Universiteit Antwerpen
Faculteit Wetenschappen
Departement Biologie

Interactions between food quantity and waterborne copper toxicity in carp, *Cyprinus carpio*.

Interacties tussen de hoeveelheid beschikbaar voedsel en de toxiciteit van koper in het water voor de karper, *Cyprinus carpio*.

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen aan de Universiteit Antwerpen

Te verdedigen door **Shodja Hashemi**

Promotor: prof. dr. Gudrun De Boeck

Co-promoter: prof. dr. Ronny Blust

Antwerpen, 2007

What is food to one man, maybe fierce poison to others

Lucretius (95– 55 B.C.)

Acknowledgements

I would like to thank my promoter Prof. Dr. Gudrun De Boeck for her inspirations, constructive comments, her patient scientific guidance and fruitful discussions.

I am also grateful to Prof. Dr. Ronny Blust for standing as my Co-promoter and giving me the opportunity to join his group.

I would also like to extend my gratitude to all staff and technical members of laboratory of Eco-physiology, biochemistry, and Toxicology for their help and support, and sharing their knowledge and skills.

I am indebted to my wife “Zivar” for her support and patience through my PhD.

This study was carried out at the laboratory of Eco-physiology, biochemistry, and Toxicology of University of Antwerp, and was funded by Ministry of science of Iran and University of Antwerp.

Contents

List of abbreviations

AHR	Acclimated high ration
ALR	Acclimated low ration
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BLM	Biotic Ligand Model
BW	Body weight
Ca	Calcium
Cu	Copper
EPA	US Environmental Protection Agency
ESCPB	European society for Comparative Physiology and Biochemistry
FAO	Food and agriculture organization of United Nations
H_2O_2	Hydroperoxide
HR	High ration
ICP-AES	Inductively coupled plasma atomic emission spectrophotometry
K	Potassium
LC 50	Median lethal concentration
LMW	Low molecular weight
LR	Low ration
LT 50	Median lethal time
Mg	Magnesium
MT	Metallothionein
Na	Sodium
NAHR	Non-acclimated high ration
NALR	Non-acclimated low ration

OECD	Organization for Economic Co-operation and Development
pH	Negative logarithm of the concentration (mol/L) of the H3O+ [H+] ion; scale range from 0 to 14
SD	Standard deviation
SEB	Society for Experimental Biology
SETAC	Society of Environmental Toxicology and Chemistry
TSK	Trimmed Spearman-Karber
TOC	Total organic carbon
UNEP	United Nations Environmental Programme
UNESCO	United Nations Educational, Scientific and Cultural Organization

1

Introduction

Copper is generally present in freshwater with a natural background from 0.20 to 30 µg/L (Bowen, 1985). Human activities like mining, and production of manmade products such as leather, electronic equipments, fabricated metal products, and agricultural wastes are known to discharge additional copper to surface waters (Patterson et al., 1998). Copper components are also introduced to aquaculture systems as a prophylactic and therapeutant agents (Schlenk et al., 1998).

Water quality is an issue of concern in a densely populated and largely industrialized area like Flanders (Belgium, 445 inhabitants per km^2). However, progress has been made recently in the improvement of water quality in Flanders as a result of increased purification of municipal discharges by sewage plants, but problems still remain numerous. According to the fish index, only 0.4 % of sampling stations were fully conform to the European Water Framework Directive (Dumortier et al., 2006), and the water quality in Belgium rivers was found the lowest quality in Europe (UNESCO, 2003). Exceeding levels of copper were recorded in Flanders rivers (Bervoets et al., 2005, Bervoets and Blust, 2003), and Cu levels in almost 5% of routine measurements were found to be higher than 50 µg/L which stands as Flemish quality criteria for copper in surface waters (The Flemish Government, 2000). The Flemish copper standard value is 45.5, 5.5 fold higher than the Netherlands quality criteria (Warmer and Van Dokkum, 2002) and the US

Environmental protection agency standard range (US Environmental protection Agency, 1999) respectively, which highlights concerns over copper pollution in Flanders water resources.

Increased levels of copper cause concern for the health of aquatic organisms. Scientific studies are required to investigate the adverse effects of exceeding copper levels on aquatic organisms. Previous studies traditionally focused on acute toxicity which is manifested within a relatively short period, and sublethal effects which are long term physiological effects, were widely overlooked. Despite of remarkable progress made in acute toxicity studies, which led to the development of the Biotic Ligand Model (BLM) which predicts the bioavailability and toxicity of copper to aquatic organisms under site-specific conditions (EPA, 2007), studies on the chronic toxicity are limited, and the mechanisms of the chronic Cu toxicity in fish have not been studied to a great extend. Previous studies dealt mostly with dietary copper exposure or waterborne Cu exposure under changing environmental factors and water quality (Kamunde and Wood, 2003; Taylor et al., 2000). The influence of the condition of the fish and the effect of food rations as an important factor for toxicity have not yet been investigated. Particularly, no studies have been conducted to ascertain the interaction between food quantity and waterborne copper exposure in carp. In this research, a new approach to copper toxicity was introduced by focusing mainly on the response of fish to environmentally realistic, sublethal waterborne copper concentrations under different feeding conditions over time.

1.1 Copper as an essential element

Copper is a vital trace element in vertebrates and plays a role in many important enzymes and compounds (Harris, 1991) which act like catalysts in the metabolism (Cousins, 1985). Copper is also crucial for

many functions in body like maintenance of the nervous system and development of bone tissues as well as the production of red blood cells. In addition, copper plays a central role in the conversion of iron to its useable ferric (Fe III) form and also helps transport iron around the body (Abdel- Mageed and Oehme, 1990). Copper deficiency can result in reduced growth, anemia, and disturbance of the nervous system. Dietary copper requirements for carp are 3 mg/kg of diet (National Research Council, 1993).

1.2 Copper uptake and accumulation

Similar to other heavy metals, copper could be accumulated in fish tissues from the dissolved water (Yamamoto et al., 1997, De Boeck et al., 2004). Fish gills representing a large exposure surface area (over 50 percent of the total surface area of a fish) and providing a short diffusion distance between the internal and external environment, are the primary target of copper contamination (Brungs et. al., 1973, Buckley et al., 1982). Branchial Cu uptake is responsible for the internal Cu status of fish (Kamunde et al., 2001, 2002). 30 to 70% of whole-body copper accumulation was obtained from the water by gills in rainbow trout, which was increased with increasing copper concentration (Grosell and Wood, 2002). Copper uptake was also found to be passive, involving diffusion down to its concentration gradient created by the absorption into tissues (Pilgaard et al., 1994). Accumulation of copper in the gills is due to binding to haemopoetic tissue mucus and to methallothioneins. Branchial copper transport via an apical Na^+ channel was also reported by Grosell and Wood (2002) using ^{64}Cu application. Absorption on to membranes and cells can be associated with impaired physiological function (Stagg and Schuttleworth, 1982). Copper accumulation mainly occurs in the target tissues such as gills and liver.

1.3 Copper toxicity

Despite of its role as an essential trace element, copper is a potent toxicant. It becomes toxic in fish at elevated levels (Furness and Rainbow, 1990). Toxicity of copper alters from system to system and even from one time to another time in same system (Sylva, 1976). Cu^{2+} and $CuOH^{+}$ are two major toxic forms of copper in waterborne metal uptake (Pagenkopf et al., 1974). Multitude inorganic and organic factors affect the toxicity of copper. Water quality as pH (Sylva, 1976), alkalinity (Lauren and McDonald, 1986), water hardness (Miller and Mackay, 1980), salinity (Birdsong and Avault, 1971) and water temperature (Lydy and Wissing, 1988) were recognized as effective factors in copper toxicity. Organic substances can also affect the copper toxicity (Zitko et al., 1973, Hollis et al., 1997). In addition, size related differences in Cu toxicity in fish were reported by Lauren and McDonald (1986) and Howarth and Sprague (1978).

Copper toxicity, accumulation, and excretion are species specific (Taylor et al., 2003, De Boeck et al., 2004) and even differ in different life stages (Kazlauskiene and Stasiunaite, 1999). Increased levels of copper lead to great alterations in whole systems of fish including: behavior, physiology, reproduction, histopathology and hematology (Sorensen, 1991). Copper is an ionoregulatory toxicant, which induces dysfunction by causing a large net loss of Na^{+} and inhibition of branchial Na^{+}/ K^{+}-ATPase activity (Taylor et al., 2003, Li et al., 1998).

Physical gill injuries (Wilson and Taylor, 1993, De Boeck et al., 2001), increasing coughing frequencies (Drummond et al., 1973), and reduction in swimming performance (De Boeck et al., 2006) are the most reported effects of copper exposure in fish.

1.4 Carp as a test organism

Carp originate in Asia. They belong to the largest freshwater fish family "Cyprinidae", which dominates the freshwater fish fauna of Eurasia and North America. They were introduced to European watersheds possibly at the time of ancient Rome. Carp were present in Flanders aquatic ecosystems over 100 years ago, and they have established a permanent population (Dumortier et al., 2006).

Carp have obtained a worldwide standing in the freshwater aquaculture. According to the Food and Agriculture Organization of United Nations (FAO) dataset, farmed carp production was almost 14% of total global freshwater production (FAO, 2006). Carp have been stocked successfully worldwide with the exception of Antarctica (Huet, 1971). These omnivorous fish are known to be tolerant against water quality changes. Some adverse ecological effects of carp on endemic fish species were observed. Since this commercial species survives under a broad range of conditions, and can be easily handled under laboratory conditions, it was recommended as a test organism for toxicological experiments by OECD (1992a, 1992b).

Most available data are from studies with another commercially important family, the salmonids, and only few other species are examined (tilapia, fathead minnow, yellow perch). It has been shown that copper toxicity and accumulation are species-specific, and results of one species are hardly expandable to other species even within the same family. Therefore the study of individual species is advisable for copper toxicity studies.

1.5 Interaction between food rations and copper toxicity

Fish growth and performance are generally affected by feeding rates (Bert, 1979). Toxicants can also disturb growth of fish either directly or

indirectly via effects on feeding or by increasing maintenance costs (Kooijman and Bedaux, 1996). More attention has been given to the interaction between food and metal toxicity in recent studies. It is suggested that the nutritional status, fish size, and growth rates should be considered when comparing tissue Cu concentrations for biomonitoring and risk assessment. Segner (1987) found higher copper accumulation in the liver of starved roach (*Rutilus rutilus*) compared to fed ones following a one week sublethal copper exposure, and suggested the nutritional status of fish as an important factor modifying the response of fish to sublethal Cu contamination. Significant interactions between food rations, food composition, and copper accumulation were also found in rainbow trout during dietary copper exposure, and a low feeding regime resulted in higher Cu concentrations in the tissues (Kamunde and Wood, 2003). Furthermore, an antagonistic competition between Cu and Na^+ which share the same channel for entry into epithelial cells was reported by Grosell and Wood, 2002, and Pyle et al., 2003. It was suggested that dietary sodium inhibited waterborne copper uptake (Pyle et al., 2003) and reduces Cu accumulation and toxicity of waterborne Cu in the rainbow trout (Kamunde et al., 2005). These studies were mainly focused on the toxicity of Cu available through the food and they compared its importance to waterborne Cu exposure. Recently some studies have also focused on the effect of food composition and food availability on the performance of fish during waterborne Cu exposure (Niyogi et al. 2006), or they combined both aspects in their study (Kamunde and Wood 2003; Kjoss et al. 2005a, b). Most of these studies are conducted on trout, and no studies investigated this approach in other important species such as cyprinids.

1.6 Metallothionein induction

Metallothionein (MT) is a low molecular weight non-enzymatic and non-aromatic protein which includes high cysteine levels. The thiol (-SH) groups of cystein residues allow MT to bind heavy metals. MT was identified for the first time in equine renal cortex by Margoshes and Vallee (1957). MT was also found in liver, gills, and kidney of freshwater fish (De Boeck et al., 2003). It plays a key role in the regulation of homeostasis of essential metals within cells. MT was introduced as biomarker of environmental metal pollution by United Nations Environmental Programme (UNEP/ RAMOGE, 1999). It is widely used as a cost-effective molecular biomarker for the monitoring of copper contamination. MT induction was reported both in starvation and copper exposure processes in fish. While significant differences in the levels of MT mRNA between starved and control cod (*Gadus morhua*) were reported by McNamara and Buckley (1994), increased levels of MT were associated with escalated tissue copper accumulation in some freshwater fish (De Boeck et al., 2003). MT is likely involved in metal detoxification process (Roesijadi et al., 1982). MT induction was introduced as a defense mechanism against metal pollution in aquatic species including fish (Roesijadi, 1994). Higher MT induction in starved fish probably also can affect both copper accumulation and fish sensitivity to copper exposure. There are few studies reporting higher levels of MT in the starved fish, but to our knowledge, no reports exist about effect of starving on MT induction in the carp. Furthermore, time dependent alterations in MT induction levels during a long term sublethal waterborne copper exposure in fish are still subjects of investigation.

1.7 Aims and objectives of study

In this study, the interactions between food rations and chronic and acute waterborne copper exposure in carp were studied. Physiological and metabolic modifications in carp during long term waterborne copper exposure including acclimatization, accumulation, sensitivity, growth, energy stores, and metabolic waste changes were investigated as well as the involved toxic mechanisms.

The aims of this study are:

1: Investigating of effects of different food rations during sublethal Cu exposure on growth, energy stores, and metabolic waste in carp

2: Analyzing copper bioaccumulation in different tissues of carp under different feeding regimes during chronic and acute waterborne Cu exposure

3: Evaluating of sensitivity of starved and fed carp against acute waterborne copper exposure

4: Assessing the effects of a long term acclimatization to a sublethal Cu exposure on copper toxicity and copper accumulation in carp

5: Exploring the differences between toxicity mechanisms in fed and starved carp during waterborne copper exposure

In the first study, the effects of different food rations combined with sublethal Cu exposure on growth in common carp were assessed. In addition, the effects of copper exposure on hepatic energy stores and metabolic waste products between the low and high feeding treatments were investigated. The results of this study are discussed in chapter two which is based on the published paper in Archives of Environmental Contamination and Toxicology. Hashemi, S., Blust, R., De Boeck, G., 2007. Combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp. Archives of

Environmental Contamination and Toxicology 10.1007/s00244-007-9017-1.The results of this study were also presented at the 12th Benelux congress of Zoology, Wageningen, The Netherlands, 2005.

In the second study, the bio-accumulation of copper in different tissues of common carp was compared under different feeding regimes during a chronic waterborne copper exposure. The results of this research are explained in chapter three which is based on the published paper by Hashemi S., Blust R., De Boeck G., 2007. The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. Journal of Environmental Toxicology and Chemistry 26(7):1507-1511. The results were also presented at the annual meeting of the Society for Experimental Biology (SEB) at Canterbury, United Kingdom, 2006.

In the third study, the sensitivity of starved and fed fish against acute waterborne copper exposure was investigated. Furthermore, the copper uptake and the effects of long term acclimatization to a sublethal Cu concentration were studied. The relationship between copper accumulation and toxicity was established. The results of this study are explained in chapter four, based on the paper by Hashemi S., Blust R., De Boeck G., 2007. The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp, Accepted by Aquatic Toxicology. The results of this study were presented at the 24th annual meeting of the European Society for Comparative Physiology and Biochemistry (ESCPB), Antwerp, Belgium, 2006, and 13th Benelux congress of Zoology in Leuven, Belgium, 2006.

Eventually the mechanisms involved in copper uptake and toxicity during sublethal waterborne copper exposure in carp were investigated in the

last study. The results of this study are introduced in chapter five, which is based on manuscript by Hashemi S., Blust R., De Boeck G., 2007. Differential metallothionein induction patterns in fed and starved carp during waterborne copper exposure which was submitted to the Journal of Environmental Toxicology and Chemistry. An overview of this research was presented in 17th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC) Europe in Porto, Portugal, 2007.

References

Abdel- Mageed, A.B., Oehme, F.W. 1990. A review of biochemical roles, toxicity and interaction of zinc, copper and iron: II. Copper. Vet. Hum. Toxicol 32(3): 230-234.

Bert, J.R. 1979. Environmental factors and growth. In: Hoar WS, Randall DJ, Bertt JR (ed) Fish Physiology, vol VIII. Bioenergetic and Growth. Academic press, London, pp. 599-675.

Bervoets, L., Blust, R. 2003. Metal concentrations in water, sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor, Environ. Pollut. 126: 9-19.

Bervoets, L., Knaepkens, G., Eens, M., , Blust, R. 2005. Fish community responses to metal pollution. Environ. Pollut. 138(2): 338-349.

Birdsong, C.L., Avault, J.W. 1971. Toxicity of certain chemicals to juvenile pompano. The Progressive Fish Culturist 33:76-80.

Bowen, H.J.M. 1985. In D. Hutzinger (ed.), The Handbook of Environmental Chemistry, Vol. 1, Part D: The natural environment and biogeochemical cycles, Springer-Verlag, New York. p. 1-26.

Brungs, W.A., Leonard, E.N., Mckim, J.M. 1973. Acute and long term accumulation of copper by the brown bullhead, *Ictalurus nebulosus*. J. Fish. Res. Board. Can. 30: 583-586.

Buckley, J.T., Roch, M., McCarter, J.A., Rendell, C.A., Matheson, A.T. 1982. Chronic exposure of coho salmon to sublethal concentration of copper –I. Effect on Growth, on Accumulation and distribution of copper, and on copper tolerance. Comp. Biochem. Physiol. 72: 15-19.

Cousins, R.J. 1985. Absorbtion, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol. Rev. 65(2): 238-309.

De Boeck, G., Vlaeminck, A., Balm, P.H.M., Lock, R.A.C., de Wachter, B., Blust, R. 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environ. Toxicol. Chem. 20: 374-381.

De Boeck, G., Ngo, T.T., Van Campenhout, K., Blust, R. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. Aquat. Toxicol. 65, 413-424.

De Boeck, G., Meeus, W., De Coen, W., Blust, R. 2004. Tissue-specific Cu bioaccumulation patterns and differenced in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Onchorynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). Aquat. Toxicol. 70: 179-188.

De Boeck, G**.**, van der Ven, K*.,* Hattink, J., Blust, R. 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat. Toxicol. 80(1): 92-100.

Drummond, R.A., Spoor, W.A., Olson, G.F. 1973. Some short-term indicators of sublethal effects of copper on brook trout, *Salvelinus fontinalis*. J. Fish. Res. Board. Can. 30: 689-701.

Dumortier, M., De Bruyn, L., Hens, M., Peymen, J., Schneiders, A., Van Daele, T., Van Reeth, W., Weyembergh, G., Kuijken, E. 2006. Biodiversity indicators 2006. State of nature in Flanders (Belgium). Research Institute for Nature and Forest, Brussels.

Food and Agriculture Organization of United Nations (FAO), 2006. State of world fisheries and aquaculture, Rome, Italy, P 180.

Furness, R.W., Rainbow, P.S. 1990. Heavy metals in the Marine Environment. CRC Press, Florida, United States.

Grosell, M., Wood, C.M. 2002. Copper uptake across rainbow trout gills: mechanism of apical entry. J. Exp. Biol. 205: 1179-1188.

Harris, E.D. 1991. Copper transport: an overview. Proc. Soc. Exp. Med. 192: 130-140.

Higashimoto, M., Sano, M., Kondoh, M., Sato, M. 2002. Different responses of metallothionein and leptin induced in the mouse by fasting stress. Biol. Trace Elem. Res. 89:75-84.

Hollis, L., Muench, L., Playle, R.C. 1997. Influence of dissolved organic matter on copper binding, and calcium on cadmium binding, by gills of rainbow trout. J. Fish Biol. 50: 703-720.

Howarth, R.S., Sprague, J.B. 1978. Copper lethality to rainbow trout in waters of various hardness and pH. Water Res. 12 (7): 455- 462.

Huet, M. 1971. Textbook of fish culture, breeding and cultivation of fish. Farnham, Surrey, Fishing News (Books) Ltd., 454 p.

Kamunde, C., Grosell, M., Lott, J.N., Wood, C.M. 2001. Copper metabolism and gut morphology in rainbow trout (*Onchorynchus mykiss*) during chronic sublethal copper exposure. Can. J. Fish. Aquat. Sci. 58: 293-305.

Kamunde, C., Grosell, M., Higgs, D., Wood, C.M. 2002. Copper metabolism in actively growing rainbow trout (*Onchorynchus mykiss*): interaction between dietary and waterborne copper uptake. J. Exp. Biol. 205:279-290.

Kamunde, C., Wood, C.M. 2003. The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss* . Aquat. Toxicol. 62 (3): 235-254.

Kamunde, C.N., Niyogi, S., Wood, C.M. 2005. Interaction of dietary sodium chloride and waterborne copper in rainbow trout (*Onchorynchus*

mykiss): Copper toxicity and sodium and chloride homeostasis. Can. J. Fish .Aquat. Sci. 62:390–399.

Kazlauskiene, N., Stasiunaite, P. 1999. The lethal and sublethal effect of heavy metal mixture on rainbow trout (*Onchorynchus mykiss*) in its early stages of development. Acta Zoologica Lituanica. Hydrobiologia 9(2): 47-55.

Kjoss, V.A., Grosell. M., Wood, C.M. 2005a. The influence of dietary Na on Cu accumulation in juvenile rainbow trout exposed to combined dietary and waterborne Cu in soft water. Arch. Environ. Contam. Toxicol. 49 (4): 520-527.

Kjoss, V.A., Kamunde, C.N., Niyogi, S., Grosell, M., Wood, C.M. 2005b. Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. J. Fish Biol. 66 (2): 468-484.

Kooijman, S.A.L.M., Bedaux, J.J.M. 1996. Analysis of toxicity tests on fish growth. Water Res. 30: 1633-1644.

Lauren, D.J., McDonald, D.G. 1986. Influence of water hardness, pH and alkalinity on the mechanism of copper toxicity in juvenile rainbow trout, *Salmo gairdneri.* Can. J. Fish. Aquat. Sci. 43: 1488-1496.

Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., lock, R.A.C. 1998. Effects of waterborne copper on branchial chloride cells and Na^+/ K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquat. Toxicol. 43, 1-11.

Lydy, M.J., Wissing, T.E. 1988. Effect of sublethal concentrations of copper on the critical thermal maxima (CTMax) of the fantail (*Etheostoma flabellare*) and johnny (*E. nigrum*) darters. Aquat. Toxicol. 12(4): 311-322.

Margoshes, M., Vallee, A. 1957. A cadmium protein from equine kidney cortex. J. Am. Chem. Soc 79: 4813-4814.

McNamara P.T., Buckley, L.J. 1994. Identification and characterization of metallothionein cDNA from mRNA transcripts induced by starvation in Atlantic cod (*Gauds morhua*). Mol. Mar. Biol. Biotechnol. 3(5), 252-260.

Miller, T.G., Mackay, W.C. 1980. The effects of hardness, alkalinity, and pH of test water on the toxicity of copper to rainbow trout (*Salmo gairdneri*). Water Res. 14(2): 129-133.

National Research Council (U.S.A), 1993. Nutrient requirements of fish. National academy press. United States p. 128.

Niyogi, S., Kamunde, C.N., Wood, C.M. 2006. Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu exposure. Aquat. Toxicol. 77 (2): 210-221.

Organisation for Economic Co-operation and Develompent (OECD), 1992a. Fish Early-life stage test, toxicity test. Test guideline 210, Paris, France.

Organisation for Economic Co-operation and Development (OECD) 1992b. Fish, Acute toxicity test. Test guideline 203, Paris, France.

Pagenkopf, G.K., Russo, R.C., Thurston, R.V. 1974. Effects of complexation on toxicity of copper to fishes. J. Fish. Res. Board. Can. 31: 462-465.

Patterson, J.W., Minear, R.A., Gasca, E., Petropoulou, C. 1998. Industrial discharges of metals to water. In: H.E. Allen, A.W. Garrison and G.W. Luther III (Eds.). Metals in Surface Waters. Ann Arbor Press, Chelsea, MI. pp. 37-66.

Pilgaard, L., Malte, H., Jensen, F.B. 1994. Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Onchorynchus*

mykiss) under normoxic and hypoxic conditions. Aquat. Toxicol. 29:197-212.

Pyle, G.G., Kamunde, C., Mcdonald, D.G., Wood, C.M. 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Onchorynchus mykiss*). J. Exp. Biol. 206:609–618.

Roesijadi, G., Drum, A.S., Thomas, J.M., Fellingham, G.W. 1982. Enhanced mercury tolerance in marine mussels and relationship to low weight, mercury-binding proteins. Mar. Pollut. Bull. 13(7): 250-253.

Roesijadi, G. 1994. Metallothionein induction as a measure of response to metal exposure in aquatic animals. Environ. Health Prospect. 102 (Suppl 12), 91-96.

Taylor, L.N., McGeer, J.C., Wood, C.M., McDonald, D.G. 2000. The Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. *Environ Toxicol Chem* 19, 2298-2308.

Schlenk, D., Gollon J.L., Griffin, B.R. 1998. Efficacy of copper sulfate for the treatment of Ichthyophthiriasis in channel catfish. J. Aquat. Anim. Health 10: 390-396.

Segner, H. 1987. Response of fed and starved roach, *Rutilus rutilus*, to sublethal copper contamination. J. Fish Biol. 30: 423-437.

Sorensen, Elsa M. B. 1991. Metal Poisoning in Fish. CRC Press, Boca Raton, Florida, United states, P 374.

Stagg, R.M., Schuttleworth, T. J. 1982. The accumulation of copper in *Plaichthys fleus L.* and its effects on plasma electrolyte concentrations. . J. Fish Biol. 20:491-500.

Sylva, M.J. 1976. The Environmental chemistry of copper (II) in aquatic systems. Water Res. 10: 789-792.

Taylor, L. N. , Wood, C.M., McDonald, D.G. 2003. An evaluation of sodium loss and gill metal binding properties in rainbow trout and yellow

perch to explain species differences in copper tolerance. Environ. Toxicol. Chem. 22: 2159–2166.

The Flemish government, 2000. Besluit van de Vlaamse regering van 1 juni 1995 houdende vaststelling van het Vlaamse reglement betreffende de milieuvergunnung (Vlarem), zoals gewijzigd bij besluit van 17 juli 2000. Belgisch Staatsblad.

United Nations Educational, Scientific and Cultural Organization (UNESCO), 2003. The 1st United Nations world water development report: water for people, water for life, P 576.

United Nations Environmental Programme (UNEP/ RAMOGE), 1999. Manual on the biomarkers recommended for the MED POL biomonitoring programme. Greece, Athens P 92.

US Environmental Protection Agency, 1999. National recommended water quality criteria—correction: EPA 822/Z-99-001.Washington, DC., United States.

US Environmental Protection Agency (EPA), 2007. Aquatic life ambient freshwater quality criteria copper. EPA 7440-50-8. Washington, DC., United States, 204p.

Warmer, H., Van Dokkum, R. 2002. Water pollution control in the Netherlands, policy and practice. RIZA report, Lelystad, The Netherlands, 76p.

Wilson, RW, Taylor; E.W. 1993. The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. B 163: 38-47.

YamamotoY., Ishii, T., Ikeda, S. 1997. Studies on copper metabolism in fishes II. The site of copper accumulation in the tissues of carp. Bull. Jap. Soc. Scient. Fish. 43:1327-1332.

Zitko, P., Carson, W.V., Carson, W.G. 1973. Prediction of incipient lethal levels of copper to juvenile Atlantic salmon in the presence of

humic acid by cupric electrode. Bull. Environ. Contam. Toxicol. 10: 265-271.

2

Combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp

Shodja Hashemi, Ronny Blust, Gudrun De Boeck

Published in Archives of Environmental Contamination and Toxicology
10.1007/s00244-007-9017-1

Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Groenenborgerlaan171, 2020 Antwerp, Belgium

Abstract

Common carp (Cyprinus carpio) were fed two different rations: 0.5% body weight (low ration, LR) and 5% body weight (high ration, HR) throughout acclimation, sublethal (64 µg/L) Cu exposure for 28 days, and a subsequent 2 week recovery period. Growth, liver water content and liver energy stores were assessed during this period. Growth rates were elevated in HR fish compared to LR fish, as was the hepatic lipid content. This was associated with a higher water content in the livers of LR fish. Liver glycogen levels were similar in both feeding treatments and remained relatively stable during Cu exposure. Cu exposure caused a significant reduction in growth in both HR and LR fish. Reduction in growth coincided with significant changes in liver and blood composition. Liver lipid levels dropped significantly during the first days of the exposure in both feeding treatments, and the difference between LR and HR lipid levels disappeared during Cu exposure. During the first week of Cu exposure, the difference in liver water content disappeared as well, with a significant reduction of the water levels in the LR fish. A rise of hepatic protein was observed after two and four weeks of Cu exposure in the LR carp and after 4 weeks of exposure in the HR fish. Cu exposure led to pronounced increases in plasma ammonia concentrations in HR carp. Growth and energy stores recuperated during the two weeks recovery period in both feeding treatments. The observed changes during Cu exposure are probably related to physiological disturbances like hypoxia and stress, as well as an inhibition of ammonia excretion.

2.1 Introduction

Sublethal long-term exposure of fish to Cu involves a succession of physiological and metabolic adjustments or acclimations that allow long-term survival (McGeer et al. 2000, Flik et al. 2002). These acclimations are usually reversible, and fish recover when the exposure ends (Handy 2003). Studies on chronic toxicity are limited, and the mechanisms of chronic Cu toxicity in fish have not been studied as well as the mechanisms of acute toxicity. Feeding rates are one of the most important factors affecting fish performance and growth (Bert 1979), and toxicants can affect growth of fish either directly or indirectly via effects on feeding or by increasing maintenance costs (Kooijman and Bedaux 1996). Segner (1987) also noted that the nutritional status of roach (*Rutilus rutilus*) is an important factor modifying the response to sublethal Cu contamination.

Recently, more attention has been given to the interaction between food and metal toxicity. Most of these studies focused on the toxicity of Cu available through the food and compare its importance to waterborne Cu exposure (Handy et al. 1999; Bielmyer et al. 2005; Kjoss et al. 2006; Shaw and Handy 2006). Recently some studies have also focused on the effect of food composition and food availability on the performance of fish during waterborne Cu exposure (Niyogi et al. 2006), or combine both aspects in their study (Kamunde and Wood 2003; Kjoss et al. 2005a, b). Kamunde and Wood (2003) suggested that the nutritional status, fish size, and growth rates should be considered when comparing tissue Cu concentrations for biomonitoring and risk assessment.

A parallel study using common carp fed two different food rations (0.5 and 5.0% bodyweight [BW], respectively) showed substantial differences in the response to sublethal waterborne Cu exposure (Hashemi et al.

2007). Fish fed on the high food ration accumulated less Cu in their tissues but suffered more mortality when exposed to increased Cu concentration, indicating that some indirect effects of the Cu exposure might play an important role (Hashemi et al. unpublished results). Differences in metabolic rate, available energy, and growth are to be expected between the two feeding rations and might affect the response to Cu exposure. Therefore, the objective of this study was to assess the effects of different food rations combined with sublethal Cu exposure on growth in common carp. Since growth is energy demanding process, we also investigated whether hepatic energy stores were affected and whether metabolic waste products such as ammonia differed between the two feeding treatments.

2.2 Materials and methods

2.2.1 Animal holding

Common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Wageningen University, The Netherlands and kept at the University of Antwerp at 23±1°C in softened Antwerp city tap water. For exposure experiment, fish of comparable size were selected (mean initial weight of 16.3±0.3 g) and were randomly distributed in a flow-through system consisting of six 200 L tanks with a density of 55 fish per tank. Fish were acclimated to test conditions for 52 days before Cu exposure started and were pre-acclimated to either 0.5% (low ration, LR) or 5% (high ration, HR) BW food rations with Trouvit (Trouw nutrition, Fontaine-les-Vervins, France) divided over two equal feedings per day. A passive integrated transponder system (PIT-Tag, Trovan unique, ID 100 implantable) was injected into the abdominal cavity of the fish 2 weeks before exposure started to allow calculation of individual growth rates.

PIT tags have a 100% retention rate in carp (Basavaraju et al. 1998), which was confirmed in our study.

2.2.2 Experimental set up

Toxicity tests were conducted at the Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Antwerp, Belgium, according to guideline 210 of the OECD (1992). The photoperiod was set at 12 L: 12 D. Water quality was checked daily, the pH was 7.5-8.0, oxygen concentration remained above 7 mg/L, and ammonia concentration below 0.1 mg/L at all times. Water hardness was 270 mg $CaCO_3$/L, and ion concentrations in the water as follows: 79.3 mg/L Ca^{2+}, 7.4 mg/L Mg^{2+}, 27.8 mg/L Na^{+}. Both feeding treatments of fish were exposed to a sublethal total concentration of 64 µg/L Cu in the flow-through system for a period of 28 days. The tanks were predosed with Cu $(NO_3)_2 \cdot 2H_2O$ to bring the concentration to the nominal level. A stock copper nitrate solution (Cu $(NO_3)_2 \cdot 2H_2O$ 0.722 g/L) (Merck, Darmstadt, Germany) was added into the tanks with the inflowing water using a peristaltic pump (Watson Marlow 505 S) during the entire experiment. Each feeding treatment was split into two replicate tanks with Cu exposure plus one without exposure. The amount of food fed to each exposure tank was adjusted weekly for fish growth and sampling. Fish were sampled at regular intervals (day 0, 3, 7, 14, 28, and 2 weeks after the end of exposure). Control and exposure tanks were cleaned daily to minimize the accumulation of feces and uneaten food. The fish were not fed the day of sampling to allow for gut clearance and minimize weight differences between fish caused by feeding and gut clearance.

2.2.3 Sampling procedure and sample analysis

Every week all fish were collected from the tanks for weighing. Growth data are presented as (W2-W1)/W1, where W1 and W2 were weight of fish in previous week and weight of fish at the time of measuring respectively. Growth data were subsequently converted to percentage of growth per week. According to Kooijman and Bedaux (1996) weight measurements are usually more accurate than length measurements.

Eight fish were removed from the exposure and control tanks at each sampling period. Fish were quickly netted and killed by an overdose of buffered MS 222 (1 g/L at pH 7.5, Acros Organics, Geel, Belgium). All fish were weighed and immediately dissected on ice. Liver tissue was rinsed with physiological saline (0.9% NaCl), frozen in liquid nitrogen, and stored at -20°C for biochemical determination. A blood sample was collected from caudal blood vessel using a heparinized syringe. Blood was immediately centrifuged, and aliquots of plasma were frozen in liquid nitrogen and later stored at -80°C for ammonia determination.

Bradford's (1976) reagent was used for protein analysis, glycogen was determined using Anthrone reagent (Roe and Dailey, 1966), and total lipids were extracted and measured following Bligh and Dyer (1959). Total plasma ammonia (sum of NH_3 and NH_4^+) was determined using an enzymatic kit (r-Biopharm 11 112 732 035 Boehringer Mannheim Darmstadt, Germany). For determination of the water content of the liver, sub-samples were weighed, dried at 60°C for 72 h, and reweighed afterward.

2.2.4 Statistical Analysis

All values are presented as mean± S.D. Data were evaluated by analysis of variance (ANOVA), Tukey multiple comparison procedure, and Kruskal-Wallis test via GraphPad Instat. Bonferroni's test was used when there were missing samples. All data were natural logarithm transformed

to stabilize the variance and to approximate normal distribution prior to use in further statistical analysis. Data were considered significant when $p<0.05$. Control animals did not show significant differences in hepatic protein, glycogen, lipid or water content over time, and for clarity of the figures all control values were averaged, and these average values are shown at day 0.

2.3 Results

Fish feeding on 5% BW performed significantly better in terms of weight gain and growth compared to the fish fed on 0.5% BW (Fig. 1). Average growth rates for control HR fish were 11.5% per week compared to 1.3% per week for LR fish. Copper exposure induced a significant reduction in growth in both feeding treatments (Fig. 1). Growth reduction started at the first week of exposure in the HR exposed carp and continued during the entire exposure period. In the LR fish, growth rates were low in both control and exposed animals and a significant reduction in growth only occurred in the second week when fish actually lost weight (Fig. 1). At the end of the one month exposure period, weight of the HR and LR exposed fish was reduced by 19.17% and 17.01% compared to their respective controls.

The weight loss was accompanied by biochemical changes in liver and blood of the exposed fish. In control fish, the hepatic lipid content in the HR fish was significantly elevated compared to the LR group. Liver lipid levels dropped significantly during the first 3 days of Cu exposure in both feeding treatments (Fig. 2). As the Cu exposure continued, the differences between hepatic lipid content in LR and HR fish disappeared, mainly because of the low lipid levels in HR fish. By the end of the

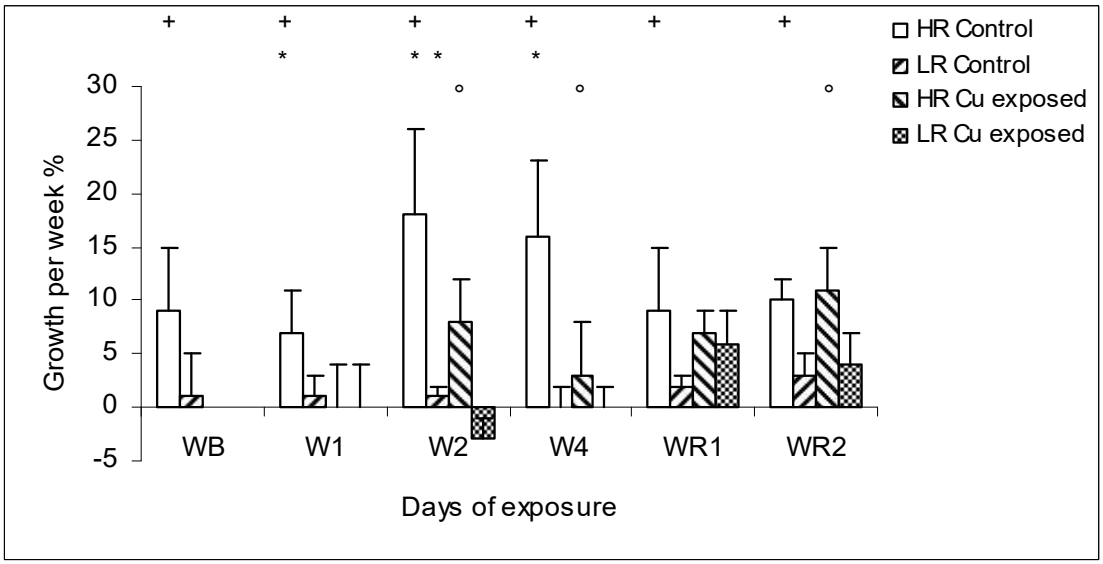

Fig. 1**:** Growth rates in carp fed two different food rations during a 28-day exposure to 64 µg/L Cu followed by a 2-week recovery period. LR, 0.5 % body weight (BW); HR, 5% BW. WB: week before the exposure started; W1-W4, weeks of exposure, WR1-WR2, weeks of recovery, (+) Significant difference between control LR and HR carp; (°) significant difference between exposed LR and HR carp; (*) significant difference between exposed carp and its respective control

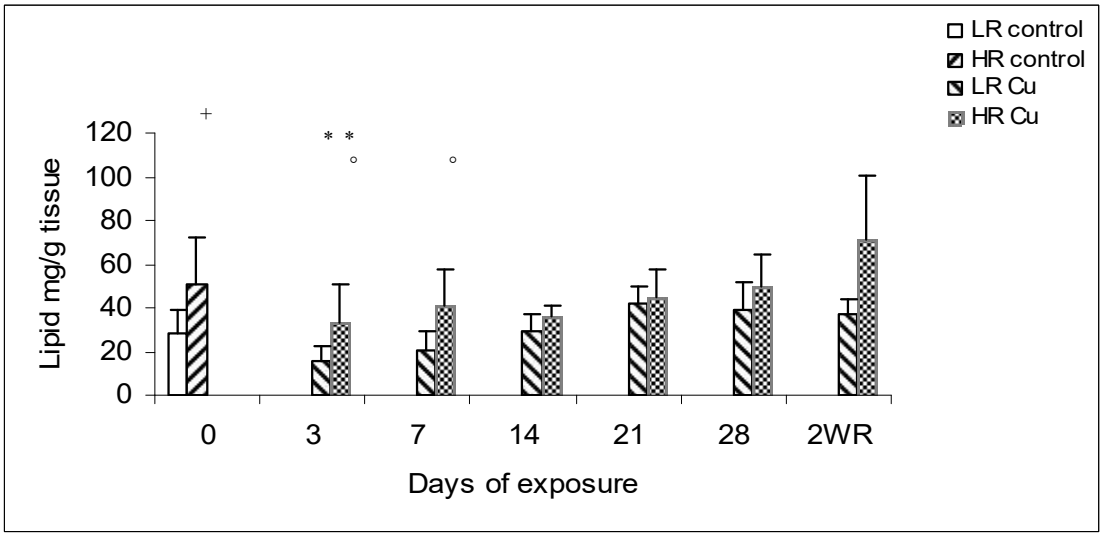

Fig. 2**:** Lipid content in liver tissue of carp fed two different food rations (LR, 0.5 % body weight [BW], HR, 5% BW) during a 28-day exposure to 64 µg/L Cu and after 2 weeks recovery (2WR). Significant difference in lipid content (°) between the exposed LR and exposed HR carp, (*) between the exposed carp and its respective control, and (+) between the control LR and control HR carp

exposure, and during recovery the difference in lipid levels seemed to restore, although they had not yet returned to significant levels at the end of our experiment. Variations in the lipid content were correlated to changes in the water content of fish livers (Fig. 3), and a negative relationship could be demonstrated between liver lipid and water content ($P<0.0001$ for all fish). Hepatic glycogen levels did not differ between control LR and HR fish (Fig. 4). In the exposed fish, liver glycogen in the LR fish was significantly lower than in the HR fish after three days of the exposure, but subsequently it peaked in the LR group after which it slowly recovered to control levels. This peak in glycogen coincided with a significant drop in water content after one week of exposure in this same group. Various feeding rates had no significant effect on the hepatic protein levels in the controls, but in the exposed LR fish protein levels changed over time. Liver protein dropped in the LR group during the first days, and increased significantly thereafter. A significant increase in

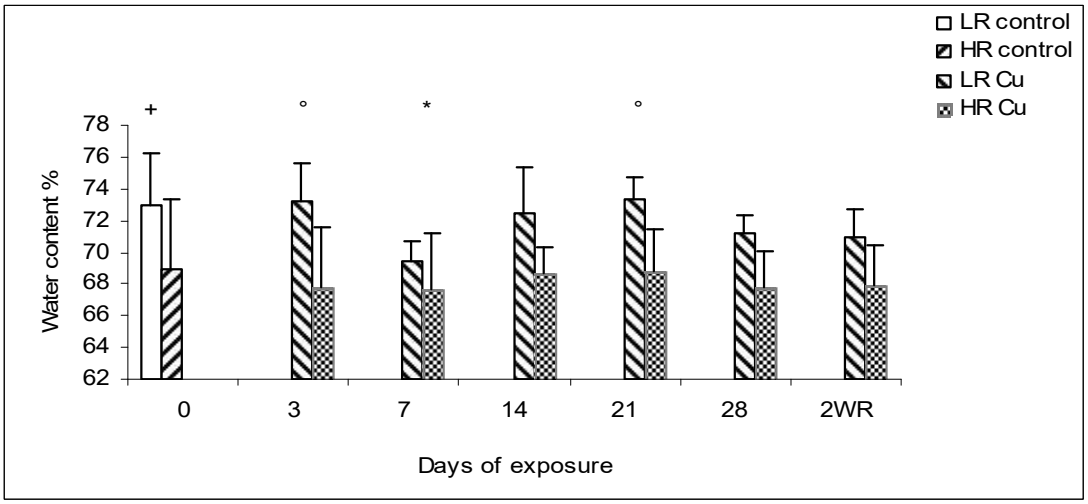

Fig. 3**:** Water content in liver tissue of carp fed two different food rations (LR, 0.5 % body weight [BW], HR, 5% BW) during a 28-day exposure to 64 µg/L Cu and after 2 weeks recovery (2WR). Significant difference in water content (°) between the exposed LR and exposed HR carp, (*) between the exposed carp and its respective control, and (+) between the control LR and control HR carp

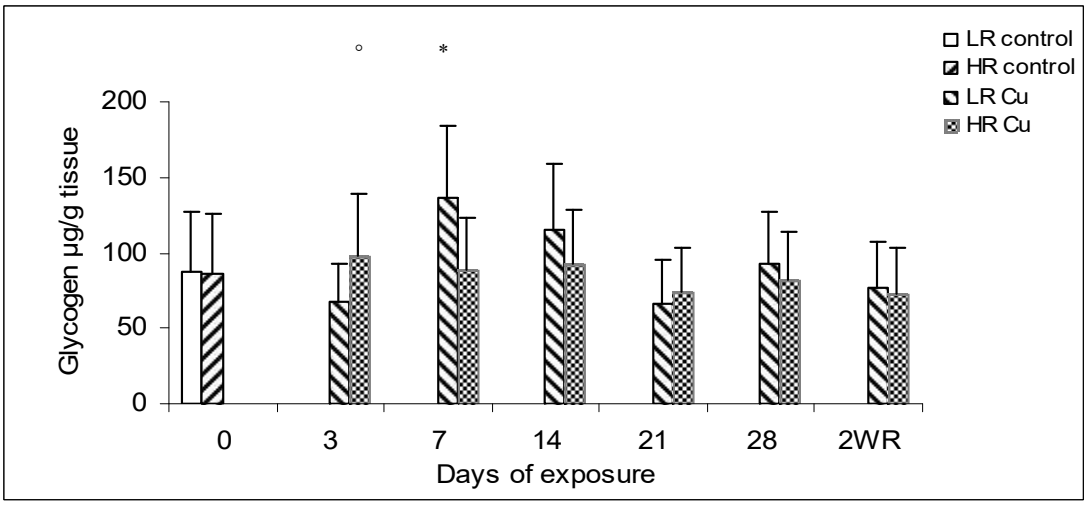

Fig. 4. Glycogen content in liver tissue of carp fed two different food rations (LR, 0.5 % body weight [BW], HR, 5% BW) during a 28-day exposure to 64 µg/L Cu and after 2 weeks recovery (2WR). Significant difference in glycogen content (°) between exposed LR and exposed HR carp and (*) between the exposed carp and its respective control

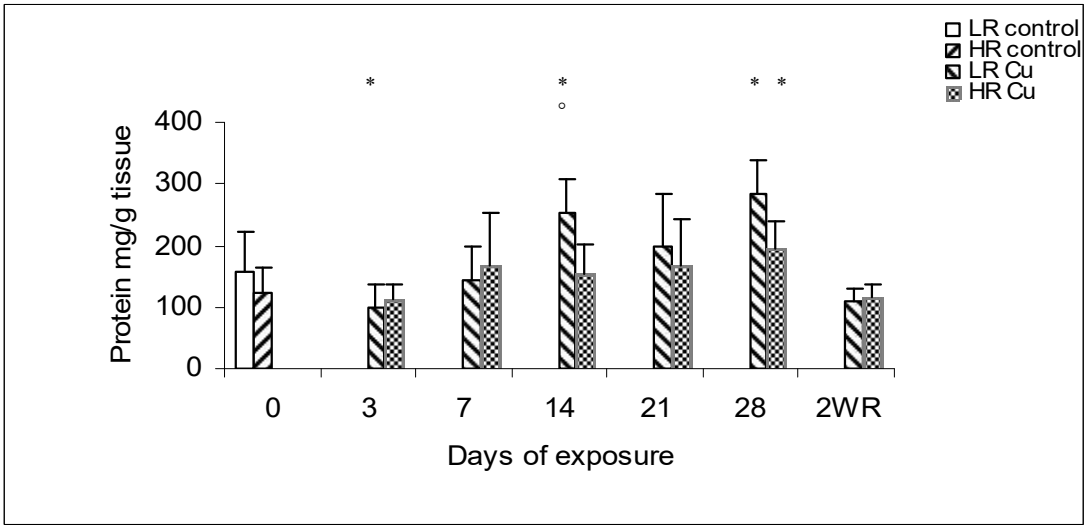

Fig. 5. Protein content in liver tissue of carp fed two different food rations (LR, 0.5 % body weight [BW]; HR, 5% BW) during a 28-day exposure to 64 µg/L Cu and after 2 weeks recovery (2WR). Significant difference in protein content (°) between the exposed LR and exposed HR carp and (*) between the exposed carp and its respective control

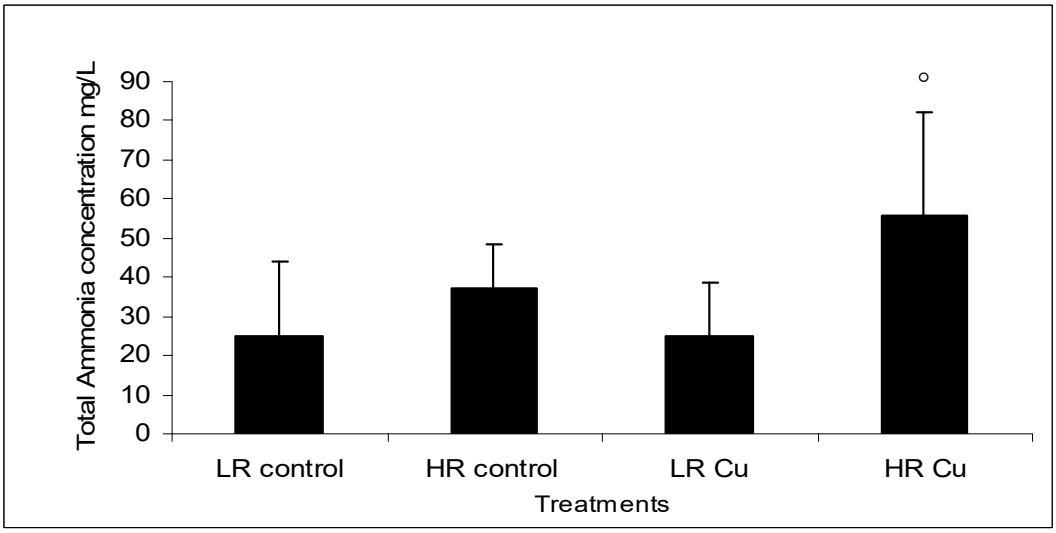

Fig. 6**.** Pooled data for plasma ammonia in carp fed two different food rations (LR, 0.5 % body weight [BW]; HR, 5% BW) during a 28-day exposure to 64 µg/L Cu and after 2 weeks recovery (2WR). Significant difference in plasma ammonia (°) between the exposed LR and exposed HR carp

protein occurred in the HR fish during the last week of exposure as well (Fig. 5). Copper exposure led to a pronounced increase in plasma ammonia concentration in the HR carp. While plasma ammonia concentrations in the HR carp always tended to be higher than in the LR fish, the deviation was intensified during Cu exposure (Fig. 6).

2.4 Discussion

Feeding carp with a high food ration led to increased growth rates and liver lipid stores. It is well known that body lipids increase with increasing amounts of food (Weatherley and Gill 1987; Jobling 1994; Shearer 1994). Food ration did not affect hepatic glycogen or protein content in accordance with Abdelghani and Ahmad (2002), who also did not find a difference in protein levels when carp were fed at 0%, 1%, 3%, or 5% BW. Exposure to 64 µg/L of waterborne Cu affected growth and weight gain of both the HR and LR carp significantly. Similar results were obtained earlier (De Boeck et al. 1997) with carp fed *ad libitum*

once a day during a 28-day exposure to 0, 12.7, 35 and 50.8 µg/L of waterborne Cu. At 50.8 µg/L of Cu, the exposure concentration closest to our exposure level, the authors observed a reduced food intake during the first two weeks of Cu exposure and a decreased growth rate over the entire exposure duration. This suggests that the reduced growth rates were not only a direct effect of the reduced food intake but also an indirect effect of an increased energy demand under Cu exposure. This finding was confirmed at 35 µg/L where food intake was not reduced but growth rates slowed down, and at 12.7 µg/L, where growth rate was not affected but food uptake was increased to maintain this growth rate. Our study showed that the effect was most pronounced in the HR fish, where the reduction in growth rate lasted for the entire exposure period, while in the LR fish growth was only reduced during the second week of exposure. Since food uptake was probably only affected during the first days to weeks of the exposure (De Boeck et al. 1997), the extended reduction in growth in the HR fish confirms that an extra cost is associated with sublethal Cu exposure. Possibly, the low growth rates and a corresponding lower metabolism of the LR carp protected them from the damaging effects that Cu exerts, which include disturbances in oxygen and ion uptake.

Gill epithelia are the primary target for waterborne Cu exposure (Sola et al. 1995; Taylor et al. 1996), and the disruption of gill epithelia has been observed in both the carp and salmonids (Benedeczky et al. 1986; Marek et al. 1991; Kirk and Lewis 1993; Wilson and Taylor 1993). Gill injury includes hypertrophy and hyperplasia, as well as increased mucus excretion, resulting in increased diffusion distances. In common carp, the damage was most severe in the shock phase during the first few days of exposure, with a recovery thereafter (De Boeck et al. 2007). Gill damage was combined with reduced ventilation, leading to reduced oxygen

consumption rates and decreased arterial oxygen pressures (De Boeck et al. 2006, 2007). Therefore, the HR fish with high growth rates and metabolism were probably more affected by the disturbed oxygen uptake while fish with low growth rates combined with a low metabolic cost could possibly still sustain this low metabolism, which resulted in reduced effects of Cu exposure in LR fish.

Hansen and co-workers (2002) suggested that both physiological and behavioral stress cause a reduction in growth of fish during exposure to a toxicant. Wendelaar Bonga (1993) pointed out that the release of stress catecholamines and corticosteroid hormones such as adrenaline, noradernaline, and cortisol is the first response to a stressor such as Cu exposure. Catecholamines and corticosteroids cause a secondary stress response, including a series of biochemical and physiological changes such as hyperglycemia, hyperlacticemia, and a depletion of glycogen tissue reserves, lipolysis, and inhibition of protein synthesis. A transitory release of cortisol has previously been observed in Cu exposed carp (De Boeck et al. 2001) and coincides with the first days of exposure, when feeding was reduced and lipolysis occurred. Thus, the fact that fish primarily used hepatic lipid stores during this study was probably triggered both by the reduced food intake and the increased levels of stress during the first days of exposure.

Changes in liver glycogen were limited, as we only observed an initial reduction followed by a peak of hepatic glycogen in the Cu exposed LR carp. Gluth and Hanke (1985) found an increase in liver glycogen concentration in carp before it reduced after chronic exposure. Possibly, these responses in liver glycogen are in fact responses to the induced hypoxia. LR fish, with limited energy stores, might use their glycogen store more readily compared to HR fish. The subsequent increase in glycogen could be a defense mechanism against the hypoxia and

indication of an increasing anaerobic metabolism (De Boeck et al. 1995). We did not observe an inhibition of protein synthesis but, on the contrary, found an increase in liver protein levels in the second half of the exposure. This is in agreement with earlier studies (Hilmy et al. 1987; Gill et al. 1992), where increases in hepatic protein have been found as the most conspicuous response to metal toxicity. An increase in protein synthesis could also be a physiological response to stress (Handy 2003).

Ammonia, which is the major breakdown product of protein catabolism was slightly higher in HR fish and increased further during Cu exposure. Since ammonia is toxic, the observed ammonia accumulation might actually contribute significantly to the increased sensitivity of HR fish. An increase in cortisol is believed to increase plasma ammonia accumulation (Hogstrand et al. 1999), and ammonia excretory capacity can be impeded during Cu exposure (Grosell et al. 2002; Beaumont et al. 2003; De Boeck et al. 2006). De Boeck and co-workers (2006) reported an irregular and prolonged peak of plasma ammonia during the first days of Cu exposure in carp, followed by a lasting increase in muscle ammonia. According to Taylor et al. (1996) physical damage to gills may be responsible for accumulation of plasma ammonia, but also a disturbance in Na transport including effects on the Na^+/NH_4^+ exchanger might play a role (Grosell et al. 2002). The relatively high plasma ammonia values observed here are related to the blood sampling technique. When using caudal blood sampling, a mixture of arterial and venous blood is sampled, and ammonia may have diffused from muscle to venous blood. Also, fish need to be removed from the water before sampling, which may cause changes in blood composition. This effect was also discussed by other researchers comparing caudal blood sampling with sampling through chronic indwelling catheters in the dorsal aorta (Beaumont et al. 2003; De Boeck et al. 2006).

We conclude that carp fed a high ration regime have increased growth rates and high hepatic lipid stores. Despite this extra energy store, copper exposure has a more distinct effect on their growth rates compared to those of carp fed on a low ration. HR fish also suffer from a more pronounced plasma ammonia accumulation, which could possibly lead to additional toxic effects. It might therefore be advisable to starve fish briefly prior to an expected copper exposure; such as a treatment with copper containing herbicides.

Acknowledgments. This research was funded by grant 42/5/23003 from the Ministry of Science, Research and Technology of Iran and BOF project 20337 by the University of Antwerp-Belgium.

References

Abdelghani AE, Ahmad MH (2002) Effects of feeding rates on growth and production of Nile tilapia, common carp, and silver carp polyculture in fertilized ponds. Aquaculture Res 33: 415-423

Basavaraju Y, Renuka Devi BS, Mukthayakkz G, Reddy Purushotham L, Mair GC, Rodeick EE, Penman DJ (1998) Evaluation of making and tagging methods for genetics studies in carp. J Biosci 23: 585-593

Beaumont MW, Butler PJ, Taylor EW (2003) Exposure of brown trout, *Salmo trutta*, to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation. J Exp Biol 206: 153-162

Benedeczky I, Nemcsok J, Halasy K (1986) Electronmicroscopic analysis of the cytopathological effect of pesticides in the liver, kidney and gill tissue of carp. Acta Biologica Szegediensis 32: 66-91

Bert JR (1979) Environmental factors and growth. In: Hoar WS, Randall DJ, Bertt JR (ed) Fish Physiology, vol VIII. Bioenergetic and Growth. Academic press, London, pp. 599-675

Bielmyer GK, Gatlin D, Isely JJ, Tomasso J, Klaine SJ (2005) Responses of hybrid striped bass to waterborne and dietary copper in freshwater and saltwater. Comp Biochem Physiol C 140 (1): 131-137

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 249-254

De Boeck G, De Smet H, 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

De Boeck G, Valaeminck A, Blust R (1997) Effects of sublethal copper exposure on copper accumulation, food consumption, Growth, Energy stores, and Nucleic acid contents in Common carp. Arch Environ Contam Toxicol 33: 415-422

De Boeck G, Vlaeminck A, Balm PHM, Lock RAC, de Wachter B, Blust R (2001) Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environ Toxicol Chem 20: 374-381

De Boeck G, Van der Ven K, Hattink J, Blust R (2006) Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat Toxicol 80(1): 92-100

De Boeck G, Van derven K, Meeus W, Blust R (2007) Sublethal copper exposure induces respiratory stress in common and gibel carp but not in rainbow trout. Comp Biochem Physiol C 144: 380-390

Flik G, Stouthart XJHX, Spanings FAT, Lock RAC, Fenwick JC, Wendelaar Bonga SE (2002) Stress response to waterborne Cu during early life stage of carp, *Cyprinus carpio*. Aquat Toxicol 56:167-176

Gill TS, Tewari H, Pande J (1992) Short and long term effects of copper on the rosy barb (*Puntius conchonius Ham*.). Ecotoxicol Environ Saf 23: 294-306

Gluth G, Hanke W (1985) A comparison of physiological changes in carp, *Cyprinus carpio*, induced by several pollutants at sublethal concentrations. I. The dependency on exposure time. Ecotoxicol Environ Saf 9(2): 179-188

Grosell M, Nielsen C, Bianchini A (2002) Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Comp Biochem Physiol 133C (1-2): 287-303

Handy RD (2003) Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process. Comp Biochem Physiol A 135: 25-38

Handy RD, Sims DW, Giles A, Campbell HA, Musonda MM (1999) Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout (*Oncorhynchus mykiss*) during chronic dietary exposure to copper. Aquat Toxicol 47 (1): 23-41

Hansen JA, Welsh PG, Lipton J, Cacela D (2002) Effects of copper exposure on growth and survival of juvenile Bull Trout. Trans Am Fish Soc 131: 690-697

Hashemi S, Blust R, De Boeck G (2007) The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. Environ Toxicol Chem 26(7):1507-1511

Hilmy AM, El-Domiaty NA, Daabees AY, Abde-latief HA (1987) Some physiological and biochemical indicies of Zinc toxicity in two freshwater fishes *Clarias lazera* and *Tilapia zilii.* Comp Biochem Physiol 87C: 297-301

Hogstrand C, Ferguson EA, Galvez F, Shaw JR, Webb NA, Wood CM (1999) Physiology of acute silver toxicity in the starry flounder (*Platichthys stellatus*) in seawater. J Comp Physiol B 169: 461-473

Jobling M, (ed.) (1994) Fish Bioenergetics. Chapman and Hall, London

Kamunde C, Wood CM (2003) The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Oncorhynchus mykiss*. Aquat Toxicol 62 (3): 235-254

Kirk RS, Lewis JW (1993) An evaluation of pollutant induced changes in the gills of rainbow trout using scanning electron microscopy. Environ Tech 14: 577-585

Kjoss VA, Grosell M, Wood CM (2005a) The influence of dietary Na on Cu accumulation in juvenile rainbow trout exposed to combined dietary and waterborne Cu in soft water. Arch Environ Contam Toxicol 49 (4): 520-527

Kjoss VA, Kamunde CN, Niyogi S, Grosell M, Wood CM (2005b) Dietary Na does not reduce dietary Cu uptake by juvenile rainbow trout. J Fish Biol 66 (2): 468-484

Kjoss VA, Wood CM, McDonald DG (2006) Effects of different ligands on the bioaccumulation and subsequent depuration of dietary Cu and Zn in juvenile rainbow trout (*Oncorhynchus mykiss*). Can J Fish Aquat Sci 63 (2): 412-422

Kooijman SALM, Bedaux JJM (1996) Analysis of toxicity tests on fish growth. Water Res 30: 1633-1644

Marek J, Szulkowska-Wojaczek E, Czarna Z (1991) The effect of copper Cu^{+2} on carp (*Cyprinus carpio L.*) juveniles in a laboratory experiments. Pol Arch Hydrobiol 38: 485-494

McGeer JC, Szebedinszky C, McDonald DG, Wood CM (2000) Effect of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 1: Iono-regulatory disturbance and metabolic costs. Aquat Toxicol 50: 231-243

Niyogi S, Kamunde CN, Wood CM (2006) Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu exposure. Aquat Toxicol 77 (2): 210-221

OECD (1992) OECD test guideline 210, Fish, Early-life stage test, Toxicity test. Adopted 17th July 1992

Roe JH, Dailey RE (1966) Determination of glycogen with the anthrone reagent. Anal Biochem 15: 245-250

Segner H (1987) Response of fed and starved roach, *Rutilus rutilus*, to sublethal copper contamination. J Fish Biol 30: 423-437

Shaw BJ, Handy RD (2006) Dietary copper exposure and recovery in Nile tilapia, *Oreochromis niloticus*. Aquat Toxicol 76 (2): 111-121

Shearer KD (1994) Factor affecting the proximate composition of cultured fishes emphasis on Salmonids. Aquaculture 119: 63-88

Sola F, Isaia J, Masoni A (1995) Effects of copper on gill structure and transport function in the rainbow trout, *Oncorhynchus mykiss*. J Appl Toxicol 15: 391-398

Taylor EW, Beaumont MW, Butler PJ, Mair J, Mujallid MSI (1996) Lethal and sublethal effects of copper on fish: a role for ammonia toxicity. In: Taylor EW (ed) Toxicology of aquatic pollution. Cambridge University press, Cambridge, pp 85-114

Weatherley AH, Gill HS (1987) The biology of fish growth. Academic press, London

Wendelaar Bonga SE (1993) Endocrinology. In: Evan DH (ed) The physiology of the fishes. CRC Press, Baco Raton, FL, pp 469-502

Wilson RW, Taylor EW (1993) The physiological responses of freshwater rainbow trout, *Oncorhynchus mykiss*, during acutely lethal copper exposure. J Comp Physiol B 163: 38-47

3

The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*

Shodja Hashemi*, Ronny Blust, Gudrun De Boeck

Published in Environmental Toxicology and chemistry 26(7): 1507-1511

Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Groenenborgerlaan171, 2020 Antwerp, Belgium

Abstract

Common carp (Cyprinus carpio) were fed to two different food rations, 0.5% body weight (low ration, LR) and 5% body weight (high ration, HR) and were exposed to sublethal (1µM) copper levels for 28 d in softened Antwerp city tap water (Ca^{2+}, 79.3 mg/L, Mg^{2+}, 7.4 mg/L, Na^{+}, 27.8 mg/L , pH, 7.5-8.0). Copper accumulations in the liver, gills, kidney, anterior intestine, posterior intestine, and muscle were determined. Copper accumulation in the gills, liver, and kidney of LR fish was significantly higher than in HR fish. The only time copper uptake in HR fish was significantly higher than LR fish was in the posterior intestine after two weeks of exposure. No difference was found between the two rations in the anterior intestine. Copper accumulation in the liver of both feeding treatments occurred in a time-dependent manner and did not reach steady in any treatment. On the contrary, copper concentration in the gills reached a steady state for both HR and LR exposed fish within the first week of exposure. No copper accumulation was found in muscle tissues of either treatment. Copper concentration dropped to control levels in all tissues, except in liver tissue, two weeks after the exposure ended. Our studies indicated that copper uptake was influenced by the food ration in carp. The difference in copper accumulation probably is related to the amount of dietary NaCl and different rates of metallothionein synthesis. Low food availability provides less Na^{+} influx and leads to increased brachial uptake of Na^{+} and copper. In addition, it has been shown that starved animals show increased levels of metallothionein, possibly causing higher copper accumulation.

3.1 Introduction

Copper is an essential element for fish [1], but it can be toxic at higher levels [2]. Similar to other heavy metals, copper could be accumulated in fish tissues not only from the dissolved water phase but also from the food [3]. Some studies have examined the accumulation of copper in carp tissues [4, 5], but to our knowledge, no studies have been conducted to ascertain the effect of different feeding rations on copper accumulation in tissues of carp during sublethal waterborne exposure. Previous studies were conducted on other species, and dealt mostly with dietary copper exposure [6], or changing environmental factors and water quality [7]. However, the work by Kamunde and Wood [6] and by Kamunde et al. [8] demonstrated significant interactions between food rations, food composition, and copper accumulation. The condition of the fish and the effect of food rations as an important factor for toxicity have yet not been investigated. Significant differences in metabolic response to sublethal waterborne copper exposure were found in a parallel experiment using common carp fed two different food rations (0.5 and 5%, body weight). While growth rates and hepatic lipid stores were increased in carp fed a high ration (HR) regime, but copper exposure affected them to a greater extend. Additionally, a reduction in growth coincided with a loss of hepatic lipid stores and increased plasma ammonia levels (unpublished data). Therefore, in the present survey, bio-accumulation of copper in different tissues of common carp under different feeding regimes were measured and compared during a chronic waterborne copper exposure to find out whether or not tissue-specific copper accumulation also was affected.

3.2 Materials and methods

3.2.1 Animal holding – Experimental set up

Common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Wageningen University (The Netherlands) and kept at the University of Antwerp (Belgium) at 23 ±1 °C in softened Antwerp city tap water. Water hardness was 270 mg $CaCO_3$/L, and ion concentrations in the water were as follows: Ca^{2+}, 79.3 mg/L, Mg^{2+}, 7.4 mg/L; and Na^{+}, 27.8 mg/ L. The experiment was conducted at 23±1°C in a flow-through system consisting of six tanks (200- L each) using the same softened Antwerp city tap water. Water quality was checked daily. The pH was 7.5-8, oxygen concentration remained above 7 mg/L, and ammonia concentration was below 0.1 mg/L at all times. The photoperiod was set at 12:12-h light:dark. Total organic carbon (TOC) ranged from 3.14±1.25 ppm in 0.5% feeding tanks to 4.77±1.24 ppm in 5% feeding tanks, and no significant differences were found in terms of TOC in different feeding treatments. The fish had a mean initial wet weight of 16.3±03 g and were randomly distributed in 6 tanks at a ratio of 55 fish per tank. Fish were acclimated to the test conditions for 52 d before Cu exposure began and preacclimated to either 0.5% (low ration [LR]) or 5% (high ration [HR]) body weight food rations with Trouvit (Trouw Nutrition, Fontaine-les-Vervins, France) divided over two feedings per day.

The copper and sodium contents of the food were 0.012 ±0.001 mg/g (mean ± standard deviation [SD], *n*=8) and 4.529±.147 mg/g (mean ±SD, n=8), respectively. Experimental setup and exposure conditions conformed to the Organisation for Economic Co-operation and Development (OECD) guidelines 210 [9]. Each feeding treatment was split into two replicate tanks with exposure plus one without exposure. The tanks were predosed with Cu $(NO_3)_2 \cdot 2H_2O$ to bring the concentration

to the nominal level. A stock copper nitrate solution (Cu $(NO_3)_2 \cdot 2H_2O$ 0.722 g/l; Merck, Darmstadt, Germany) was then added into the tanks with the inflowing water using a peristaltic pump (Watson Marlow 505 S, Falmouth, Cornwall, UK) during the entire experiment. Both feeding treatments of fish were exposed to a sublethal total concentration of 1µM Cu for 28 d. Control and exposure tanks were cleaned daily to minimize any fecal ingestion. The amount of food fed to each exposure tank was adjusted daily for fish growth and sampling. Fish were weighed weekly to assess weight gain. The fish were not fed the day of sampling to allow for gut clearance. Fish were sampled at regular intervals (day 0, 3, 7, 14, 21, 28, and 2 weeks after the end of exposure). A passive integrated transponder system (PIT-Tag; Trovan unique; ID 100 implantable; Hessle, East Yorkshire, UK) was injected into the abdominal cavity of the fish using the applicator needle supplied with the tag 2 weeks before exposure started. Passive integrated transponder tags have a 100% retention rate in carp [10], which allowed determining individual growth rates.

3.2.2 Sampling Procedure

At the different sampling times, eight fish were removed from the exposed LR and HR tanks. They were quickly netted and killed by an overdose of buffered MS 222 (1 g/L at pH 7.5, Across Organics, Geel, Belgium). All fish were weighed, and immediately dissected on ice. Gill lamellae, liver tissue, anterior intestine, posterior intestine, kidney, and muscle tissues were rinsed with physiological saline (0.9% NaCl), snap-frozen in liquid nitrogen, and stored at -20 °C for copper determination. Tissues from each fish were placed into preweighed tubes, dried in a 60 °C drying oven for a minimum of 72h, and after cooling in a desiccator,

reweighed to obtain the dry weight of the tissue. Subsequently, they were dissolved in 70 % HNO_3, placed in a microwave oven [11] until total digestion had occurred, and diluted 10-fold with Milli-Q grade water (Millipore, Bedford, MA, USA) based on their dry weight and the volume of acid used for digestion. Total copper concentration was determined using inductively coupled plasma-atomic emission spectrophotometry (ICP-AES).

3.2.3 Data analysis

All values are given as means ± SD. Data was evaluated by analysis of variance (ANOVA) followed by Tukey multiple-comparison posttest. All data were natural logarithm transformed to stabilize the variance and to approximate normal distribution prior to use in further statistical analysis. Differences were considered to be significant at $p< 0.05$.

3.3 Results

No mortality occurred during the exposure in either the exposed fish or controls. Fish fed a high food ration had significantly higher performance in terms of weight gain and growth. As expected, Cu accumulation was found in tissues of the exposed fish. The liver of both LR and HR treatments showed dramatic increases in Cu level, and over time, copper accumulation in the liver of both exposed treatments was significantly higher than in their respective controls (Fig. 1). In the exposed fish, copper concentration in the liver of the LR fish was significantly higher than in HR fish (Fig. 1). Surprisingly, copper accumulation in the liver of the LR control fish also was significantly higher than that in the control HR fish (Fig. 1). Copper accumulation in

the liver of both feeding treatments occurred in a time-dependent manner and did not reach steady state.

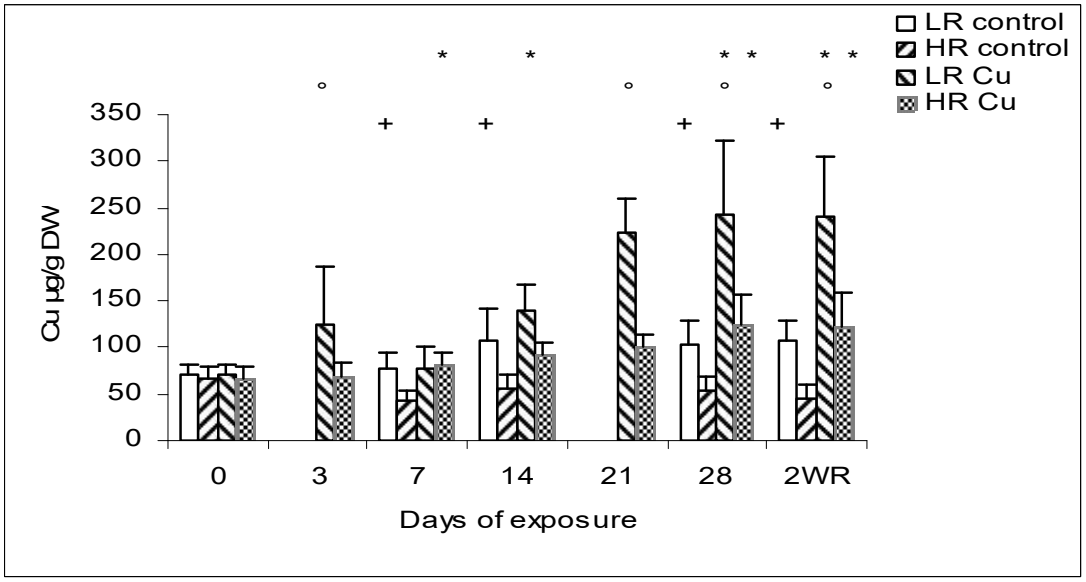

Fig. 1. Copper accumulation in liver tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). °= significant difference between exposed LR and exposed HR carp, *= significant difference between exposed carp and its respective control, += significant difference between control LR and control HR carp.

The largest changes in copper level were observed in the gills of the exposed fish **(**Fig. 2**)**. Copper levels significantly increased 6.9 and 4.6-fold respectively, in the LR and HR exposed fish (Fig. 2) and reached a steady state after one week. Copper levels in the gills of the LR exposed carp were significantly higher than in the HR exposed fish. Although in the LR exposed fish copper concentration slightly increased through the exposure, no statistical differences were detected. Gills of the LR control fish also showed significantly higher copper accumulation compared to the HR control at the last week of exposure (Fig. 2).

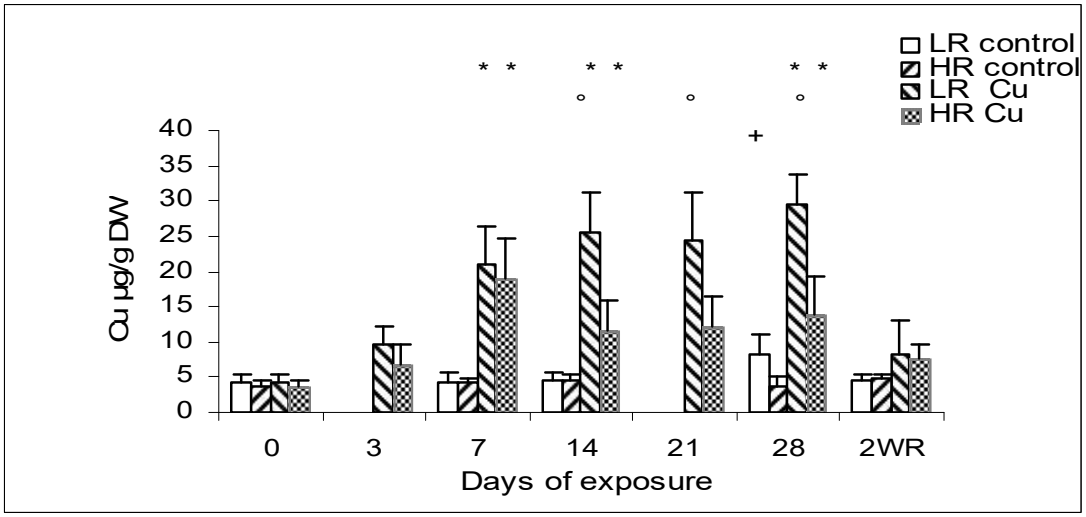

Fig. 2. Copper accumulation in gills tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). °= significant difference between exposed LR and exposed HR carp, *= significant difference between exposed carp and its respective control, += significant difference between control LR and control HR carp.

A significant increase in copper accumulation occurred in the anterior intestine of the exposed LR (at days 7 and 14) and HR (at day 7) fish compared to their respective controls (Fig. 3), but copper concentration in the anterior intestine between the LR and HR control or exposed fish did not show a significant difference (Fig. 3). Copper concentration rose significantly in the posterior intestine of the HR exposed fish compared to its control (Fig. 4). Copper level in the posterior intestine of the exposed HR carp differed significantly from the LR exposed one (Fig. 4), and this was the only time that we observed a higher copper accumulation in the HR fish compared to the LR fish. Copper concentration in the kidney of HR and LR exposed fish was significantly different even in controls, but no significant increase was found in the total copper concentration in exposed treatments compared

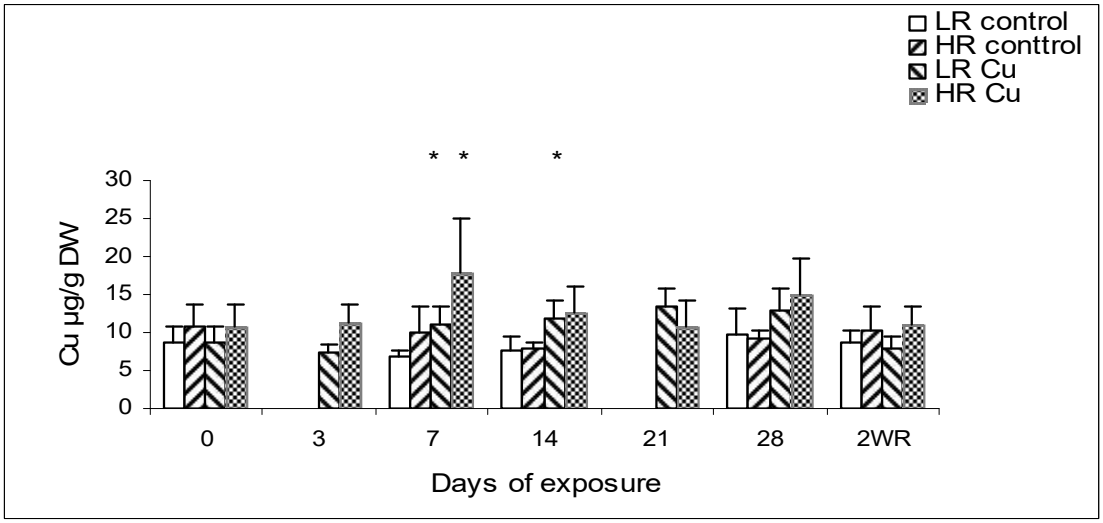

Fig. 3. Copper accumulation in anterior intestine tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). *= significant difference between exposed carp and its respective control.

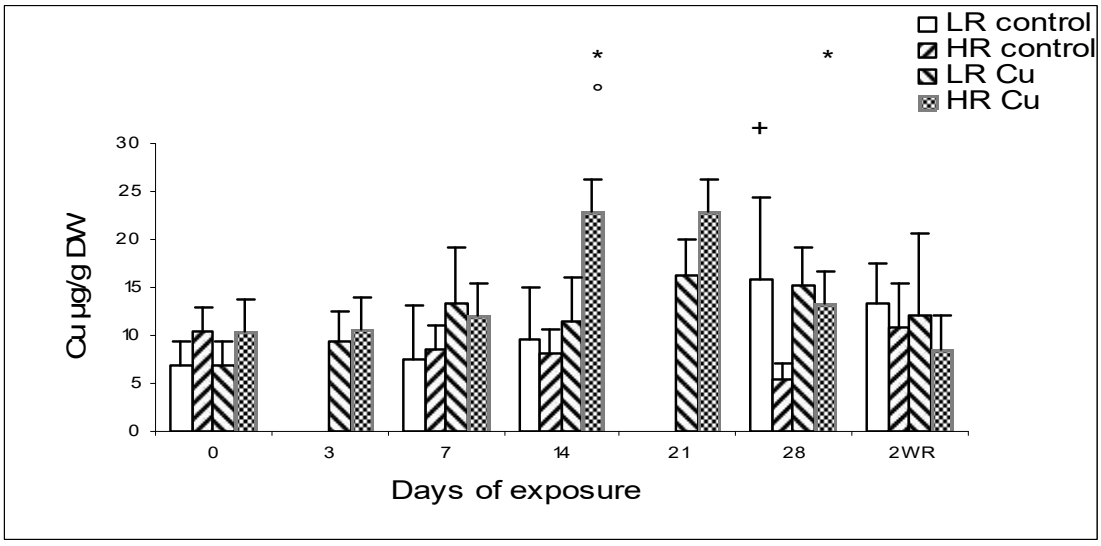

Fig. 4. Copper accumulation in posterior intestine tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). °= significant difference between exposed LR and exposed HR carp, *= significant difference between exposed carp and its respective control, += significant difference between control LR and control HR carp.

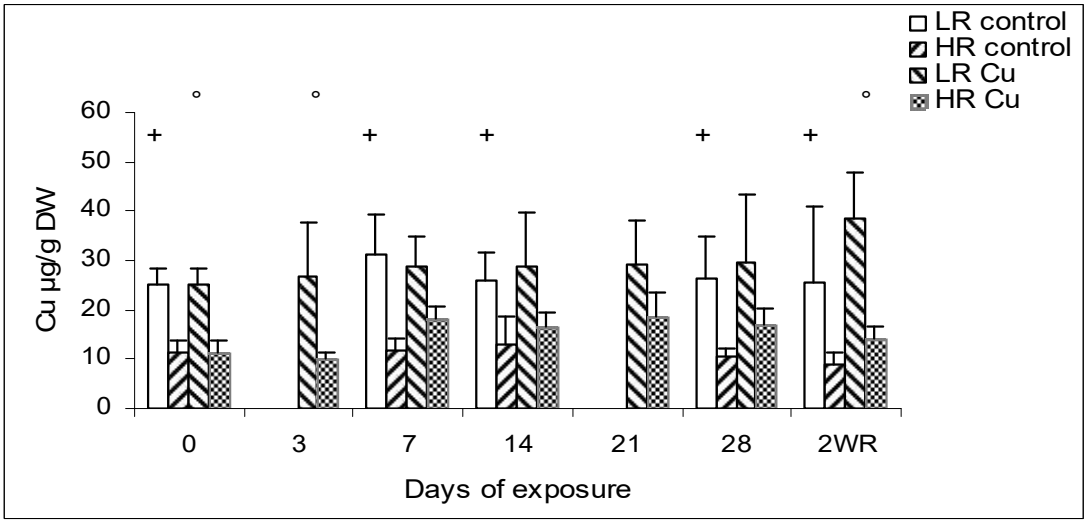

Fig. 5. Copper accumulation in kidney tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). °= significant difference between exposed LR and exposed HR carp, += significant difference between control LR and control HR carp.

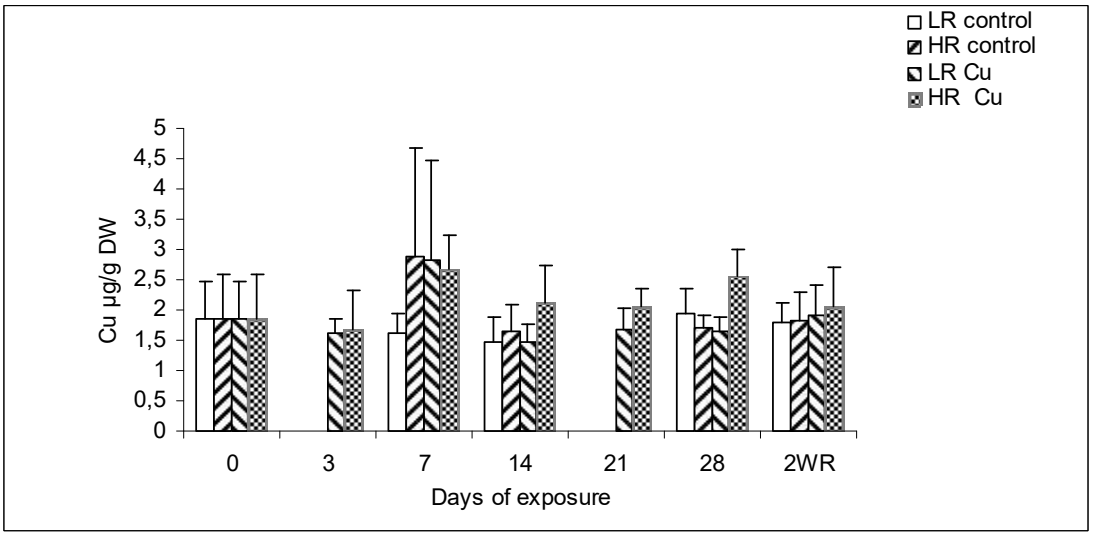

Fig. 6. Copper accumulation in muscle tissue of common carp (*Cyprinus carpio*) fed two different food rations 0.5% body weight (low ration [LR]) and 5% body weight (high ration [HR]) during a 28-d exposure to 1µM copper and after two weeks recovery (2WR). No significant differences were found.

to controls (Fig. 5). No significant copper accumulated in muscle tissues in the exposed LR and HR fish (Fig. 6). Copper concentration dropped in all tissues of the exposed fish and reached the levels of the control fish two weeks after the end of exposure except in the liver. In liver tissue, copper concentration was still significantly different between the HR exposed and LR exposed fish when compared to each other as well as when compared to their respective controls.

3.4 Discussion

The present study is, to our knowledge, the first to describe the interaction between food quantity and waterborne copper exposure in carp. Copper accumulation mainly occurred in the primary target tissues, gills and liver, both in HR and LR carp. Differential uptake patterns of copper have been previously found in different tissues [5], and these were critically dependent on the characteristics of structure and function inherent to each tissue [12]. Additionally, the present results showed significant differences in copper accumulation among the tissues of the exposed carp, in agreement with the results of earlier work [5, 13-16], but we also found significant differences in copper accumulation between the tissues of LR and HR. The present results are consistent with the results of Kamunde and Wood [6], who found that a low feeding regime resulted in higher Cu concentration in the tissues of rainbow trout (*Onchorynchus mykiss*) during dietary copper exposure. Furthermore Segner [17] found similar results in fed and starved roach (*Rutilus rutilus*) exposed to sublethal copper for 7 d.

Fish gills represent a large exposure surface area with only a short diffusion distance between the internal and external environment, which

makes them a primary target of copper contamination [18, 19]. Branchial copper uptake is responsible for the internal copper status of fish [20, 21]. Taylor et al. [7] noted that gill copper is a more sensitive tissue indicator than liver copper for waterborne copper exposure. Accumulation of copper in the gills is caused by binding to haemopoetic tissue mucus and to metallothioneins (MTs) involved in detoxification and excretion. Absorption onto membranes and cells possibly is associated with impaired physiological function [22]. According to McDonald and Wood [23] ionoregulatory functions of gills are disturbed by metal exposure. Pyle et al. [24] presented the hypothesis that Na^+ and copper share a common branchial uptake route in fish, probably through an apical sodium channel. This hypothesis was supported by the results of Grosell et al. [25] and Kamunde et al. [8]. The need for branchial Na^+ uptake is determined by the dietary uptake, as Smith et al. [26] found out that almost all of the Na^+ taken up from diet was absorbed rapidly. Considering this, copper uptake might be prohibited in the gills via the higher dietary NaCl absorption (available from higher food ration) in HR carp compared to LR fish. Our findings coincide well with those of Pyle et al. [24], who found that dietary sodium inhibited waterborne copper uptake in rainbow trout. Likewise, Kamunde et al. [27] pointed out that dietary NaCl reduced accumulation and toxicity of waterborne copper in the rainbow trout.

As expected, the bulk storage of copper occurred in the liver of both feeding treatments. In the rainbow trout, the liver was responsible for 75% of Cu body burden during dietary and waterborne exposure [21]. In the present study, copper accumulation in the liver of the LR and HR carp was significantly different, with 68.9% and 43% of total body burden respectively, of total body burden. Also Segner [17] found that

copper concentrations in the liver of starved roach were higher than in fed ones. According to Stagg et al. [22] the rise of copper concentration was accompanied by an increase in low–molecular–weight protein (MT) levels in the liver. These proteins bind heavy metal and reduce their toxicity, and strong relationships between liver MT and copper residues have been demonstrated before [13, 28, 29]. In addition, De Boeck et al. [13] reported a significant correlation between copper and MT levels in the gills and a moderate correlation between copper and MT levels in the muscle of carp. These studies led us to conclude that the level of MT in the liver, gills and kidney of LR fish are probably much higher than in exposed HR carp. This is supported by results of McNamara and Buckley [30], who found that the level of MT mRNA in starved cod (*Gadus morhua*) was sevenfold higher than in control cod. Significantly increased levels of MT also were found in fasting mice [31]. The elevated copper accumulation in the posterior intestine of the HR carp may be related to the increased consumption of food, as seen in rainbow trout [6]. The present study indicated that the muscle tissue showed no Cu accumulation. According to Lauren and McDonald [32], copper accumulation in the muscle becomes important only when the maximum storage capacity of liver is reached, a finding confirmed by Villegas–Navarro and Villarreal-Trevino [33].

3.5 Conclusion

In conclusion, the present study confirmed that copper uptake was influenced by diet ration in carp. Dietary NaCl and MT induction likely were the main component contributing to this difference, but this remains to be investigated. Less food availability provides less Na^+ influx and, possibly, leads to high brachial uptake of Na^+ and copper. Finally, induction of higher levels of MT in starving fish could cause higher

copper accumulation. Administration of the different uncontaminated food rations alone caused increased Cu uptake in the posterior intestine and kidney, indicating that the different rations also caused a direct effect on copper accumulation.

Acknowledgement

This research was funded by grant 42/5/23003 from the Ministry of Science, Research and Technology of Iran and Bijzonder Onderzoeksfonds (BOF) project 20337 by the University of Antwerp-Belgium. The technical assistance of Marcel Selens is gratefully acknowledged.

References

1. Ogino C, Yang GY. 1980. Requirements of carp and rainbow trout of dietary manganese and copper. *Bull Jap Soc Sci Fish* 46: 455-458.

2. Handy RD. 1996. Dietary exposure to toxic metals in fish. In Taylor EW, eds, Toxicology of Aquatic Pollution: Physiology, Cellular and Molecular Approaches. Cambridge University Press, Cambridge, UK, pp 29-60.

3. Dallinger R, Kautzky H. 1985. The importance of contaminated food for the uptake of heavy metals by rainbow trout (*Salmo gairdneri*): a field study. Oecologia 67:82-89.

4. De Boeck G, Valaeminck A, Blust R.1997. Effects of sublethal copper exposure on copper accumulation, food consumption, Growth, Energy stores, and Nucleic acid contents in Common carp. *Arch Environ Contam Toxicol* 33: 415-422.

5. De Boeck G, Meeus W, De Coen W, Blust R. 2004. Tissue-specific Cu bioaccumulation patterns and differenced in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Onchorynchus mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). *Aquat Toxicol* 70: 179-188.

6. Kamunde C, Wood CM. 2003. The influence of ration size on copper homeostasis during sublethal dietary copper exposure in juvenile rainbow trout, *Onchorynchus mykiss*. *Aquat Toxicol* 62: 235-254.

7. Taylor LN, McGeer JC, Wood CM, McDonald DG. 2000. The Physiological effects of chronic copper exposure to rainbow trout (*Oncorhynchus mykiss*) in hard and soft water: evaluation of chronic indicators. *Environ Toxicol Chem* 19:2298-2308.

8. Kamunde CN, Pyle GG, McDonald DG, Wood CM. 2003. Influence of dietary sodium on waterborne copper toxicity in rainbow trout *Onchorynchus mykiss*. *Environ Toxicol Chem* 22:342-350.

9. Organisation for Economic Co-operation and Development 1992. Fish, early-life stage test, toxicity test. Test guideline 210. Paris, France.

10. Basavaraju Y, Devi BSR, Mukthayakk G, Reddy LP, Mair GC, Roderick EE, Penman DJ. 1998. Evaluation of making and tagging methods for genetics studies in carp. *J Biosci* 23:585-593.

11. Blust R, Vanderlinden A, Verheyen E, Decleir W. 1988. Evaluation of microwave-heating digestion and graphite-furnace atomic-absorption spectrometry with continuum source background correction for the determination of iron, copper, and cadmium in brine shrimp. *J Anal At Spectrom* 3:387-393.

12. Villarreal-Trevino CM, Villegas-Navarro A. 1987. Differential accumulation of lead by soft tissues of rabbit. *Bull Environ Contam Toxicol* 39: 334-342.

13. De Boeck G, Ngo TT, Van Campenhout K, Blust R. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquat Toxicol* 65:413-424.

14. Yamamoto Y, Ishii T, Ikeda S. 1997. Studies on copper metabolism in fishes II. The site of copper accumulation in the tissues of carp. *Bull Jap Soc Sci Fish* 43:1327-1332.

15. Toth L, Juhasz M, Varga T, Csikkel- Szolnoki A, Nemcsok J. 1996. Some Effect of $CuSO_4$ on carp. *J Environ Sci Health Pt B* 31: 625-635.

16. Nemcsok J, Albers C, Benedeczky J, Gotz KH, Schickers K, Kufcsak O, Juhasz M. 1991. *Bioindicators and Environmental Management. Academic*, New York, NY, USA.

17. Segner H. 1987. Response of fed and starved roach, *Rutilus rutilus,* to sublethal copper contamination. *J Fish Biol* 30:423-437.

18. Brungs WA, Leonard EN, Mckim JM. 1973. Acute and long term accumulation of copper by the brown bullhead, *Ictalurus nebulosus*. *J Fish Res Board Can* 30: 583-586.

19. Buckley JT, Roch M, McCarter JA, Rendell CA, Matheson AT. 1982. Chronic exposure of coho salmon to sublethal concentration of copper –I. Effect on Growth, on Accumulation and distribution of copper, and on copper tolerance. *Comp Biochem Physiol* 72: 15-19.

20. Kamunde C, Grosell M, Lott JN, Wood CM. 2001. Copper metabolism and gut morphology in rainbow trout (*Onchorynchus mykiss*) during chronic sublethal copper exposure. *Can J Fish Aquat Sci* 58: 293-305.

21. Kamunde C, Grosell M, Higgs D, Wood CM. 2002. Copper metabolism in actively growing rainbow trout (*Onchorynchus mykiss*): interaction between dietary and waterborne copper uptake. *J Exp Biol* 205:279-290.

22. Stagg RM, Schuttleworth TJ. 1982. The accumulation of copper in *Plaichthys fleus L.* and its effects on plasma electrolyte concentrations. *J Fish Biol* 20:491-500.

23. McDonald DG, Wood CM. 1993. Branchial mechanism of acclimation to metals in freshwater fish. In Rankin JC, Jesen FB, eds, Fish ecophysiology. Chapman & Hall, London, UK, pp 297-321.

24. Pyle GG, Kamunde C, Mcdonald DG, Wood CM. 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Onchorynchus mykiss*). *J Exp Biol* 206:609-618.

25. Grosell M, Wood CM. 2002. Copper uptake across rainbow trout gills: mechanism of apical entry. *J Exp Biol* 205: 1179-1188.

26. Smith N, Eddy F, Talbot C. 1995. Effect of dietary salt load on transepthelial Na^+ exchange in freshwater rainbow trout (*Onchorynchus mykiss*). *J Exp Biol* 198:2359-2364.

27. Kamunde CN, Niyogi S, Wood CM. 2005. Interaction of dietary sodium chloride and waterborne copper in rainbow trout (*Onchorynchus*

mykiss): copper toxicity and sodium and chloride homeostasis. *Can J Fish Aquat Sci* 62: 390-399.

28. Marr JC, Bergman HL, Lipton J, Hogstrand C. 1995. Differences in relative sensitivity of naive and metals-acclimated brown trout exposed to metals representative of the Clark Fork River, Montana. *Can J Fish Aquat Sci* 52:2016-2030.

29. Hogstrand C, lithner G, Haux C. 1991. The importance of metallothionein for the accumulation of copper, zinc, and cadmium in environmentally exposed perch, *Perca fluviatilis*. *Pharmacol Toxicol* 68:492-501.

30. McNamara PT, Buckley LJ. 1994. Identification and characterization of metallothionein cDNA from mRNA transcripts induced by starvation in Atlantic cod (*Gauds morhua*). *Mol Mar Biol Biotechnol* 3(5):252-260.

31. Higashimoto M, Sano M, Kondoh M, Sato M. 2002. Different responses of metallothionein and leptin induced in the mouse by fasting stress. *Biol Trace Elem Res* 89:75-84.

32. Lauren DJ, McDonald DG. 1987. Acclimation to copper by rainbow trout, *Salmo gaidneri*, biochemistry. *Can J Fish Aquat Sci* 44:105-111.

33. Villegas-Navarro A, Villarreal-Trevino M. 1989. Differential uptake of Zinc, copper, and the Leads in Texas Cichlid *(Cichlasoma cyanoguttatum*). *Bull Environ Contam Toxicol* 42:761-768.

4

The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp

Shodja Hashemi *, Ronny Blust, Gudrun De Boeck

Accepted in Aquatic Toxicology

Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Groenenborgerlaan171, 2020 Antwerp, Belgium

Abstract

Common carp (Cyprinus carpio) were fed two different food rations: 0.5% body weight (low ration, LR) and 5% body weight (high ration, HR) and were either acclimated to sublethal copper (1µM) for 28 days in softened Antwerp city tap water or not acclimated. Fish were exposed for 10 days to high Cu levels using 4 different concentrations (3.5, 6, 10, and 15 µM) before and after the Cu acclimation. Fish tolerance against Cu exposure was evaluated, and gill, liver, and carcass Cu and sodium levels were measured in dead and surviving fish. HR fish were twice as sensitive as LR fish in both tests. The 96h median lethal concentration (LC50) values for the non-acclimated LR and HR fish were 8.46±2.79 µM and 4.34±0.82 µM respectively. The fish became more resistant to low Cu concentrations after Cu acclimation and the LC50 values were slightly increased, reaching 9.20±1.56 µM and 5.01±1.93 in LR and HR fish accordingly. Cu concentrations in the gills, liver, and carcass were significantly elevated in response to the short-term Cu exposure, and were significantly higher in LR fish than in HR fish. High Cu levels caused a net loss of sodium resulting in a severe ion regulatory disturbance. The rate of sodium loss increased linearly with increasing exposure concentrations. Cu acclimation resulted in reduced sodium loss and increased the resistance and tolerance to Cu toxicity.

4.1 Introduction

Copper is generally present in freshwater with a natural background from 0.20 to 30 µg/L (Bowen, 1985). Although it is an essential element supporting physiological processes in fish, it may become inhibitory and ultimately toxic at higher concentrations (Furness and Rainbow, 1990). Copper levels between 4-160,000 µg/L were considered toxic for carp depending on water quality and organism condition (Kaur and Dhawan, 1994; Deshmukh and Marathe, 1980). Some forms of copper are reactive and potentially hazardous (Sposito, 1986). Copper can accumulate in fish tissues (Yamamoto et al., 1997, De Boeck et al., 2003, 2004). Copper toxicity, accumulation, and excretion are species specific (Taylor et al., 2003, De Boeck et al., 2004) and even differ in different life stages (Kazlauskiene and Stasiunaite, 1999). Food quantity has recently been proposed to be considered among different external and internal factors that can affect copper bioavailability in carp (Hashemi et al., 2007a). A significant difference in copper accumulation in target organs between fed and starved carp during a sublethal waterborne copper exposure was reported (Hashemi et al., 2007a). Furthermore, substantial differences in metabolic rate, available energy, growth rate, and metabolic waste handling were established between fed and starved fish during sublethal waterborne copper exposure (Hashemi et al., 2007b). Therefore, differences in toxicity and copper uptake during acute waterborne copper exposure were likely to occur. In this study, the sensitivity of starved and fed fish against acute waterborne copper exposure was investigated. In addition, copper uptake, Na loss, and the effects of a long-term acclimation to a sublethal Cu concentration (1 µM) were studied. The relationships between copper accumulation, Na loss and Cu toxicity were determined.

4.2 Material and methods

4.2.1 Animal holding- Experimental set up

Common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Wageningen University, The Netherlands, and kept at the University of Antwerp at 23 ±1 °C in softened Antwerp city tap water. The fish used in the tests had a mean initial wet weight of 16.3±0.3 g (mean ± standard deviation (SD), N=160) and were randomly distributed in 6 tanks. Fish were acclimated to the test conditions and food rations for 52 days before Cu acclimation started. They were pre-acclimated to either 0.5% or 5% body weight (BW) food rations with Trouvit (Trouw Nutrition, Fontaine-les-Vervins, France) divided over two equal feedings a day. These two feeding treatments were named as LR (low ration, 0.5% BW) and HR fish (high ration, 5%BW). A passive integrated transponder system (PIT-Tag, Trovan unique, ID 100 implantable, Hessle, East Yorkshire, United Kingdom) was injected into the abdominal cavity of the fish using the applicator needle supplied with the tag 2 weeks before Cu acclimation started. PIT tags have a 100% retention rate in carp (Basavaraju et al., 1998), which was confirmed in our study.

The Cu acclimation was conducted at 23±1°C in a flow-through system consisting of 6 tanks (200 L each). The photoperiod was set at 12 L: 12 D. Water quality was checked daily. The pH was 7.5 to 8, oxygen concentration remained above 7 mg/L, and ammonia concentration was below 0.1 mg/L at all times. Water hardness was 270 mg $CaCO_3$/L, and ion concentrations in the water were as follows: 79.3 mg/L Ca^{2+}, 7.4 mg/L Mg^{2+}, 27.8 mg/L Na^{+}. Total organic carbon (TOC) ranged from 3.14±1.25 ppm (N=5) in 0.5% feeding tanks to 4.77±1.24 ppm (N=5) in

5% feeding tanks, and there was no significant difference in terms of TOC between different feeding treatments. The Cu and Na contents of the food were 0.012 ±0.001 mg/g and 4.529±0.147 mg/g (N=8) respectively. The Cu acclimation conditions conformed to the OECD test guideline 210 (OECD, 1992). Each feeding treatment was split into two replicate exposure tanks plus one control tank. The tanks were predosed with Cu $(NO_3)_2 \cdot 2H_2O$ to bring the concentration to the nominal level. A stock copper nitrate solution ($Cu(NO_3)_2 \cdot 2H_2O$ 0.722 g/L) (Merck, Darmstadt, Germany) was added into the tanks with the inflowing water using a peristaltic pump (Watson Marlow 505 S, Falmouth, Cornwall, United Kingdom) during the entire experiment, and both feeding treatments were acclimated to a sublethal total concentration of 1µM Cu for 28 days. Cu acclimation tanks were cleaned daily to minimize any fecal ingestion. The amount of food fed to each exposure tank was adjusted daily for fish growth. Fish were weighed weekly to assess weight gain. Eventually, 128 Cu-acclimated and non-acclimated starved and fed fish were exposed for 10 days to high Cu levels using 4 different concentrations (3.5, 6, 10, and 15 µM) via static exposure using 8 aquaria (40 L, 2 per concentration) for each test. Water was changed daily, and the appropriate amount of Cu was added after water renewal. Copper levels were monitored daily, and reached the nominal levels. The fish were not fed during the acute tests. Control fish were taken from acclimation tanks during acute study.

4.2.2 Sampling procedure

Mortality was recorded every 2 hours during daytime and once at nighttime, and fish were collected upon death. Surviving fish were collected at the end of the acute exposure. All fish were weighed, and immediately dissected on ice. Full gill lamellae and full liver tissue were

separated from the carcass, rinsed with physiological saline (0.9% NaCl), and snap frozen in liquid nitrogen and stored at -20 °C for later Cu and Na analysis. Carcasses were homogenized, and a subsample was stored at -20 °C for Cu and Na determination. Samples from each fish were placed into prelabled, preweighed tubes or bullet tubes, dried in a 60 °C drying oven for a minimum of 72h, and reweighed after cooling in a desiccator to obtain the dry weight of the tissue. Subsequently they were digested in 70 % HNO_3, placed in a microwave oven (Blust et al., 1988) until total digestion had occurred and diluted with Milli-Q grade water (Millipore, Bedford, MA, USA). Total Cu and Na concentrations were determined using inductively coupled plasma atomic emission spectrophotometry (ICP-AES, Varian, Liberty, series II, Mulgrave-Victoria, Australia). The accuracy of copper analysis was verified by including blank and certified reference material.

4.2.3 Statistical analysis

Data are expressed as mean ± SD. Data were evaluated by ANOVA followed by Tukey multiple comparison procedure. All data were natural logarithm transformed to stabilize the variance and to approximate normal distribution prior to use in further statistical analysis. Acute lethal concentration (LC50) and their 95% confidence intervals were calculated by the trimmed Spearman-Karber method (Hamilton et al., 1977) using TSK program version 1.5 (Environmental Monitoring Systems Laboratory, U.S. EPA). Differences were considered significant at $p < 0.05$.

4.3 Results

No mortality occurred during the sublethal Cu acclimation. The HR fish were almost twice as sensitive as the LR fish in both acute tests

(Fig. 1). The 96h median lethal concentration (LC50) values for the non-acclimated LR and HR fish were 8.46±2.79 µM and 4.34±0.82 µM, respectively. After Cu acclimation the LC50 values were slightly increased in LR and HR fish, and LC50 values were 9.20±1.56 µM and 5.01±1.93, subsequently. Excess mucus secretion was observed at the gills in the higher copper concentration.

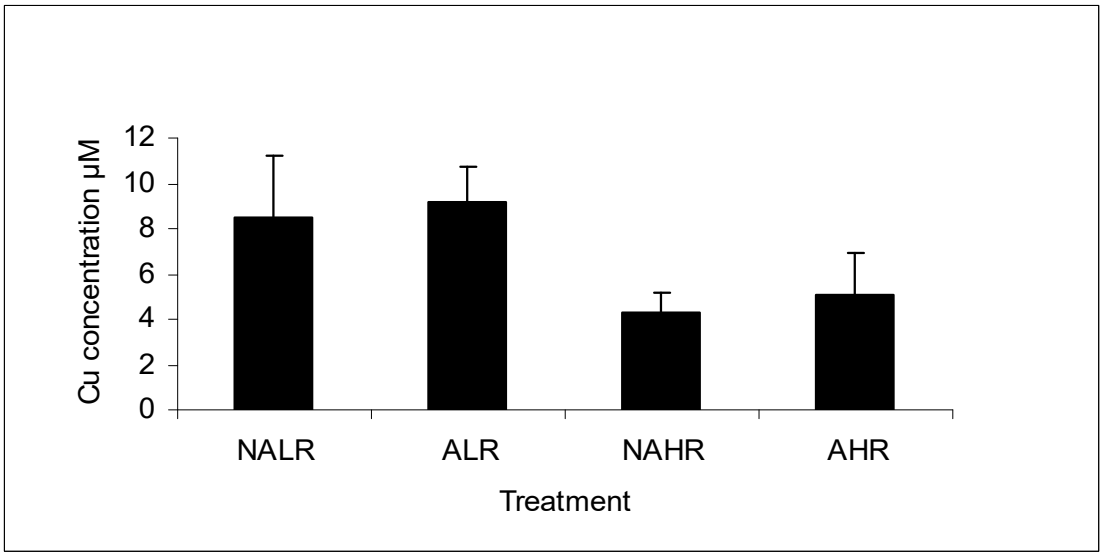

Fig. 1. The 96h median lethal concentration estimated for the starved and fed acclimated and non-acclimated carp (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW).

HR fish reached the median lethal time (LT50) earlier than LR fish in both tests as well. The HR and LR non-acclimated fish reached LT50 in 30h and 111h, respectively in the highest Cu concentration (15 µM). The LT50 in both acclimated LR and HR fish increased 1.8 fold in all treatments except in highest concentration (15 µM), where acclimated LR and HR fish reached the LT50 earlier than non-acclimated ones. Fish became more tolerant to low Cu concentrations in the second LC50 test

after Cu acclimation. All acclimated LR fish in 6 µM of copper exposure survived (Fig. 2), and the initial LT50 of 38.2h and 36.2h in non-acclimated LR and HR fish in 10 µM Cu elevated to 64.5h and 43.92h, respectively after the acclimation.

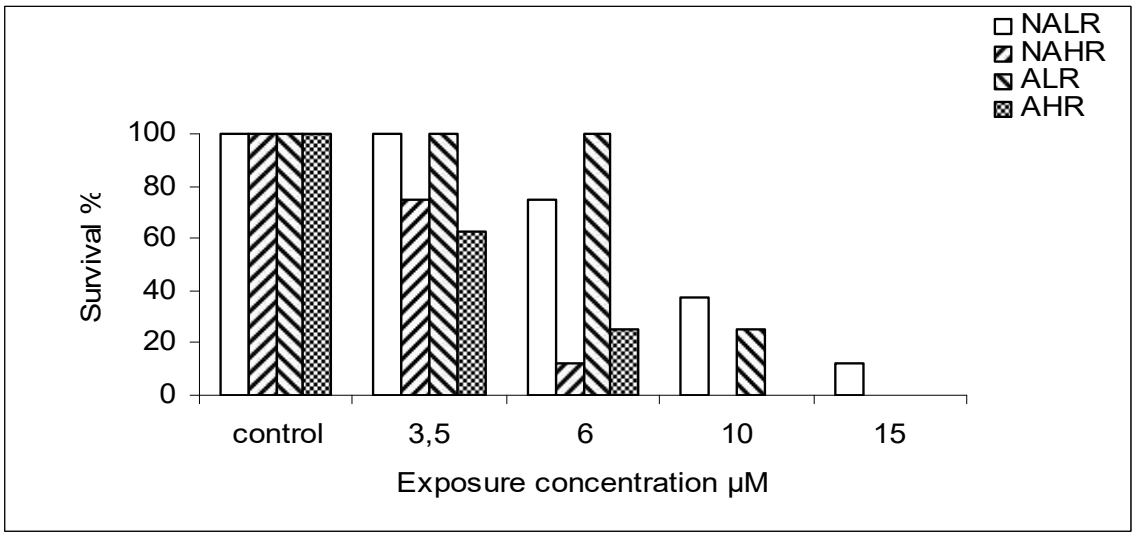

Fig. 2. Survival of Cu acclimated and non-acclimated carp fed two different food rations after a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW).

Copper concentrations in the gills, liver, and carcasses were significantly elevated by copper exposure (Fig. 3, 4, 5). Copper concentrations in the gills, liver and carcasses of the LR fish were in general significantly elevated compared to the HR fish (Fig. 3, 4, 5). Copper accumulation in the liver of the LR carp was clearly influenced by survival time, and was lower at higher Cu exposure concentrations. In the HR fish this effect was less obvious (Fig. 4). Cu acclimation only marginally increased Cu levels in the carcass, but exposure to higher Cu levels clearly induced Cu accumulation in the remaining carcass as well. Again, Cu levels in LR fish were in general higher than in HR fish except

in gills of fish exposed to 15 µM of Cu where non-acclimated and acclimated HR carp accumulated more than LR fish.

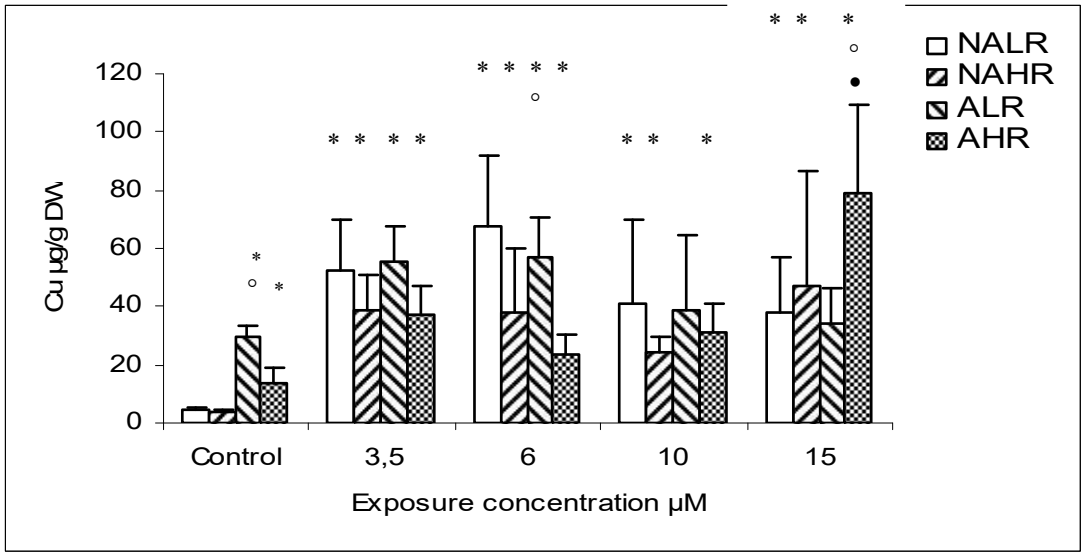

Fig. 3. Copper accumulation in the gill tissues of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non acclimated carp fed 0.5 % body weight (BW), NAHR: Non acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, ●: significant difference between the acclimated and non- acclimated carp, °: significant difference between the acclimated LR and HR carp. Note that survival times, and thus sampling times differed between fish.

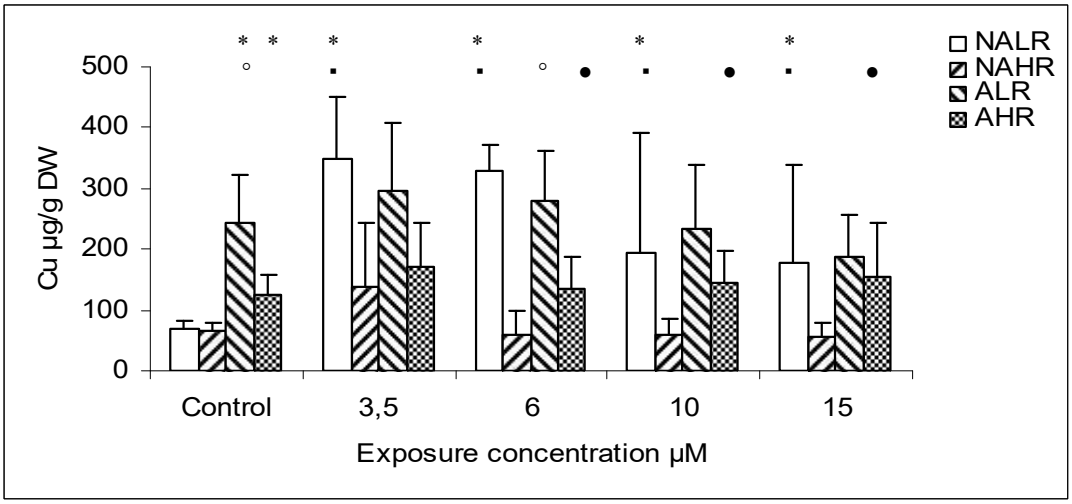

Fig. 4. Copper accumulation in the liver tissue of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, ●: significant difference between the acclimated and non- acclimated carp, °: significant difference between the acclimated LR and HR carp, ▪: significant difference between the non-acclimated LR and HR carp. Note that survival times, and thus sampling times differed between fish.

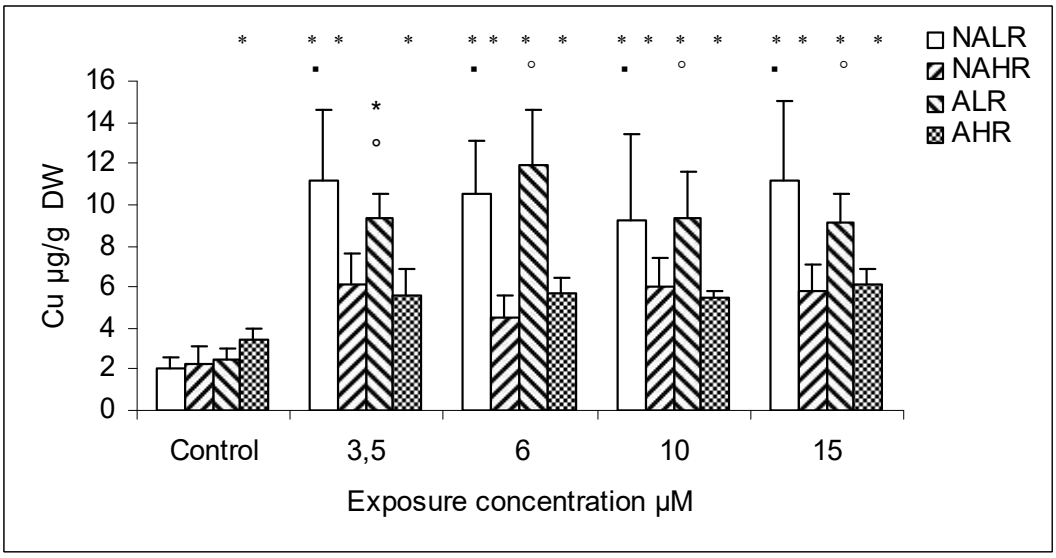

Fig. 5. Copper accumulation in the carcass of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, °: significant difference between the acclimated LR and HR carp, ▪: significant difference between the non-acclimated LR and HR. Note that survival times, and thus sampling times differed between fish.

Na concentration in the gills, liver, and carcasses dropped significantly with increasing copper exposure (Fig. 6, 7, 8). The rate of sodium loss increased linearly with the exposure concentration. Na levels in gills and carcasses of non-acclimated LR fish were higher than Na levels in HR fish. After Cu exposure Na concentration in the non-

acclimated HR carp remained significantly lower than the non-acclimated LR carp. Acclimated fish lost less Na from gills and carcasses compared to non-acclimated carp. There was a significant positive correlation between Cu and Na levels in LR and HR control fish, but this was disrupted following acute copper exposure for low ration fish and became negative after acute exposure for high ration fish (Fig. 9).

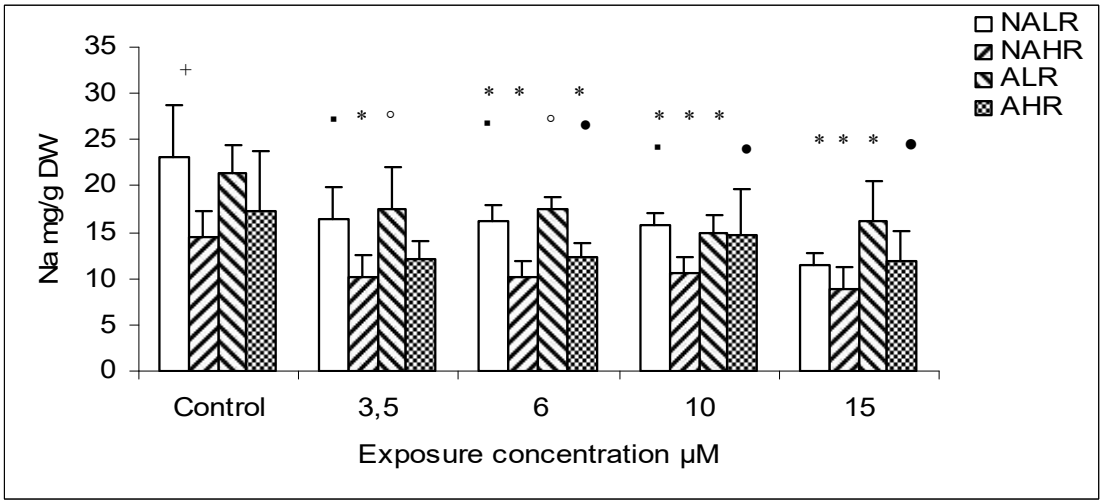

Fig. 6. Na^+ levels in the gill tissues of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). +: significant difference between controls of non-acclimated LR and HR carp, *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, ●: significant difference between the acclimated and non- acclimated carp, °: significant difference between the acclimated LR and HR, ▪: significant difference between the non-acclimated LR and HR. Note that survival times, and thus sampling times differed between fish.

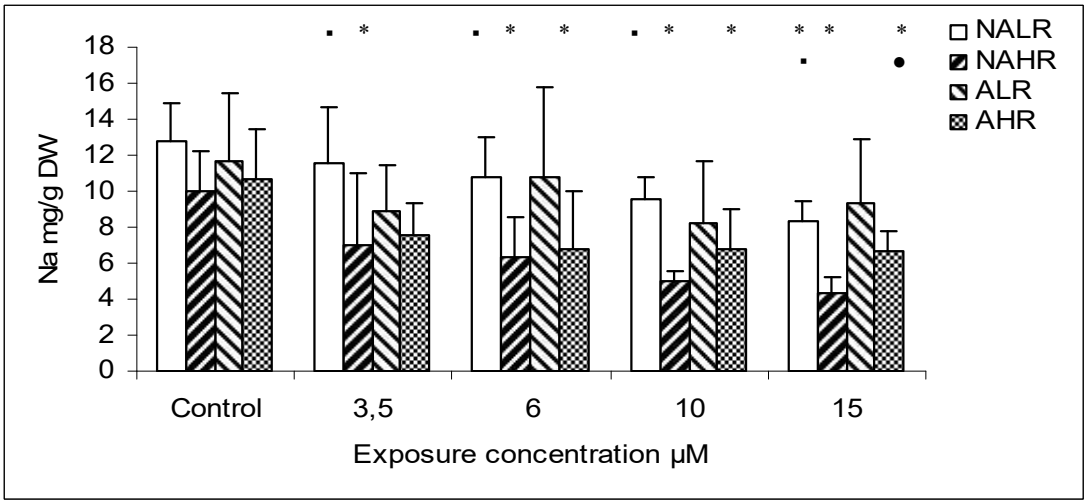

Fig. 7. Na^+ levels in the liver tissue of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, ●: significant difference between the acclimated and non- acclimated carp, ▪: significant difference between the non-acclimated LR and HR. Note that survival times, and thus sampling times differed between fish.

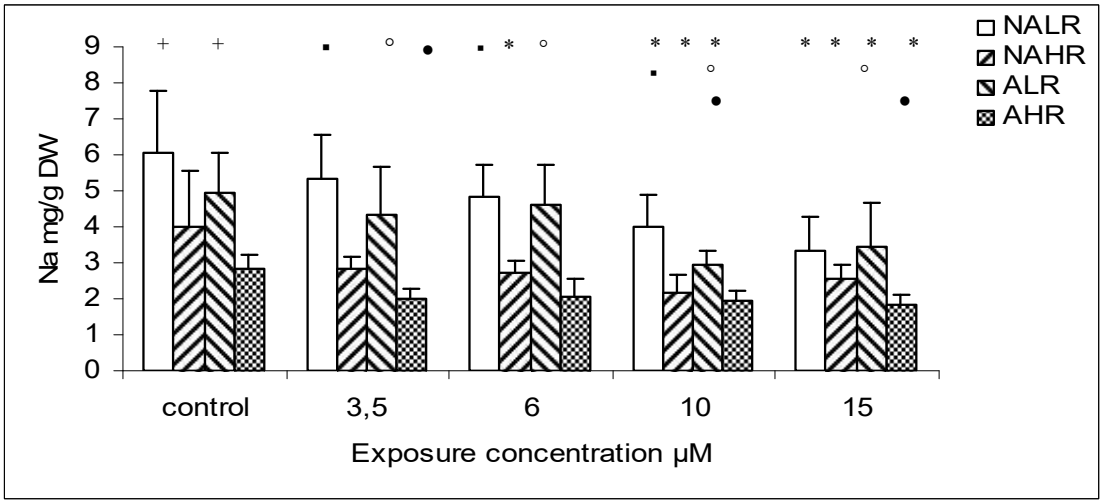

Fig. 8. Na^+ levels in the carcass of Cu acclimated and non-acclimated carp fed two different food rations (NALR: Non-acclimated carp fed 0.5 % body weight (BW), NAHR: Non-acclimated carp fed 5% BW, ALR: Acclimated carp fed 0.5 % BW, AHR: Acclimated carp fed 5 % BW) during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM. +: significant difference between controls of acclimated, non-acclimated LR and acclimated, non-acclimated HR carp, *: significant difference between the acclimated or non-acclimated LR and HR carp and their respective controls, ●: significant difference between the acclimated and non-acclimated carp, °: significant difference between the acclimated LR and HR, ▪: significant difference between the non-acclimated LR and HR. Note that survival times, and thus sampling times differed between fish.

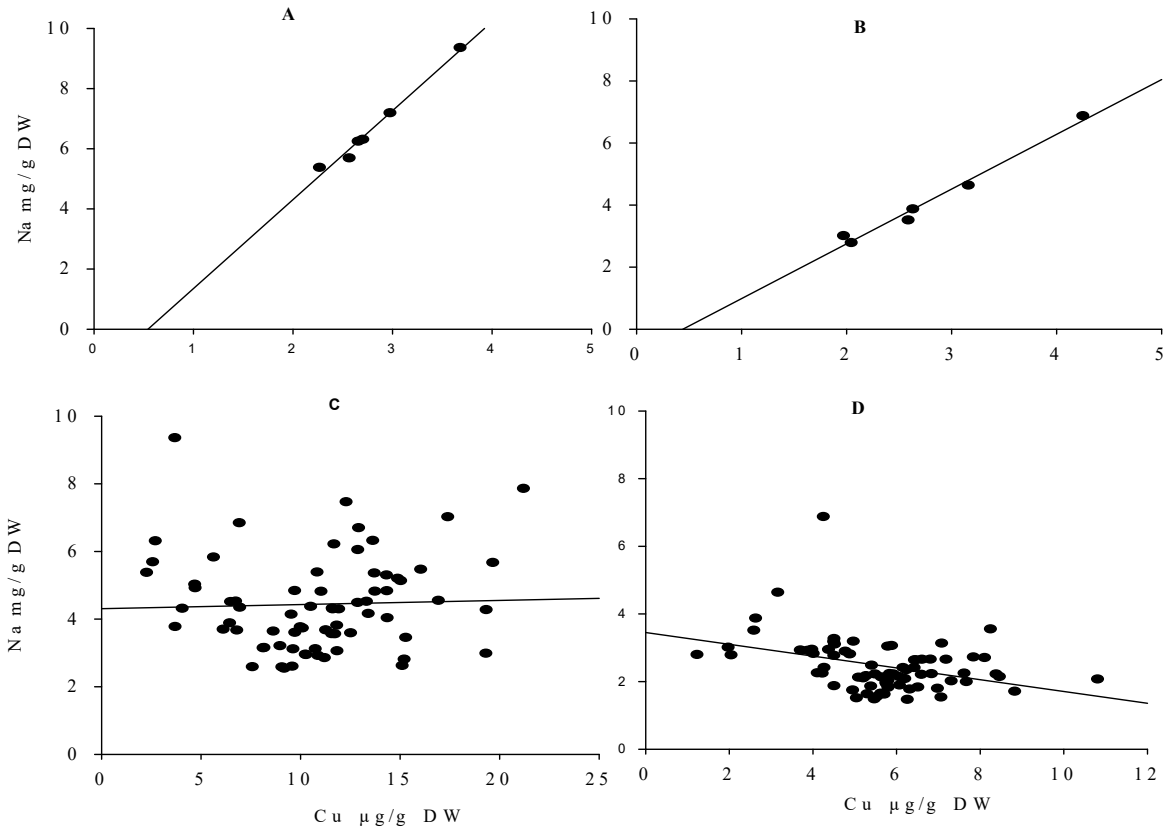

Fig. 9. Correlation between Cu and Na in the whole body of Cu acclimated and non-acclimated carp fed two different food rations during a 10-day exposure to 4 different Cu concentrations (3.5, 6, 10, and 15 µM). A: Non-acclimated control carp fed 0.5 % body weight (BW), B: Non-acclimated control carp fed 5% BW, C: Acclimated and non-acclimated carp fed 0.5 % BW after acute Cu exposure, D: Acclimated and non-acclimated carp fed 5% BW after acute Cu exposure.

4.4 Discussion

Our study determined that the sensitivity of the HR and LR carp against acute waterborne copper exposure was significantly different.

Despite the higher copper accumulation in target organs of the LR fish, it appeared that the HR carp were almost twice as sensitive to copper. Cu acclimation in the LR and HR carp greatly affected the LT50, but had only a moderate effect on the LC50. Acclimation to copper during chronic exposure was observed before in trout (Dixon and Sprague, 1981) and an elevated hepatic Cu elimination into the bile in the Cu acclimated trout was reported by Grosell et al., (1997) suggesting Cu excretion as an acclimation technique.

Cu exposure induces Na^+ loss through the gills, liver and likely kidney. The fact that more sensitive carp (HR) lost more Na^+ at a faster rate in both tests could implicate that mortality is related to quantity and rapidity of Na^+ loss. Declines of Na^+ levels during copper exposure were also reported by Taylor et al., (2003) and Wilson and Taylor (1993). According to Smith and co-workers (1989) Na^+ loss through the gills is more important than through other routes, and it is likely related to inhibited gill Na^+/K^+- ATP activity (Lorz and McPherson, 1976, Lauren and Mcdonald, 1986, Li et al., 1998). There is strong evidence for an antagonistic competition between Cu and Na^+ which share the same channel for entry into epithelial cells as well (Grosell and Wood, 2002, Pyle et al., 2003). Furthermore, higher copper concentrations cause structural damage to the gills of fish (De Boeck et al., 2001). Eventually the ability of the fish to ionoregulate breaks down, and sodium level progressively falls (Taylor et al., 2003, Wilson and Taylor, 1993) which causes a decline in plasma osmotic potential (Milligan and Wood, 1982) ending in the death of the fish. According to Wood (1989) rainbow trout dies during acid exposure, which shares the same mechanism with copper toxicity (Lauren and McDonald, 1985), once sodium concentration falls below 70% of the normal level. In the current study, fish death occurred when sodium concentration fell below 63% of normal level. A 30-40%

whole body Na^+ loss in rainbow trout and yellow perch was considered as a toxicity threshold by Taylor et al., (2003) as well.

Our result showed that copper accumulation in the tissues of acutely exposed LR fish was much higher than in the HR fish. These results are in agreement with our previous experiment using a chronic exposure (Hashemi et al., 2007a). In mouse a remarkable increase of hepatic metallothionein (MT) during continuous fasting periods has been reported by Higashimoto et al., (2002). Therefore, it was suggested that the level of MT in the starved fish could also be higher than in the fed fish (Hashemi et al., 2007a). MT induced by metals has shown to detoxify harmful heavy metals (Karin, 1985, Kagi and Kojima, 1987, De Boeck et al., 2003). In an attempt to investigate the differential metallothionein patterns in three freshwater fish, it became obvious that more tolerant species have a better correlation between tissue Cu levels and tissue MT levels (De Boeck et al., 2003). Considering these facts, it is likely that a higher amount of metallothionein in the starved fish protects the fish against the copper exposure.

The elevated Na levels in the LR fish are likely related to the positive correlation between Na and Cu in the whole body of fish which was also observed by Kamunde et al., (2003). LR carp may have more sodium channels as a result of their acclimation to starvation which necessitates that a larger portion of essential elements is absorbed from the water, and therefore allows both higher Na^+ and Cu influx. For the HR fish, it seems that in addition to the lower amount of Na channels, the Na^+/ NH_4^+ exchange pathway is disturbed as is shown by a remarkable increase in plasma ammonia concentration (Hashemi et al., 2007b) and ammonia excretion blockage (De Boeck et al. 2006). Higher plasma ammonia possibly also makes the HR fish more susceptible against acute copper exposure (Hashemi et al., 2007b).

The unusual accumulation of copper in the gills of the HR carp in the highest copper concentration (15 μM) could be related to severe damage of the gills at the first day of the acute exposure (Kirk and Lewis, 1993, McDonald and Wood, 1993, De Boeck et al., 2001). The LT50 was reached in less than 2 days which indicates that osmoregulatory disturbance was severe. This could lead to ruptured epithelia, with a free influx of Cu and efflux of Na as a consequence. A series of other physiological disturbances before death have been reported by other researchers. Taylor and co-authors (1996) pointed out that a fish dies because of an integrated response to physiological events culminating in its destruction. A release of catecholamine into the plasma of acid exposed rainbow trout was observed by Ye and Randall (1991), and Wells and Weber (1991) reported an increase in blood viscosity. According to Wilson and Taylor (1993), cardiac failure is the main reason of death during acute copper toxicity, and their experiment showed that heart rate and blood pressure increased after 20 h of lethal copper exposure in rainbow trout.

4.5 Conclusion

In conclusion it becomes obvious that both a reduced food intake and acclimation can decrease the effect of acute copper exposure. The quantity of copper in the tissues is not directly related to the death of the fish, as LR fish showed higher tissue Cu levels but a lower sensitivity to acute exposure. A reduced and slow Na^+ loss and probably a higher level of metallothionein in the starved carp helped these fish to cope with acute copper exposure. Therefore Na^+ loss and the way copper is handled intracellularly determine the toxic effect. This is in agreement with Taylor et al., (2003) who recorded more copper accumulation and less Na^+ loss in more resistant yellow perch compared to less tolerant rainbow

trout. As a result of this survey, we suggest considering food quantity as an effective factor in acute toxicity tests for fish.

Acknowledgements

This research was funded by grant 42/5/23003 from the Ministry of Science, Research and Technology of Iran, and Bijzonder Onderzoeksfonds (BOF) project 20337 by the University of Antwerp-Belgium. The technical assistance of Mr. M. Selens is gratefully acknowledged.

References

Basavaraju, Y., Devi, B.S.R., Mukthayakk, G., Reddy, L.P., Mair, G.C., Roderick, E.E., Penman, D.J., 1998. Evaluation of making and tagging methods for genetics studies in carp. J. Biosci. 23, 585-593.

Blust, R., Vanderlinden, A., Verheyen, E., Decleir, W., 1988. Evaluation of microwave-heating digestion and graphite-furnace atomic-absorption spectrometry with continuum source background correction for the determination of iron, copper, and cadmium in brine shrimp. JAAS. 3, 387-393.

Bowen, H.J.M., 1985. In D. Hutzinger (ed.), The Handbook of Environmental Chemistry, Vol. 1, Part D: The natural environment and biogeochemical cycles, Springer-Verlag, New York. p. 1-26.

De Boeck, G., Vlaemink, A., Balm, P.H.M., De Wachter, B., Lock, R.A.C., Blust, R., 2001. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during copper exposure: direct effects of Cu^{+2} or indirect effects of plasma cortisol evaluation? Environ. Toxicol. Chem. 20, 347-381.

De Boeck, G., Ngo, T.T., Van Campenhout, K., Blust, R., 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. Aquat. Toxicol. 65, 413-424.

De Boeck, G., Meeus, W., De Coen, W., Blust, R., 2004. Tissue specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Onchorynchys mykiss*), common carp (*Cyprinus carpio*), and gibel carp (Carassius *auratus gibelio*). Aquat. Toxicol. 70, 179-188.

De Boeck, G., Van der Ven, K., Hattink, J., Blust, R., 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat. Toxicol. 80(1), 92-100.

Deshmukh, S.S., Marathe, V.B., 1980. Size related toxicity of copper and mercury to *Lebistes reticulates* (Peter) labeo rohita (Ham.) and *Cyprinus carpio* (Linn). Indian J. Exp. Biol. 18,421-423.

Dixon, D.G., Sprague, J.B., 1981. Acclimation to copper by rainbow trout (Salmo *gairdneri*) –A modifying in toxicity. Can. J. Fish. Aquat. Sci. 38, 880-888.

Furness, R.W., Rainbow, P.S., 1990. Heavy metals in the Marine Environment. CRC Press, Florida.

Grosell, M.H., Hogstrand, C., Wood, C.M., 1997. Cu uptake and turnover in both Cu-acclimated and non-acclimated rainbow trout (*Oncorynchus mykiss*). Aquat. Toxicol. 38, 257-279.

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179-1188.

Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimmed Spearman-Karber method for estimating median lethal concentration in toxicity bioassay. Environ. Sci. Technol. 11, 714-719.

Hashemi, S., Blust, R., De Boeck, G., 2007a. The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. Environ. Toxicol. Chem. 26(7): 1507-1511.

Hashemi S., Blust R., De Boeck G., 2007b. Combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp. Arch. Environ. Contam. Toxicol. 10.1007/s00244-007-9017-1.

Higashimoto, M., Sano, M., Kondoh, M., Sato, M., 2002. Different responses of metallothionein and leptin induced in the mouse by fasting stress. Biol. Trace. Elem. Res. 89, 75-84.

Kamunde, C.N., Pyle, G.G., McDonald G., Wood C.M., 2003. Influence of dietary sodium on waterborne copper toxicity in rainbow trout, *Oncorynchus mykiss*. Environ Toxicol. Chem. 22(2), 342-350.

Karin, M., 1985. Metallothioneins: proteins in search of a function. Cell 41(1), 9-10.

Kagi, J.H.R., Kojima, Y., 1987. Metallothionein II, Experiential supplementum 52, 63-82.

Kaur, K., Dhawan, A., 1994. Metal toxicity to different life stages of *Cyprinus carpio* Linn. Indian J. Ecol. 21, 93-98.

Kazlauskiene, N., Stasiunaite, P., 1999. The lethal and sublethal effect of heavy metal mixture on rainbow trout (*Onchorynchus mykiss*) in its early stages of development. Acta Zoologica Lituanica. Hydrobiologia 9(2), 47-55.

Kirk, R.S., Lewis, J.W., 1993. An evaluation of pollutant induced changes in the gills of rainbow trout using scanning electron microscopy. Environ. Tech. 14, 577-585.

Lauren, D.J., McDonald, D.G., 1985. Effects of copper on branchial ionoregulation in the rainbow trout *Salmo gairdneri* Richardson: modulation by water hardness and pH. J. Comp. Physiol. B 155, 635-644.

Lauren, D.J., McDonald, D.G., 1986. Influence of water hardness, pH, and alkalinity on mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. Can. J. Fish. Aquat. Sci. 43, 1488-1495.

Li, J., Quabius, E.S., Wendelaar Bonga, S.E., Flik, G., lock, R.A.C., 1998. Effects of waterborne copper on branchial chloride cells and Na^+/ K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquat. Toxicol. 43, 1-11.

Lorz, H., McPherson, B.P., 1976. Effects of copper and zinc in fresh water on the adaptation to sea water and ATPase activity, and the effects

of copper on migratory disposition of coho salmon (*Onchorynchus mykiss*). J. Fish. Res. Board Can. 33, 2023.

McDonald, D.G., Wood, C.M., 1993. Branchial mechanism of acclimation to metals in freshwater fish. In: Rankin, J.C., Jesen, F.B., (Eds.), Fish ecophysiology. Chapman & Hall, London, pp. 297-321.

Milligan, C. l., Wood, C.M., 1982. Disturbance in haematology, fluid volume, distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 99, 397-415.

OECD, 1992. OECD test guideline 210, Fish, Early-life stage test, Toxicity test (adopted 17th July 1992).

Pyle, G.G., Kamunde, C., Mcdonald, D.G., Wood, C.M., 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Onchorynchus mykiss*). J. Exp. Biol. 206, 609-618.

Smith, N., Talbot, C., Eddy, F.B., 1989. Dietary salt intake and its relevance to ionic regulation in fresh water salmonids. J. Fish Biol. 35, 749-753.

Sposito, G., 1986. Distribution of potentially hazardous trace metals. In: Sigel, H. (Eds.), Trace metals in Biological systems, 20. Marcel Dekker Inc., New York, pp. 1-20.

Taylor, E.W., Beaumont, M.W., Butler, P.J., Mair, J., Mujallid, M.S.I., 1996. Lethal and sublethal effects of copper upon fish: a role for ammonia toxicity. In: Taylor, E.W., Toxicology of aquatic pollution, Cambridge University Press, Cambridge, pp. 85-113.

Taylor, L. N. , Wood, C.M., McDonald, D.G., 2003. An evaluation of sodium loss and gill metal binding properties in rainbow trout and yellow perch to explain species differences in copper tolerance. Environ. Toxicol. Chem. 22, 2159–2166.

Wells, R.M.G., Weber, R.E., 1991. Is there an optimal haematocrit for rainbow trout, *Onchorynchus mykiss* (Walbaum)? An interpretation of recent data based on blood viscosity measurements. J. Fish Biol. 38, 53-65.

Wilson, R.W., Taylor, E.W., 1993. The physiological responses of freshwater rainbow trout, *Onchorynchus mykiss*, during acutely lethal copper exposure. J. Comp. Physiol. B 163, 38-47.

Wood, C.M., 1989. The physiological problems of fish in acid waters. In: Morris, R., Taylor, E.W., Brown, D.J.A., Brown, J.A. (Eds.), Acid Toxicity and Aquatic Animals. Cambridge University Press, New York, pp. 124-152.

Yamamoto, Y., Ishii, T., Ikeda, S., 1997. Studies on copper metabolism in fishes II. The site of copper accumulation in the tissues of carp. Bull. Jap. Soc. Scient. Fish 43, 1327-1332.

Ye, X., Randall, D.J., 1991. The effect of water pH on swimming performance in rainbow trout (*Salmo gairdneri*). Fish Physiol. Biochem. 9, 15-21.

5

Differential metallothionein induction patterns in fed and starved carp during waterborne copper exposure

Shodja Hashemi, Prabesh Kunwar, Ronny Blust, Gudrun De Boeck

Submitted to Environmental Toxicology and chemistry

Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp, Groenenborgerlaan171, 2020 Antwerp, Belgium

Abstract

Starved and fed carp (Cyprinus carpio) were exposed to sublethal waterborne copper exposure (1µM) for 28 days in softened Antwerp city tap water. Copper accumulation in liver and gill tissues was determined, and the changes in branchial Na^+/K^+ ATPase activity and metallothionein (MT) induction in gill and liver tissues were investigated. Gill Na^+/ K^+ ATPase activity in both the starved and fed exposed fish was at its lowest values after 3 days of exposure, after which it slowly recovered to pre-exposure values. No significant differences in branchial Na^+/ K^+ ATPase activity were found between the starved and fed fish. Copper accumulation in the liver and gills of the exposed starved carp was significantly higher than in the exposed fed carp. The highest MT induction was found in liver tissues. Different patterns of MT induction were observed in the starved and fed carp during copper exposure. Even before exposure, MT levels in the livers of the starved fish were significantly higher than in the fed ones. Copper exposure significantly induced MT levels in the liver of the fed fish, but no changes occurred in the starved fish. In contrast, copper exposure induced MT levels in the gills of the starved fish in the first week of exposure, while only a slight increase in MT levels in the gills of the fed fish was observed. When taking into account the role of feeding status in MT induction, hepatic MT shows to be a more relevant indicator for long-term monitoring of copper pollution in carp, while gill MT provided useful information on short-term copper toxicity.

5.1 Introduction

Copper exposure generally disturbs physiological functions in fish [1]. It accumulates in fish tissues and predominantly leads to ionoregulation disorders [2, 3, and 4]. Ionoregulatory disruption is usually attributed to Na loss through gill tissues, mainly because of the inhibition of branchial Na^+/K^+ ATPase activity [5, 6]. Copper accumulation is mostly ascribed to stimulate metallothionein (MT) induction as a physiological defense mechanism [7]. Increased levels of MT were associated with elevated tissue copper accumulation in some freshwater fish [8], and MT induction is widely used as a cost-effective molecular biomarker for monitoring copper contamination. Nevertheless, in contrast to its copper binding affinity, it was believed that copper was not a strong MT inducer [9].

Recent studies showed that Na loss was more severe in fed carp compared to starved fish during acute copper exposure [10]. Despite the higher copper accumulation in the target organs of starved carp during lethal and chronic copper exposure, starved carp were two fold more tolerant against acute copper toxicity compared to fed fish [4, 10]. These studies suggested that branchial Na^+/K^+ ATPase inhibition and MT induction are likely involved in these differences. Disruption of ionoregulation makes fish more vulnerable to copper toxicity, and MT plays a key role in the detoxification of copper in fish. Significantly elevated levels of MT have been found in fasting mice [11], and also in starved cod (*Gadus morhua*) the level of MT mRNA increased [12]. Since starved fish rely more on the branchial uptake mechanisms to acquire the necessary ions, differences in ionoregulation might be expected as well. Therefore we hypothesized that differences in MT

induction levels and branchial Na^+/K^+ ATPase activity were likely to be observed between the starved and fed fish, and that the starved fish may be benefited from an additional MT induction, and a more efficient branchial Na^+/K^+ ATPase activity. We strived to investigate whether or not feeding rations affect MT induction and branchial Na^+/K^+ ATPase during sublethal waterborne copper exposure in carp. Furthermore, we examined if MT induction correlated with copper accumulation.

5.2 Material and methods

5.2.1 Animal holding- Experimental set up

Common carp, *Cyprinus carpio*, were obtained from the fish hatchery at the Wageningen University, The Netherlands, and kept at the University of Antwerp at 23 ±1 °C in softened Antwerp city tap water. The fish used in the tests had a mean initial wet weight of 5.14±1.40 g and were randomly distributed in 4 tanks. Fish were acclimated to the test conditions and food rations for 30 days before Cu acclimation started. They were pre-acclimated to either 0.5% or 5% body weight (BW) food rations with Trouvit (Trouw Nutrition, Fontaine-les-Vervins, France) divided over two feedings a day. These two feeding treatments were named as the starved (0.5% BW) and fed fish (5% BW). The photoperiod was set at 12: 12-h light:dark. Water quality was checked daily, and 80% of the water was renewed three times a week. The temperature was set at 23±1°C, pH was 7.5 to 8, oxygen concentration remained above 7 mg/L, and ammonia concentration was below 0.1 mg/L at all times. Water hardness was 270 mg $CaCO_3$/L, and ion concentrations in the water were Ca^{2+}, 79.3 mg/L; Mg^{2+}, 7.4 mg/L; and Na^+ , 27.8 mg/L. Cu and Na contents of the food were 0.012 ±0.001 mg/g (mean ± standard deviation [SD], n=8) and 4.529±0.147 mg/g (mean ±SD, n=8) respectively. Cu acclimation conditions via static exposure conformed to the OECD

guidelines 210 [13]. Each feeding treatment was split into two tanks: one with exposure plus one without exposure. The tanks were predosed with Cu $(NO_3)_2 \cdot 2H_2O$ to bring the concentration to the nominal level. A copper nitrate stock solution (Cu $(NO_3)_2 \cdot 2H_2O$ 1M) (Merck, Darmstadt, Germany) was added into the tanks during the entire experiment, and both feeding treatments of fish were acclimated to a sublethal total concentration of 1µM Cu for 28 days. Tanks were cleaned daily to minimize any fecal ingestion. The amount of food fed to each exposure tank was adjusted weekly for fish growth.

5.2.2 Sampling procedure

Eight fish were sampled at regular intervals (days 0, 0.5, 1, 3, 7, 14, and 28). They were quickly netted and killed by an overdose of buffered MS 222 (1 g/l at PH 7.5, Across organics, Geel, Belgium). All fish were weighed, and immediately dissected on ice. Gill lamellae and liver tissue were rinsed with physiological saline (0.9% NaCl), and snap frozen in liquid nitrogen and stored at -80 °C for later analysis.

5.2.3 Na^+/ K^+ ATPase analysis

Gills Na^+/ K^+ ATPase activity was measured based on disappearance of NADH, and differences between the enzymatic activity in the presence and absence of ouabain were used for the calculation of Na^+/ K^+ ATPase activity as explained by McCormick [14].

5.2.4 Cu analysis

Subsamples of each tissue were dried in a 60 C° drying oven for a minimum of 72h, and after cooling in a desiccator, reweighed to obtain the dry weight of the tissue. Subsequently, they were digested in 70 %

HNO_3, placed in a microwave oven [15] until total digestion had occurred and diluted with Milli-Q grade water (Milliopre, Bedford, MA, USA). Total copper concentrations were determined using inductively coupled plasma atomic emission spectrophotometry (ICP-AES, Varian, Liberty, series II, Mulgrave-Victoria, Australia). The accuracy of copper analysis was verified by including blank and certified reference material.

5.2.5 MT analysis

Liver and gills samples were thawed and homogenized using disposable tissue-ruptor probes (Qiagen, Hilden, Germany) in 4 volumes of buffer A (10 mM tris-HCl, 85 mM NaCl, pH 7.4). Tissues were centrifuged at 16000 g at 4°C for 20 min (Eppendorf 5415R, Hamburg, Germany), and supernatant aliquots were stored at -80°C. Cu-containing MT was measured by the ^{109}Cd saturation method as described by Klein et al. [16, 17], and an assumption of a molar ratio of Cd to MT of 7 was used for MT calculation. According to Bienengraber et al. [18] the Cd saturation procedure accords well with the Ag-saturation assay or the Elisa method.

5.2.6 Statistical analysis

Data are presented as mean ± SD. Data were compared using analysis of variance (ANOVA) followed by Tukey multiple comparison. Bonferroni's test was used when there were missing samples. Regression analysis was performed to determine the relationship between MT and Cu concentrations. Differences were considered significant at $p< 0.05$. Control fish did not show significant differences over time in branchial Na^+/ K^+ ATPase activity, copper accumulation in the gills and liver tissues, or gills MT levels. For clarity of the figures all control values were averaged, and these average values were shown at the day 0. Liver

MT levels did show significant differences over time for control fish, therefore all control values are shown.

5.3 Results

Gill Na^+/ K^+ ATPase activity decreased within the first days of exposure with more than 50% reduction in activity in both the exposed starved and fed fish at the third day of Cu exposure, after which Na^+/ K^+ ATPase activity recovered to pre-exposure levels (Fig. 1). No significant differences in branchial Na^+/ K^+ ATPase activity were found between the starved and fed fish.

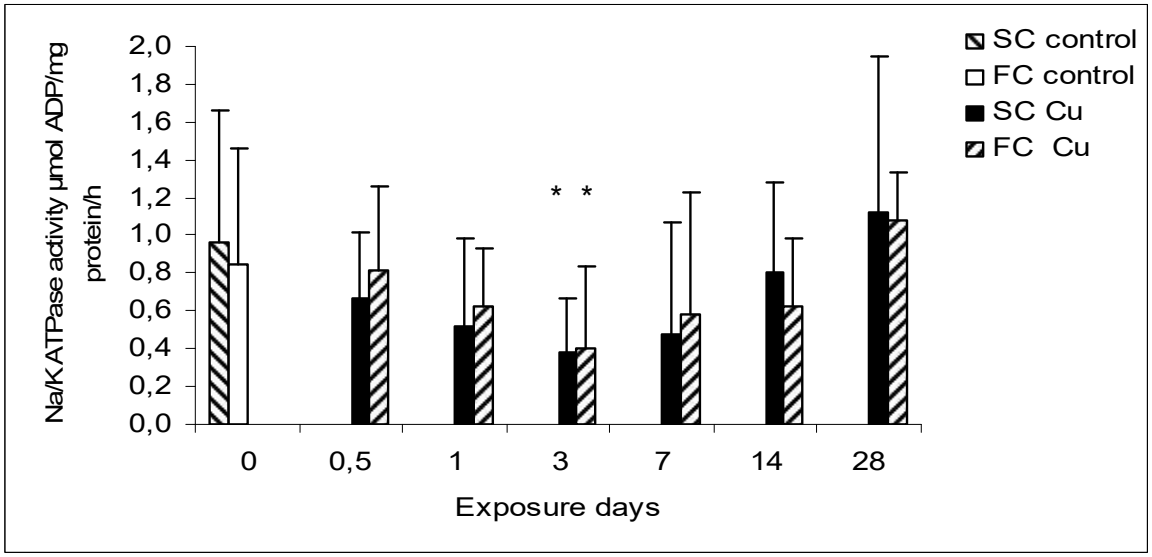

Fig. 1. Na^+/ K^+ ATPase activity in gill tissues of starved (SC) and fed (FC) carp (*Cyprinus carpio*) during a 28-day exposure to 1 µM of copper. *: significant difference between exposed carp and its respective control.

Copper levels in the liver of the starved control carp were significantly higher than in the fed controls (Fig. 2), while no differences were seen in gill Cu accumulation between starved and fed control fish (Fig. 3). Copper exposure increased copper accumulation in the liver of fed fish, while copper levels in the livers of starved fish did not change

significantly. By the end of the 4 weeks exposure, liver Cu levels in starved fish were still significantly higher compared to fed fish. In gills tissue, Cu levels were significantly increased from the start of the

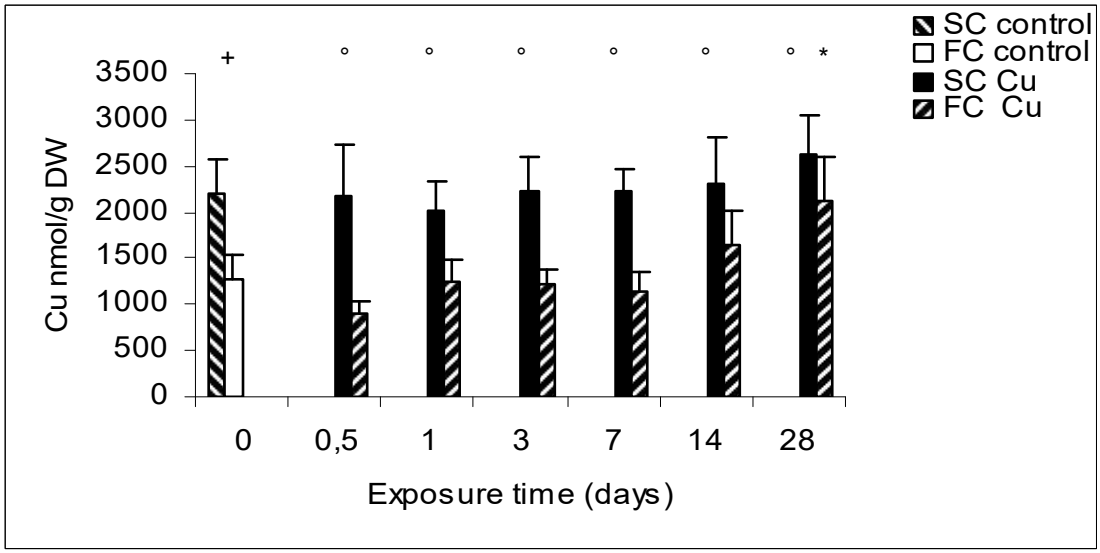

Fig. 2. Copper accumulation in liver tissue of starved (SC) and fed (FC) common carp (*Cyprinus carpio*) during a 28-day exposure to 1 µM copper. °: significant difference between exposed SC and exposed FC, *: significant difference between exposed carp and its respective control, +: significant difference between control SC and control FC.

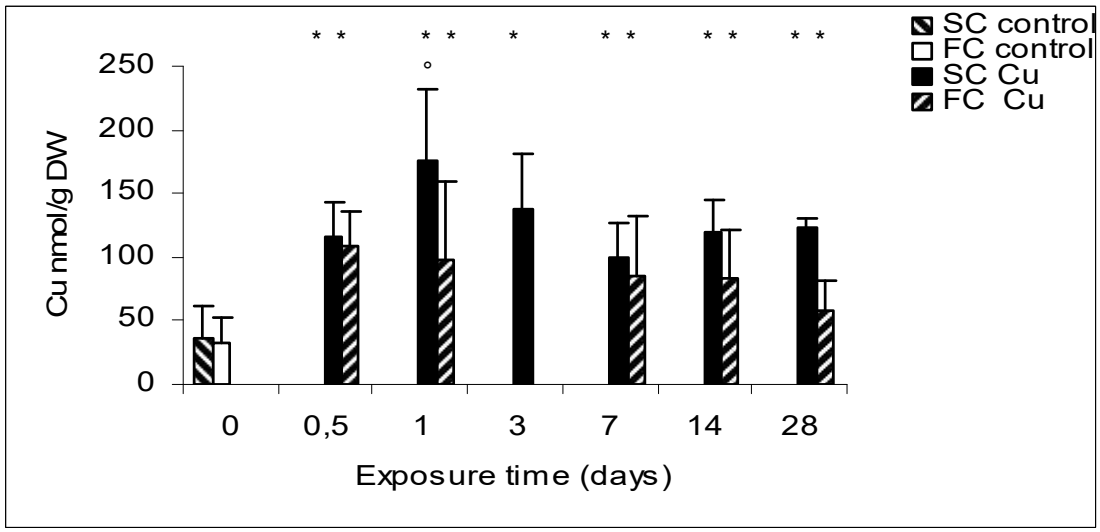

Fig. 3. Copper accumulation in gill tissues of starved (SC) and fed (FC) common carp (*Cyprinus carpio*) during a 28-day exposure to 1 µM copper. °: significant difference between exposed SC and exposed FC, *: significant difference between exposed carp and its respective control.

exposure onwards in both starved and fed fish (Fig. 3). Copper accumulation in the gills of the starved exposed carp was significantly higher than in fed ones after 1 day of exposure. Copper levels in the liver of fish were 16.3 fold higher than in gills.

As for liver Cu levels, also liver MT levels were significantly higher in starved control carp compared to the fed control fish (Fig. 4). During the experiment, MT levels in control fed fish approached the levels in the starved fish, and by day 28, the difference between the two groups was no longer significant. Copper exposure significantly induced MT levels in the liver of the fed fish, but not in the liver of starved fish. MT levels in the liver of both the starved and fed fish were remarkably higher than in the gills in either the control or exposed fish.

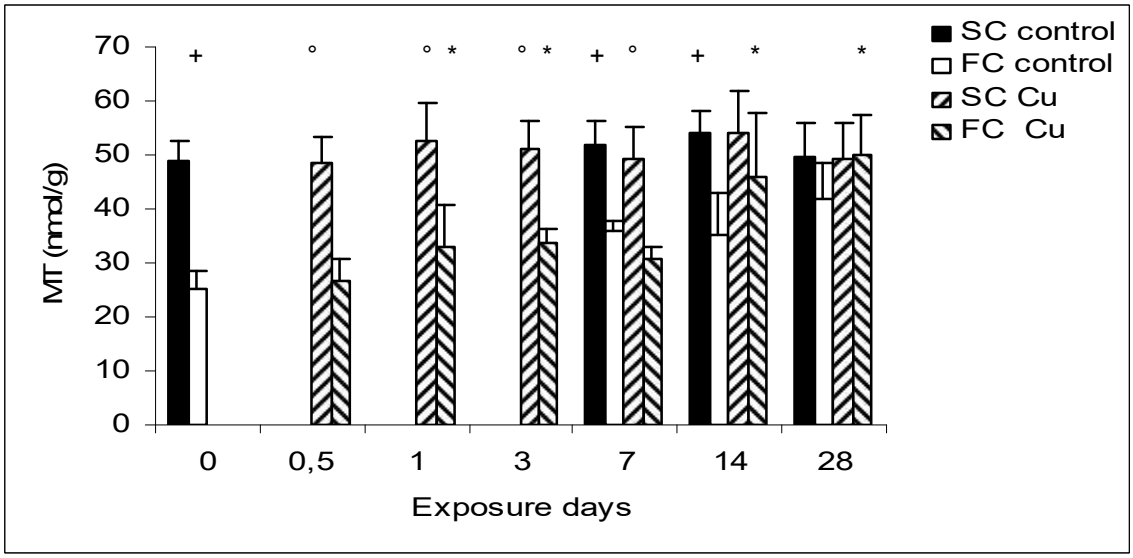

Fig. 4. Metallothionein levels in liver tissue of starved (SC) and fed (FC) carp (*Cyprinus carpio*) during a 28-day exposure to 1 µM of copper. °: significant difference between exposed starved and exposed fed carp, *: significant difference between exposed carp and its respective control, +: significant difference between control starved and control fed carp.

There was no difference between MT levels in the gills of fed and starved control fish, and MT levels in control fish remained stable during the duration of the experiment. In contrast, MT levels in the gills of the starved fish doubled within the first week of Cu exposure, but no significant changes were seen in MT levels in the gills of the exposed fed fish (Fig. 5). MT concentration in the gills of both the exposed starved and fed fish dropped and reached the control levels after two weeks of exposure. A significant linear relationship was established between liver Cu and MT concentrations (Fig. 6), whereas in the gills Cu and MT levels were not correlated.

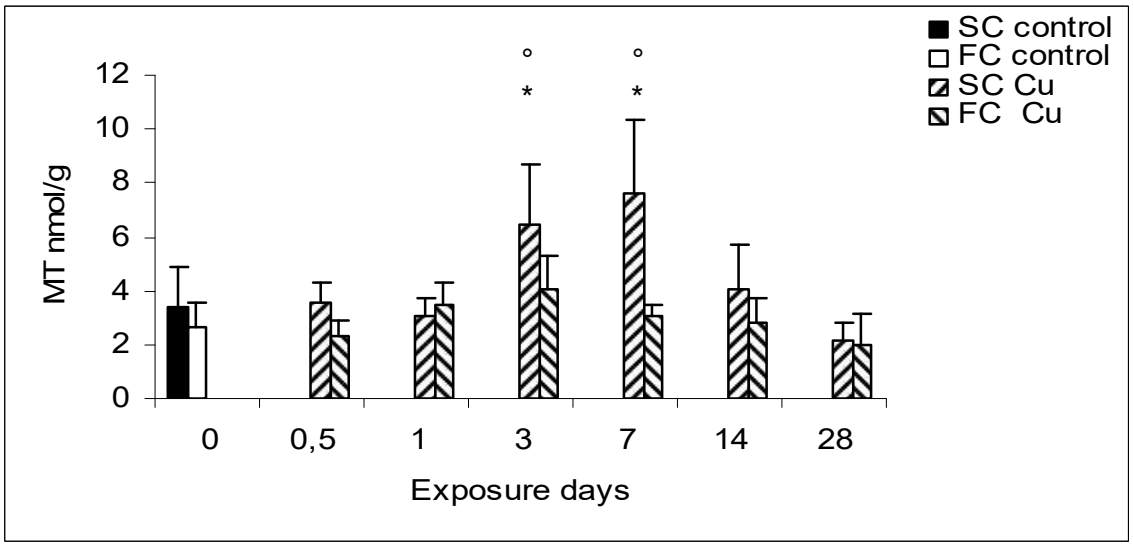

Fig. 5. Metallothionein levels in gill tissues of starved (SC) and fed (FC) carp (*Cyprinus carpio*) during a 28-day exposure to 1 µM of copper. °: significant difference between exposed starved and exposed fed carp, *: significant difference between exposed carp and its respective control.

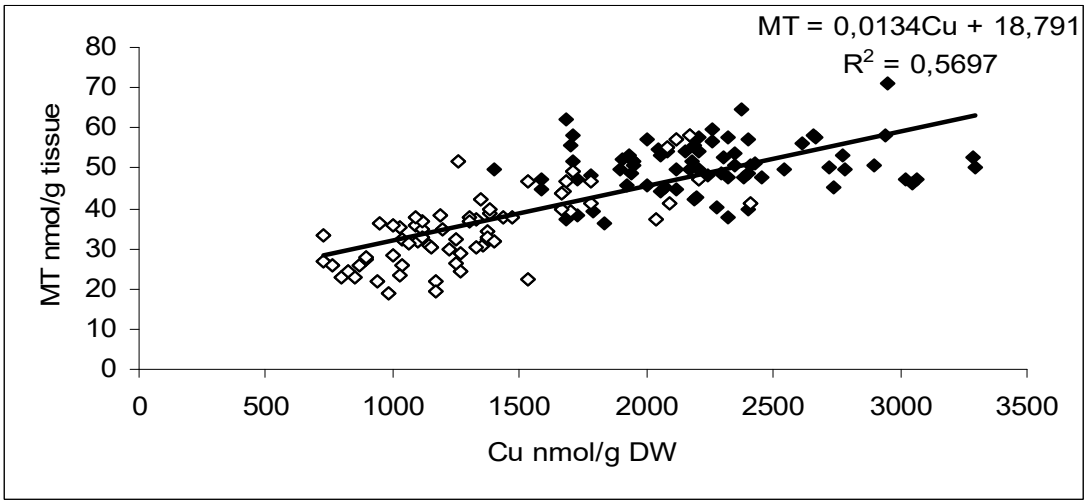

Fig. 6. The relationship between MT and Cu concentrations in liver tissue of starved (♦) and fed (◊) carp (*Cyprinus carpio*) during a 28-day exposure to 1 μM of copper.

5.4 Discussion

Copper exposure disrupts Na^+/ K^+ ATPase activity due to its high affinity to sulfhydral groups found in transport enzymes [5, 19] and its interaction with Mg^{+2}-binding sites [20]. The observed inhibition of branchial Na^+/ K^+ ATPase activity during the first days of sublethal copper exposure and recovery afterwards was documented before [1, 6, 21, and 22]. This finding indicates that carp are capable of acclimating to sublethal waterborne copper exposure. The rapid decrease in Na^+/ K^+ ATPase coincides with loss of whole body Na^+ [10]. No significant differences in Na^+/ K^+ ATPase activity were found between the starved and fed carp in the current study, which suggests that food rations did not affect Na^+/ K^+ ATPase activity. The observed differences in Na concentration and Na losses between starved and fed fish in a previous study could be related to the disturbance of Na^+/ NH_4^+ exchange

pathways in the fed fish since increased levels of plasma ammonia concentrations were observed in these fish as well [23, 24].

Different patterns of copper accumulation and MT induction were found between the fed and starved carp during copper exposure. Tissues specific variations in MT induction were noticed, similar to what was already reported for copper accumulation during waterborne copper exposure [3, 4]. MT levels were significantly increased in the liver of unexposed fish during starvation. There are a few studies reporting higher levels of MT in the starved animals, but to our knowledge, this is the first direct report on the effect of starving on MT induction in the carp. According to McNamara and Buckley [12] the level of MT mRNA in starved cod (*Gadus morhua*) was 7-fold higher than in control cod. Higashimoto et al. [11] also reported significantly elevated levels of MT in fasting mice. In the current study, liver MT levels in the starved control carp were 1.7 fold higher than in the fed control carp. In contrast, a decrease in liver MT levels in mummichog (*Fundulus heteroclitus*) was reported under restricted feeding by Ferraro and co-workers [25], which impels that MT induction is likely species-specific. Starvation is known as a stress factor in fish [26, 27]. It also leads to increased levels of cortisol [28]. The inducibility of MT by cortisol was reported by Hyllner et al. [29], Olsson et al. [30] and Wu et al. [31]. Elevated levels of MT in the control starved fish are likely related to stimulated levels of cortisol.

In the current study similar trends were observed when comparing Cu accumulation and MT data. Copper levels in the liver of carp were almost 16.3-fold higher than in the gills, and a similar trend was observed for MT levels in the same tissues, with 12.3-fold higher MT levels in the liver compared to the gills. MT induction in the liver of exposed fed fish was time related which was observed for Cu accumulation as well [4 and this study]. A positive correlation between MT levels and copper

accumulation in the liver of fish was established. This finding is in agreement with De Boeck et al. [8] who found strong correlations between MT and Cu levels in the liver of carp, but no MT induction in the gills. The current study showed MT induction in the gills of starved fish only, and not in the fed fish, which provides a better protection during the initial phase of Cu exposure in the starved fish.

More resistant starved carp accumulated more copper in their target organs than the less resistant fed ones during the chronic and acute waterborne copper exposure [4, 10]. The higher MT level in the liver of the starved fish is likely one of the explanations of their increased tolerance to Cu exposure since MT's are involved in the detoxification and accumulation of copper in fish. The strongest MT induction during waterborne copper exposure occurred in the liver tissues of the fed fish. Moreover, MT levels reached a plateau in the liver compared to the transient increase in the gills. Therefore, the hepatic MT represents a more relevant indicator for long-term monitoring of copper pollution in carp, and the branchial MT provide useful information on short-term copper pollution.

5.5 Conclusion

This study demonstrates that MT induction levels play a key role in copper toxicity and accumulation in carp. Starvation as well as copper exposure induces MT in carp. Food rations did not affect branchial Na^+/K^+ ATPase activity. As MT is widely used as a metal pollution indicator [7, 32, 33], it can be concluded that the feeding condition of fish should be taken into account when using MT as a biomarker for copper contamination. Since fish are poikilotherm and their feeding habits largely depend on surrounding environmental temperature, in situ field studies are required to investigate the seasonal variations of MT in fish

tissues. As copper accumulation and toxicity are dose-related, further studies need to find out whether or not this fact also affects MT induction levels. These findings would help pave the way for further improvements in biomonitoring plans involving MT levels as biomarker for metal pollution.

References

1. Pelgrom SMGJ, Lock RAC, Balm PHM, Wendelaar Bonga SE. 1995. Integrated physiological responses of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. *Aquat Toxicol* 32: 303-320.

2. McDonald DG, Wood CM. 1993. Branchial mechanism of acclimation to metals in freshwater fish. In Rankin JC, Jesen FB, eds. *Fish ecophysiology*. Chapman & Hall, London, United Kingdom, pp. 297-321.

3. De Boeck G, Meeus W, De Coen W, Blust R. 2004. Tissue specific Cu bioaccumulation patterns and differences in sensitivity to waterborne Cu in three freshwater fish: rainbow trout (*Onchorynchys mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*). *Aquat Toxicol* 70: 179-188.

4. Hashemi S, Blust R, De Boeck G. 2007. The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. *Environ Toxicol Chem* 26(7): 1507-1511.

5. Lauren DJ, McDonald DG. 1986. Influence of water hardness, pH, and alkalinity on mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*. *Can J Fish Aquat Sci* 43: 1488-1495.

6. Li J, Quabius ES, Wendelaar Bonga SE, Flik G, lock RAC. 1998. Effects of waterborne copper on branchial chloride cells and Na^+/ K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). *Aquat Toxicol* 43: 1-11.

7. Roesijadi G. 1994. Metallothionein induction as a measure of response to metal exposure in aquatic animals. *Environ Health Prospect* 102 (Suppl 12): 91-96.

8. De Boeck G, Ngo TT, Van Campenhout K, Blust R. 2003. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquat Toxicol* 65: 413-424.

9. Olsson PE. 1996. Metallothionein in fish: induction and use in environmental monitoring. In: Taylor, E.W., *Toxicology of aquatic pollutant, cellular and molecular approaches*. Cambridge University Press, New York, United States, pp. 187-203.

10. Hashemi S, Blust R, De Boeck G. 2007. The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp. *Aquat Toxicol,* in press.

11. Higashimoto M, Sano M, Kondoh M, Sato M. 2002. Different responses of metallothionein and leptin induced in the mouse by fasting stress. *Biol Trace Elem Res* 89: 75-84.

12. McNamara PT, Buckley LJ. 1994. Identification and characterization of metallothionein cDNA from mRNA transcripts induced by starvation in Atlantic cod (*Gauds morhua*). *Mol Mar Biol Biotechnol* 3(5): 252-260.

13. Organization for Economic Co-operation and Development. 1992. OECD test guideline 210, Fish, Early-life stage test, Toxicity test (adopted 17th July 1992).

14. McCormick SD. 1993. Methods for nonlethal gill biopsy and measurement of Na^+/ K^+ ATPase. *Can J Fish Aquat Sci* 50: 656-658.

15. Blust R, Vanderlinden A, Verheyen E, Decleir W. 1988. Evaluation of microwave-heating digestion and graphite-furnace atomic-absorption spectrometry with continuum source background correction for the determination of iron, copper, and cadmium in brine shrimp. J. *Anal At Spectrom* 3: 387-393.

16. Klein D, Bartch R, Summer KH. 1990. Quantification of Cu-containing metallothionein by Cd-saturation method. *Anal Biochem* 189: 35-39.

17. Klein D, Sato S, Summer KH. 1994. Quantification of oxidized methallothionein in biological material by a Cd saturation method. *Anal Biochem* 221: 405-409.

18. Bienengraber M, Forderkunz S, Klein D, Summer KH. 1995. Determination of Cu-containing metallothionein: Comparison of Ag saturation assay, thiomolybdate assay, and enzyme-linked immunosorbent assay. *Anal Biochem* 228: 69-73.

19. Stekhoven FS, Bonting SL. 1981. Transport adenosine triphosphatase: properties and function. *Physiol Rev* 61: 1-76.

20. Li J, lock RAC, Klaren PHM, Swarts HGP, Schuuramans Stekhoven FMAH, Wendelaar Bonga SE, Flik G. 1996. Kinetics of Cu^{+2} inhibition of Na^{+}/ K^{+}-ATPase. *Toxicol Lett* 67: 31-38.

21. Pyle GG, Kamunde CN, Mcdonald DG, Wood CM. 2003. Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Onchorynchus mykiss*). *J Exp Biol* 206: 609-628.

22. Stagg RM, Shuttleworth TJ. 1982. The effects of copper on Ionic regulation by the gills of the seawater-adpoted flounder (*Platichthys flesus*). *J Comp Physiol* 149: 83-90.

23. Hashemi S, Blust R, De Boeck G. 2007. Combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp. *Arch Environ Contam Toxicol* 10.1007/s00244-007-9017-1.

24. De Boeck G, Van der Ven K, Hattink J, Blust R. 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. *Aquat Toxicol* 80(1): 92-100.

25. Ferraro M, Curetsy K, Crivello JS. 2003. The effects of feeding restriction on metallothionein levels ethoxyresorufin-O- deethylase activities in mummichog. *J. Aquat Anim Health* 15: 31-38.

26. Morales EM, Perez-Jimenez A, Hidalgo MC, Abellan E, Cardenete G. 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. *Comp Biochem Physiol C- Toxicol Pharmacol* 139: 153-161.

27. Ruane NM, Huisman EA, Komen J. 2002. The influence of feeding history on the acute stress response of common carp (*Cyprinus carpio*). *Aquaculture* 210: 245–257.

28. Vijayan MM, Moon TW. 1992. Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). *Can J Fish Aquat Sci* 49: 2260– 2266.

29. Hyllner SJ, Andersson T, Haux C, Olsson PE. 1989. Cortisol induction of metallothionein in primary culture of rainbow trout hepatocytes. *J Cell Physiol* 139: 24-28.

30. Olsson PE, Hyllner SJ, Zafarullah M, Andersson T, Gedamu L. 1990. Differences in metallothionein gene expression in primary cultures of rainbow trout hepatocyts and the RTH-149 cell line. *BBA* 1049: 78-82.

31. Wu S, Chou Y, Deng A. 2002. Effects of exogenous cortisol and progesterone on metallothionein expression and tolerance to waterborne cadmium in tilapia (Oreochromis *mossambicu*). *Zool Stud* 41(1): 111-118.

32. Henning HFKO. 1986. Metal binding proteins as metal pollution indicator. *Environ Health Perspect* 65: 175-178.

33. George SG, Olsson PE. 1994. Metallothioneins as indicators of trace metal pollution. In: Kramer, K.J.M., Editor, *Biomonitorig of coastal waters and estuaries*, CRC Press, Boca Raton, Florida 151-171.

6

General conclusions and future research prospective

Although copper is an essential element supporting physiological processes in fish, it can be toxic to fish when exceeding natural background levels. Human activities discharge additional copper to surface waters, which endangers aquatic organisms' health. Although most aspects of acute copper toxicity on fish were studied, sublethal effects have not been studied to such a great extend. Literature data show that copper toxicity in fish alters depending on fish species, exposure route, water composition, temperature and time, but a little attention was given to the importance of the organisms' condition prior to or during the copper exposure. This research presents a novel approach to copper toxicity in fish by focusing mainly on long term effects of sublethal waterborne copper on carp taking into account the feeding status of the fish. Carp (*Cyprinus carpio*) were chosen as test organism due to their economical importance in the aquaculture and fisheries industry in the world, and their suitability as test organism in eco-toxicological studies. Long-term experiments were performed to achieve five objectives of this research, which included:

I: Investigating effects of different food rations before and during sublethal Cu exposure on growth, energy stores, and metabolic waste products in carp

II: Analyzing copper bioaccumulation in different tissues of carp under different feeding regimes during chronic and acute waterborne Cu exposure

III: Evaluating sensitivity of starved and fed carp against acute waterborne copper exposure

IV: Assessing the effects of a long term acclimatization to a sublethal Cu exposure on copper toxicity and copper accumulation in carp

V: Exploring the differences between physiological toxic mechanisms in fed and starved carp during waterborne copper exposure

Generally, carp were fed two different food rations: 0.5% body weight (low ration, LR) and 5% body weight (high ration, HR) and were chronically exposed to an environmentally realistic copper concentration (1µM) for 28 days. Fish were sampled during the entire exposure duration and a subsequent 2 week recovery period. Short- term acute waterborne copper exposures were conducted using four different copper concentrations. Physiological responses of the fish were studied, and the interaction between food quantity and waterborne copper exposure was investigated. Sublethal waterborne Cu exposure caused substantial physiological changes, which were significantly different between the fed and starved carp. Remarkable interactions between food rations, food composition and Cu accumulation were found and summarized below.

In the first study, combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp were studied. Differences were established in growth, available energy, and metabolic waste handling. Fish fed a high food ration had a significantly higher performance in terms of weight gain and growth. Feeding carp a

high food ration also resulted in increased lipid stores. Exposure to 1µM waterborne copper exposure affected growth and weight gain of both HR and LR carp significantly. Reduced growth rate was an indirect effect of an increased maintenance cost in fish under copper exposure. These effects were more pronounced in the HR fish. The HR fish were probably more affected by a disturbed oxygen uptake, while fish with low growth rates could still sustain their low metabolism, a hypothesis that has recently been confirmed. Food ration did not affect hepatic glycogen and protein. The fish primarily used lipid metabolism in this study, and hepatic lipid stores reflected both the reduced food intake and increased levels of stress during the first days of exposure. The limited changes in glycogen most likely were a response to a mild hypoxia induced by the copper exposure. The increase in hepatic protein content was possibly a physiological response to stress during copper exposure. Plasma ammonia concentration in HR fish always tended to be higher than in LR fish, and the difference was amplified during copper exposure. The ammonia accumulation might actually contribute to the increased sensitivity of the HR fish to copper toxicity. Higher ammonia accumulation could be related to an increased level of cortisol, inhibited excretory capacity, disturbance in Na^+/ NH_4^+ exchange, and physical gill damage resulting in increased diffusion distances. This study showed that the physiological changes during the copper acclimation are reversible, and fish recover when the exposure ends.

In the second study, the effects of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio* were studied. Copper accumulation in the liver, gills, kidney, anterior intestine, posterior intestine, and muscle were determined. Copper accumulation was found to be tissue specific and

related to exposure time during waterborne copper exposure. Significant differences in copper accumulation among the tissues of exposed carp were observed. Copper accumulation mainly occurred in the primary target tissues, the gills and liver, in both HR and LR carp. Furthermore, copper accumulation in the tissues between the LR and HR fish were significantly different. Copper accumulation in gills, liver and kidney of the LR fish was significantly higher than in the HR fish. The only time copper uptake in the HR fish was significantly higher than in the LR fish was in the posterior intestine which was probably food related rather than being caused by waterborne Cu exposure. No difference was found between the two rations in the anterior intestine. Muscle tissue showed no copper accumulation in either treatment. Although copper accumulation in the gills reached a steady state after first week of exposure, copper accumulation in the liver of both feeding treatments occurred in a time-dependent manner and did not reach a plateau in any treatment. Copper concentration dropped to control levels in all tissues except liver tissue 2 weeks after the exposure ended. This study indicated that copper accumulation was significantly affected by food ration in carp.

In the third study, the effects of starving and feeding on acute copper toxicity and copper uptake in Cu acclimated and non-acclimated carp were investigated. Common carp (*Cyprinus carpio*) were exposed to high Cu levels for 10 days using 4 different concentrations (3.5, 6, 10, and 15 μM) before and after Cu acclimation to a sublethal 1 μM concentration for 28 days. Fish tolerance against Cu exposure was evaluated, and gill, liver, and carcass Cu and sodium accumulations were measured in dead and surviving fish. Copper accumulation and toxicity were both found to be time related and dose depended. The sensitivity of the HR and LR carp against acute waterborne copper exposure was significantly

different. In general, fish fed on the high food ration accumulated less Cu in their tissues but suffered more mortality when exposed to increased Cu concentrations. The starved carp were twice as tolerant against acute copper exposure as the fed fish. Cu acclimation in the starved and fed carp greatly affected the LT50, and had only a moderate effect on the LC50. High Cu levels caused a net loss of sodium resulting in a severe ion regulatory disturbance. The rate of sodium loss increased linearly with increasing exposure concentration. More sensitive carp (HR) lost more Na^+ at a faster rate in both tests which implicates that mortality is related to quantity and rapidity of Na^+ loss rather than tissue copper levels. Na^+ loss and the way copper is handled intracellularly determine the toxic effect. In the current study, fish death occurred when sodium concentration fell below 63% of the normal level. Cu acclimation resulted in reduced sodium loss and increased the resistance and tolerance to Cu toxicity. There was a significant positive correlation between Cu and Na levels in the whole body of the LR and HR control fish, but this was disturbed following acute copper exposure for low ration fish and became negative after acute exposure for high ration fish. This study shows that starvation and acclimation can decrease the effect of copper as a toxicant, and the amount of copper uptake does not play a direct role in death of fish.

In the last study, physiological mechanisms of copper toxicity between the fed and starved fish were compared. Copper accumulation in the liver and gill tissues were related to changes in brancial Na^+/K^+ ATPase activity, and metallothionein (MT) induction in gill and liver tissues. Gill Na^+/ K^+ ATPase activity in both the exposed starved and fed fish decreased towards the lowest values after 3 days of exposure, and slowly recovered to pre-exposure values after 2 weeks of exposure. This

indicates that carp are able to acclimate to sublethal copper exposure which confirms our findings in the previous studies. No significant differences in branchial Na^+/ K^+ ATPase activity were found between the starved and fed fish which suggest that food ration did not affect Na^+/ K^+ ATPase activity. The observed differences in Na concentration between the starved and fed fish are likely related to the disturbance of Na^+/ NH_4^+ exchange pathway in the fed fish because of increased levels of plasma ammonia concentrations, ammonia excretion blockage, and physical gill damage. It is also suggested that the starved fish have more sodium channels than the fed ones. Different patterns of MT induction were observed in the starved and fed carp during copper exposure. The highest MT levels were found in liver tissues. MT levels in the livers of the starved fish were significantly higher than in the fed ones. Copper exposure significantly induced MT levels in the liver of the fed fish, but no changes occurred in the starved fish. In contrast, copper exposure induced MT levels in the gills of the starved fish in the first week of exposure, while only a slight increase in MT levels in the gills of the fed fish was observed. Our study suggests that MT induction is also tissue specific. A positive correlation between MT levels and copper accumulation in the liver of fish was observed in this study. The strongest MT induction due to waterborne copper exposure occurred in the liver tissues. While the hepatic MT appears to be more relevant for long-term monitoring of copper pollution in carp, the gill MT provided useful information on short-term copper toxicity. Starvation has significantly increased MT levels in the liver of fish. Elevated levels of MT in the control starved fish are likely related to stimulated levels of cortisol.

To sum up, this research demonstrated that fed fish are more vulnerable to copper exposure than starved ones. Food quantity was found as an

important factor affecting copper accumulation and toxicity in carp during waterborne copper exposure. The research also showed that both a reduced food intake and acclimation to sublethal Cu levels can decrease the effect of acute copper exposure. The fact that more resistant starved carp accumulated more copper in their target organs than less resistant fed ones during the chronic and acute waterborne copper exposure, and higher MT levels which were found in the liver of the starved fish suggest that MT is very efficient in the detoxification and accumulation of copper in fish, at least at sublethal levels. Therefore it might be advisable to briefly starve fish prior to an expected copper exposure; such as a treatment with copper containing herbicides. As fish are poikilotherm, and their feeding habits largely depend on surrounding environment temperature, in situ field studies are required to investigate the seasonal variations of copper accumulation and MT induction in fish as well as seasonal copper toxicity. Furthermore, as copper accumulation and toxicity are dose-related, further studies also need to find out whether or not this fact also affects MT induction levels. In addition, more detailed studies need to study the interaction between feeding and the Na^+/ NH_4^+ exchange pathway. This study also suggests that the feeding status of fish should be investigated before extrapolating MT levels to metal pollution.

7

Summary

Common carp (*Cyprinus carpio*) were fed two different food rations: 0.5% body weight (low ration, LR) and 5% body weight (high ration, HR) and were exposed to an environmentally realistic copper concentration (1µM Cu) for 28 days. Fish were monitored during the exposure and during a subsequent 2 week recovery period. Two short-term acute waterborne copper exposures were conducted before and after copper acclimation using four different copper concentrations (3.5, 6, 10, and 15 µM). Physiological responses of fish were studied, and the interaction between food quantity and waterborne copper exposure was investigated.

Remarkable interactions between food rations, food composition and Cu accumulation were found. Differences in growth, available energy stores (especially lipids), copper accumulation rate, sodium loss, handling of metabolic wastes, physiological defense mechanisms, and sensitivity to acute copper toxicity were observed between fed and starved fish. Cu exposure increased maintenance cost in fish. The adverse effects were most pronounced in the HR fish. It appeared that the fed carp were almost twice as sensitive to acute copper exposure. Fed fish also suffered from a more pronounced plasma ammonia accumulation, which could possibly lead to additional toxic effects. Copper accumulation and copper toxicity were found to be tissue specific, time related, and dose depended.

Copper uptake was influenced by food ration. In general, fish fed on the high food ration accumulated less Cu in their tissues but suffered more mortality when exposed to increased Cu concentration. Cu acclimation resulted in reduced sodium loss and increased the resistance and tolerance to Cu toxicity; it greatly affected the LT50 in the starved and fed carp, but had only a moderate effect on the LC50. No significant differences in branchial Na^+/ K^+ ATPase activity were found between the LR and HR fish which suggest that food ration did not affect Na^+/ K^+ ATPase activity. Different patterns of MT induction were observed in the LR and HR carp during copper exposure. Starvation significantly increased MT levels in the liver of fish, even before copper exposure. A positive correlation between MT levels and copper levels was observed in the liver of fish but no correlation was seen in the gills. Since more resistant starved carp accumulated more copper in their target organs than less resistant fed ones during the chronic and acute waterborne copper exposure, the fact that higher MT levels were found in the liver of the starved fish, and the additional induction of gill MT in starved fish, suggests that MT contributes significantly to the detoxification and accumulation of copper in fish. Na^+ loss and the way copper is handled intracellularly determine the toxic effect that Cu exerts. Furthermore, our study indicates that carp are able to acclimate to sublethal copper exposure and that effects are reversible, and fish recover when the exposure ends.

Samenvatting

Gewone karpers (*Cyprinus carpio*) werden onderworpen aan twee verschillende voedselregimes: 0.5% lichaamsgewicht (laag rantsoen, LR) en 5% lichaamsgewicht (hoog rantsoen, HR) en werden vervolgens via het water blootgesteld aan een relevante koperconcentratie (1µM Cu) gedurende 28 dagen. De vissen werden gevolgd tijdens deze blootstellingsperiode en een herstelperiode van 2 weken na de blootstelling. Twee korte acute blootstellingen (3.5, 6, 10, and 15 µM Cu) werden uitgevoerd voor en na de 28 dagen acclimatisatie aan koper. De fysiologische effecten van de blootstellingen werden gevolgd en de interacties tussen de hoeveelheid voedsel en de koperblootstelling onderzocht.

Er werden enkele verrassende interacties tussen de voedsel rantsoenen en de Cu accumulatie gevonden. Ook vonden we verschillen in groei, beschikbare energiereserves (in het bijzonder vetten), koper accumulatie, natrium verlies, stikstofmetabolisme, fysiologische verdedigings-mechanismen, en de gevoeligheid aan acute Cu blootstelling tussen de LR en HR vissen. Cu blootstelling verhoogde het basaal energiemetabolisme. Deze effecten waren het meest uitgesproken in de HR karpers. De HR vissen waren bijna twee keer zo gevoelig aan acute Cu blootstelling. Ze vertoonden ook een meer uitgesproken ammonia accumulatie in hun plasma, wat mogelijk bijdroeg aan de waargenomen toxische effecten. Koper accumulatie en koper toxiciteit waren weefsel specifiek en afhankelijk van tijd en dosis. De opname van koper werd beinvloed door het voedsel rantsoen. Algemeen gezien vertoonden de HR vissen een lagere koper accumulatie in hun weefsels, maar een hogere

mortaliteit bij acute blootstellingen. Acclimatisatie aan lage koper concentraties reduceerde het natrium verlies en verhoogde de weerstand tegen acute blootstelling. Het had een uitgesproken effect op de LT50 (blootstellingstijd die lethaal is voor 50% van de populatie) bij zowel LR als HR vissen, en slechts een klein effect op de LC50 (blootstellingsconcentratie die lethaal is voor 50% van de populatie). Er werden geen significante verschillen gevonden in de Na^+/K^+ ATPase aktiviteit in de kieuwen van LR en HR vissen, wat suggereert dat de beschikbare hoeveelheid voedsel niet bepalend is voor de Na^+/K^+ ATPase aktiviteit. Er werden wel verschillen gevonden in de inductie van metaalbindende eiwitten (metallothioneinen, MT) tussen de LR en HR karpers tijdens de koperblootstelling. Een laag voedsel rantsoen verhoogde de MT gehalten in de lever, zelfs zonder koperblootstelling. Er werd een positieve correlatie gevonden tussen MT gehalten en Cu gehalten in de lever van de karpers, maar er werd geen verband tussen beiden gevonden in de kieuwen. Aangezien de LR karpers meer koper accumuleerden, maar toch meer resistentie vertoonden tijdens de acute blootstelling, blijken de hoge lever MT gehalten en de additionele inductie van MT in de kieuwen van de LR vissen een belangrijke rol te spelen bij de accumulatie, maar ook de detoxificatie van koper in deze karpers. Het verlies aan Na^+ samen met de manier waarop Cu intracellulair gebonden wordt bepaalden de toxiciteit van Cu. Deze studie bevestigde eveneens dat karpers kunnen acclimatiseren aan lage koperconcentraties, en dat de effecten van koperblootstelling reversibel zijn, daar de vissen volledig herstelden als de blootstelling werd gestopt.

Publications

1-Hashemi S., Blust R., De Boeck G., 2007. The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*. Environmental Toxicology and Chemistry 26(7): 1507-1511.

2-Hashemi S., Blust R., De Boeck G., 2007. Combined effects of different food rations and sublethal copper exposure on growth and energy metabolism in common carp. Archives of Environmental Contamination and Toxicology. 10.1007/s00244- 007-9017-1.

3-Hashemi S., Blust R., De Boeck G., 2007. The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp. Accepted in Aquatic Toxicology.

4- Hashemi S., Blust R., De Boeck G., 2007. Differential metallothionein induction in fed and unfed carp during copper exposure. Submitted to Environmental Toxicology and Chemistry.

5-Hashemi S., Maes J., 2005. Herring and sprat migration predictive model in The Scheldt Estuary. Journal of Environmental, Agricultural and Food Chemistry 4(4).

6- Hashemi S., 2007. Variation in energy content of Herring and Sprat during estuarine residency. Submitted to Journal of Aquatic Ecology.

7- Hashemi S., 2000. Limnology and Environmental assessment of Sofi and Lighvan Rivers. Environmental organization of Iran and Tabriz industrial research center.

8- Hashemi S., Ahmadi M.R., 1997. Limnology and Environmental assessment of Mordogh River. Environmental organization of Iran and Tabriz industrial research center.

9- Hashemi S., Azari Takami G., 1997. Assessment of *Artemia urmiana* cysts and investigation of rising of its hatching rate. University of Tarbiat Modarres.

10- Hashemi S., 1997. Internal gonads in Salmonidae. Iranian Aquaculture bulletin.

11- Hashemi S., Keyvan A., 1995. Ichthyology of Aras River. Tehran University.

Selected presentations

1- Poster presentation entitled "Physiological responses of carp to waterborne copper exposure under different feeding rations" in 17th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC) Europe in Porto, Portugal 2007.

2- Oral presentation entitled "The effect of starving and feeding on copper toxicity and uptake in Cu acclimated and non-acclimated carp" in 13th Benelux congress in Leuven-Belgium 2006.

3- Oral presentation entitled "Variation in the energy content of Herring and Sprat during estuarine residency in the Scheldt Estuary" in International conference of Biology, Tehran-Iran 2006.

4- Poster presentation entitled "The effect of food rations on the tissue specific copper accumulation patterns of sublethal waterborne exposure in *Cyprinus carpio*" presented at the SEB meeting at Canterbury-England 2006.

5- Poster presentation entitled" Effects of sublethal copper exposure on Growth and Energy metabolism in common carp with different feeding

rations" presented at the 12th Benelux congress of Zoology, Wageningen, The Netherlands, 2005.