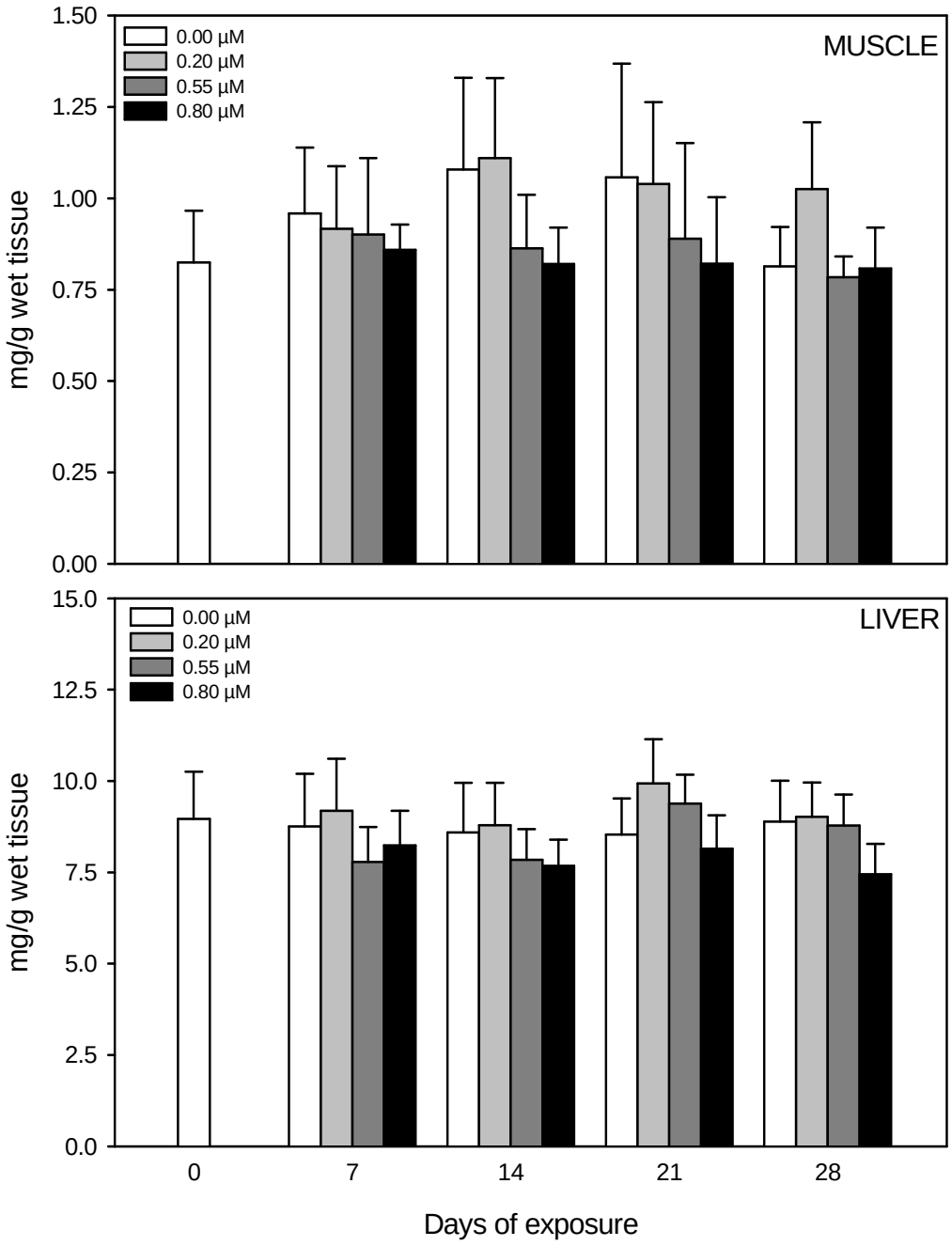

Figure 3.6. RNA content of muscle and liver tissue from copper exposed carp. Values are means ± S.D. (n=8), no significant differences were found.

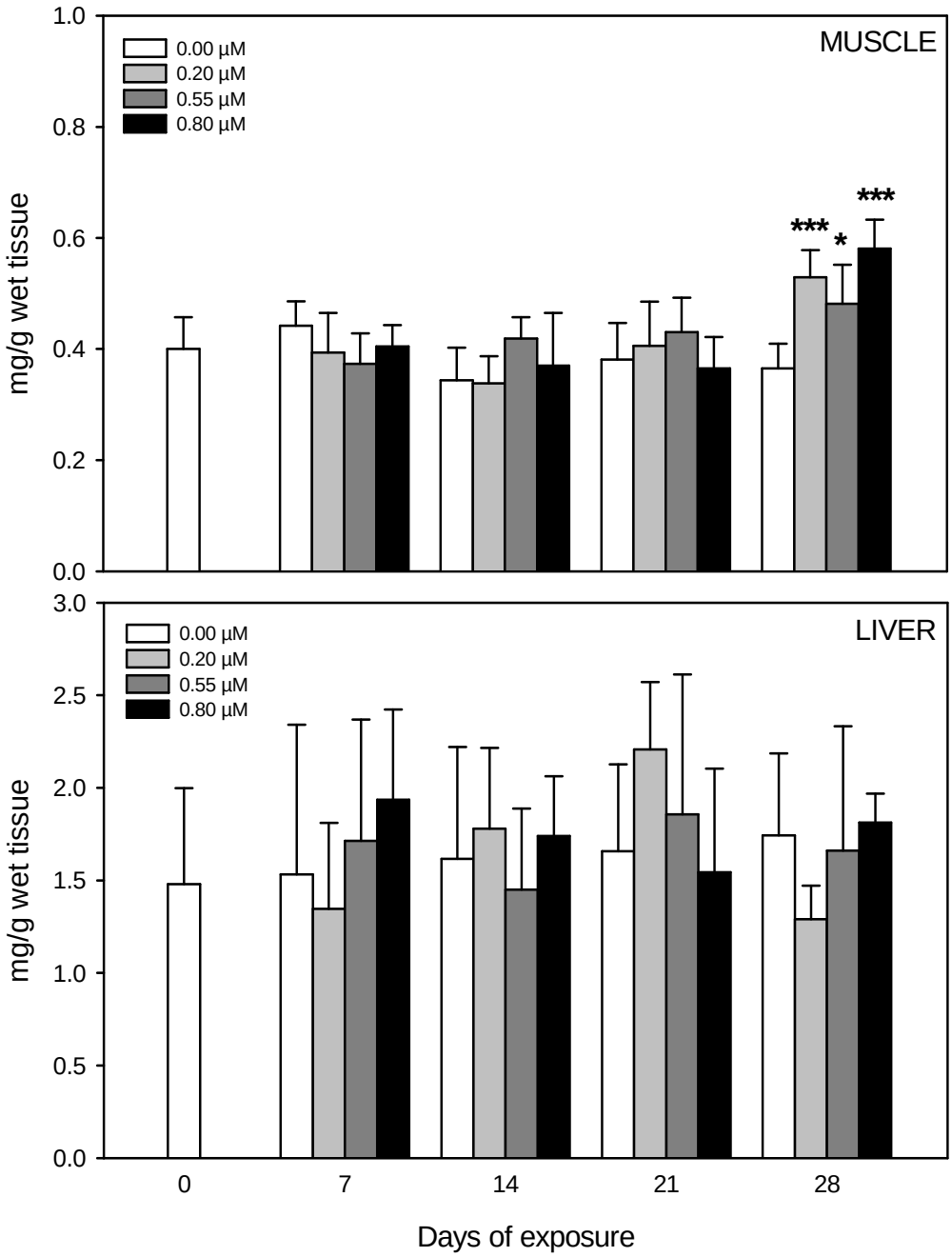

Figure 3.7. DNA content of muscle and liver tissue from copper exposed carp. Values are means ± S.D. (n=8), significant differences compared to control value at the same day are indicated (: P<0.05; **: P<0.01; ***: P<0.001).*

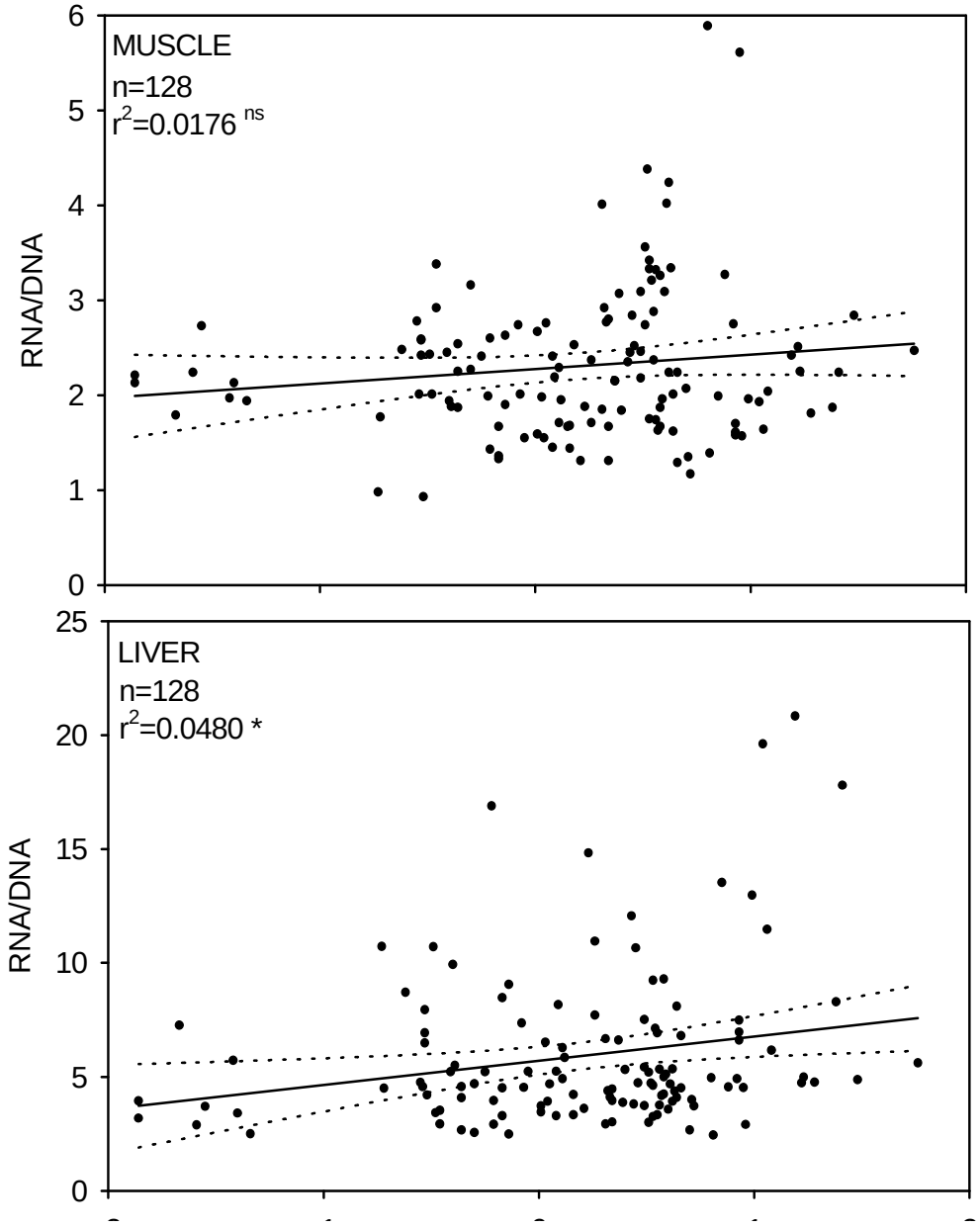

Figure 3.8. RNA/DNA ratios of muscle and liver tissue compared to % growth per day measured for the week preceding the sampling of tissues. Values are from all carp used in this study.

3.5. Discussion

One of the first marked changes exposing common carp to sublethal copper concentrations was the immediate reduction of feeding in fish exposed to 0.80 µM of copper, whereafter the feeding rate slowly recovered to control levels during the next weeks of exposure. Consequently, also growth was reduced in this high exposure group, and carp lost weight during the first three weeks of exposure. Loss of appetite and reduced growth rate followed by recovery to rates approaching control levels have also been seen in salmonid fishes exposed to copper (Lett *et al*., 1976 (rainbow trout); Drummond *et al*., 1973 (brown trout); Buckley *et al*., 1982 (coho salmon)). Whereas feeding levels of the carp exposed to 0.55 µM of copper remained unchanged, growth did not. The fish in this exposure group lost weight during the first three weeks of copper exposure, whereafter they slowly started gaining weight again. Despite the increased food consumption of the lowest exposure group (0.20 µM of copper), no increase in growth rate was observed here either. Thus, it appears that the copper exposed carp spent more energy to sustain their normal metabolism, leaving less energy available for growth. This effect has also been observed in copper exposed perch (Collvin, 1985), and coho salmon (Buckley *et al*., 1982). Presumably, the increase in metabolic rate may have been associated with tissue repair, development of defence systems and with copper excreting mechanisms.

Tissue damage may also be, at least partially, cause of the reduced appetite seen in copper exposed fish. Low-level copper exposure in rainbow trout revealed degeneration of the olfactory system of the fish (Julliard *et al*., 1993) and responses to a stimulus (L-alanine) on the olfactory receptors of atlantic salmon appeared to be disturbed under copper exposure (Winberg *et al*., 1992; Bjerselius *et al*., 1993). In the study by Julliard *et al*. (1993) signs of neural regeneration were reported during the time course of the exposure, indicating some form of acclimation, which agrees with the recovery of feeding rate seen. Other neural mechanisms may also be involved in copper induced loss of appetite: copper exposure has been noted to reduce

acetylcholinesterase activity in carp, the consequent increase in acetylcholine contents in nerve endings may disrupt synaptic transmissions between neurones (Nemcsók *et al*., 1984; Nemcsók and Hughes, 1988). Also indications for the involvement of monoamine neurotransmitters exist (De Boeck *et al*., 1995a: see chapter VI).

Copper accumulation at the two highest copper concentrations (0.55 and 0.80 µM) clearly occurred mainly in liver tissue. This corroborates the view that this organ is the major storage and regulatory organ in copper homeostasis. After induction by an environmental metal exposure, major concentrations of metallothionein can be found in the liver (Roesijadi, 1992). Induction of metallothioneins in these organs leads to metal accumulation in the cells, but the specific binding to the metallothionein molecules keep the free concentrations of metal extremely low, which protects cellular proteins from metal induced damages. From the first week of exposure to the highest copper concentration on, copper concentrations in the tissue were doubled, and after the third week, copper concentration in the tissue were almost tripled. Then, the accumulation rate appeared to slow down and copper levels after four weeks of exposure are comparable to those after three weeks of exposure. Also in brown bullhead, equilibrium concentrations of copper in the liver were reached in 30 days and no great difference was apparent between tissue levels after 30 days or 20 months at copper exposure concentrations of 0.77 to 1.00 µM (Brungs *et al*., 1973). In coho salmon, this equilibrium was coincident with recovery in growth rate (Buckley *et al*., 1982), a fact confirmed by our results.

Copper accumulation in white muscle tissue has been seen in common carp exposed to copper (Nemcsók *et al*., 1987; Marek *et al*., 1991), usually at higher copper exposure concentrations than the ones used here (0.80 to 626 µM). The fact that no rise in white muscle copper levels has been seen here agrees with the view that accumulation in muscle becomes only important when the maximum storage capacity of the liver is reached (Laurén and McDonald, 1987). The blood brain barrier seems to protect the brain rather well from copper toxicity, no rise in copper levels was seen in

telencephalon or hypothalamus, and in brain stem only a temporary increase in copper level was noted.

One of the first responses to a stressor such as copper exposure is the release of so called stress hormones adrenaline, noradrenaline and cortisol (Wendelaar Bonga, 1993). The release of these catecholamines and cortisol triggers a broad suite of biochemical and physiological changes known collectively as secondary stress responses. The metabolic effects may include hyperglycemia, hyperlacticaemia, depletion of glycogen tissue reserves, lipolysis and inhibition of protein synthesis. There may also be increased catabolism of muscle protein, and alterations in the plasma levels of amino acids, free fatty acids and cholesterol (Jobling, 1994). The cessation of feeding, accompanied by the catabolic effects of the catecholamines and corticosteroids on the energy reserves stored in the body tissues, must result in reduced growth in stressed fish.

The changes seen in muscle tissue agree well with the general picture of secondary stress responses. The muscle glycogen store is depleted during the first three weeks of copper exposure. During the fourth week of exposure, when glycogen levels in the white muscle recover, a significant decrease in protein levels appears at the two highest copper concentrations. This protein catabolism is not unusual. Whereas catecholamines are thought to cause the initial elevation in plasma glucose levels by mobilising the glycogen reserves (glycogenolysis), the corticosteroids may contribute to the maintenance of hyperglycemia via the stimulation of gluconeogenesis from amino acids and thus stimulate protein catabolism. In addition to maintaining hyperglycemia, this increase in synthesis of glucose from amino acids could, in the longer term, also result in the restoration of glycogen levels (Jobling, 1994), as was seen here for muscle tissue. Concerning this gluconeogenesis, even protein deficiency (necessitating amino acid conservation) does not suppress gluconeogenesis since starving fish can exhibit quite high rates of gluconeogenesis (Cowey and Sargent, 1979). The fact that protein levels decrease during the fourth week of exposure to the two highest copper concentrations does not have to be in contradiction to the fact that

growth rate recovered at this moment. Although protein content per gram of tissue decreased, total protein content of the organism might have increased. The rise in DNA in muscle tissue at this moment indicates that a reduction of cell size appeared, probably due to cell division considering the regained capacity for growth.

In the liver however, a completely different pattern can be seen. After an insignificant decrease in glycogen content during the first week, glycogen levels start to rise at the highest copper exposure concentration. One possible explanation for this event is that carp exposed to the highest copper concentration were experiencing some form of hypoxia. If a fish is kept in oxygen-deficient water for an extended period of time it will become more hypoxia tolerant. This improved tolerance is attributable to several changes that occur during long-term exposure to non-lethal hypoxia. Besides an improved efficiency with which the fish can extract oxygen from the water, there may also be an increase in the tissue glycogen reserves and an elevation in the levels of several enzymes in the liver, leading to a higher gluconeogenic and anaerobic capacity following acclimation to hypoxia (Jobling, 1994). Carp in this highest exposure group could very well experience hypoxia since copper has been shown to disrupt gill epithelia in carp and in salmonids (Benedeczky *et al*., 1986; Marek *et al*., 1991, Kirk and Lewis, 1993, Wilson and Taylor, 1993). Collapse and fusion of lamellae, lifting of lamellar epithelium away from pillar cells and swelling of the epithelial cells has been observed. In addition to this ultrastructural damage, an increase in the secretion of mucus and concomitant swelling of the mucus layer around the gill increases the diffusion distance for oxygen. Whereas structural gill damage repairs gradually, swelling of the epithelial cells and thickening of the mucus layer remains for a more extended period. Other experiments showed an impaired oxygen consumption in common carp exposed to similar copper concentrations (De Boeck *et al*., 1995b: see chapter IV) and increased lactate levels were seen indicating an increase in anaerobic metabolism (De Boeck *et al*., 1995a: see chapter VI). Increased liver glycogen levels are therefor suggested to be a consequence of a defence mechanism against this hypoxic condition.

Protein levels are slightly reduced in the liver after the second and third week of exposure to 0.80 µM. Possibly, this muscle catabolism was caused by the need for gluconeogenesis. Lipid stores in muscle as liver tissue do not appear to be a used extensively under these circumstances. Also Lett *et al.* (1976) found no changes in lipid content of rainbow trout during a 40 day exposure period to copper, although growth rates were also initially depressed and recovered subsequently. The rise in DNA in muscle tissue indicates that a reduction of cell size appeared during the last week of copper exposure. Since growth rates also recovered during this last week, active cell division could be the cause of this compactness of cells.

In our study, a poor correlation was found between growth rate and RNA/DNA ratios and no correlation was found between growth and protein/RNA or protein/RNA/DNA ratios. Other studies have not been able to establish these relationship either. In rainbow trout liver, Jürss *et al.*, 1987 found no relation between the RNA/DNA quotient and growth. Also Satomi and Tanaka (1973) found that there is no close correlation between the RNA/DNA quotient and the growth of rainbow trout. Protein synthesis is probably controlled faster and more comprehensively by translation than by the amount of RNA (Jürss *et al.*, 1987), and levels of ribosomal RNA could mask changes in messenger RNA. According to Knowles and McKee, (1989) it is important to measure the nucleic acids at or immediately preceding periods of rapid growth to allow maximum resolution of toxicant effects on RNA ratios but they also acknowledge that from a practical point of view, this is virtually impossible.

In conclusion, we can say that copper exposure to 0.80 µM immediately affected as well growth as feeding behaviour in common carp, whereafter feeding and growth rates slowly recovered. At 0.55 µM, growth is affected despite the normal food consumption. Even at the lowest copper concentration (0.20 µM), metabolic demand for the fish is increased challenging the carp with an increased demand for food. Copper accumulation mainly occurred in the liver, reaching an equilibrium between uptake and excretion after one month of exposure. Substantial biochemical changes were observed at the two highest copper exposure concentrations, but no good

correlation was found between growth rate and RNA/DNA ratio. Therefor the use of the RNA/DNA ratio as a sensitive biomarker is questionable.

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CHAPTER IV: THE EFFECT OF SUBLETHAL LEVELS OF COPPER ON OXYGEN CONSUMPTION AND AMMONIA EXCRETION IN THE COMMON CARP, *CYPRINUS CARPIO*[*]

4.1. Summary

Common carp (*Cyprinus carpio*) of 15-30g body weight were exposed to copper levels of 0.22±0.07, 0.34±0.12 and 0.84±0.35 μmol.l^{-1}. Oxygen consumption and nitrogen excretion were determined repeatedly for up to two weeks of exposure to copper. Critical oxygen concentrations for oxygen consumption as well as for ammonia excretion were determined after one week of exposure to copper. Oxygen consumption dropped significantly immediately after exposure to 0.34 and 0.84 μmol.l^{-1} of copper whereas nitrogen excretion remained stable. After one week of continuous exposure to 0.34 μmol.l^{-1} of copper the oxygen consumption showed an apparent recovery, while the ammonia quotient (AQ = mole to mole ratio of ammonia excreted to oxygen consumed) did not. At a copper concentration of 0.84 μmol.l^{-1}, no recovery was observed. The critical oxygen concentration for oxygen consumption shifted from 45 μmol.l^{-1} (1.4 mg.l^{-1}) in copper-free water to 126 μmol. l^{-1} (3.9 mg.l^{-1}) at a copper concentration of 0.34 μmol.l^{-1}. At 0.84 μmol.l^{-1}, regulation of oxygen consumption was lost. Also ammonia excretion showed a decline at lower oxygen concentrations and a critical oxygen concentration for ammonia excretion was determined. For the nitrogen excretion, loss of regulation already occurred at copper concentrations of 0.34 μmol.l^{-1}. For the AQ, no critical oxygen concentration was found.

[*] Based on the manuscripts by De Boeck, G., De Smet, H. and Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. *Aquat. Toxicol.* **32**: 127-146 and by Blust, R., G. De Boeck, R. Borger and W. Decleir, 1993. Effects of changing environmental conditions on the energy metabolism of aquatic organisms. Proceedings of the Global Change Symposium, Belgian Science Policy Office: 141-168.

The results obtained in this study suggest that measurements of oxygen consumption in combination with measurements of nitrogen excretion can be useful indicators of stress. Furthermore it is shown that a critical oxygen concentrations for ammonia exists in carp and that the critical oxygen concentration for oxygen consumption and for ammonia excretion are affected by exposure to copper.

4.2. Introduction

To assess the effects of a toxic compound on an aquatic organism, responses to sublethal levels of this compound should be studied rather than performing acute toxicity tests. Sublethal concentrations of toxic compounds may cause biochemical, physiological, morphological and genetic changes, affecting e.g. development, growth and reproduction. Before changes in viability occur, it is likely that changes in the energy status of the organism appear.

In fish, the relative use of protein, lipid and carbohydrate as energy source can be influenced by both internal and external factors. As most of the nitrogenous end products of freshwater fish originate from protein catabolism, with ammonia as the principal end product, the contribution of protein catabolism to the total energy production of the fish can be assessed by determination of the ammonia quotient (AQ = mole to mole ratio of ammonia excreted to oxygen consumed) (Brett and Zala, 1975; Kutty, 1972, 1978; Kutty and Peer Mohamed, 1975; Van Waarde, 1983).

The metabolic response to changes in oxygen availability may vary, depending on the physiological state of the animal, level of activity and temperature (Grieshaber *et al*., 1988; Burggren and Roberts, 1991). As most fish, carp are oxygen regulators meaning that they maintain their oxygen consumption at a constant level along a gradient of environmental oxygen concentrations, until a critical oxygen concentration (C_c) is reached, below which oxygen consumption begins to fall. Under conditions of stress, this C_c is likely to increase, reflecting the decreased capacity of the fish to cope with

environmental perturbations. For carp and trout, a shift in C_c to higher oxygen concentration has been observed when exposed to low pH (Ultsch *et al*., 1980).

To evaluate the use of oxygen consumption, ammonia excretion, AQ and the C_c as measures for stress in fish, common carp (*Cyprinus carpio*) were exposed to different sublethal levels of copper. Exposure to copper causes a number of effects in fish and lethal copper concentrations for carp can vary depending on fish size and water composition (Sörensen, 1991). Alam and Maughan (1992) found 96 hour LC50 values varying from 4.7 to 15.8 $\mu mol.l^{-1}$ depending on fish size whereas Peres and Pihan (1991) found 48 hour LC50 values for carp juveniles (3.5-5.5 g) between 1.9 and 11.8 $\mu mol.l^{-1}$ depending on water hardness. Based upon these values, three sublethal copper concentrations were chosen and acute effects (from first to tenth hour of exposure) as well as effects on a longer term (one to two weeks) on oxygen consumption and ammonia excretion were studied.

4.3. Materials and methods

Juvenile (1 month) common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Agricultural University of Wageningen, The Netherlands. They were grown at the University of Antwerp at the optimal temperature of 25°C (Guderley & Blier, 1988) in softened Antwerp city tap water (Ca 0.875 $mmol.l^{-1}$, Mg 0.145 $mmol.l^{-1}$, pH 7.0±0.5). Water was filtered with a trickling filter and water quality was checked weekly with Visicolor Test Kits (Macherey-Nagel, Düren) for ammonia, nitrite and nitrate. 50% of the water was renewed when levels exceeded 0.1 $mg.l^{-1}$, 1.0 $mg.l^{-1}$ and 20 $mg.l^{-1}$ respectively. Two weeks before starting the experiments, carp weighing between 15 and 30 g were transferred into 50 l aquaria filled with standard moderately hard water according to Standard Methods (American Public Health Association, 1989: $CaSO_4.2H_2O$: 0.348 $mmol.l^{-1}$; $MgSO_4.2H_2O$: 0.500 $mmol.l^{-1}$; $NaHCO_3$: 1.143 $mmol.l^{-1}$; KCl: 0.054 $mmol.l^{-1}$; pH: 7.8-8.0). The standard water was well aerated during at least 24 hours before use. During the first week the temperature in the aquaria was gradually

adjusted to 20°C (±1°C) and the photoperiod was set at a 14 hour light, 10 hour dark period. Carp were fed to satiation once a day with 'Pond Sticks' (Tetrapond, Henckel), and excess food was removed 15 minutes after feeding. Water was filtered with an Eheim filter filled with activated charcoal (Calgon Carbon) and Rivalon synthetic filter wadding. Water quality was checked daily for ammonia, nitrite and nitrate and 50% of the water was replaced twice a week.

For exposure of the carp to the stressor, 250, 125 or 62.5 mg copper per liter standard water was added as a copper nitrate standard solution (Merck, 1 $g.l^{-1}$ $Cu(NO_3)_2.2H_2O$). The water was again aerated and mixed for at least 24 hours before use. In the aquaria the water was filtered with an Eheim filter filled with Rivalon synthetic filter wadding and 75% of the water was replaced with new copper containing standard water twice a week. This resulted in copper concentrations in the aquaria of 0.84±0.35, 0.34±0.12 and 0.22±0.07 $\mu mol.l^{-1}$ respectively (means ±S.D.). Samples for copper measurements were taken 5 times a week and copper measurements were made using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAS 703 and HGA 500).

Measurements of oxygen consumption and ammonia excretion were made in a respirometer (Fig. 4.1.) consisting of a small measurement unit (885 ml) which was connected to two larger circulation units (ca. 25 l each). The measurement unit included a small circulation pump, a vessel containing the fish, and four holders for electrodes. Time necessary for total replacement of the water in the measurement unit with water of one of the circulation units was determined spectrophotometricaly using methylene blue and was 2-3 minutes. The electrodes used were a gold-silver oxygen electrode (Syland scientific, type 400-1E), an ion-selective ammonia electrode (Ingold, type 15 223 300) with reference electrode (Ingold, type 373-90-WTE-ISE-S7) and a pH electrode (Ingold, type U457-S7/110). The oxygen electrode was calibrated weekly using the Winkler method (American Public Health Association, 1989). Calibration curves for the ammonia electrode were made weekly in the respirometer by adding NH_4Cl to water with the same composition as the water used during the experiment. In the appropriate range (0-80

μmol.l^{-1} NH_4^+) the ammonia electrode showed a non linear response according to the formula: $mV=(c_1.[NH_4^+])/(c_2+[NH_4^+])+c_3$ with a correlation coefficient varying between 0.997 and 0.999.

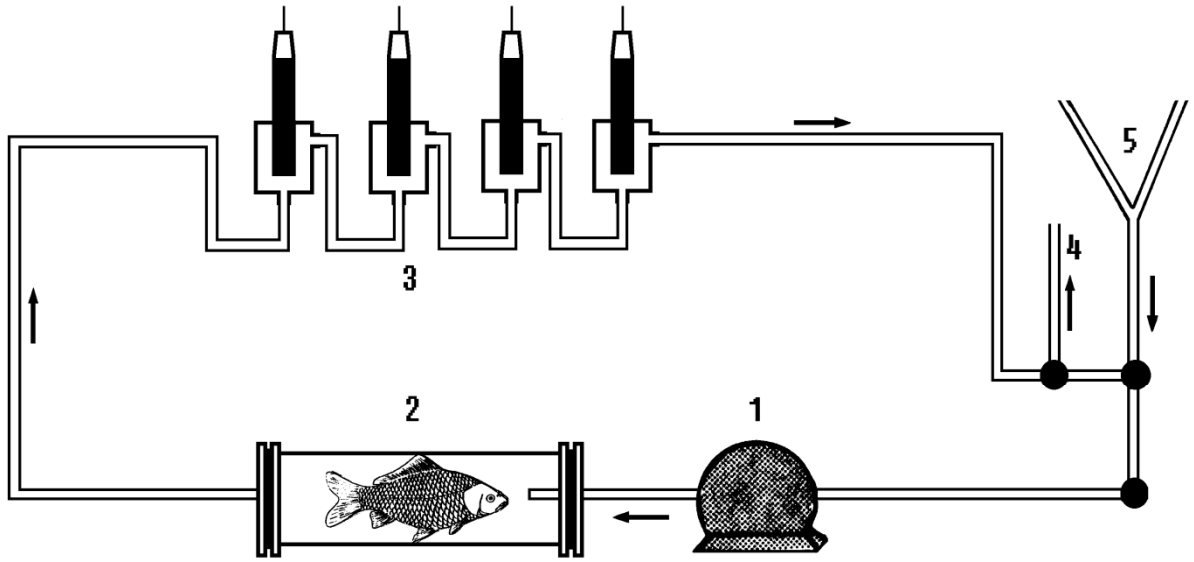

Figure 4.1. The respirometer as used in the experiments: 1. circulation pump; 2. vessel containing the fish; 3. electrode vials; 4. outflow when connected to circulation units; 5. connection to two different 25l circulation units.

Chakraborty *et al.* (1992) examined ammonia excretion peaks in common carp after feeding and found that ammonia excretion returned to routine levels 24 hours after feeding. Therefore carps in our experiment were not fed for two days before measurement. The fish were positioned in the respirometer the day before measurements, allowing them to adapt to the situation overnight and avoiding handling stress on the day of measurements. Overnight, the fish were provided with well aerated water from one of the circulation units. Electrodes were put in place at least 1 h before measurements

started and possible air bubbles were removed through the air outlet without disturbing the fish. Five minutes before measurements started, water provision was switched to the second circulation unit allowing replacement of the water with ammonia free, well aerated water which could be either copper-free or copper containing standard water. As the measurements started, the tap to the circulation unit was closed, and the water circulated in the measurement unit only.

A first set of preliminary tests was performed to determine optimal measuring conditions. The effects of measurement time, measurement frequency and oxygen concentration of the water were determined in standard copper-free water in order to assess the possible influences of these conditions on oxygen consumption and ammonia excretion. Based on the results of the preliminary experiments, all further measurements were made 3 to 4 times a day for a period of one hour. In between measurements fish were allowed to recover for two hours provided with water from one of the circulation units. For the determinations of the critical oxygen concentrations, measurements continued until fish lost equilibrium due to the depletion of the oxygen in the water. During this procedure, ammonia concentrations in the measurement unit reached maximum values of 25 $\mu mol.l^{-1}$.

In each of the experiments, 5 fish were marked with small cuts in dorsal, caudal or pectoral fins and acclimated to the standard water for 2 weeks. Each day during the third week, oxygen consumption and nitrogen excretion were measured three times a day for one of these fish. The next week, the fish were measured again, but this time they were exposed to standard water containing 0.84, 0.34 or 0.22 $\mu mol.l^{-1}$ of copper. The exposure started five minutes prior to the first measurement during which all the water in the respirometer was replaced.

Measurements were made during the first, fourth, seventh and tenth hour of exposure and subsequently fish were kept in the copper containing standard water. After one week of exposure, fish were measured again three times a day and the critical oxygen concentration was determined during the last measurement. For the copper concentration

of 0.84 μmol.l^{-1}, no signs of recovery were found after this first week of exposure and measurements were repeated three times a day after a second week of exposure.

All values are given as means ±S.D.. For statistical analysis the Kolmogornov-Smirnov and the Bartlett test were used to assess the conditions for an ANOVA test. If these conditions were not fulfilled, the Kruskal-Wallis and Dune test were used. Critical oxygen concentrations were calculated according to the method of Ultsch *et al*. (1980).

4.4. Results

During measurements, pH varied from 7.8 to 7.0. Under the given circumstances of temperature and ionic strength, more than 98% of the ammonia was present as the ionised form (NH_4^+) in this pH range (calculated from Martell and Smith, 1982). Therefore, measured ammonia concentrations are referred to as total ammonia (T_{amm}).

The effects of different copper exposure concentrations on oxygen consumption, ammonia excretion and AQ, are shown in Fig. 4.2. Exposure to 0.84 μmol.l^{-1} of copper (Fig 4.2. A-C) caused an immediate significant rise of 60% in the AQ (Fig. 4.2. A), increasing from 0.081±0.009 to 0.212±0.050 within the first hour. During the next nine hours of exposure, a distinct, although only partial, recovery was observed. Nevertheless, this recovery was only temporary and the AQ stabilised after one week of exposure at a level close to that seen during the first hour (0.184±0.044). A similar AQ was also seen after 2 weeks of copper exposure (0.216±0.061, results not shown). Fish also showed

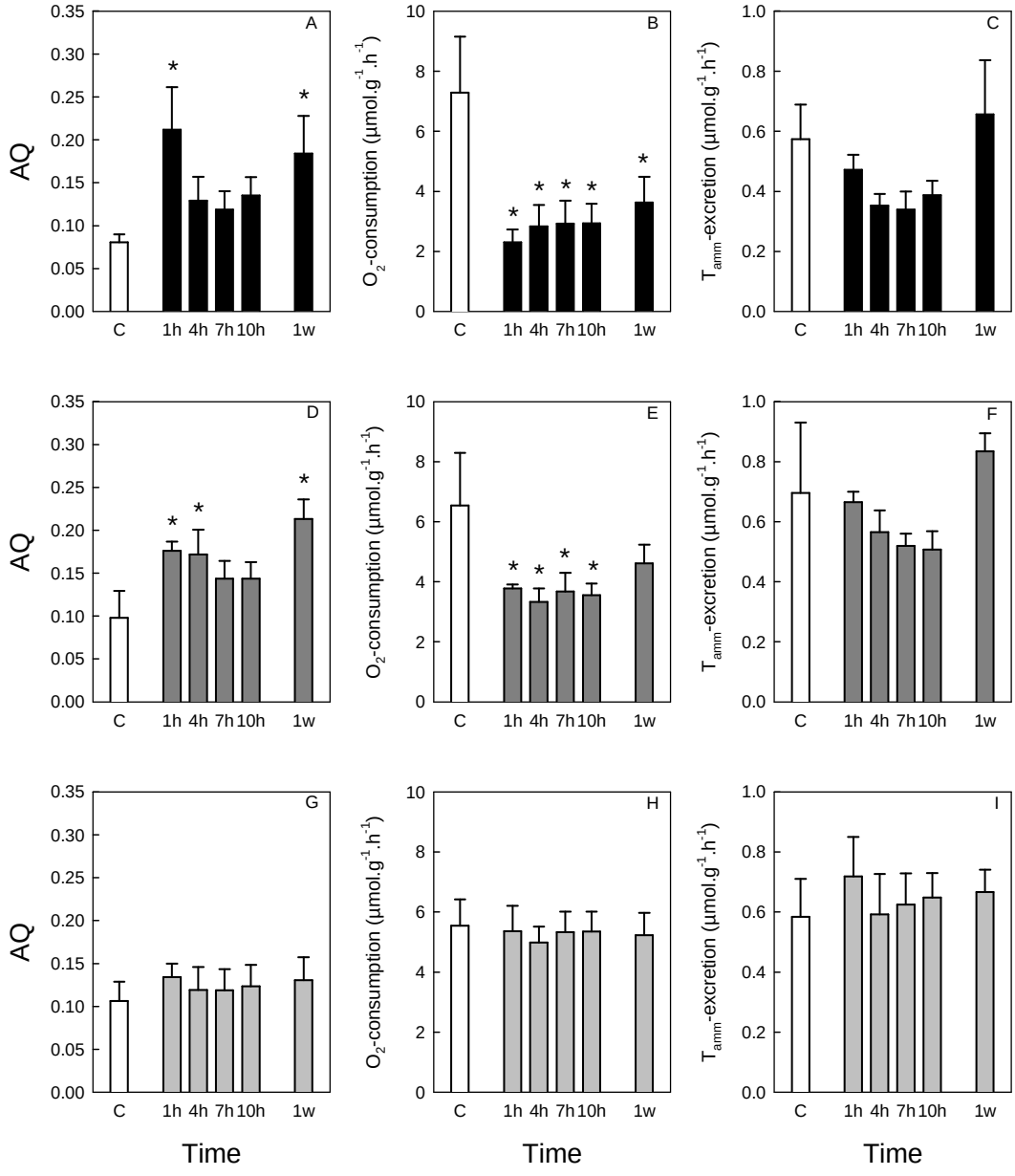

*Figure 4.2. AQ, oxygen consumption and ammonia excretion rates for carp before (C) and after 1-10 hours and one week of exposure to different copper concentrations (A-C: 0.84 µmol.l^{-1} copper; D-F: 0.34 µmol.l^{-1} copper; G-I: 0.22 µmol.l^{-1} copper). Data (N=5) are given as means ±S.D. * = P <0.05 compared to control.*

slow apathetic movements and food intake stopped. The sudden increase in the AQ during the first hours was mainly caused by a striking drop in oxygen consumption falling from 7.3±1.9 μmol.g^{-1}.h^{-1} to 2.3±0.4 μmol.g^{-1}.h^{-1} during the first hour (Fig. 4.2. B). Ammonia excretion only showed a small, non-significant decrease from 0.6±0.1 μmol.g^{-1}.h^{-1} to a minimum of 0.3±0.1 μmol.g^{-1}.h^{-1} during the seventh hour of exposure (Fig. 4.2. C). One week later, ammonia excretion had recovered to previous levels (0.7±0.2 μmol.g^{-1}.h^{-1}), whereas oxygen consumption remained significantly lower (3.6±0.9 μmol.g^{-1}.h^{-1}).

Figures 4.2. D-F show the results of exposure to copper concentrations of 0.34 μmol.l^{-1}. Here, the pattern was similar to that seen with 0.84 μmol.l^{-1}, with a smaller but still significant initial rise in the AQ of 45% (Fig. 4.2. D). Although the AQ after one week of exposure was still significantly higher than before exposure, the oxygen consumption had possibly partially recovered (Fig. 4.2. E) and was no longer significantly different from the value seen before exposure. Nevertheless, food intake and activity remained on a lower level when compared to levels before exposure. At a copper concentration of 0.22 μmol.l^{-1}, none of the measured physiological responses of the fish were significantly different from control values (Figs. 4.2. G-I), and no difference could be seen in food intake or in activity level.

To compare the AQ from fish exposed to different copper concentrations, the control value for each group was set to 100% (Fig. 4.3.). The results showed that after 1 h of copper exposure, the AQ of the three groups all differed significantly from each other, clearly indicating a dose response relationship. During the fourth hour of exposure, the AQ in the 0.34 μmol.l^{-1}-group was significantly higher than the AQ in the group exposed to 0.22 μmol.l^{-1}, while the AQ for the group of 0.84 μmol.l^{-1} was not. During the seventh hour of exposure, no significant differences were found between the three copper exposed groups. During the tenth hour of exposure, the AQ of both the 0.34 μmol.l^{-1} and 0.84 μmol.l^{-1} group were significantly higher than the AQ in the exposure group of 0.22 μmol.l^{-1}. This situation remained unchanged after one week of copper exposure.

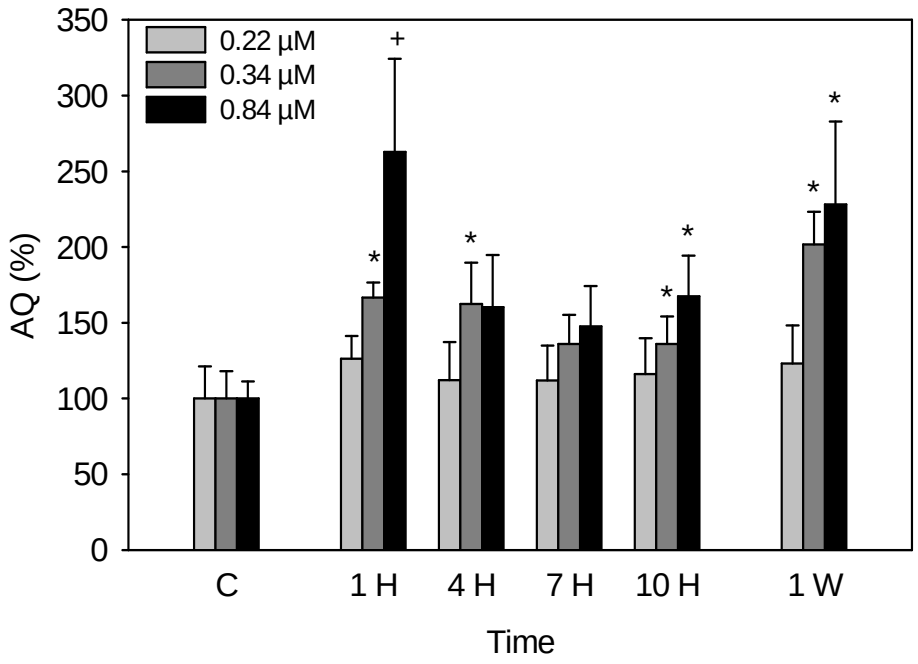

Figure 4.3. Comparison of the AQ for carp exposed to the different copper concentrations. The mean value of the controls of each exposure group was set to 100%. Data (N=5) are given as means ±S.D. ∗ indicates that results are significantly different (P<0.05) from the 0.22 µmol.l^{-1} group and + indicate that results are significantly different (P<0.05) from the 0.22 µmol.l^{-1} group and from the 0.34 µmol.l^{-1} group.

Fig. 4.4. shows the determinations of the critical oxygen concentration for oxygen consumption in common carp. Critical oxygen concentration for common carp under normal conditions was rather low, i.e. the oxygen consumption remained constant until the oxygen concentration reached 45 µmol.l^{-1}, wereupon the carp lost their ability to maintain their oxygen uptake (Fig. 4.4. A). The critical oxygen concentration was strongly affected by 1 week of exposure to the two highest copper concentrations. Thus,

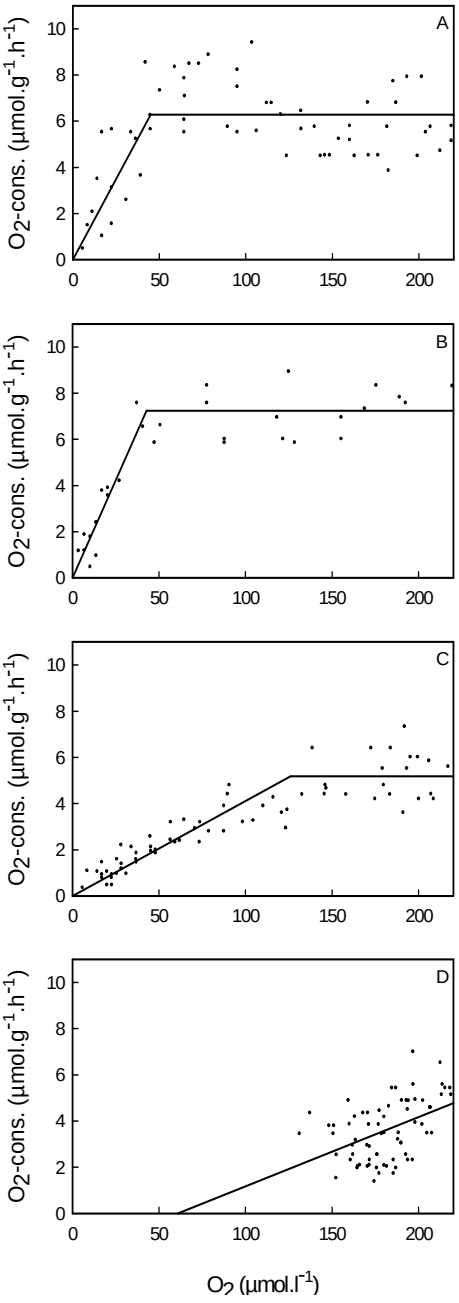

Figure 4.4. Oxygen consumption rate of carp in relation to oxygen concentration in the water after one week of exposure to copper concentration of A: 0.00 µmol.l^{-1} (C_c= 45 µmol.l^{-1}); B: 0.22 µmol.l^{-1} (C_c= 43 µmol.l^{-1}); C: 0.34 µmol.l^{-1} (C_c= 126 µmol.l^{-1}); D: 0.84 µmol.l^{-1} (C_c could not be determined).

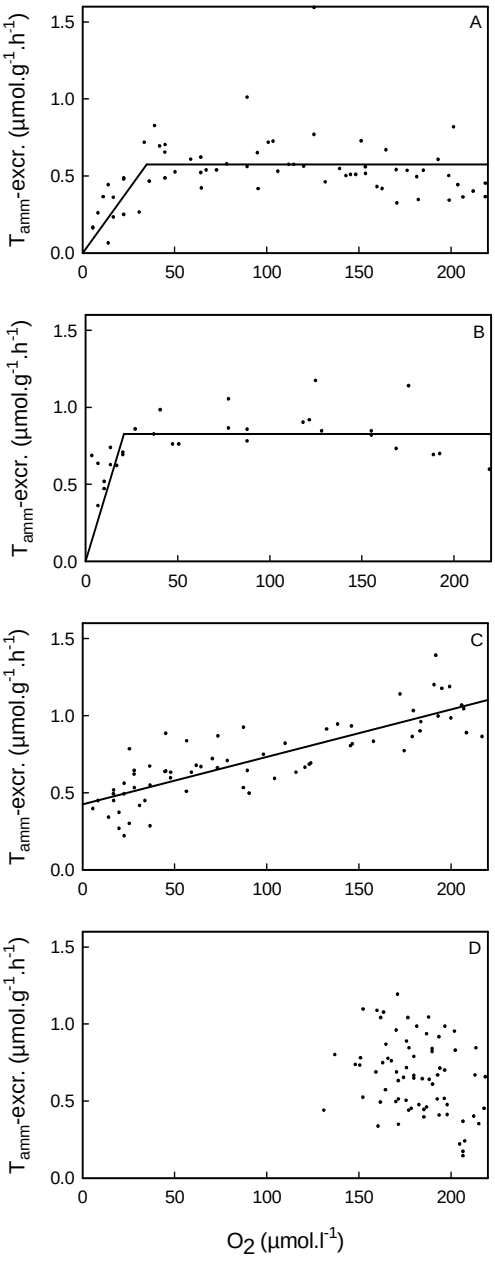

Figure 4.5. Ammonia excretion rate of carp in relation to oxygen concentration in the water after one week of exposure to copper concentration of A: 0.00 µmol.l^{-1} (C_c= 35 µmol.l^{-1}); B: 0.22 µmol.l^{-1} (C_c= 21 µmol.l^{-1}); C: 0.34 µmol.l^{-1} and D: 0.84 µmol.l^{-1} (C_c could not be determined).

for the 0.34 μmol.l^{-1}-group, a critical oxygen concentration of 126 μmol.l^{-1} was found (Fig. 4.4. C), and the influence of a copper concentration of 0.84 μmol.l^{-1} was even more severe, as no critical oxygen concentration could be determined (Fig 4.4. D). In fact, at this high copper concentration, regulation of oxygen consumption was totally lost as the water oxygen level declined. Moreover, these fish already showed difficulties in maintaining their equilibrium at rather high oxygen concentrations (125 to 150 μmol.l^{-1}). Therefore, measurements were stopped.

Ammonia excretion showed the same pattern as oxygen consumption in the copper-free water, quickly declining below an oxygen concentration of 35 μmol.l^{-1} (Fig 4.5. A). Under control conditions and at the lowest copper concentration, ammonia excretion remained constant until a critical oxygen concentration was reached. At a copper concentration of 0.34 μmol.l^{-1} ammonia excretion fell gradually in response to the declining oxygen level (Fig. 4.5. C), and at the highest copper concentration all regulation appeared to be lost (Fig 4.5. D).

Because of the similarity in oxygen consumption and ammonia excretion in the responses to falling oxygen levels, no critical oxygen concentrations were indicated in the AQ (Fig. 4.6.). Whereas in copper-free water the AQ remained stable, exposure to copper caused an increase of the AQ in function of falling oxygen concentration, the Spearman's correlation coefficients being -0.805 ($P<0.001$), -0.449 ($P<0.001$) and -0.478 ($P<0.001$) for copper concentrations of 0.22, 0.34 and 0.84 μmol.l^{-1} respectively (Fig 4.6. B-D).

4.5. Discussion

The respirometer used in this study allowed us to measure rates of oxygen consumption which were close to the standard metabolic rate for carp determined by Ultsch *et al.* (1980). The mean rate of oxygen consumption presently seen in copper-free water was 6.4±1.7 μmol.g^{-1}.h^{-1}. Ultsch *et al.* (1980) determined a standard metabolic

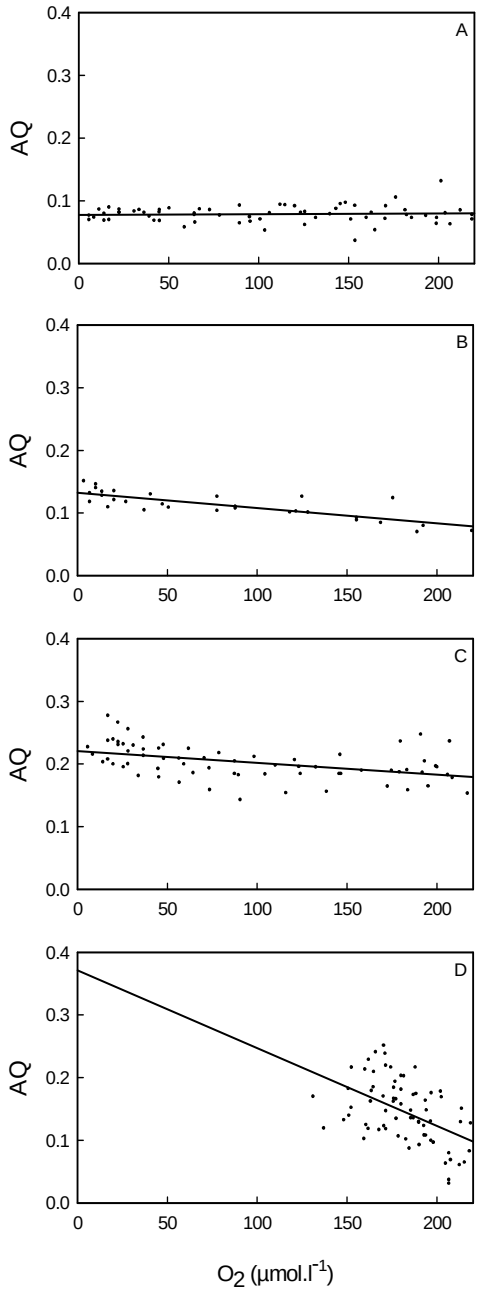

Figure 4.6. AQ of carp in relation to oxygen concentration in the water after one week of exposure to copper concentration of A: 0.00 µmol.l^{-1}; B: 0.22 µmol.l^{-1}; C: 0.34 µmol.l^{-1} and D: 0.84 µmol.l^{-1}.

rate for carp of 1.8 μmol.g^{-1}.h^{-1} and a routine metabolic rate up to 5.8 μmol.g^{-1}.h^{-1} using carps of ca 1960 g at a temperature of 15 °C. If we correct these values for weight according to the formula for carp given by Konstantinov (1981) ($Q=0.343.W^{0.85}$), and for temperature according to the Q_{10} of 2.0 for carp given by Hughes *et al*. (1983) we obtain values of 5.3 μmol.g^{-1}.h^{-1} for the standard metabolic rate and 16.8 μmol.g^{-1}.h^{-1} for the routine metabolic rate for carp of 25 g at 20°C. These results indicate that stress caused by our experimental procedure was minimal in copper-free standard water.

For mammalian tissue, the maximal theoretical AQ for a 100% aerobic protein degradation was determined to be 0.27. This value has also been used as the maximal AQ for aerobic protein metabolism in fish tissue (Kutty, 1972; Van den Thillart and Kesbeke, 1978; Van Waarde, 1983). In 1975, Kutty and Peer Mohamed suggested that for fish tissue this value could be an underestimate, and Kutty (1978) gives a maximal AQ of 0.33 for fully aerobic protein degradation in fish tissue. Based on this value, it can be estimated that the fraction of total energy use covered by protein oxidation changes from 25-32% in copper-free standard water to 65% after one week exposure to 0.34 μmol.l^{-1} of copper and after two weeks of exposure to copper levels of 0.84 μmol.l^{-1}. All presently seen AQ values remained below the maximal AQ of 0.33 suggesting that steady state conditions prevailed during the experiments.

The critical oxygen concentration for oxygen consumption determined in the copper-free water was 45 μmol.l^{-1}, which also falls into the range of critical oxygen concentrations determined by Ultsch *et al*. (1980). Furthermore, our data demonstrated for the first time that also a critical oxygen concentration for ammonia excretion exists. There are three possible mechanisms for the excretion of ammonia: passive NH_3 flux, ionic exchange of NH_4^+ for Na^+, and passive NH_4^+ flux. The most significant of these three routes is without any doubt the passive diffusion of NH_3, and this excretion is positively correlated with its partial pressure gradient (Cameron and Heisler, 1983; Randall and Wright, 1987; Wright and Wood, 1985; Randall *et al.*, 1991). The rate of ammonia release to the water is therefore closely related to the production of ammonia by the fish. The major source of ammonia in fish is protein catabolism; thus, it appears

that protein catabolism closely follows oxygen consumption in carp, and that the rate of protein breakdown rapidly falls when the oxygen concentration in the water falls below a critical level. In fact, oxygen consumption and ammonia excretion rates followed each other so closely that the AQ remained linear over the whole range of ambient oxygen levels studied, indicating that the fraction of oxygen consumption related to protein oxidation remained stable. This also suggests that the carp did not switch to an anaerobic degradation of proteins or amino acids in response to falling oxygen levels, but instead increased their use of carbohydrates as fuel. This agrees well with the generally accepted view that carbohydrates are the only major sources of fuel for energy metabolism during severe hypoxia and anoxia (Hochachka and Somero, 1984). The possible effect of ammonia accumulation in the closed respirometer can not be ignored. However, this factor is unlikely to have had any appreciable effects on the results since maximum ammonia concentrations in the respirometer remained below 10% of normal plasma concentrations in carp (Vellas and Serfaty, 1974). Moreover, an effect of the partial pressure gradient of NH_3 would be more gradual and can therefore not explain the sudden drop seen in ammonia excretion.

Exposure to copper concentrations of 0.34 $\mu mol.l^{-1}$, or higher, resulted in an immediate drop in oxygen consumption. Despite this drop in oxygen consumption, ammonia excretion rates remained stable under normoxic conditions. Although lower mean ammonia excretion rates occurred at the fourth, seventh and tenth hour of exposure to copper concentrations of 0.34 $\mu mol.l^{-1}$ and 0.84 $\mu mol.l^{-1}$, none of these differences were significant when compared with the ammonia excretion rate before exposure. Thus, although oxygen consumption is reduced by copper exposure, protein catabolism appears to remain constant, or is at least less affected, and becomes relatively more important.

Possible causes of the impaired ability of copper exposed fish to extract oxygen from the water include gill damage and secretion of mucus by the gills and body surface. At 0.84 $\mu mol.l^{-1}$, an excess of mucus could be observed. Mucus production is a general defence mechanism against metal toxicity (reviewed by McDonald and Wood, 1993).

Pärt and Lock (1983) showed that mucus not only binds metals but also significantly retards their rate of diffusion.

After one week of exposure to copper, a recovery of the oxygen consumption was observed in the group exposed to 0.34 μmol.l^{-1} copper but the AQ remained significantly higher. This recovery could indicate repair of gill tissue during this period. In a study on brook trout exposed to 75 μmol.l^{-1} of Al, Mueller *et al.* (1991) showed that gill damage was most pronounced at day four of exposure, with necrosis, hyperplastic epithelial cells separating from the basal lamina and extensive lamellar fusion, after which a considerable progressive repair of the gills occurred.

In the group exposed to 0.84 μmol.l^{-1} copper, no recovery in oxygen consumption or the AQ occurred, not even after two weeks of exposure. In addition to mucus secretion and gill damage, a third phenomena could account for a lower oxygen consumption. With increasing copper concentrations, the carp became more and more apathetic and lost appetite. Such effects may contribute to a decrease in metabolic activity.

The threshold for the effect of copper exposure on common carp measured in this investigation lies between 0.22 and 0.34 μmol.l^{-1}. Only a few previous studies have examined physiological effects of copper exposure on common carp. Effects of copper sulphate on enzyme activities have been examined at the József Attila University in Hungary where concentrations of 1 to 50 ppm of copper sulphate (6.25 to 312.5 μmol.l^{-1}) were used (Nemcsók *et al*., 1984; Asztalos, 1986; Asztalos *et al*., 1990). O'Neill (1981) showed that antibody levels of *Cyprinus carpio* and *Salmo trutta* decreased after 38 weeks of exposure to 0.29 ppm of copper (4.56 μmol.l^{-1}). Other effects were reduced hematocrits, reduced serum protein levels and weight loss.

In conclusion, with increasing copper concentrations, carp lose more and more of their ability to regulate their oxygen consumption, and the critical oxygen concentration for both oxygen consumption and ammonia excretion shifts to higher oxygen concentrations. In copper-free water, they are able to regulate their oxygen consumption until oxygen concentrations drop as low as 45 μmol.l^{-1}. At a copper concentration of 0.34 μmol.l^{-1} this has shifted to an oxygen concentration of 126 μmol.l^{-1} and at a copper

concentration of 0.84 μmol.l^{-1} the ability to regulate oxygen consumption is totally lost. This means that exposure to these copper concentrations strongly disturbed the energy metabolism of carp at oxygen concentrations which could occur in their natural environments. Thus, the characteristic ability of carp to survive under low oxygen conditions is likely to be strongly compromised in the presence of copper. As also ammonia excretion is clearly disturbed at this point, the carp are submitted to an even more demanding situation.

Our results suggest that measurements of oxygen consumption in combination with measurements of ammonia excretion can be used to assess the effects of environmental perturbations on the energy metabolism of fish. The determination of the critical oxygen concentrations for regulation of oxygen consumption and ammonia excretion provides important information on the physiological condition of the organism. Since the procedure is *in vivo* and non-invasive, the measurements can be performed repeatedly on the same animal over periods of time so that acclimation processes can be followed.

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CHAPTER V: EFFECTS OF SUBLETHAL COPPER EXPOSURE ON THE ENERGY METABOLISM OF COMMON CARP, MEASURED BY ^{31}P-NMRS*

5.1. Summary

The effects of shock and subchronic exposure (1 week) to 0.36±0.08 and 1.31±0.22 µM of copper on the energy metabolism of common carp (*Cyprinus carpio*) were studied by means of *in vivo* ^{31}P-Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMRS). During the experiments, fish were submitted to an additional hypoxic exercise and recovery from this exercise was followed during 6 hours. During all experiments ATP levels remained stable. Whereas under control conditions, levels of phosphocreatine (P_{Cr}) and inorganic phosphate (P_i) recovered fast after the exercise, no full recovery was observed after shock copper exposure. Also intracellular pH (pH_i) did not recover from the exercise after shock exposure. After one week of exposure the fish had clearly developed some tolerance towards copper. At both copper concentrations, P_{Cr} and P_i levels returned to resting levels after the exercise, but at the highest copper concentration P_{Cr}/P_i ratios were significantly lower than P_{Cr}/P_i ratios in the control group and levels of P_{Cr} and P_i were very unstable. At this high copper concentration, also pH_i was clearly decreased compared to the control group even before the exercise.

* Based on the manuscript by De Boeck, G., Borger, R., Van der Linden, A. and Blust, R., 1996. Effects of sublethal copper exposure on the energy metabolism of common carp, measured by ^{31}P-NMRS. *Environ. Toxicol. Chem.*, submitted.

5.2. Introduction

Due to human activities, trace metal levels in aquatic habitats have increased considerably during the last decades. The current rate of world-wide industrial inputs greatly exceeds the baseline burdens of trace metals in the average lake and river, with the principal sources of pollutant metals in natural waters being the discharge of domestic and industrial (including mining and smelter) wastewater and the dumping of sewage sludge. Anthropogenic inputs of copper in the aquatic ecosystems are estimated to be up to 190 10^6 kg yr^{-1} (Nriagu and Pacyna, 1988). In all of these aquatic ecosystems, sublethal concentrations of these metals may cause biochemical, physiological, morphological and genetic changes, affecting e.g. development, growth and reproduction.

In fish, the gills are the most important organs for ion regulation and oxygen uptake. The branchial epithelium forms a large and vulnerable contact area with the environment, making gills the first organs to be affected by metals (Laurén and McDonald, 1985; Mueller *et al.*, 1991; McDonald and Wood, 1993). Also the skin is in intimate contact with the environment and responds rapidly to pollutants such as copper (Iger *et al.*, 1994). Following uptake across these surfaces, physiological effects of copper, even at sublethal concentrations, are substantial. Reduction of appetite and growth under copper stress are long known effects (Benoit, 1975; Lett *et al.*, 1976; Buckley *et al.*, 1982). Also elevated plasma cortisol levels (Schreck and Lorz, 1978) and plasma glucose and ammonia levels (Laurén and McDonald, 1985; Nemcsók and Hughes, 1988) were reported. Furthermore, acetylcholinesterase activity was depressed (Nemcsók and Hughes, 1988) while lactate dehydrogenase activity was elevated (Asztalos, 1986). Besides elevated glucose levels Heath (1991) also reported depressed liver ATP levels, an effect which was still enhanced under hypoxic conditions. This indicates that both the aerobic and the anaerobic capacity of the energy metabolism were affected. In our own lab, exposing common carp to low levels of copper (0.20-0.84 µM) showed reduced growth (De Boeck *et al.*, 1996, see chapter

III), reduced oxygen consumption and higher critical oxygen concentrations, indicating a decreased capacity to cope with environmental perturbations (De Boeck *et al.*, 1995a, see chapter IV). Also substantial changes in the levels of the brain neurotransmitters serotonin and dopamine, lower locomotor activity and depressed food intake were observed (De Boeck *et al.*, 1995b, see chapter VI).

In order to further elucidate the effects of sublethal copper concentrations on the energy metabolism of common carp, this paper presents results obtained by *in vivo* ^{31}P-Nuclear Magnetic Resonance Spectroscopy (^{31}P-NMRS) of lateral muscle in copper exposed carp. *In vivo* ^{31}P-NMRS allows simultaneous and continuous measurements of the phosphorous compounds involved in the energy metabolism. Inorganic phosphate (P_i), phosphocreatine (P_{Cr}) and three phosphorous resonances of the nucleoside phosphates (NP) can be monitored in function of time in a non-invasive way. ^{31}P-NMRS has been used before to study the energy metabolism in fish muscle under normoxic, hypoxic and anoxic conditions (Van den Thillart *et al.*, 1989a; Van den Thillart and Van Waarde, 1991; Van Waarde *et al.*, 1991) and after acid exposure (Van Waarde *et al.*, 1990). So far, no ^{31}P-NMRS studies have focused on effects of chemical stressors in fish. In the mollusc *Haliotis* (Abalones) different salinities (Higashi *et al.*, 1989) and a combination of pentachlorophenol and temperature (Tjeerdema *et al.*, 1993) caused a decrease in levels of both ATP and phosphoarginine while P_i levels were elevated. Because *in vivo* ^{31}P-NMRS allows continuous measurements of the metabolites in a non-invasive way, it has the advantage that the exposed organisms can be followed over a period of time. In order to be able to evaluate the capacity of the carp to cope an additional mild exercise, a short period of hypoxia was included during the measurements and recovery was followed.

5.3. Material and methods

5.3.1. Animal holding and copper exposure

Common carp, *Cyprinus carpio*, were obtained from Vanstalle fish farm (Belgium). They were held in large tanks (800 l softened Antwerp tab water: Ca: 0.875mM, Mg: 0.145 mM, pH = 7.0-8.0) at the optimal temperature of 25°C (Elliott, 1981) for at least 2 months before use. The water was filtered with a trickling filter and its quality was checked every week with Visicolor Test Kits (Macherey-Nagel, Düren) for ammonia, nitrite and nitrate to ensure that levels never exceeded 0.1 mg l^{-1}, 1 mg l^{-1} or 20 mg l^{-1}, respectively. The carp were fed ad libitum once a day with 'Pond Sticks' (Tetrapond, Henckel).

Three weeks before starting the experiments, 20 carp weighing 80±6 g were selected and transferred to two 150 l aquaria filled with standard moderately hard water (STW) according to Standard Methods (American Public Health Association, 1989: $CaSO_4.2H_2O$: 0.348 mM; $MgSO_4.2H_2O$: 0.500 mM; $NaHCO_3$: 1.143 mM; KCl: 0.054 mM; pH: 7.4-7.8). The STW was well aerated for at least 24 hours before use. During the first week the temperature in the aquaria was gradually adjusted to 20°C (± 1°C) and the photoperiod was set at a 14 hour light period, 10 hour dark period. Carp were again fed ad libitum once a day with 'Pond Sticks' and water was filtered with trickling filters and partly (50%) renewed twice a week. Following this procedure levels of excretory products did never exceed levels mentioned above and pH was 7.7 ±0.1 for all groups.

Copper was added to the STW as a copper nitrate standard solution (Merck, 1 g l^{-1} $Cu(NO_3)_2.2H_2O$) and the water was aerated and mixed for at least 24 hours before use. During copper exposure, all activated charcoal was removed from the filters. After equilibration the addition of 1 or 4 µM of copper resulted in concentrations in the aquaria of 0.36±0.08 and 1.31±0.22 µM of copper respectively. Copper

measurements were made using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAS 703 and HGA 500).

5.3.2. ^{31}P-NMR spectroscopy

In vivo ^{31}P-NMR spectra of carp muscle were obtained on a SMIS NMR instrument (Gildford, U.K.), with a field strength of 7 tesla, an horizontal accessible bore of 8 cm diameter and shielded gradients with a strength of 100 mT/m. The signal of the fish muscle was picked up with a surface coil of 20 mm diameter, which was double tuned to the hydrogen and phosphorous frequencies (300 MHz and 121 MHz, respectively). A glass capillary tube filled with a solution of methylenediphosphonate (MDP) was mounted on the surface of the coil, opposite to the fish and served as standard. For time course studies, free induction decay (FID) signals were acquired under the following conditions: a 60° pulse of 100 µs, a 10 kHz spectral width, a 3750 ms repetition time, and 2048 sample points. Each spectrum was averaged over 48 scans, which resulted in a total acquisition time of 3 minutes. The B_0 field was optimised by shimming on the ^{1}H (proton) signal until the full width at half maximum of the ^{1}H signal was less than 0.2 ppm. In this set of experiments, relative (compared to the external standard (MDP)) quantification was obtained using peak integration since the peak area is directly proportional to the concentration of the chemical compound (Van Waarde and Van den Thillart, 1994). Thus, the levels of P_i, P_{Cr} and NP in fish could be monitored by determining the relative resonances intensity (RRI). The intracellular pH (pH_i) was calculated from the difference in chemical shift between P_i and P_{Cr} as standardised by Van den Thillart *et al.* (1989a). Since pH_i could only be determined when a clear P_i peak was observed, and since under normoxic steady state conditions the P_i peak was barely visible, extrapolations were made for the missing data of individual fish.

5.3.3. Experimental setup

Carp were anaesthetised untill loss of equilibrium with 100 mg l^{-1} of MS-222 and mounted in a flow cell, which was a slightly modified version of the one described by Van den Thillart et al. (1989b). The animal was pressed gently against the flat side of the flow cell and immobilised by an inflatable plastic bag filled with water. The flow cell was mounted horizontally in the bore of the spectrometer and the fish remained immobilised during the entire measuring period. A constant irrigation of the gills (500 ml/min) was realised with experimental water provided by one out of two aquaria near the NMR spectrometer. Both aquaria contained different experimental waters; one was filled with copper free the STW, the other one contained STW with the appropriate copper concentration. A switch between experimental waters could easily be made by a control valve. Since experimental water contained no anaesthetics the fish woke up rapidly and remained conscious during the entire experiment. Handling of carp prior to experimentation revealed an increase in P_i, a decrease in P_{Cr} levels and a drop in pH_i. It has been demonstrated before that fish could recover from handling stress when allowed to remain for a few hours in aerated water conditions (Van den Thillart *et al.*, 1989b). Experiments were started when effects of handling stress had disappeared according to the maximal P_{Cr} resonance, the minimum P_i resonance, and the stable pH_i.

During a first two hour period, ^{31}P-NMR spectra were acquired under fully aerated conditions. When exposed to a copper shock, the switch to the copper containing water was made after the first hour of NMR acquisition. Following this two hour period, the fish were submitted to a short intermitted period of hypoxia obtained by bubbling the medium with nitrogen. This period of hypoxia created an extra load on the energy metabolism and was considered as an exercise. Oxygen content of the water dropped from 9.3-9.2 mg l^{-1} to 1.1-1.0 mg l^{-1} within 10 minutes. The common carp is known to withstand short periods of hypoxia rather well (Smith and Heath, 1980; Van den Thillart and Van Waarde, 1985) and these periods of hypoxia represent only a

minor challenge to the energy metabolism of the fish. The hypoxia period to which each individual carp was exposed lasted until the P_{Cr} resonance dropped with 50%. This procedure ensured an equivalent moderate load on the energy metabolism to all the individuals used. Hereafter the carp were allowed to recover from the hypoxia stress under fully aerobic conditions and the NMRS acquisition proceeded up to six hours after the hypoxia was ended.

Five groups of fish were studied: a 'control group' which remained in the STW during the entire experiment, two 'copper shock exposure groups' in which the switch to copper containing water was made after the first hour of observations (0.36 or 1.31 µM of copper respectively), and two 'subchronic copper exposure groups' which had been in copper containing STW at the same copper concentrations for one week before being measured.

5.3.4. Data processing

In this study the phosphorous compound levels of ATP, P_{Cr} and P_i were expressed as peak areas after the spectra had been normalised using the external standard (MDP) as reference peak. Also P_{Cr}/P_i ratios were calculated as an indication of the use of P_{Cr} pool to stabilise the ATP level. Indeed, when ATP is used ($ATP \leftrightarrow ADP+P_i$), the creatine kinase equilibrium shifts to ATP formation ($P_{Cr}+ADP+H^+ \leftrightarrow Cr+ATP$) resulting in a net reaction of $P_{Cr}+H^+ \rightarrow Cr+P_i$.

To analyse the results we have determined resting levels before hypoxia, maximal deviations during hypoxia and mean levels after hypoxia for the phosphorous compounds and for the pH_i. The resting levels are the means of the values obtained before the hypoxia load is induced. The maximal deviation refers to the minimal (P_{Cr}, P_{Cr}/P_i) or maximal (P_i) value that was measured due to the hypoxia load. The levels after hypoxia are the means of the values during recovery from hypoxia, calculated for every hour starting from the moment that the hypoxia load was ended until six hours later. Furthermore, we looked at the percentage of recovery of the phosphorous

compounds. For P_{Cr} and P_i, this is calculated from the RRI prior and after hypoxia. The resting level is considered equal to 100% and the maximal deviation equal to 0%. Thus the level of recovery is defined as follows :

$$L_R = \frac{A - M}{R - M} \cdot 100\%$$

(L_R = level of recovery, A = level after hypoxia, R = resting level and M = maximal deviation caused by hypoxia).

5.3.5. Statistics

Statistics were performed using Statistica for Windows (StatSoft, Microsoft) using two-way analysis of variance with repeated measurements and results were considered significant when $P<0.05$, $P<0.01$ and $P<0.001$. Post hoc comparisons were made using the least significant difference test for planned comparisons. Homogeneity of variances was tested using the univariate test.

5.4. Results

5.4.1. Changes in ATP content of carp muscle

Levels of ATP did not differ between the different exposure groups (RRI: 1.43±0.17) and remained at a constant level throughout the experiments. Since the hypoxia was chosen to be a mild exercise, it never caused ATP levels to drop, not even in the fish exposed to the highest copper concentration before, during or after hypoxia.

5.4.2. Changes in P_{Cr} content of carp muscle

In the control group, normal RRI levels of P_{Cr} were 3.44±0.03 (Fig. 5.1.a). Levels of P_{Cr} remained constant during the two hours of continuous measurements

before the hypoxic exercise was initiated. The hypoxic exercise lasted until P_{Cr} levels dropped with about 50% (e.g. the maximal deviation for the control group was 1.82±0.16) whereafter fish were allowed to recover in air saturated water. Recovery in the control group was very fast, during the first hour a recovery of 82% was reached (Table 1) and full recovery was attained during the second hour after the exercise (recovery 100%).

For the copper shock exposure groups, initial levels of P_{Cr} and maximal deviations were similar, but the recovery after the hypoxia exercise differed substantially (Fig. 5.1.b and 5.1.d). Immediately after exposure to the lowest copper concentration, recovery from the hypoxic exercise was greatly impaired, with only 19% recovery during the first hour following hypoxia, and no more than 46% recovery during the next five hours. Although the P_{Cr} levels during the entire recovery period were significantly reduced compared to the resting levels before hypoxia ($P<0.001$) and thus no full recovery occurred, recovery levels differed from the corresponding control group levels only during the first hour after hypoxia ($P<0.05$). This is probably caused by the results of one fish who had very high P_{Cr} levels during the entire experiment (clearly shown in fig. 5.1.b), even though ATP and P_i levels were normal. The P_{Cr} levels of this fish also showed a higher rate of recovery compared to the other fish in the low copper shock exposure group. After one week of exposure to this low copper concentration, fish appear to have acclimated and show, as in the control group, a full recovery from the hypoxia (97%) after two hours as far as P_{Cr} is concerned (Fig. 5.1.c).

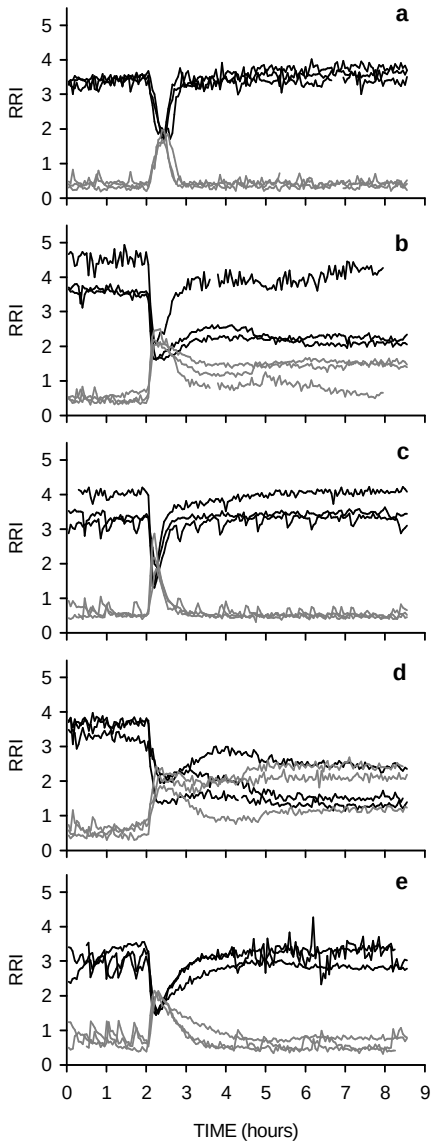

Figure 5.1. Relative resonance intensities (RRI) of P_{Cr} (black lines) and P_i (grey lines) measured in lateral muscle of common carp. After two hours the hypoxic exercise started until P_{Cr} levels dropped with 50% and recovery of the carp in fully air saturated water was followed for 6 more hours. Each graph presents data from 3 individuals: a) control group; b) low copper shock exposure group; c) low subchronic copper exposure group; d) high copper shock exposure group; e) high subchronic copper exposure group.

Although this acclimation occurred, their was no significant difference between the low copper shock exposure group and the low subchronic copper exposure group, again caused by this one aberrant fish.

In the high copper shock exposure group, P_{Cr} levels of the fish did not recover at all and remained significantly lower ($P<0.001$) than the resting levels during the entire measuring period (Fig. 5.1.d). After a first very small recovery during the first two hours after hypoxia (no more than 14% recovery, $P<0.05$ compared to control group), P_{Cr} levels even dropped to 13% below the maximal deviation level obtained during the hypoxic exercise ($P<0.01$ compared to control group). Thus, a further decline of their condition was observed. However, no mortalities were observed during the experiment, and after one week of exposure to the high copper concentration, resting RRI levels of P_{Cr} (3.11 ± 0.29) were comparable to the resting levels of the control group (Fig. 5.1.e). Resting RRI levels of the two subchronic copper exposure groups were not very stable compared to the resting RRI levels of the control group and, certainly at the highest copper concentration, drops in P_{Cr} levels were observed regularly (Fig. 5.1.e). When we tested the homogeneity of variances over this first two hours of measurements, both the group exposed to the low as well as the group exposed to the high copper concentration showed a significantly higher variation ($P<0.05$ and $P<0.001$ respectively). Except for the first hour, where the recovery level of the P_{Cr} was still significantly lower than the recovery level of the control group ($P<0.05$) fish exposed subchronically to the high copper level, had apparently also regained the capacity to recover from the hypoxic exercise. Full recovery to resting levels occurred starting from the second hour after hypoxia (95%). From the third hour on, recovery levels were significantly higher than in the high copper shock exposure group, indicating that some form of acclimation had occurred.

*Table 5.1. Mean values and recovery levels after hypoxia of PCr in white muscle of common carp immedately after exposure to a low (Cu: 0.36 µM) or high (Cu: 1.31 µM) copper shock and mean values and recovery levels after one week of exposure to these coppere concentrations (n=3; *: mean values are significantly lower than resting level in the same group; •: mean values are significantly lower than corresponding values in control group; °: mean values are significantly higher than corresponding values in the shock group at the same copper concentration; *: p<0.05; **: p<0.01; ***: p<0.001).*

	RESTING LEVEL	MAXIMAL DEVIATION	1ST HOUR	2ND HOUR	3TH HOUR	4TH HOUR	5TH HOUR	6TH HOUR
CONTROL								
Mean (RRI)	3.44±0.03	1.82±0.16	3.17±0.21	3.44±0.11	3.54±0.17	3.54±0.19	3.61±0.18	3.58±0.20
Recovery level (%)	100	0	82	100	106	106	110	108
Statistics			***					
LOW SHOCK								
Mean (RRI)	3.88±0.57	1.91±0.22	2.34±0.73	2.89±0.90	2.82±0.88	2.75±0.99	2.81±1.12	2.85±0.18
Recovery level (%)	100	0	19	46	43	39	42	43
Statistics			***; •	***	***	***	***	***
LOW WEEK								
Mean (RRI)	3.52±0.45	1.66±0.32	2.96±0.31	3.44±0.31	3.54±0.36	3.60±0.40	3.61±0.39	3.62±0.40
Recovery level (%)	100	0	71	97	102	105	106	107
Statistics			***					
HIGH SHOCK								
Mean (RRI)	3.53±0.25	1.92±0.25	2.05±0.53	2.17±0.64	1.93±0.61	1.78±0.62	1.76±0.62	1.73±0.58
Recovery level (%)	100	0	7	14	-1	-11	-12	-13
Statistics			***; •	***; •	***; ••	***; ••	***; ••	***; ••
HIGH WEEK								
Mean (RRI)	3.11±0.29	1.60±0.09	2.29±0.23	2.99±0.26	3.10±0.15	3.17±0.24	3.11±0.24	3.16±0.31
Recovery level (%)	100	0	48	95	101	107	103	107
Statistics			***; •		°	°	°	°

*Table 5.2. Mean values and recovery levels after hypoxia of Pi in white muscle of common carp immedately after exposure to a low (Cu: 0.36 µM) or high (Cu: 1.31 µM) copper shock and mean values and recovery levels after one week of exposure to these coppere concentrations (n=3; *: mean values are significantly lower than resting level in the same group; •: mean values are significantly lower than corresponding values in control group; °: mean values are significantly higher than corresponding values in the shock group at the same copper concentration; *: p<0.05; **: p<0.01; ***: p<0.001).*

	RESTING LEVEL	MAXIMAL DEVIATION	1ST HOUR	2ND HOUR	3TH HOUR	4TH HOUR	5TH HOUR	6TH HOUR
CONTROL								
Mean (RRI)	0.38±0.07	1.81±0.18	0.53±0.09	0.40±0.05	0.41±0.08	0.39±0.05	0.40±0.06	0.40±0.06
Recovery level (%)	100	0	89	99	98	99	99	98
Statistics			***					
LOW SHOCK								
Mean (RRI)	0.49±0.13	2.20±0.22	1.74±0.13	1.18±0.32	1.25±0.26	1.31±0.35	1.23±0.45	1.20±0.53
Recovery level (%)	100	0	25	58	54	50	54	55
Statistics			***; •••	***; •	***; •	***; ••	***; •	***; •
LOW WEEK								
Mean (RRI)	0.55±0.06	2.18±0.49	1.00±0.15	0.55±0.05	0.51±0.03	0.51±0.02	0.52±0.03	0.50±0.02
Recovery level (%)	100	0	73	101	102	102	102	103
Statistics			***; •. °	°	°	°	°	°
HIGH SHOCK								
Mean (RRI)	0.58±0.13	2.01±0.20	1.79±0.46	1.63±0.63	1.82±0.67	1.89±0.69	1.91±0.66	1.92±0.64
Recovery level (%)	100	0	16	28	14	10	8	7
Statistics	•		***; •••	***; ••	***; •••	***; •••	***; •••	***; •••
HIGH WEEK								
Mean (RRI)	0.78±0.17	2.10±0.06	1.48±0.22	0.79±0.27	0.71±0.10	0.68±0.14	0.72±0.16	0.66±0.17
Recovery level (%)	100	0	49	101	107	110	107	111
Statistics	•••; °°		***; •••	°	°°	°°	°°	°°

5.4.3. Changes in P_i content of carp muscle

Changes in the RRI levels of P_i are similar but opposite to the changes in P_{Cr} RRI levels (Fig. 5.1.a-e). As with P_{Cr} RRI levels in the control group, recovery from the hypoxic exercise was already complete during the second hour (Table 2; 99%). The effects of the shock exposure on P_i RRI levels were slightly smaller than the effects on P_{Cr} RRI levels with recovery levels of 25% during the first hour and between 50 and 58% during the next hours. P_i levels in the low exposure group were significantly higher during the entire recovery period ($P<0.001$ during the first hour, $P<0.05$ or $P<0.01$ during the next hours) since none of the fish showed to have irregular P_i values (Fig. 5.1.b). After one week of exposure to the low copper concentration, again acclimation appeared to have occurred and recovery from the hypoxia was already complete during the second hour. Still, during the first hour, P_i levels were significantly higher than in the control group. The acclimation was also reflected in the fact that P_i levels of the low subchronic copper exposure group were significantly lower than those of the low copper shock exposure group, and this during the entire recovery period (Table 2, $P<0.01$ during the first hour, $P<0.05$ during the next hours).

Shock exposure to the high copper concentration again caused very profound effects. P_i levels remained very high ($P<0.001$) compared to resting levels during the entire recovery period. Also compared to the recovery levels in the control group, P_i levels remained significantly higher ($P<0.001$ exept during the second hour where $P<0.01$). Although after one week of exposure to this higher copper concentration some form of acclimation could be observed, resting RRI levels of P_i were still significantly elevated compared to control levels (0.78 ± 0.17, $P<0.001$) and P_i levels showed a very irregular and unstable pattern. Exept for the first hour of recovery, where P_i was almost three times as high as in the control group ($P<0.001$), P_i levels returned to the resting levels (101% during the second hour) and were no longer significantly higher than levels in the control group. Starting from the second hour of

recovery, P_i levels were also significantly lower than P_i levels in the copper shock exposure group at the same copper concentration ($P<0.001$ except during the second hour where $P<0.01$) and thus some form of acclimation had occurred.

Resting P_i levels were comparable in all groups except for the subchronic exposure group at the high copper concentration. Small elevations were found in P_i resting levels in the low subchronic copper exposure group and the high copper shock exposure group ($P<0.05$), but P_i resting levels in the high subchronic exposure group were clearly elevated ($P<0.001$). Also the variation in P_i resting levels of the groups which had been exposed to copper subchronically differed significantly from the variation in the resting levels of the control group ($P<0.001$ for both groups).

5.4.4. Changes in P_{Cr}/P_i content of carp muscle

More pronounced effects appeared when the P_{Cr}/P_i ratios were calculated (Table 3). For the control group, recovery of the P_{Cr}/P_i ratio following the hypoxic exercise was again complete after the first hour. As for P_{Cr} and P_i for the low copper shock exposure group, the P_{Cr}/P_i ratio did not recover from the hypoxic exercise compared to the resting level during the measuring period ($P<0.001$), and remained also significantly lower than levels of the control group during this entire period ($P<0.001$ except during the 5th hour where $P<0.01$).

After one week of exposure to the lowest copper concentration, P_{Cr}/P_i ratios recovered after one hour compared to the resting level, but remained significantly lower than control levels for the first four hours ($P<0.001$ during the first hour, $P<0.05$ for the next three hours). The recovery after hypoxia in the low subchronic copper exposure group was significantly better than in the low copper shock group ($P<0.01$ or $P<0.001$), indicating that the fish had partially acclimated to the exposure conditions.

*Table 5.3: Mean values and recovery levels after hypoxia of PCr/Pi ratios in white muscle of common carp immedately after exposure to a low (Cu: 0.36 µM) or high (Cu: 1.31 µM) copper shock and mean values and recovery levels after one week of exposure to these coppere concentrations (n=3; *: mean values are significantly lower than resting level in the same group; •: mean values are significantly lower than corresponding values in control group; °: mean values are significantly higher than corresponding values in the shock group at the same copper concentration; *: p<0.05; **: p<0.01; ***: p<0.001).*

	RESTING LEVEL	MAXIMAL DEVIATION	1ST HOUR	2ND HOUR	3TH HOUR	4TH HOUR	5TH HOUR	6TH HOUR
CONTROL								
Mean (RRI)	9.69±2.10	1.01±0.13	7.28±1.11	9.19±1.17	9.18±1.84	9.51±0.72	9.54±1.45	9.26±0.96
Recovery level (%)	100	0	73	96	95	100	99	97
Statistics			***					
LOW SHOCK								
Mean (RRI)	8.50±2.47	0.87±0.04	1.53±0.75	2.75±1.67	2.48±1.40	2.41±1.67	2.91±2.49	3.35±3.30
Recovery level (%)	100	0	7	22	19	19	24	28
Statistics			***; •••	***; •••	***; •••	***; •••	***; ••	***; •••
LOW WEEK								
Mean (RRI)	6.65±1.38	0.80±0.31	3.91±0.74	6.49±0.61	7.09±0.72	7.18±0.96	7.15±0.85	7.39±0.89
Recovery level (%)	100	0	54	100	110	111	111	115
Statistics			***; •••; °°	•; °°	•; °°	•; °°°	°°	°°°
HIGH SHOCK								
Mean (RRI)	6.57±2.11	0.97±0.17	1.30±0.71	1.68±1.32	1.32±1.03	1.16±0.92	1.12±0.87	1.07±0.79
Recovery level (%)	100	0	4	9	3	1	0	0
Statistics			***; •••	***; •••	***; •••	***; •••	***; •••	***; •••
HIGH WEEK								
Mean (RRI)	4.35±1.42	0.76±0.07	1.81±0.52	4.16±1.45	4.60±0.90	4.97±1.35	4.64±1.33	5.13±1.61
Recovery level (%)	100	0	35	112	122	136	125	143
Statistics	•••		***; •••	•••; °	•••; °	•••; °°	•; °	•••; °°°

The same pattern could be observed at the high copper concentration. For the copper shock exposure group, P_{Cr}/P_i ratios were significantly lower than resting levels and levels of the control group after the hypoxic exercise ($P<0.001$ during the entire recovery period). After one week of exposure, resting levels of P_{Cr}/P_i ratio were significantly lower than P_{Cr}/P_i ratio of the control group, indicating that energy supply was compromised, even without an additional exercise. Although some acclimation seemed to have occurred compared to the shock exposure after one week of exposure ($P<0.05$ except during the last hour where $P<0.001$), P_{Cr}/P_i ratios remained significantly lower than control group levels ($P<0.001$ except for the 5th hour were $P<0.05$) after the hypoxic exercise.

5.4.5. Changes in intracellular pH

Figure 5.2. shows the changes in pH_i of the different exposure groups during the experiments. Resting pH_i level of the control group was 7.26±0.03 and resting levels of the copper shock exposure groups before the exercise were very similar (7.29±0.02 and 7.24±0.03). For the group exposed for one week to the low copper concentration, mean resting pH_i levels were slightly decreased (7.17±0.05), but for the group exposed for one week to the high copper concentration mean resting pH_i levels were significantly lower (6.89±0.05, $P<0.001$). Under control conditions, pH_i declined quickly during the hypoxic exercise and continued to drop for a while after reoxygenation of the water had occurred so that the minimum level was reached 6 to 9 minutes after reoxygenation of the water (6.87 for the control group). After this, pH_i recovered rapidly and one hour after the hypoxic exercise pH_i was fully recovered to 7.26. The same trend was observed for the fish exposed to the low copper concentration for one week, with a full recovery after the first hour. Although the levels remained slightly lower than in the control group, this difference was not significant. In the copper shock exposure groups, no recovery of the pH_i was observed during the measuring period ($P<0.001$ for the whole recovery period). For the low

copper shock exposure group a minimum level (6.85) was reached 45 minutes after reoxygenation whereafter a stabilisation occured. For the high copper shock exposure group pH_i continuously dropped with a minimum level of 6.72 during the last hour measured. The group exposed to the high copper concentration for a week, showed a recovery to resting levels two hours after the exercise although the pattern remained very irregular with values between 6.71 and 6.99 and clearly below the levels of the control group ($P<0.01$ compared to control group during the 1th, 3th, 4th and 5th hour of recovery; $P<0.001$ during the 2nd hour and $P<0.05$ during the last hour).

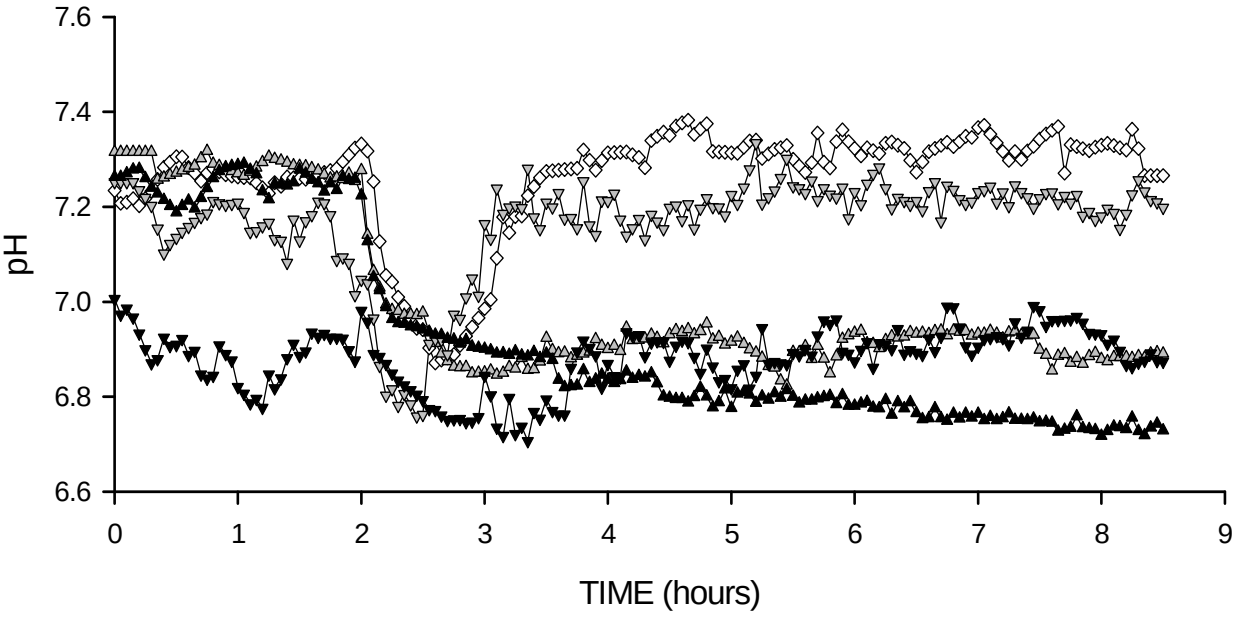

Figure 5.2. Mean pH_i values in lateral muscle of common carp for each of the experimental groups: ◊: control group, Δ: low copper shock exposure group, ∇: low subchronic copper exposure group, Δ: high copper shock exposure group, ∇: high subchronic copper exposure group.

5.5. Discussion

The stability of the ATP levels during these experiments reflects the importance of this high energy compound in the energy metabolism of living organisms. Van den Thillard and Van Waarde (1993) found that for goldfish, a P_{Cr} drop of 70% was necessary to cause a significant drop in ATP levels, and for common carp phosphagen energy stores had to be exhausted by more than 85% (Van Waarde and Van den Thillard, 1994). These values are considered to be indicative for the limits of anoxia resistance. Since in our experiments, P_{Cr} levels always remained well above these limiting values, the buffering capacity of the P_{Cr} pool on the ATP level due to the activity of creatine kinase was never exceeded and thus ATP levels remained stable.

Under control conditions, resting RRI levels of P_{Cr} remained continuously high and resting RRI levels of P_i were very low, indicating the high cytosolic phosphorylation potential (the index of the energy status of a cell in terms of potential transferable phosphate groups calculared as $ATP/(ADP.P_i)$). Shock exposure to copper alone did not appear to destabilise these resting levels and P_{Cr} and P_i remained at their original level after the switch to copper containing water. A mild additional exercise was necessary to show the profound effects caused by the copper shock. After one week of exposure however, differences could be observed even without the exercise: P_{Cr} and P_i RRI levels were no longer constant and at the high copper concentration P_{Cr}/P_i ratios were reduced compared to those of the control group. This lower ratio denotes a reduced cytosolic phosphorylation potential, even when fish were at rest, and thus a lowered capacity of these animals to cope with additional environmental perturbations.

The events taking place in white carp muscle during hypoxia have been described extensively before (Van den Thillart and Van Waarde, 1993). In short, ATP hydrolysis, phophagen depletion and anaerobic glycolysis occur. The absence of sufficient oxygen will cause free ADP and AMP levels together with P_i levels to rise due to ATP hydrolysis. An increase of P_i and AMP levels will then stimulate

glycolysis, resulting in acidosis. The acidosis together with the elevated levels of free ADP will shift the creatine kinase equilibrium towards P_{Cr} hydrolysis. Through the coupling of acidotic glycolysis and the alkalotic P_{Cr} hydrolysis, acidotic effects of lactate production are partially compensated. After reoxygenation, the fast reactions of mitochondrial respiration cause a rapid drop of free ADP, which shifts the creatine kinase equilibrium back to P_{Cr} synthesis despite the low pH_i. This acidotic reaction induces the pH_i to drop a little further before lactate and H^+ elimination and lactate oxidation restore pH_i values to control levels. Basically, this is what has been shown in the control group: a rapid decline in P_{Cr} levels accompanied by an increase of P_i and a rapid decrease of pH_i. When reoxygenation occurred, P_{Cr} and P_i quickly restored to the resting levels and pH_i showed a small delay and recuperated only after an initial further decline.

Although after shock exposure to copper apparently nothing appeared to happen before the exercise, recovery from the mild hypoxia exercise was compromised. P_{Cr} and P_i levels did not at all recover to resting levels during the measuring period, showing a severe reduction of the cytosolic phosphorylation potential.

Several possible mechanisms of metal toxicity could be involved here. First, a direct effect of copper on the activity of creatine kinase could cause these effects. In a previous study using similar copper concentrations (De Boeck *et al.*, 1995b, see chapter VI), copper accumulation was only observed in liver, not in brain or white muscle and also other studies with copper exposed fish showed that muscle does not accumulate much copper (Sörensen, 1991). Furthermore, copper accumulation in tissues requires time, while the effects observed here appear already one hour after copper exposure. This indicates that a direct effect of copper on this mechanism can only be of minor importance. More likely, the low cytosolic phosphorylation potential is a secondary effect, caused in consequence of other effects of copper toxicity.

One of the first lines of defence against metal exposure is secretion of mucus by the gills. There is general agreement that production of mucus is an important mechanism for protecting gill tissues from toxic metals (McDonald and Wood, 1993),

not only by binding the metals, but also by retarding their rate of diffusion (Pärt and Lock, 1983). Besides retarding the diffusion rate of copper through the gills, also the oxygen diffusion rate will be lowered. Earlier experiments with common carp (De Boeck *et al.*, 1995a, see chapter IV) showed that at a copper concentration of 0.34 µM the critical oxygen concentration for oxygen consumption was almost three times as high as under control conditions (respectively 126 µM and 45 µM O_2). At 0.84 µM of copper the capacity to regulate oxygen consumption was lost and no critical oxygen concentration could be determined. Furthermore, oxygen consumption during the first ten hours after exposure dropped dramatically, even at normoxic oxygen concentrations. In another study we showed a significant rise in plasma lactate after one week of exposure to copper concentrations of 0.55 and 0.80 µM (De Boeck *et al.*, 1995b, see chapter VI). These results clearly indicate the lowered capacity of common carp for oxygen uptake under these conditions, creating anaerobic conditions in muscle tissue. Apparently, after shock exposure, fish were still able to cover their energy expenses aerobically during the resting phase since P_{Cr} levels and pH_i remained stable before the hypoxic exercise. The mild exercise, which under control conditions resulted only in a short disturbance of the aerobic metabolism followed by a fast recovery, showed that muscle tissue remained hypoxic, with a low pH_i and only a partial or no recovery of P_{Cr} and P_i levels.

After one week of exposure, carp at the lowest copper concentration appeared to have developed a tolerance to copper. The cytosolic phosphorylation potential of the muscle tissue was restored and pH_i, P_{Cr} and P_i levels, although slightly less stable than in the control group, quickly recovered to resting levels after the exercise. Only P_{Cr}/P_i ratios took a significantly longer time to recover to levels of the control group. At 0.34 µM of copper, also oxygen consumption was reported recovered at least partially after one week of copper exposure (De Boeck *et al.*, 1995a, see chapter IV). If fish survive the initial shock phase of metal exposure, a phase of compensation and repair follows even in the continued presence of the metal. At least three mechanisms are believed to contribute to this phenomenon: 1) alterations to the barrier properties of the tissue that

act to decrease the net rate of metal entry, 2) an increase in the storage and detoxification of the metal once it has entered the fish, and 3) an increase in the resistance of metal-sensitive processes like ion transport mechanisms (McDonald and Wood, 1993). It is clear that at the high copper concentration however, the development of this tolerance is not sufficient, or at least not yet complete resulting in a low pH_i and P_{Cr}/P_i ratio. At 0.84 µM, no recovery of oxygen consumption was observed either (De Boeck *et al*., 1995a, see chapter IV).

In short, ^{31}P-NMRS in combination with a mild hypoxic exercise showed to be a useful tool to study the effects of copper exposure on the energy metabolism of common carp. The effect on the utilisation of aerobic or anaerobic pathways could be evaluated, and processes of recovery could be followed in a non-invasive *in vivo* way. Shock exposure to copper greatly reduced the cytosolic phosphorylation potential and although after one week some acclimation to the metal was obvious, aerobic capacities of the energy metabolism were still reduced.

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CHAPTER VI: BRAIN MONOAMINE LEVELS AND ENERGY STATUS IN COMMON CARP (*CYPRINUS CARPIO*) AFTER EXPOSURE TO SUBLETHAL LEVELS OF COPPER [*]

6.1. Summary

Serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) are two major monoamine neurotransmitters with a multitude of functions in the vertebrate brain. In fish, the 5-HT system has been shown to be sensitive to various forms of stress, but very few studies have examined the effects of toxic metals on these monoamine systems.

Juvenile common carp were exposed to copper levels of 0.22, 0.34 and 0.84 μM during one week. In telencephalon, dose dependent falls in 5-HT and DA levels were observed, with approximately 50% losses of these neurotransmitters at the highest copper concentration. Although less dramatic, falls were also seen in 5-HT and DA levels in hypothalamus and brain stem. No changes in either 5-hydroxyindoleacetic acid (5-HIAA, the main 5-HT metabolite), AMP, ADP, ATP, adenylate energy charge or lactate levels were observed in brain. However, lactate levels in blood plasma increased with copper concentration. A significant copper accumulation only occurred in the liver, while no changes in brain or muscle were seen.

It is concluded that copper exposure of common carp causes decreased brain 5-HT and DA levels, two neurotransmitters involved in, for example, feeding behaviour and locomotor control in fish. In fact, in telencephalon a fall in 5-HT levels was seen already at a copper concentration below that where food intake and movement were impaired.

[*] Based on the manuscript by De Boeck, G., Nilsson G.E., Elofsson, U., Vlaemick, A. and Blust, R., 1995. Brain monoamine levels and energy status in common carp (*Cyprinus carpio*) after exposure to sublethal levels of copper. *Aquat. Toxicol.* **33**: 265-277.

6.2. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA) are two monoamine neurotransmitters that occur in the brain of all vertebrates. Among the functions of these monoamines that have been studied in fish, can be mentioned locomotor control (Fingerman, 1976; Genot *et al.*, 1984; Winberg and Nilsson, 1992: Winberg *et al.*, 1993b), feeding (Smith, 1984), central control of hormone release (Chang *et al.*, 1985; Van der Kraak *et al.*, 1986, Olivereau *et al.*, 1987; Somoza *et al.*, 1988), and modulation of social behaviour (Winberg and Nilsson, 1993).

5-HT activity, measured as the quotient of 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT, has proven to be highly sensitive to different stressors in fish. Thus, stressful experiences like repetitive netting (Winberg *et al.*, 1992a), intra-species aggression (Winberg *et al.*, 1992b), and predator exposure (Winberg *et al.*, 1993a) have all been found to cause elevated 5-HIAA/5-HT quotients in fish. Although these findings suggest that the brain 5-HT system in fish could be of particular interest from a toxicological point of view, only a few studies explore the possibility of using the brain serotonergic system as an indicator of toxic effects on the central nervous system of fish. As most of these studies focus on effects of organic compounds (Thomas *et al.*, 1981; Gopal *et al.*, 1985; Rozados *et al.*, 1991), even fewer experimental studies assess the effects of metals on the monoamine metabolism. Katti and Sathyanesan (1986) found elevated levels of whole brain serotonin in catfish (*Clarias batrachus*) exposed to lead, and Weber *et al.* (1991) obtained similar results with fathead minnows (*Pimephales promelas*).

An involvement of the brain in copper toxicity is suggested by previous studies. Thus, exposure of brook trout, *Salvelinus fontinalis* (Drummond *et al.*, 1973), rainbow trout, *Salmo gairdneri* (Lett *et al.*, 1976), and common carp, *Cyprinus carpio* (De Boeck *et al.*, 1995, see chapter IV), to sublethal levels of copper results in changed locomotor activity and depressed food intake.

The aim of the present study was to correlate these effects of sublethal copper exposure with changes in the levels of 5-HT, 5-HIAA and DA, as well as with 5-HIAA/5-HT ratios in the brain of common carp.

6.3. Materials and methods

6.3.1. Animal holding and copper exposure

Juvenile (1 month old) common carp, *Cyprinus carpio,* were obtained from the fish hatchery at the Agricultural University of Wageningen, The Netherlands. They were grown at the University of Antwerp at the optimal temperature of 25°C (Guderley and Blier, 1988) in softened Antwerp city tap water (Ca 35 mg l^{-1}, Mg 3.5 mg l^{-1}, pH 7.0-8.0). Water was filtered with a trickling filter and water quality was checked weekly with Visicolor Test Kits (Macherey-Nagel, Düren) for ammonia, nitrite and nitrate. 50% of the water was renewed when levels exceeded 0.1 mg l^{-1}, 1.0 mg l^{-1} or 20 mg l^{-1} respectively.

Two weeks before starting the experiments, 32 carp weighing between 20 and 30 g were transferred into four 150 l aquaria filled with standard moderately hard water according to Standard Methods (American Public Health Association, 1989: $CaSO_4.2H_2O$: 60 mg l^{-1}; $MgSO_4$: 60 mg l^{-1}; $NaHCO_3$: 96 mg l^{-1}; KCl: 4 mg l^{-1}; pH: 7.8-8.0). The standard water was well aerated during at least 24 h before use. During the first week the temperature in the aquaria was gradually adjusted to 20°C (± 1°C) and the photoperiod was set at a 14 h light, 10 h dark period. Carp were fed until satiation twice a day (1 and 8 h after light period started) with 'Pond Sticks' (Tetrapond, Henckel). Water was filtered with trickling filters filled with activated charcoal (Calgon Carbon) and Rivalon synthetic filter wadding. Water quality was checked daily for pH, ammonia, nitrite and nitrate and 50% of the water was renewed

twice a week. Following this procedure levels of excretory products did never exceed levels mentioned above and pH was 7.7 ±0.1 for all groups.

Copper was added to the standard moderately hard water as a copper nitrate standard solution (Merck, 1 g l^{-1} $Cu(NO_3)_2.2H_2O$). Charcoal was removed from the filters before the copper was added. During the first day of exposure, the copper nitrate standard solution was slowly dripped in the aquaria over a period of 2 h. Starting from the next day, 75% of the water in the aquaria was renewed twice a week with copper containing standard water which had been aerated and mixed for at least 24 h after addition of the copper nitrate standard solution. Each time, water of the control group was renewed in exactly the same way with copper free standard water. Following this procedure, the addition of 1, 2 or 4 μmol of copper per liter water resulted in a rather constant copper exposure with copper concentrations in the aquaria of 0.22±0.07, 0.34±0.12 and 0.84±0.35 μM respectively (means ±S.D.). Samples for copper measurements were taken 5 times a week and copper measurements were performed using graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAS 703 and HGA 500).

A second group of carp were copper exposed in the same way in order to analyse blood lactate levels and determine copper accumulation in the brain, muscle and liver. Copper concentrations in the aquaria during this exposure experiment were 0.20±0.08, 0.55±0.15 and 0.80±0.39 μM respectively (means±S.D.).

6.3.2. Sampling of tissue

At the day the brain tissue was sampled, fish were not fed because sampling started at their normal feeding time (1 h after light period started). Each fish was killed by decapitation. The brain (excluding the olfactory bulbs) was rapidly removed and dissected into 3 parts, viz. telencephalon, hypothalamus and brain stem (i.e. the remaining parts of the brain). Mean weights of each brain region: telencephalon: 0.0236±0.0025 g; hypothalamus: 0.0244±0.0030 g; brain stem: 0.1935±0.0141 g (means ±S.D.). The brain regions were frozen in liquid nitrogen within 1 min of decapitation (telencephalon within 10 s) and stored at -80°C.

In the second exposure group, fish were rapidly anaesthetised (MS222) and blood plasma samples were taken by caudal puncture, centrifuged immediately and plasma was frozen (-80°C). For the copper accumulation measurements, brain parts and additional pieces of muscle and liver tissue were dissected. Weights of the different tissues were: telencephalon: 0.0222±0.0029 g; hypothalamus: 0.0222±0.0025 g; brain stem: 0.1555±0.0162 g; muscle: 0.1171±0.0386 g and liver 0.0711±0.0195 g (means ±S.D.).

6.3.3. HPLC-assay of monoamines and their metabolites

After being weighed, the frozen brain samples were sonicated or homogenized at 0°C in 0.2 - 1.2 ml of 4 % (w/v) ice-cold perchloric acid containing 2 mg/ml EDTA, 0.5 mg/ml sodium bisulphite and 40 ng/ml epinine (deoxyepinephrine, the internal standard) using an MSE 100 W Ultrasonic Disintegrator (for telencephalon and hypothalamus) or a Potter-Elvehjem homogenizer (for brain stem). The amount of monoamines present in 100 µl aliquots of the supernatants obtained after centrifugation (14 000 g for 10 min at 4°C) were quantified using reversed-phase ion-pair HPLC (high performance liquid chromatography) with electrochemical detection as

described by Nilsson, 1989a. In short, the HPLC system consisted of a 6000 A solvent delivery system and a U6K injector (both from Waters Associates Inc., Milford, Massachusetts, USA), a reversed phase column (4.6 x 125 mm, Nucleosil 120, C18, 3 μm, from Macherey-Nagel, Düren, Germany) kept at 40°C, and a LC-3 electro-chemical detector with a glassy carbon working electrode, which was set at +750 mV vs. an Ag/AgCl reference electrode (all from Bioanalytical Systems, West Lafayette, Indiana, USA). The flow rate was 1.1 ml/min and the mobile phase consisted of 100 mM NaH_2PO_4, 0.2 mM EDTA, 0.63 mM sodium octylsulphate, and 9 % (v/v) methanol, pH 3.6. Monoamines, their metabolites and epinine used for HPLC standards were obtained from Sigma Chemicals (St. Louis, MO, U.S.A.).

The monoamine contents are given in relation to the wet weights of the tissues.

6.3.4. Measurements of adenosine phosphates and lactate

The levels of ATP, ADP and AMP in the supernatants were analyzed using HPLC with spectrophotometric detection as described by Van der Boon *et al.* (1992). The HPLC system consisted of an LDC Consta Metric III pump, an LDC/TSP Spectro Monitor 4100, and a reversed phase column (4 x 125 mm, Nucleosil C18, 3 μm). The mobile phase consisted of 100 mM NaH_2PO_4, 10% acetonitrile, and 5 mM tetrabutyl ammonium bromide (pH set to 6.0 with NaOH).

Lactate in brain was analyzed with Boehringer Mannheim Lactic acid kit (Cat. No. 139 084). Lactate levels were only analyzed in brain stem samples. After analysing monoamine levels and adenosine phosphates, the aliquots remaining of the telencephalon and hypothalamus extracts were to small to allow lactate measurements. Lactate measurements in blood plasma were performed using a Sigma Lactate Reagent kit (Cat. No. 735-10).

6.3.5. Copper measurements in tissue

All measurements were performed with graphite furnace atomic absorption spectrophotometry (Perkin Elmer AAS 703 and HGA 500). For copper measurements in brain, liver and muscle, tissue samples were weighed and 250 ml of nitric acid (67%) was added to each sample. Samples were left at room temperature overnight and microwaved in a closed box for further destruction (5 minutes at 140, 210, 280 and 350 W with 5 minute intervals). Samples were weighed again to calculate evaporation and 1 ml of MQ water was added. Liver samples were diluted up to 20 times when necessary.

6.3.6. Statistics

All values are given as means ±S.D. Statistics were performed with GraphPad InStat, using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test if significant differences were found.

6.4. Results

Significant copper accumulation could only be detected in the liver (Table 6.1.). In the different brain parts and muscle, no significant increase in copper concentration was found.

Brain 5-HT concentrations showed a clear dose dependent decrease in telencephalon (Fig. 6.1.), the drop in the 5-HT level being significant, even at the lowest copper concentration (0.22 μM, $P<0.05$). At a copper concentration of 0.34 μM, the level of 5-HT had dropped by 27% and was significantly lower compared to controls ($P<0.001$) and to the lowest exposure group ($P<0.05$). At the highest exposure

Table 6.1. Copper concentrations in different brain parts, liver and muscle tissue and plasma lactate of common carp after one week of exposure to different copper concentrations. Values are means and S.D. from 8 individuals for each exposure group (: P<0.05; **: P<0.01; ***: P<0.001).*

	0 µM	0.20 µM	0.55 µM	0.80 µM
Cu telencephalon (µg/g)	1.24 (±0.26)	1.33 (±0.23)	1.39 (±0.12)	1.42 (±0.27)
Cu hypothalamus (µg/g)	1.84 (±0.23)	1.88 (±0.19)	1.96 (±0.23)	2.12 (±0.18)
Cu brain stem (µg/g)	1.63 (±0.24)	1.71 (±0.18)	1.71 (±0.12)	1.80 (±0.03)
Cu liver (µg/g)	18.94 (±5.38)	25.42 (±6.65)	24.06 (±3.99)	38.85 (±8.05)***
Cu muscle (µg/g)	0.48 (±0.11)	0.39 (±0.10)	0.42 (±0.08)	0.38 (±0.10)
Plasma lactate (mM)	2.88 (±0.35)	3.92 (±1.25)	4.71 (±1.20)*	5.26 (±1.53)**

concentration (0.84 μM), 5-HT levels had fallen by 48% compared to controls and were significantly lower than 5-HT levels in all other groups ($P<0.001$). In hypothalamus and brain stem, a significant decrease in 5-HT levels was found only at the highest copper concentration (for hypothalamus: $P<0.05$ compared to control, $P<0.001$ compared to lowest exposure group, $P<0.01$ compared to the median exposure group; for brain stem: $P<0.001$ compared to controls and lowest exposure group).

For 5-HIAA, no significant differences were found between the different exposure groups. As a result, 5-HIAA/5-HT ratios for telencephalon were higher in the group exposed to 0.34 μM of copper ($P<0.001$ compared to controls, $P<0.05$ compared to 0.22 μM of copper) as well as in the group exposed to 0.84 μM of copper ($P<0.001$ compared to controls and lowest exposure group). Also in hypothalamus and brain stem, significant rises in 5-HIAA/5-HT ratio could be observed in the group exposed to the highest copper concentration (for hypothalamus: $P<0.05$ compared to controls and lowest exposure group; for brain stem: $P<0.01$ compared to controls and lowest exposure group, $P<0.05$ compared to the median exposure group).

As for 5-HT, the levels of DA in the telencephalon seemed to show a dose dependent response to copper exposure, falling by 8, 16 and 48% respectively at copper exposure concentrations of 0.22, 0.34 and 0.84 μM, the last one being significantly different from controls ($P<0.001$). In brain stem, the same tendency could be observed, with a 45% decrease in the group exposed to the highest copper concentration ($P<0.001$ compared to all other groups). For hypothalamus, the effect of copper exposure was less clear. As the DA level in the control group was rather low, the decrease in DA concentration in the high exposure group was significantly different only from the lowest ($P<0.001$) and the median ($P<0.01$) exposure groups. The DA metabolites homovanillic acid and dihydroxyphenylacetic acid, which could be detected with the HPLC-system used, were found to be below the detection limit

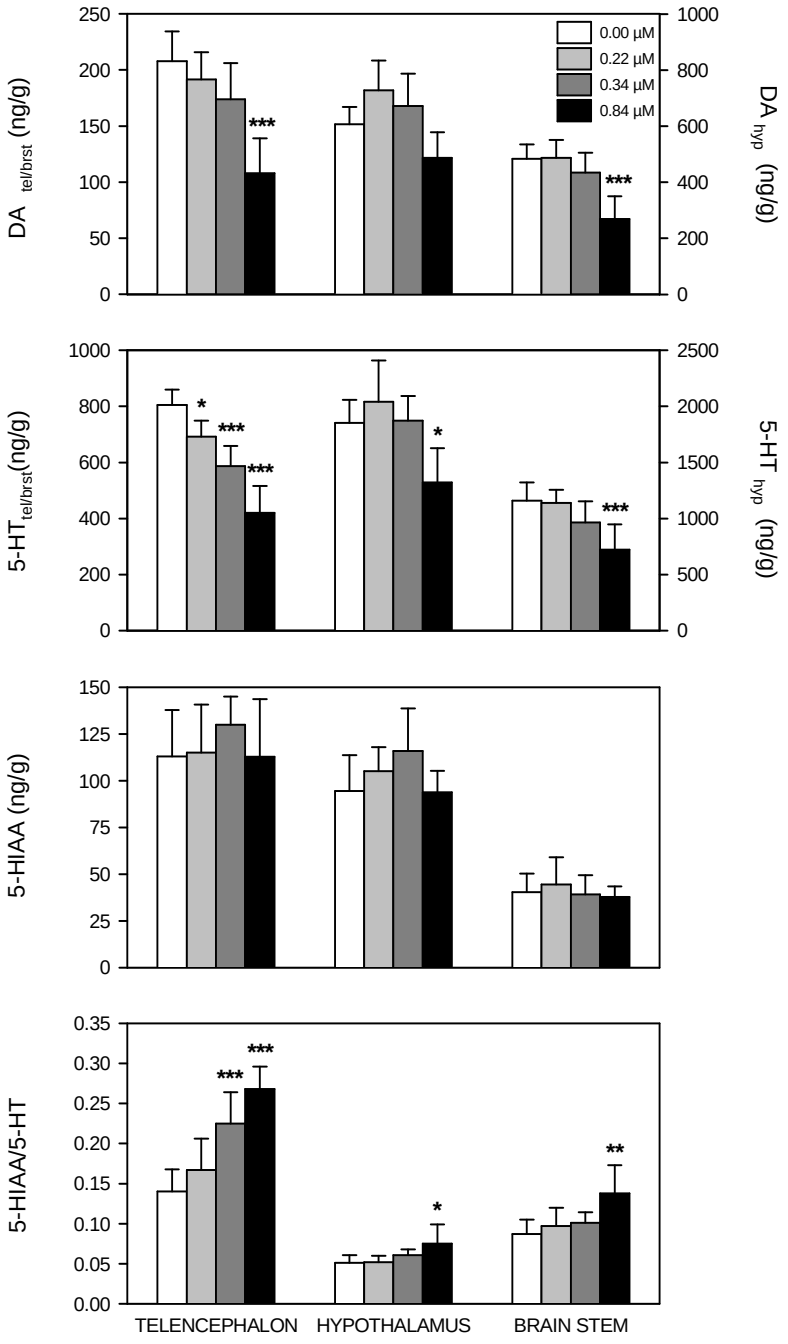

Figure 6.1. Concentrations of DA, 5-HT, 5-HIAA and 5-HIAA/5-HT ratios in the different brain regions of common carp after one week of exposure to different copper concentrations. Values are means and S.D. from 8 individuals for each exposure group (: P<0.05; **: P<0.01; ***: P<0.001).*

(ca 10 $ng.g^{-1}$). Only in brain stem, small peaks indicating the presence of dihydroxyphenylacetic acid could be observed in the chromatograms of some fish.

The ATP levels presently measured (Fig. 6.2.) were similar to the *ca.* 1.2 mM found in the *in situ* frozen goldfish whole brain, a tissue where the ATP concentration remains stable at room temperature for 30 min after decapitation (McDougal *et al.*, 1968). Although the common carp brain is probably not as ischemia tolerant as its relative, the goldfish, it is unlikely that the 10-60s that presently elapsed between decapitation and freezing significantly affected the ATP levels. No significant differences were found in AMP, ADP, ATP or total adenylate phosphate levels, although a tendency to higher AMP and lower ATP levels could be observed in telencephalon and hypothalamus at the highest copper exposure concentration. Consequently, the adenylate energy charge (AEC) showed no significant differences in any of the brain parts.

As for the ATP levels, the lactate levels measured in the brain stem samples (Fig. 6.2.) were similar to those measured by McDougal *et al.* (1968) in goldfish brain (ca 5 mM) frozen *in situ*. In the present study no significant differences in the lactate levels were found between the different exposure groups. In plasma samples, lactate significantly increased in a dose dependent way (Table 1) with $P<0.05$ for carp exposed to 0.55 μM of copper and $P<0.01$ for fish exposed to 0.80 μM of copper during one week.

6.5. Discussion

The present experiments show that exposure of common carp to copper causes considerable changes in monoamine levels in different brain parts. Moreover, the decrease of 5-HT and DA in telencephalon appeared to be dose dependent, with up to 50% of loss of these neurotransmitters at the highest copper concentration. Whereas

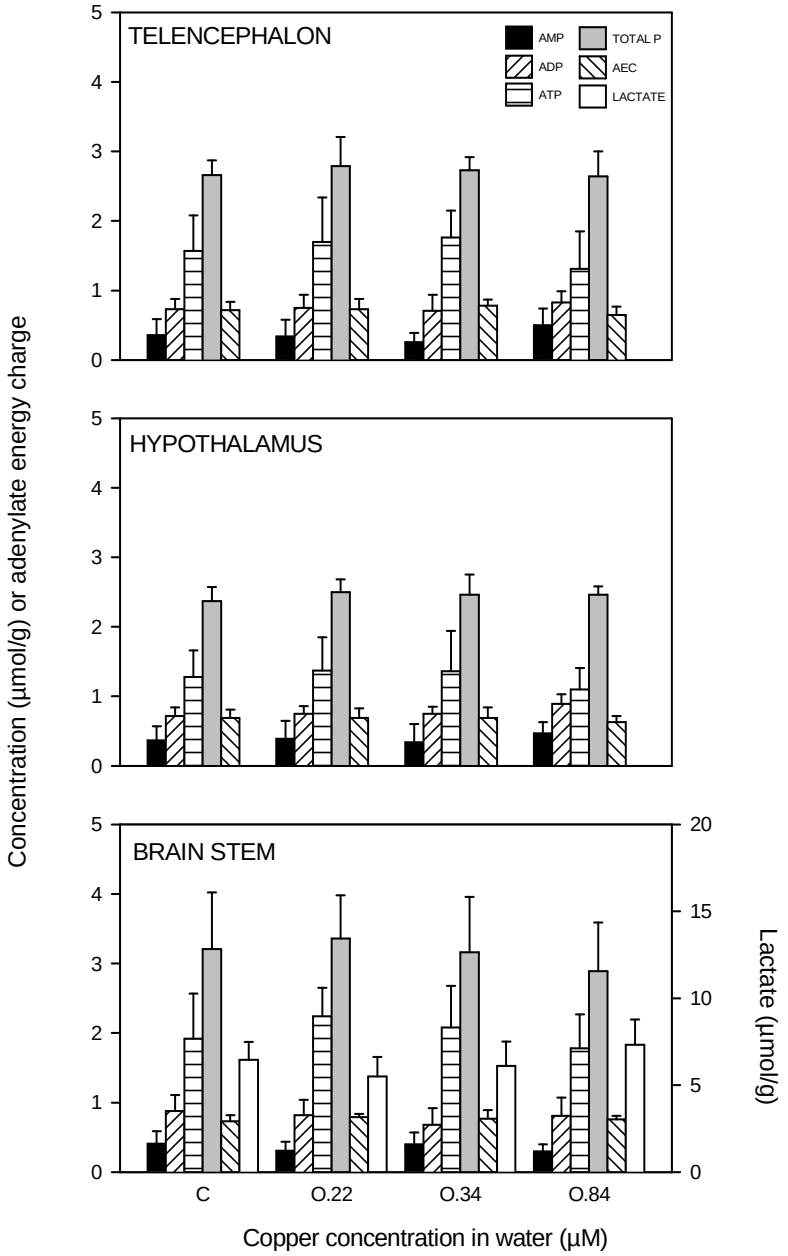

Figure 6.2. Concentrations of AMP, ADP, ATP, total adenosine phosphates (TOTAL P) and adenylate energy charge (AEC = (ATP+1/2ADP) / (ATP+ADP+AMP)) in the three brain regions of common carp after one week of exposure to different copper concentrations. For brain stem, also lactate concentrations are given. Values are means and S.D. from 8 individuals for each exposure group.

Munkittrick *et al.* (1990) found no significant effects of habitat levels of copper between 0.19 and 0.24 μM combined with levels of zinc between 3.20 and 3.87 μM on whole brain monoamine levels in the white sucker (*Catostomus commersoni*), our study revealed a significant decrease in telencephalic 5-HT in common carp at a copper concentration of 0.22 μM. However, it should be noted that Munkittrick *et al.* (1990) obtained their results by comparing fish from different lakes. In such a study it is likely that many more factors than the Cu and Zn concentrations differed between the lakes, and acclimation or even selection could affect the results.

Previous studies (see Introduction) on the effect of different stressors (repetitive netting, exposure to intra-species aggression, and predator exposure) on the 5-HT system in fish brain, have generally indicated that stress induces increases in 5-HIAA/5-HT ratios primarily because of increased 5-HIAA levels. Since 5-HIAA is the main 5-HT metabolite in brain, the increased 5-HIAA/5-HT ratios were taken as evidence for increased activities in the brain 5-HT systems (see Winberg and Nilsson, 1993 for review).

By contrast, in the present experiments with copper exposure, the increased 5-HIAA/5-HT ratios appeared mainly to be caused by falls in the 5-HT levels. This is more likely to reflect a reduced 5-HT synthesis rate rather than increased 5-HT turnover. Weber and Spieler (1994) consider several possible neurobiological mechanisms of metal neurotoxicity in fish. These include presynaptic effects on neural transmission and effects on synaptic cleft enzyme activity as well as effects on postsynaptic receptor mechanisms. In this case, a direct interference by copper on these neurobiological processes seems unlikely since we found little or no copper accumulation in any of the brain parts at the different copper exposure concentrations. Even after 1 month of exposure, no significant copper accumulation had occurred in the brain (results not shown). Nemcsók *et al.* (1987) found low levels of copper uptake in the brain of common carp, but at considerable higher copper exposure levels (6.26 to 626 μM). They suggested that the low uptake was caused by the effectiveness of the blood-brain barrier. At similar levels of exposure, they also found lower

acetylcholinesterase activity in different tissues, including the brain, which could affect metabolism, blood circulation etc. (Nemcsók *et al.*, 1984). But again, for brain, a remarkable difference occurred between *in vivo* and *in vitro* activity measurements, suggesting that the brain was well protected from copper intrusion. Therefore, we feel that at the much lower copper exposure concentrations used in our experiments, other factors must underlie the observed falls in monoamine levels.

In mammals, relatively mild hypoxia has proven to decrease tryptophan hydroxylation, the rate-limiting step in 5-HT synthesis (Brown *et al.*, 1974; Katz, 1981; Prioux-Guyonneau *et al.*, 1982; Freeman *et al.*, 1986). Copper is known to cause gill damage in fish (Baker, 1969; Bilinski and Jonas, 1973) and in a recent study on common carp (De Boeck *et al.*, 1995, see chapter IV), exposure to copper at a concentration of 0.34 μM was found to cause a significant increase in the critical oxygen concentration for oxygen consumption, indicating that the ability to take up oxygen from the water had been compromised. Unfortunately, the small size of the carp did not allow the catheterisation necessary for reliable blood P_{O2} measurements, so hypoxia could not be confirmed directly. However, the dose-dependent increase in blood lactate concentration presently observed further confirms the hypoxic state of the animals. The fact that no significant changes were observed in brain ATP or lactate level does not imply that the brain was totally protected from hypoxia, a mild form of hypoxia could still have occurred. Davis *et al.* (1973) proved for rat brain that ATP, ADP and AMP levels were stable when arterial P_{O2} remained above 30 mm Hg and lactate levels remained constant at a P_{O2} above 40 mm Hg while tryptophan hydroxylase activity was already inhibited at brain oxygen levels of 60 mm Hg.

It is possible that also the reduction in DA levels was caused by mild hypoxia, since DA is, much like 5-HT, produced in an oxygen dependent hydroxylation reaction. In mammals, no uniform response of DA levels to hypoxia has been observed (Davis and Carlsson, 1973; Prioux-Guyonneau *et al.*, 1979; Kuno *et al.*, 1981; Dalmaz *et al.*, 1988). Nilsson (1989b, 1990a) found that in crucian carp (*Carassius carassius*), anoxia causes a fall in DA levels. However, in contrast to common carp, the crucian

carp is an extremely anoxia tolerant species, so long periods (days) of total anoxia were needed to cause substantial falls in the brain DA levels in this species.

Although monoamine oxidase is also an oxygen dependent enzyme, no differences were found in concentrations of 5-HIAA. Pouilot *et al.* (1988) studied the effect of three different diets (commercial, vegetal and animal) on brain monoamines in rainbow trout under normoxia and mild hypoxia (60% air saturation). Their results showed no effects of hypoxia on 5-HIAA levels in mesencephalon and telencephalon, although for fish fed the commercial diet a slight (15%) decrease in 5-HIAA was found in hypothalamus during hypoxia. In the same study, 5-HT was stable in hypothalamus and telencephalon, but showed a 25% decrease in mesencephalon during hypoxia exposure.

Interestingly, the data suggest that there are regional differences in the sensitivity of the brain monoaminergic systems to copper exposure, the hypothalamus being less sensitive than the telencephalon. Thus, no significant changes were seen in the hypothalamic DA levels, and with regard to hypothalamic 5-HT, a significant fall was only seen in carp exposed to the highest copper concentration. One possible explanation to these differences in sensitivity could be that they reflect regional differences in the turnover of these monoamines in the carp brain, which, for example, could make regions with slower turnover rates less sensitive to a fall in oxygen availability. In experiments on crucian carp, more than 10-fold differences in 5-HT turnover rates between brain regions have been found (Nilsson, 1990b), although hypothalamus was not specifically examined in that study.

Apart from the apparent usefulness of the 5-HIAA/5-HT ratio or the 5-HT concentration as indicators of copper toxicity, the fall in the 5-HT level could have important physiological effects and contribute to the toxicity of copper. Copper exposed fish generally show a loss of appetite, and interestingly, the brain serotonergic system has been connected to feeding. In rainbow trout, treatment with the 5-HT synthesis inhibitor p-chlorophenylalanine has been found to cause reduced food intake (Johnston *et al.*, 1992). Similarly, the fall in DA levels presently seen could be

involved in the symptoms of impaired locomotor control and equilibrium problems often displayed by fish exposed to high doses of copper. In fish, the role of DA in locomotor control has been indicated in a study on Arctic charr (Winberg and Nilsson, 1992).

It is interesting to note that the 5-HT levels in telencephalon appeared to be more sensitive to copper than those in other brain parts. A lot of work has been done on the organisation of monoamine neuronal systems in fish (see Parent, 1984; Meek, 1994 for review) but the knowledge on the specific localisation of various monoamine neurotransmitter functions is still limited. In feeding behaviour, lateral hypothalamus and telencephalon appear to be important controlling regions (Peter, 1979) and also Smith (1984) mentions 5-HT in telencephalon as one of the regulators of feeding.

In conclusion, copper exposure of common carp causes decreases in 5-HT and DA levels, two neurotransmitters involved in a number of physiological processes, including feeding behaviour and locomotor control in fish. In fact, falls in the 5-HT concentration of the telencephalon occurred already at a copper concentration below that where food intake and movement were impaired.

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CHAPTER VII: GENERAL CONCLUSION

7.1. Introduction

Trace metals, such as copper, iron and zinc, are required for normal physiological functions in animals, but only at trace concentrations. When these metals are present in sufficiently high concentrations however, they will alter physiological functions and display a range of toxic effects. At low exposure concentrations, or short periods of exposure, the normal homeostatic mechanisms of the organism might still be able to cope, and no damage will be sustained. With increasing exposure concentrations or durations, initially reversible physiological changes may lead to irreversible modifications and subsequently impairment of physiological functions. Typically, the disturbances caused by metal exposure are characterised by an initial 'shock' phase of fairly short duration where the disturbances develop fairly rapidly, and a longer-term 'recovery' phase where the disturbances diminish gradually. The organism may, in some instances, fully recover in the continued presence of the metal, but more usually it reaches a new physiological steady state.

The present study examined the effects of different sublethal copper concentrations on the physiology of the common carp, *Cyprinus carpio*. When considering the biochemical functioning of organisms, copper is essential to several processes. It is an essential part in different enzymes and it is important in formation of bone and brain tissue. Thus, low levels of copper intake are elementary to vertebrate physiology. When environmental copper levels increase beyond the normal levels in an aquatic ecosystem, the metal becomes toxic to the different species inhabiting that ecosystem, and the disruptive effects of the heavy metal become apparent. Indeed, copper appears to be a very puissant toxicant when present in excessive amounts. When examining the effects of copper exposure on the physiology of an organism, it is important to consider physiological processes at different levels of biological organisation. When environmental changes affect an organism on the cellular level,

this will also reflect in organ functioning, which then can cause changes at hormonal or neural level and alter the total physiology of the organism. If a pollutant affects sensory organs, this may change release of neurotransmitters, which might then reflect in a different hormonal control of organ and cellular functioning. In this process, each level of biological organisation offers unique problems and insights and finds its explanation of mechanism in the levels below, and its significance in the levels above.

The aims of the presented research were twofold: 1) to study the effects of sublethal concentrations of copper on the physiology of the common carp, *Cyprinus carpio*, at different levels of biological organisation, and 2) to evaluate the possibility to employ the changes in these different physiological processes as sensitive biomarkers for sublethal stress. To assess the changes that occur in the physiology of the common carp under sublethal copper exposure, four series of experimental studies were conducted, studying the following processes: growth, capacity for protein synthesis, use of energy stores, total aerobic metabolism, relative protein catabolism, use of phosphorous compounds during rest and after an additional mild exercise, and changes in neurotransmitters involved in hormonal control.

1. In a first series of experiments, food consumption and growth, capacity for protein synthesis and the use of energy stores were assessed. Growth is a classic biomarker, which obviously reflects the fitness of an organism. In these studies, food consumption and growth of common carp exposed to different sublethal copper concentrations have been determined over a 28 day period as a standard to evaluate the usefulness of the other measurements as biomarkers. Protein is a major component of an organisms body mass, and RNA is necessary for the synthesis of protein. Consequently, a positive relationship between the concentration of RNA and the rate of protein synthesis has been demonstrated in many organisms. Typically, maximum RNA/DNA ratios occur during peaks of protein production. Based on these observations, protein, RNA and DNA relationships have been suggested as a promising biomarker of reduced growth. Besides protein, RNA and DNA also lipid and glycogen were quantified to determine the endogenous energy stores of the

organism. Finally, also body residues of copper were measured in different tissues. When studying the effects of copper exposure on an organism, it is indeed more important to consider the amount of copper which accumulates in the organism then the concentration of the metal in the water which does not reflect the availability of the metal to the organism.

2. In the second series of experiments, total aerobic metabolism and relative protein catabolism were assessed by measuring oxygen consumption and ammonia excretion rates, and by determining the ratio between the ammonia excreted and the oxygen consumed (ammonia quotient (AQ): mole to mole ratio of ammonia excreted to oxygen consumed). The use of the AQ is based on two principles: 1) the oxygen consumption is a measure of the total amount of energy that has been used, and 2) the excretion of nitrogen, in carp excreted mainly as ammonia, is a measure for the amount of proteins that has been used to provide this energy. The ratio between these two processes should therefore represent the relative use of proteins compared to lipids and carbohydrates. In fish, stored glycogen and especially lipids are the preferred substrate, but when these are depleted due to exercise or stress, muscle protein becomes the main energy source, which should then be reflected in the AQ. Also the critical oxygen concentration (C_c= lowest oxygen concentration at which regulation of oxygen consumption still occurs) was determined for carp exposed to the different copper concentrations. This C_c gives an indication of the capacity of the fish to regulate their oxygen consumption, and therefore of their resistance against low ambient oxygen concentrations or exercises, as burst swimming activities when escaping a predator.
3. In a third series of experiments, the use of phosphorous compounds during rest and after an additional mild exercise were assessed by means of *in vivo* phosphor nuclear magnetic resonance spectroscopy (^{31}P-NMRS). The technique of ^{31}P-NMRS allows simultaneous and continuous measurement of the phosphorous compounds involved in the energy metabolism of a living organism. In fish, the main phosphorous energy reserve is phosphocreatine. When ATP gets depleted, phosphocreatine is converted by

creatine kinase into creatine in order to restore the ATP content. During this process, inorganic phosphate increases in the fish muscle (due to ATP breakdown), while the phosphocreatine content slowly decreases. Using the ^{31}P-NMRS technique, concentrations of inorganic phosphate (P_i), phosphocreatine (P_{Cr}) and ATP can be monitored in time in a non invasive way. Furthermore, shifts in intracellular pH (pH_i), caused by lactate accumulation, can be determined.

4. In a fourh series of experiments, changes in the monoamine neurotransmitters serotonin and dopamine were assessed. Monoaminergic neurons compose a very small fraction of the neurons in the vertebrate brain, but the influence of these monoaminergic neurons on their target sites appears to go far beyond their numbers. The monoamine neurotransmitters appear to be involved in behavioral patterns (including aggression, mating, feeding and stress reactions) as well as in central regulation of autonomic and neuroendocrine functions. In fish, relationships between the monoamine brain neurotransmitters and social rank, aggression, food intake, locomotor activity, and stress have been found in salmonids. In our research, the effects of copper exposure on the content and activity of the catacholamine dopamine and the indoleamine serotonin were studied.

7.2. Results

The adverse effects of the copper exposure were most obvious from conducting the growth experiments: fish became apathetic and slow and carp exposed to the two highest copper concentrations (0.55 and 0.80 µM) dropped in weight during the first three weeks of exposure. Although food consumption in the lowest exposure group (0.20 µM) was significantly increased, growth was not. At the highest copper concentration, food consumption was significantly lowered. These effects were most prominent during the first week of exposure, whereupon some form of acclimation and repair occurred. In a ‘traditional’ stress response, the release of catecholamines and cortisol triggers a broad suite of biochemical and physiological changes known

collectively as secondary stress responses. The metabolic effects may include hyperglycemia, depletion of glycogen tissue reserves and increased catabolism of muscle protein. This is exactly what we see in the muscle tissue of the copper exposed fish. During the first three weeks the glycogen store was depleted (significant decreases at the two highest copper exposure concentrations), whereupon recovery of the glycogen level occured. At that moment, protein catabolism appeared to take over the role of energy source (significant decreases at the two highest copper exposure concentrations during the fourth week). In liver tissue, glycogen concentrations at the highest copper concentration increased, probably as a defence mechanism against hypoxic effects. Possibly, this increased level of glycogen is partly due to redistribution from muscle glycogen. No differences in RNA levels were found, while DNA levels in muscle tissue were elevated after one month of copper exposure. Copper accumulation appeared mainly in liver tissue, not in brain and muscle tissue.

In the experiments conducted to determine the ammonia quotient, a clear drop in oxygen consumption was observed, starting immediately after exposure to the two highest copper concentrations (0.34 and 0.84 µM), while ammonia excretion did not differ significantly. While at 0.34 µM of copper, a slight recovery in oxygen consumption was seen after 1 week of exposure, no recovery was observed at the highest copper exposure concentration, not even after 2 weeks of exposure. This means that, although total protein utilization remained stable (which agrees with the stable levels of protein in muscle and liver tissue during the first week of exposure), a relative larger part of aerobic metabolism was attributed to the protein utilisation during this first week of exposure (elevated AQ), leaving less aerobic capacity for lipid or glycogen utilisation. Since lipid levels also remained stable, and glycogen levels in muscle tissue already started to fall (although not yet significant), one could suggest that the metabolism should have been partly anaerobic. This suggestion is strengthened by the fact that increased levels of plasma lactate were found (see chapter VI).

The critical oxygen concentration for oxygen consumption shifted from 1.4 mg l^{-1} in copper free water to 3.9 mg l^{-1} at 0.34 µM of copper. Therefore, regulation of

oxygen consumption is compromised at oxygen concentrations that could occur in the natural habitat of the carp which obviously could jeopardise their chance of survival. At the highest copper concentration all regulation of oxygen consumption was lost, even at normoxic oxygen concentrations. Surprisingly, also ammonia excretion showed a C_c. This phenomenon has never been observed in fish before. The C_c for ammonia excretion was comparable to the C_c for oxygen consumption, indicating that, at low oxygen concentrations, protein catabolism is closely related to the amount of oxygen available to the fish and other fuels are utilised when ambient oxygen concentrations get to low. Also the C_c for ammonia excretion dissapeared when fish were exposed to copper, thus when fish are exposed to copper and low ambient oxygen concentrations at the same time, they could experience extra difficulties if they are no longer able to regulate their use of proteins as an energy source.

In vivo ^{31}P-NMRS allows simultaneous and continuous measurements of the phosphorous compounds involved in the energy metabolism as wellas measurement of the intracellular pH. Inorganic phosphate (P_i), phosphocreatine (P_{Cr}) and three phosphorous resonances of the nucleoside phosphates can be monitored as a function of time in a non-invasive way, allowing us to follow the recovery of carp from an additional exercise when exposed to different copper concentrations. When carp were exposed to a mild exercise, the energy metabolism in lateral muscle in unexposed fish recovered very fast from the exercise, whereas recovery in copper exposed carp (0.36 or 1.31 µM of copper) was not complete or absent during the first hours after the exercise. After one week of continuous exposure, fish exposed to 0.36 µM of copper seemed to have acclimated considerably to the copper. Carp recovered completely after the exercise during the monitoring period, and only in the P_{Cr}/P_i ratio a slower recovery could be observed compared to control group. At the highest copper concentration, levels of P_{Cr} as well as of P_i were unstable and the P_{Cr}/P_i ratio was already significantly lowered before the exercise. Also pH_i was significantly lower in this group, indicating lactate accumulation even before the hypoxic exercise started. Fish did recover slowly from the exercise at this high copper concentration, but P_{Cr}/P_i

ratios and pH_i remained lower than levels in the control group. Acclimation of this group therefore was only partial.

When measuring the levels of the monoamine neurotransmitters in the brain, dose dependent falls in the levels of serotonin and dopamine occurred after one week of exposure. These lower levels of dopamine and serotonin were already found at the lowest copper concentration, with close to 50% losses of these neurotransmitters at the highest copper concentration. Interestingly, these neurotransmitters are involved in feeding and locomotor control in fish, two activities that decline after copper exposure.

7.3. Conclusion

Exposing common carp to sublethal copper concentrations clearly affects these organisms. A lower metabolism (reduced oxygen consumption) and reduced food consumption (possibly caused by lower serotonin levels) resulted in a reduced growth rate. Results obtained by ^{31}P NMRS measurements and the higher C_c indicate that oxygen uptake and resistance against hypoxia are jeopardised, which reduces chances for survival. A reduction in energy stores (glycogen and proteins) and an unstable energy metabolism (^{31}P NMRS measurements) also leads to reduced fitness and survival rate. The decrease in dopamine could explain the reduced activity and mobility, which also reduces chances for survival in natural circumstances. Either of these effects (summarised in figure 7.1.) by itself could be lethal to an individual in nature, and consequences for populations and ecosystems are to be expected when copper concentrations exceed the lowest copper concentration tested here.

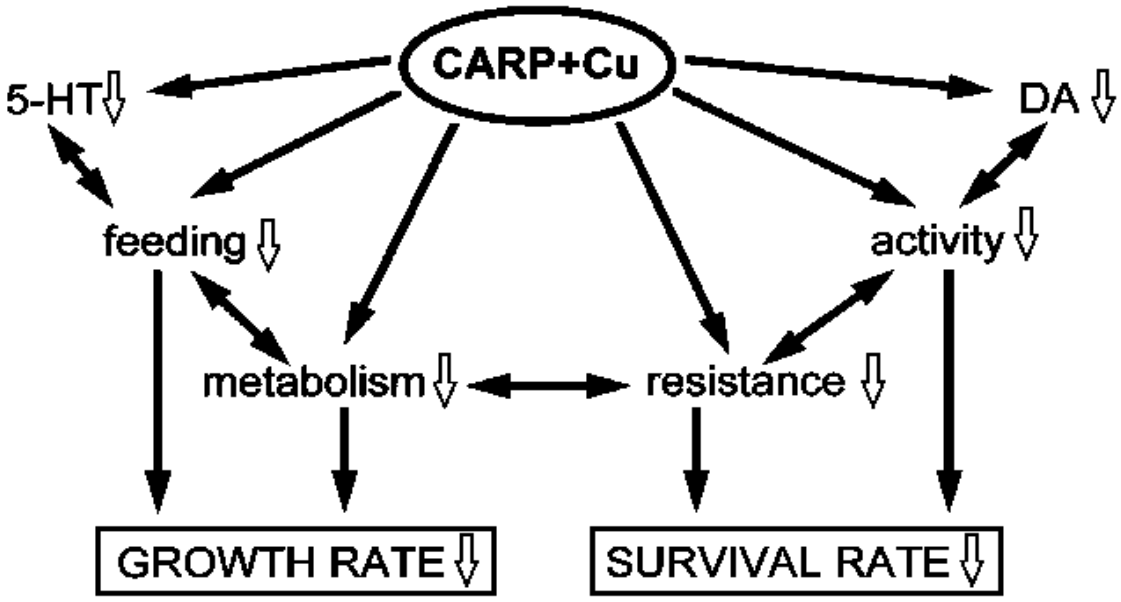

Figure 7.1. An overview of the cascade of negative events observed during copper exposure in common carp, Cyprinus carpio, and possible interrelationships between these events.

Nonetheless, fish seem to be able to escape from this cascade of negative effects after a first shock phase, since a slow process of acclimation and (partial) recovery and repair appears to occur after the first week. This process could be due to different factors: 1) alterations in the barrier properties of the exchange surfaces, 2) an increase in storage and detoxification capacities once the metal has entered the fish, 3) an increase in the resistance of metal sensitive processes like ion transport mechanisms. Further research to elucidate the roles of these different acclimation processes could provide important information concerning the ability of fish to handle copper intoxication.

The most sensitive indicator was the response at the neurotransmitter level (serotonin and dopamine), where significant changes already occurred at a copper concentration of 0.22 µM. These results however, can only be explained if additional information is available, and the high sensitivity of the indicator makes it susceptible

to other small disturbances which can only be avoided under control laboratory conditions. Measurements of oxygen consumption and AQ together with the ^{31}P NMRS measurements gave an indication of a disrupted energy metabolism starting from a copper concentration of 0.34 µM, the same copper concentration at which growth was significantly reduced and significant copper accumulation occured. Both methods also have the advantage of being *in vivo* procedures which enables us to monitor the recovery of each individual. Determination of the RNA/DNA ratio appeared to be of no value as an indicator of copper toxicity, but the determination of the energy stores of proteins and glycogen supplied useful additional information.

Compared to salmonids, common carp proved to be an equally sensitive test organism when physiological effects of copper exposure are considered. Lauren and McDonald (1985) estimated that for rainbow trout 12.5 µg l^{-1} (± 0.20 µM) can be considered as a reasonably safe concentration while McKim and Benoit (1971) considered 9.5 µg l^{-1} (± 0.15 µM) as a safe concentration for brook trout. Our studies suggest that a copper concentration of 0.20 µM still affects common carp physiology (increased food demand, decreased levels of serotonin in telencephalon) and further research would be useful to determine a no effect level. In early life stages of common carp, exposure to 0.30 µM of copper appeared to be safe at a pH of 7.6, but was toxic in slightly acid water (pH 6.3). Also in this study, a copper concentration of 0.80 µM appeared to be highly toxic at both water pH (Stouthart *et al*., 1996). When considering the official water quality criteria for copper, the Belgian normation (0.47 µM) appears to be far too high to protect organisms from the deleterious effects of copper exposure, while the guidelines used in the USA (0.10 µM) or the Netherlands (0.05 µM) seem to be more appropriate.

For further research, we would like to concentrate on mechanisms that underlie the processes of adaptation to copper. This could include alterations of the barrier properties of epithelia and changes in the resistance of metal sensitive processes like ion transport.

7.4. References

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SAMENVATTING

Tijdens de laatste decennia hebben die biologen die zich bezig hielden met waterkwaliteit voortdurend gezocht naar gevoelige indicatoren om de effecten van stress op aquatische organismen vast te stellen. In het verleden werd de invloed van stress in het aquatisch milieu vooral bestudeerd door het uitvoeren van toxiciteitstesten, waarbij mortaliteit (LC50) de bepalende factor was. Deze testen hebben het voordeel dat zij relatief eenvoudig en snel uitvoerbaar zijn en dat ze bruikbaar zijn voor zowel invertebraten als vissen. Het criterium waarop men zich baseert, namelijk mortaliteit, is echter drastisch en de duur van de experimenten is kort (meestal 24, 48 of 96 uur). Voor studies bij vissen wordt daarom voorkeur gegeven aan het zoeken naar fysiologische en biochemische indicatoren van sublethale vormen van stress. Men streeft hierbij naar het identificeren van korte-termijn responsen die in direct verband kunnen worden gebracht met de respons van het organisme op lange termijn (overleving, groei en voortplanting) in verschillende stresserende milieus.

Ook vanuit puur fysiologisch opzicht, is het bestuderen van effecten van sublethale verontreinigingen in het milieu van aquatische organismen interessant. De verontreinigingen vormen immers veranderende milieuomstandigheden waaraan deze organismen zich zullen trachten aan te passen. Hierbij worden vaak basis mechanismen in fysiologische processen blootgelegd. Daar de fysische, chemische en biologische omstandigheden in het milieu continu veranderen door toedoen van de mens, is de natuurlijke nieuwsgierigheid naar het begrijpen van deze veranderingen een doel op zich. Op lange termijn kan dit leiden tot betere theoretische modellen, wat de voorspelingen van effecten van milieuverontreinigingen in de toekomst, evenals de manier om hiertegen in te grijpen, enkel maar kan verbeteren.

In het hier voorgestelde onderzoek werd een dubbele opzet voor ogen gesteld: 1) de studie van de fysiologische effecten van sublethale koperblootstelling op de gewone karper, *Cyprinus carpio*, en 2) de mogelijkheden om de onderzochte fysiologische processen als een gevoelige indicator voor sublethale stress te gebruiken. Hierbij werden in vier reeksen van experimenten de invloed van koperblootstelling op de volgende fysiologische processen bestudeerd: groei, capaciteit tot eiwitsynthese, verbruik van energiereserves, totaal aëroob metabolisme, relatief eiwit verbruik, verbruik van energierijke fosfaatverbindingen en veranderingen in neurotransmitters in de hersenen van de karper.

De resultaten van de verschillende studies toonden een duidelijk negatief effect van de koperblootstelling op de fysiologie van de vissen aan. De karpers vertoonden een lethargisch gedrag, en bij blootstelling aan de twee hoogste koperconcentraties (0.55 en 0.80 µM) vielen de vissen af tijdens de drie eerste weken van de blootstelling. Hun behoefte aan voedsel bleek te zijn toegenomen: alhoewel de vissen blootgesteld aan de laagste koperconcentratie (0.20 µM) meer aten, veranderde hun groei niet, en de vissen van de groep blootgesteld aan 0.55 µM koper verloren gewicht terwijl hun eetlust niet verminderde. De karpers van de hoogste blootstellingsgroep verbruikten significant minder voedsel tijdens de eerste twee weken van de blootstelling, waarna hun eetlust herstelde.

De veranderingen in de samenstelling van het spierweefsel sloten aan bij het ‘klassieke’ beeld van reacties op een stressor. De vrijstelling van de ‘stress hormonen’ adrenaline en cortisol veroorzaken een waaier van secundaire effecten waaronder hyperglycemia, verbruik van glycogeen reserves en een verhoogde afbraak van proteïnen. Tijdens de eerste drie weken van de blootstelling aan de twee hoogste koperconcentraties werd in het spierweefsel dan ook een daling in het glycogeengehalte waargenomen, waarna de rol van energie bevoorrading werd overgenomen door een afbraak van de eiwitten terwijl het glycogeengehalte weer herstelde. Daarentegen vonden we in het leverweefsel een stijgend gehalte aan glycogeen, waarschijnlijk als het gevolg van een door de koper veroorzaakte

hypoxische toestand. Er werden geen veranderingen in de hoeveelheid RNA gevonden, en de RNA/DNA ratio bleek dan ook geen goede indicator voor veranderingen in groei of eiwitsynthese. Tijdens de laatste week werden in de spier verhoogde gehalten aan DNA gevonden, wat wijst op een dichtere samenpakking van kleinere cellen. Dit zou het gevolg kunnen zijn van celdeling daar de groei van de vissen ook weer hervatte. Accumulatie van koper vond vooral plaats in de lever, en niet in spier of hersenweefsel.

Als karpers werden blootgesteld aan 0.34 en 0.84 µM koper, daalde hun zuurstofverbruik onmiddellijk, maar hun stikstofexcretie bleef stabiel. Indien de vissen een week blootgesteld bleven aan deze koperconcentraties kon bij de karpers blootgesteld aan 0.34 µM koper reeds een herstel van het zuurstofverbruik waargenomen worden, maar bij de hoogste koperconcentratie niet. Dit betekent dat bij een constant verbruik van eiwitten (wat overeen komt met het stabiele eiwitgehalte in spier en leverweefsel na een week blootstelling), een relatief groter deel van de beschikbare zuurstof voor de afbraak van deze eiwitten werd gebruikt. Daar het vetgehalte stabiel bleef en het glycogeenverbruik reeds begon toe te nemen, vinden we hier al een eerste aanwijzing dat een deel van het metabolisme anaëroob verliep, een suggestie die later bevestigd werd door verhoogde lactaatgehalten in het plasma.

Naast een verminderd zuurstofverbruik, en dus een lager aëroob metabolisme, werd nog een tweede effect van koper op het zuurstofverbruik waargenomen. De kritische zuurstofconcentratie, dit is de laagste zuurstofconcentratie in het water waarbij een organisme zijn zuurstofverbruik nog kan regelen, verschoof gevoelig naar hogere waarden bij blootstelling aan 0.34 µM koper. Dit betekent dat de karpers sneller de controle over hun zuurstofverbruik verliezen, en dit reeds bij zuurstofgehalten die in het natuurlijk milieu van deze dieren kunnen voorkomen. Bij een blootstelling aan 0.84 µM koper bleken de karpers al hun controle over hun zuurstofverbruik verloren te hebben. Tot onze verbazing bleek ook de stikstofexcretie een kritische zuurstofconcentratie te vertonen, een fenomeen dat tot nu toe nog nooit

werd waargenomen. Ook deze kritische zuurstofconcentratie bleek gevoelig aan het effect van koperblootstelling.

In vivo ^{31}P Nucleaire Magnetische Resonantie Spectroscopie (^{31}P-NMRS) stelde ons in staat om de concentraties van energierijke fosfaatverbindingen en intracellulaire pH in de spier van de karper te volgen tijdens blootstelling aan koper. Uit deze metingen bleek dat de capaciteit van de karpers om snel te herstellen van een lichte oefening (hypoxie) verloren was gegaan bij acute blootstelling aan koper (0.36 en 1.31 µM). Bij een koperconcentratie van 0.36 µM bleken de vissen zich na een week blootstelling te hebben aangepast, en ze herstelden weer vlot na de oefening. Bij een koperconcentratie van 1.31 µM was deze aanpassing echter onvolledig, de gehalten aan energierijke fosfaatverbindingen waren onstabiel en de intracellulaire pH was continu verlaagd.

Ook de gehalten aan monoamine neurotransmitters serotonine en dopamine in de hersenen van de karpers werden beïnvloed door blootstelling aan koper, zelfs bij de laagste koperconcentratie (0.20 µM). Deze neurotransmitters worden onder meer in verband gebracht met de controle van voedselopname en beweeglijkheid, twee processen die door de blootstelling aan koper werden aangetast.

Indien karpers worden blootgesteld aan sublethale koperconcentraties, heeft dit een duidelijk effect op hun fysiologie. Een lager energiemetabolisme (lager zuurstofverbruik) en verminderde voedselopname (mogelijk veroorzaakt door verlaagde serotonine gehalten) resulteren in een verminderde groei. Zowel uit de metingen van de ^{31}P-NMRS als uit de veranderingen in kritische zuurstofconcentratie blijkt dat de weerstand tegen lage zuurstofconcentraties verminderd is. Dit verminderd hun overlevingskansen in de natuur. Ook de verminderde energiereserves (glycogeen en eiwitten) en een onstabiel energiemetabolisme (^{31}P-NMRS metingen) verkleint hun overlevingskansen. Bovendien geeft het lagere dopamine gehalte aanleiding tot een geringere beweeglijkheid, wat de kans dat zij ten prooi vallen aan een predator vergroot.

Toch blijken de karpers, indien zij de eerste schok van de koperblootstelling zouden overleven, in staat om zich (in beperkte mate) aan een verhoogd kopergehalte in hun milieu te kunnen aanpassen. Verdere studie zal er dan ook op gericht zijn om de mechanismen die aan de basis van deze adaptatie liggen te onderzoeken.

ADDENDUM: LIST OF PUBLICATIONS

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