EARLY LIFE STAGES OF CARP (*CYPRINUS CARPIO* L.) DEPEND ON AMBIENT MAGNESIUM FOR THEIR DEVELOPMENT

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Summary

Carp eggs, fertilized *in vitro*, were allowed to develop in fresh water with magnesium concentrations varying from 0.001 to 0.100 mmol l^{-1}. Magnesium concentrations below 0.010 mmol l^{-1} seriously impeded carp embryonic development: the incidence of deformed larvae and mortality increased steeply to 100 % at water magnesium concentrations of 0.001 mmol l^{-1}. Thus, early life stages of carp require ambient magnesium for survival and successful development. The magnesium and calcium concentrations of the developing eggs were dependent on the ambient magnesium concentration. The uptake of magnesium by eggs decreased and the uptake of calcium increased with decreasing ambient magnesium concentrations. However, the uptake of the sum of these divalent ions seemed to be independent of ambient magnesium concentration. This indicates a competition between magnesium and calcium for (passive) uptake into developing eggs.

Introduction

Magnesium is the most abundant intracellular divalent cation and in the eukaryotic cell it plays a central role in many cellular processes. For instance, magnesium has been implicated in the activation of a large number of enzymes, in hormonal signalling features, in protein synthesis and in cell division (Alvarez-Leefmans *et al.* 1987).

Our recent studies on adult freshwater fish point to the presence of magnesium regulatory mechanisms (van der Velden *et al.* 1989). Magnesium is an essential nutrient for fish and, although adult fish take up magnesium from the water *via* the gills (van der Velden *et al.* 1991*b*), intestinal magnesium uptake is of primary importance for growth and homeostasis (van der Velden *et al.* 1990).

In this study we focus on the effects of low magnesium levels in the ambient

water on the development of carp eggs fertilized *in vitro*. Fish eggs contain significant amounts of magnesium and it is thought that some of it is associated with the yolk (Hayes *et al.* 1946). Therefore, the yolk may serve as a magnesium source for the developing embryo. However, the number of cells increases rapidly before hatching and it does not seem too imprudent to postulate that developing fish eggs must extract magnesium from the ambient water to supply these cells with this essential element.

We used carp eggs to evaluate the dependence on ambient magnesium of the early life stages of fish that are not yet feeding.

Materials and methods

In vitro *fertilization*

Adult carp, *Cyprinus carpio* L., weighing 1–2 kg, were held at 23 °C in Nijmegen tap water containing around $0.2\,mmol\,l^{-1}$ magnesium. Carp gametes were obtained through hormonal induction of ovulation and spermiation by intramuscular injection of carp pituitary powder (Chaudhuri, 1976) suspended in 0.9 % NaCl solution. Gametes of both sexes were stripped and mixed in glass Petri dishes. Fertilization was induced by the addition of water (23 °C). After 5 min of incubation, the eggs were rinsed and the dishes, each containing approximately 300 eggs, were placed in an experimental unit. Every experimental unit had five incubation chambers containing 4 l of medium, in which the Petri dishes with eggs were placed, and a reservoir containing another 20 l of medium. The experimental medium was recirculated by pumps through this experimental unit. The water in the basin was kept at pH 7.8±0.1 by controlled addition of $0.01\,mol\,l^{-1}$ H_2SO_4 using pH-stat equipment. The water was thermostatted at 23 °C and was irradiated with ultraviolet light before it re-entered the basin to inhibit the development of fungi.

Experimental media

The experimental media contained $0.06\,mmol\,l^{-1}$ KCl, $0.8\,mmol\,l^{-1}$ $CaCl_2$, $3.5\,mmol\,l^{-1}$ NaCl and $0.33\,mmol^{-1}$ $NaHCO_3$ in demineralized water. To manipulate the magnesium concentration of the media, graded amounts of $MgSO_4$ were added. Inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis (plasma 200, Instrumentation Laboratory) of the media thus obtained yielded values for magnesium concentration of 0.001, 0.004, 0.007, 0.010, 0.020 or $0.100\,mmol\,l^{-1}$. All media were set at $0.1\,mmol\,l^{-1}$ sulphate using Na_2SO_4. The water magnesium concentrations varied within 10 % of the nominal concentrations during the incubation period, as determined afterwards (see below).

Experimental procedure

All experimental media were thermostatted at 23 °C. The appearance of embryonic stages with time under control conditions is thus essentially the same as recently reported for the same species by Oyen *et al.* (1991). After the fertilizatio■

procedure, unfertilized eggs were removed and the number of fertilized eggs (around 1500) per experimental medium was assessed. Every 12 h until the end of the experiment (up to 170 h after fertilization) dead and mouldy eggs and, later on, dead larvae were counted and removed. Fifty hours after fertilization, the rate of tail movement (beats min^{-1}) was determined for 20 embryos in each experimental group. As soon as hatching was observed, the number of deformed larvae was assessed every 6 h until all larvae had hatched. The identification of deformed larvae was based on gross macroscopical appearance. Larvae were designated as deformed if they were slightly crescent- to almost corkscrew-shaped. The percentage deformation was expressed as the ratio of the number of deformed larvae to the total number of larvae hatched. About 10 h after hatching the number of pigmented cells on one side of the lateral abdominal skin of 20 larvae was determined using a binocular microscope. Furthermore, the frequency of the heart beat, the occurrence of embolism and the presence of tissue necrosis were noted. Throughout the experiments 5 ml water samples were taken from the experimental units every 24 h to assess the magnesium concentration by ICP-AES analysis.

In a parallel experiment, the mineral content of the eggs under the experimental conditions was determined. Petri dishes with about 30 eggs were placed in each experimental medium. After 6, 24, 48, 63 and 76 h a dish was removed and 25 eggs were weighed, lyophilized and weighed again to assess water content and dry mass of the samples. 300 μl of concentrated HNO_3 was then added to the dried eggs. After 24 h, 1 ml of demineralized water was added and mixed and 1 ml of this solution was added to 3 ml of demineralized water. The total magnesium, calcium and sodium concentrations were measured by ICP-AES.

Statistics

Data in the text are presented as mean values ± the standard deviation, unless otherwise stated. Data were analyzed statistically using Student's *t*-test or the Mann–Whitney *U*-test, where appropriate. Statistical significance was accepted at the 5 % level.

Results

The magnesium and calcium concentrations of the eggs after 6 h of exposure to the experimental media had not changed significantly and were around 30 and 17 mmol kg^{-1} dry mass, respectively. 76 h after fertilization, eggs exposed to 0.1 mmol l^{-1} magnesium had increased their magnesium concentration to 58±3 mmol kg^{-1} and their calcium concentration to 34±5 mmol kg^{-1} ($N=3$). A concentration-dependent inhibition of this magnesium uptake was observed with decreasing water magnesium levels (Fig. 1). At 0.100 mmol l^{-1} magnesium in the water, the egg magnesium concentration almost doubled from around 30 to 58 mmol kg^{-1} between 6 and 76 h after fertilization. At 0.010 mmol l^{-1} magnesium in the water, this increase was around 70 % and at 0.004 mmol l^{-1} only around 25 %. In contrast, calcium uptake increased with decreasing ambient magnesium
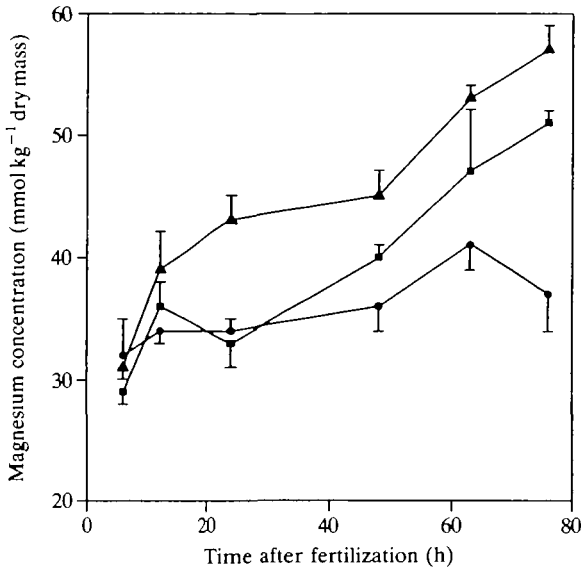
Fig. 1. The mean magnesium concentration (+ standard error of the mean) of eggs (in mmol kg^{-1} dry mass) *versus* time after fertilization of the eggs (in h) during development in different ambient magnesium concentrations (●, 0.004; ■, 0.010; ▲, 0.100 mmol l^{-1}). The number of experiments is 3; the number of eggs measured per determination is 25.

levels (Fig. 2). The calcium concentration of the eggs doubled at 0.1 mmol l^{-1} and increased around fourfold at 0.004 mmol l^{-1} magnesium in the water. The sodium concentration in the eggs after 6 h of exposure was 122 ± 10 mmol kg^{-1} ($N=18$) and had increased after 76 h of exposure to 189 ± 37 mmol kg^{-1} ($N=18$). The sodium concentration of the eggs was not affected by the experimental conditions. The water content of the eggs was not affected by any of the experimental media and was 83 ± 4 % ($N=43$). The dry mass (0.66 ± 0.1 mg per egg; $N=6$) of the eggs did not change.

The mortality of embryos and larvae and the incidence of deformed larvae under the experimental conditions are shown in Table 1. At a magnesium concentration of 0.1 mmol l^{-1} in the water, no mortality and essentially no deformed larvae (1 ± 1 %) were observed up to 170 h after fertilization. Both the mortality and relative number of deformed larvae increased with a decrease in ambient magnesium concentration, leading to 100 % mortality and 96 ± 3 % deformed larvae at a magnesium concentration of 0.001 mmol l^{-1}.

The frequency of tail movements of embryos just before hatching and the pigmentation of larvae are shown in Table 2. Both these variables decreased when the egg and larval stages were exposed to water with a low magnesium concentration. Furthermore, we observed that larvae exposed to water with a very low magnesium concentration (0.001 mmol l^{-1}) had a very low heart rate, showed
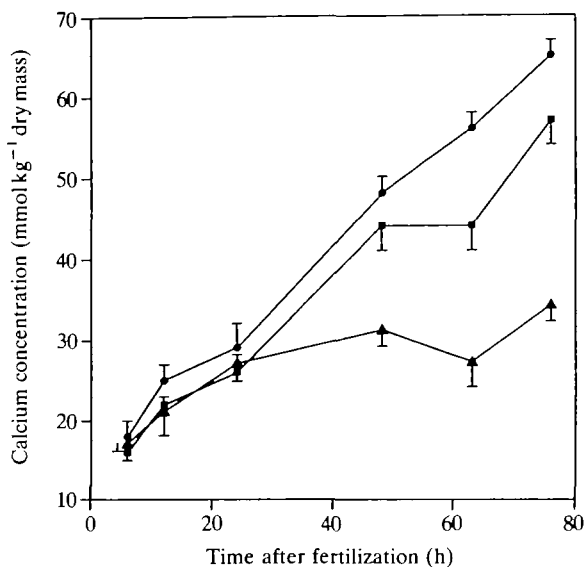
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Table 1. *Mortality of carp embryos and larvae and the percentage of deformed larvae after exposure to different ambient magnesium levels from fertilization until 170 h after fertilization*

[Mg^{2+}] (mmol l^{-1})	Mortality (%)	Deformation (%)
0.001	100±0	96±3
0.004	74±45	62±20
0.007	41±52	8±8
0.010	4±7	19±27
0.020	0±0	2±1
0.100	0±0	1±1

The values represent the mean of three separate experiments ± standard deviation (for details see Materials and methods).

embolism and, upon light microscopical observation, necrosis of brain tissue and muscle (data not shown).

Discussion

Our results show that for the development of carp low levels of magnesium in

Table 2. *The frequency of tail movements just before hatching and the pigmentation (the number of observed pigmented cells on one side of the lateral abdominal skin) 10 h after hatching of carp exposed to different ambient magnesium levels*

$[Mg^{2+}]$ $(mmol\,l^{-1})$	Tail movements $(beats\,min^{-1})$	Pigmentation (relative density)
0.001	2±2	0±0
0.004	3±3	1±2
0.007	3±4	5±4
0.010	8±7	9±7
0.020	10±9	12±8
0.100	24±3	26±7

The values represent the mean of 60 observations ± standard deviation.

the ambient water (critical lower level around $0.01\,mmol\,l^{-1}$) are essential. At magnesium levels below $0.01\,mmol\,l^{-1}$, deformation, tissue necrosis and death were observed in early life stages. As the eggs did not loose magnesium, the magnesium present in the yolk and perivitelline fluid does not suffice for successful development of the egg. Our data show that in developing eggs the magnesium concentration increased. Consequently, there is uptake of magnesium from the water. Apparently, at water magnesium levels below $0.01\,mmol\,l^{-1}$ the accumulation of magnesium is hampered and development is retarded (Fig. 1; Tables 1, 2). Thus, early life stages of carp require magnesium from the water for survival. The elemental concentrations we found for carp eggs (30, 17 and $122\,mmol\,kg^{-1}$ for magnesium, calcium and sodium, respectively) are close to values found by Chetty and Agarwal (1984) for eggs of the same species. They reported magnesium, calcium and sodium concentrations in the eggs of about 35, 25 and $109\,mmol\,kg^{-1}$ dry mass, respectively.

The uptake of ions from the water may be driven by the perivitelline potential (Peterson and Martin-Robichaud, 1986), by ion exchange (Shephard, 1987), directly by a transporting enzyme or by any combination of these. Assuming that all magnesium and sodium in the water are in their ionic form and that the equilibrium potential V between perivitelline fluid and ambient water for eggs of carp is comparable to that for eggs of freshwater Atlantic salmon (*Salmo salar* L.) under the conditions given (Peterson and Martin-Robichaud, 1986; Eddy *et al.* 1990), V will be around $-40\,mV$. Calculating the equilibrium magnesium concentration in the perivitelline fluid (C_i) at an ambient magnesium concentration (C_o) of $0.1\,mmol\,l^{-1}$ using the Nernst equation, $V = (2.3RT/zF)\log(C_o/C_i)$ or, at equilibrium, $V = -29\log(C_o/C_i)$, we arrive at a value of $2.3\,mmol\,l^{-1}$. The magnesium concentration measured in whole eggs was $5.1\,mmol\,l^{-1}$. However, by analogy with cytosolic conditions, the free magnesium concentration in the egg is predicted to be much lower than the total magnesium concentration. We conclude that, at an ambient magnesium concentration of $0.1\,mmol\,l^{-1}$, magnesium transport driven by the electrochemical potential difference is possible. At an

ambient magnesium concentration of $0.01 \, \text{mmol} \, l^{-1}$, we calculate an equilibrium concentration of $0.23 \, \text{mmol} \, l^{-1}$. Under these conditions magnesium transport, driven by the electrochemical potential difference, is unlikely. In the case of an ambient magnesium concentration of $0.004 \, \text{mmol} \, l^{-1}$, the equilibrium concentration is $0.09 \, \text{mmol} \, l^{-1}$ and potential-driven magnesium transport will be negligible. The above reasoning may explain the data presented in Fig. 1, where we show that an ambient magnesium concentration of $0.004 \, \text{mmol} \, l^{-1}$ inhibits magnesium uptake from the water almost completely.

A remarkable phenomenon observed was the stimulation of calcium uptake in eggs by decreasing ambient magnesium levels (Fig. 2). The decrease in magnesium uptake was almost compensated by the increase in calcium uptake. In other words, the increase in the sum of magnesium and calcium contents appeared to be independent of the magnesium content of the ambient water. This indicates a competition between calcium and magnesium for (passive) uptake into developing eggs. In higher vertebrates hypomagnesaemia (as a result of magnesium deficiency) may result in hypercalcaemia and in a decrease of tissue magnesium and an increase of tissue calcium concentrations (George and Heaton, 1975; Geven *et al.* 1988). Similarly, in adult tilapia (*Oreochromis mossambicus* Peters) a high magnesium concentration of the ambient water resulted in a hypermagnesaemia and hypocalcaemia (Wendelaar Bonga *et al.* 1983).

Do Mg^{2+} and Ca^{2+} compete for a carrier mechanism or does a high ambient magnesium concentration reduce the potential created by fixed anions in the organic matrix of the chorionic fluid and hence reduce calcium uptake? The observation that the uptake of the combined divalent ions Ca^{2+} and Mg^{2+} is not affected by ambient magnesium levels argues against the latter possibility, assuming that both ions interact with these fixed anions. We favour, therefore, the conclusion that Ca^{2+} and Mg^{2+} follow the same pathway for uptake into the developing egg. The rate of uptake of Mg^{2+} and Ca^{2+} appears to depend on the total divalent ion concentration in the water and is dictated by the electrochemical potential difference between the chorionic fluid and the water.

In addition to mortality and deformation, a decreased rate of tail movement and decreased pigmentation indicated the dependency on water magnesium concentration of early life stages of carp. We have also observed that the ambient magnesium level influences the heart rate of the embryo: just before hatching we observed heart rates of 29.6 ± 1.5 and 36.6 ± 2.1 in embryos developing in water containing 0.004 and $0.100 \, \text{mmol} \, l^{-1}$ magnesium, respectively ($N = 5$, $P = 0.004$). It has been shown recently in our laboratory, by evaluating the same variables, that a low water pH also hampers the development of early life stages of carp (Oyen *et al.* 1991). A decrease in heart rate and pigmentation has also been reported for early life stages of freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum)] exposed to a low pH or to a low ambient calcium concentration (Nelson, 1982). Clearly, the ionic composition of the ambient water is of importance for the development of fertilized eggs. This holds true for the magnesium concentration as well as for the calcium and proton concentrations of the water.

We conclude that ambient magnesium is required for successful embryonic and larval development of carp. The essential role of magnesium in many cellular physiological events may account for this magnesium dependency. Given the competition between Ca^{2+} and Mg^{2+}, the inhibitory effects of high calcium concentrations on embryonic development should be considered in future research.

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