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Effects of water pH on chromium toxicity to early life stages of the common carp (*Cyprinus carpio*)

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Abstract

Immediately after fertilization carp eggs were exposed to Cr(VI) concentrations of 3.9 and 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ at water pH 7.8 and 6.3. At regular intervals mortality, the incidence of spinal cord deformation, heart rate, tail movements, hatching success, whole body concentration of K, Na, Mg, Ca, and Cr, and ultrastructure of skin and gills were assessed. No effects of Cr on any of the examined parameters were observed at pH 7.8, while 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 decreased total Na concentration of larvae, and increased mortality of the larvae. Skin of larvae (144-h) exposed to 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ at pH 6.3 showed increased numbers of mucus-secreting cells. The gill epithelium had a wrinkled appearance, and dark circle-shaped spaces, probably of mucus cells, were seen. After 240 h, the larvae were almost completely covered with mucus. In controls at pH 6.3 no significant changes of any of the above parameters were observed when compared to controls at pH 7.8.

Keywords: Acid stress; Chromium; Embryonic development; *Cyprinus carpio*

1. Introduction

Chromium (Cr) is a common contaminant of surface waters (Pickering, 1980; Slooff et al., 1989). Concentrations of Cr in the micromolar range have frequently been reported in rivers and lakes (Beg et al., 1990; Alves et al., 1993; Zhang et al., 1994). This metal may either be present in the trivalent form (Cr(III)) or in the hexavalent form (Cr(VI)). The latter is considered the most toxic form to aquatic organisms (Van der Putte et al., 1981a,b). Water pH is one of the most important characteristics determining Cr toxicity to fish (Van der Putte et al., 1981a,b). The

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dominant species of Cr(III) are $\text{Cr}(\text{OH})^{2+}$ at pH 4, $\text{Cr}(\text{OH})_2^+$ at pH 6 and $\text{Cr}(\text{OH})_3$ at pH 8 (Stern, 1982). Cr(III) is largely associated with particulate matter, subject to sedimentation and filtration, making it less bioavailable. The dominant species of Cr(VI) are HCrO_4^- at pH < 6.5 and CrO_4^{2-} at pH > 6.5 (Jop et al., 1987). As Cr(VI) species are negatively charged, they do not complex with anionic particulate matter and are more bioavailable. Van der Putte et al. (1981a,b) have shown that Cr(VI) was more toxic to rainbow trout at lower pH, because the gills concentrated significantly more Cr at pH 6.5 than at pH 7.8, irrespective of exposure time and concentration.

Little information is available on the pH effects of Cr(VI) toxicity to the early life stages of fish. Most of the literature concerns results from eyed-embryos or swim-up larvae (Van der Putte et al., 1981a,b). The main objective of this study is to investigate the effects of Cr(VI) at pH 7.8 and pH 6.3 on the embryonic and larval development of carp (*Cyprinus carpio*), immediately after fertilization of the eggs. In addition to mortality, malformation, total body K, Na, Mg, Ca and Cr contents, and hatching success, attention was paid to pathological changes of skin and gill structure by using scanning electron microscopy.

2. Materials and methods

2.1. *In vitro* fertilization and incubation of eggs

Gametes were obtained after artificial fertilization of carp eggs as described by Oyen et al. (1991). Eggs from one female were divided over 6 petridishes (300–400 eggs per dish). The eggs of each petridish were fertilized by sperm from different males resulting in 6 genetically different batches of eggs ($n = 6$). This procedure was carried out for controls as well as for each Cr-exposed group. Immediately after fertilization, the petridishes with the fertilized eggs stuck to the bottom were placed in 4-l aquaria (for details see Oyen et al., 1991; Oyen, 1993). To avoid low concentrations of Cu and Al normally present in Nijmegen tapwater, we used demineralized water and added the following salts ($\text{mmol}\cdot\text{l}^{-1}$): 0.06 KCl; 0.40 NaHCO_3 ; 0.20 MgSO_4 ; 0.80 CaCl_2 . In each aquarium one petridish was used to examine mineral content, heart rate and tail movements of the developing embryos. Larvae were fed with artemia starting 72 h after hatching.

Eggs were exposed to UV-sterilized water (23°C; 8.7 ppm DOC; 12 h light/dark cycle) of pH 7.8 and pH 6.3 (controls) and to Cr (as $\text{Na}_2\text{CrO}_4\cdot 4\text{H}_2\text{O}$) in nominal concentrations of 3.9 and 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ at either pH (experimentals). Water pH was adjusted to 7.8 and 6.3 via gradual addition of solutions of either 0.01 M sodium hydrogencarbonate or 0.01 M sulphuric acid, using pH-stat equipment (variation of the water <0.1 pH units). Constant Cr concentrations were maintained via a flow-through system with partial replacement of the water, resulting in a complete turnover of the system's water content in 24 h. Desired total Cr concentrations were within 5% of the calculated values as verified every 12 h by Inductive Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo Electron, USA).

2.2. Mortality and deformation of the spinal cord

Dead eggs and larvae were counted at 6, 12, 24, 48, 58, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h after fertilization, and immediately removed to prevent fungal growth. Eggs were considered dead when parts of the content turned opaque and white, or when heart beat had stopped. The percentage of deformation of the spinal cord of the larvae (including dead ones) was determined by microscopic examination. Deformed larvae did not swim but lay on the bottom of the aquaria.

2.3. Heart rate and tail movements

Fifty hours after fertilization, heart rate ($\text{beats} \cdot \text{min}^{-1}$) and rate of tail movements ($\text{beats} \cdot \text{min}^{-1}$) were determined for 20 embryos per group.

2.4. Mineral content and chromium accumulation

To determine their mineral content, 100 eggs or larvae from each group were collected at 6, 12, 24, 48, 58, 72, 96, 120, 144, 168, 192, 216, 240 and 264 h after fertilization. To discriminate between the amount of Cr adsorbed to the chorion and the amount of Cr absorbed by the embryo, an extra 10 embryos were stripped from their chorion just before hatching (50 h after fertilization). The eggs, chorions, embryos and larvae were freeze-dried to constant weight and total K, Na, Mg, Ca and Cr contents were calculated. Tissues were dissolved during 24 h at 70°C in 65% HNO_3 . The K and Na concentrations were analyzed in a flame photometric Auto Analyzer (Technicon); Mg, Ca, and Cr(VI) were analyzed with Inductive Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo Electron, USA). All concentrations are expressed as $\mu\text{mol} \cdot \text{g}^{-1}$ dry weight.

2.5. Hatching success

Hatching success was defined as the percentage of larvae hatched every 3 h, from 51 h to 72 h after fertilization. Hatching was defined as rupture of the chorion. Fully as well as partially hatched larvae were counted.

2.6. Scanning electron microscopy (SEM) of skin and gills

Five larvae were sampled at 144 and 240 h after fertilization in each group (controls at pH 7.8 and pH 6.3 and respectively 3.9 and 9.6 $\mu\text{mol} \cdot \text{l}^{-1}$ Cr at pH 7.8 and pH 6.3). For SEM, tissues were dehydrated in increasing concentrations of ethanol, transferred to liquid CO_2 , and coated with gold in a Balzers coating unit (CPD 020, Balzers, Switzerland). Skin and gills were examined in a Jeol-JSM T 300 scanning electron microscope.

2.7. Statistical analysis

Data are expressed as means \pm s.e. ($n = 6$). A one-way analysis of variance was used to assess differences between groups. Significance of differences was tested using unpaired Student *t*-test. Significance was accepted for $P < 0.05$. Significance is expressed at the level of (a) $P < 0.05$; (b) $P < 0.02$; (c) $P < 0.01$; (d) $P < 0.001$ compared to control values.

3. Results

3.1. Mortality and deformation of the spinal cord

No difference in mortality was found between eggs and larvae exposed to Cr at pH 7.8 and to pH 7.8 alone while exposure to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 resulted in increased mortality of larvae compared to controls (Table 1). Deformation percentage was similar for Cr-exposed groups at pH 7.8 and 6.3 when compared to controls (Table 1).

Table 1

Mortality of carp eggs and larvae and percentage of deformed larvae during exposure to Cr(VI) at pH 7.8 and 6.3; mortality is expressed as percentage of total number of eggs and larvae: means \pm s.e. ($n = 6$) are given (n.d., non detectable; less than $1 \cdot 10^{-6} \mu\text{mol}\cdot\text{l}^{-1}$)

[Cr] $\mu\text{mol}\cdot\text{l}^{-1}$	Mortality of eggs (%)		Mortality of larvae (%)		Deformation of larvae (%)	
	pH 7.8	pH 6.3	pH 7.8	pH 6.3	pH 7.8	pH 6.3
n.d.	8.1 \pm 1.5	9.1 \pm 1.1	7.8 \pm 1.6	7.0 \pm 1.0	7.3 \pm 1.0	9.8 \pm 1.9
3.85	8.3 \pm 1.2	8.9 \pm 1.2	8.1 \pm 2.5	9.0 \pm 2.9	7.9 \pm 1.5	9.4 \pm 2.5
9.62	8.7 \pm 1.2	9.3 \pm 0.6	9.7 \pm 2.4	33.3 \pm 3.0 ^d	9.8 \pm 1.6	8.2 \pm 2.5

^dSignificantly different when compared to all other values on larval mortality ($P < 0.001$).

Table 2

The heart rate (beats \cdot min⁻¹) and the frequency of tail movements (beats \cdot min⁻¹) of carp embryos 50 h after fertilization exposed to different ambient Cr(VI) levels at pH 7.8 and 6.3. Data are expressed as means \pm s.e. ($n = 6$) (n.d., non detectable; less than $1 \cdot 10^{-6} \mu\text{mol}\cdot\text{l}^{-1}$)

[Cr] $\mu\text{mol}\cdot\text{l}^{-1}$	Heart rate		Tail movements	
	pH 7.8	pH 6.3	pH 7.8	pH 6.3
n.d.	116 \pm 8	103 \pm 6	11 \pm 3	14 \pm 5
3.85	95 \pm 13	101 \pm 5	13 \pm 3	12 \pm 3
9.62	99 \pm 9	99 \pm 6	14 \pm 2	11 \pm 4

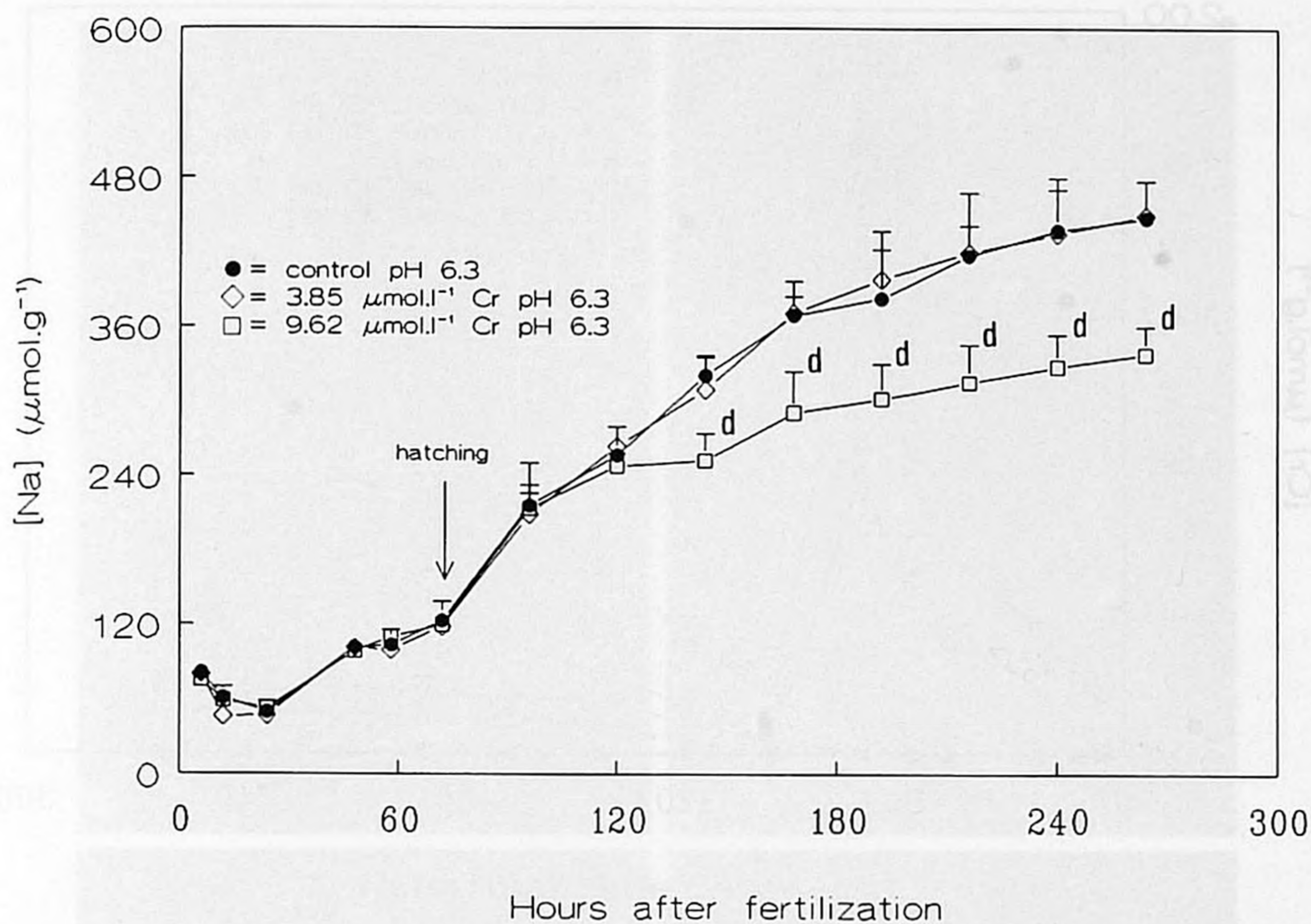


Fig. 1. Whole body Na concentrations ($\mu\text{mol}\cdot\text{g}^{-1}$ dry weight) of carp eggs and larvae exposed to Cr(VI) at pH 6.3. Data are expressed as means \pm s.e. ($n = 6$); d, significantly different from control and $3.85 \mu\text{mol}\cdot\text{l}^{-1}$ Cr values ($P < 0.001$).

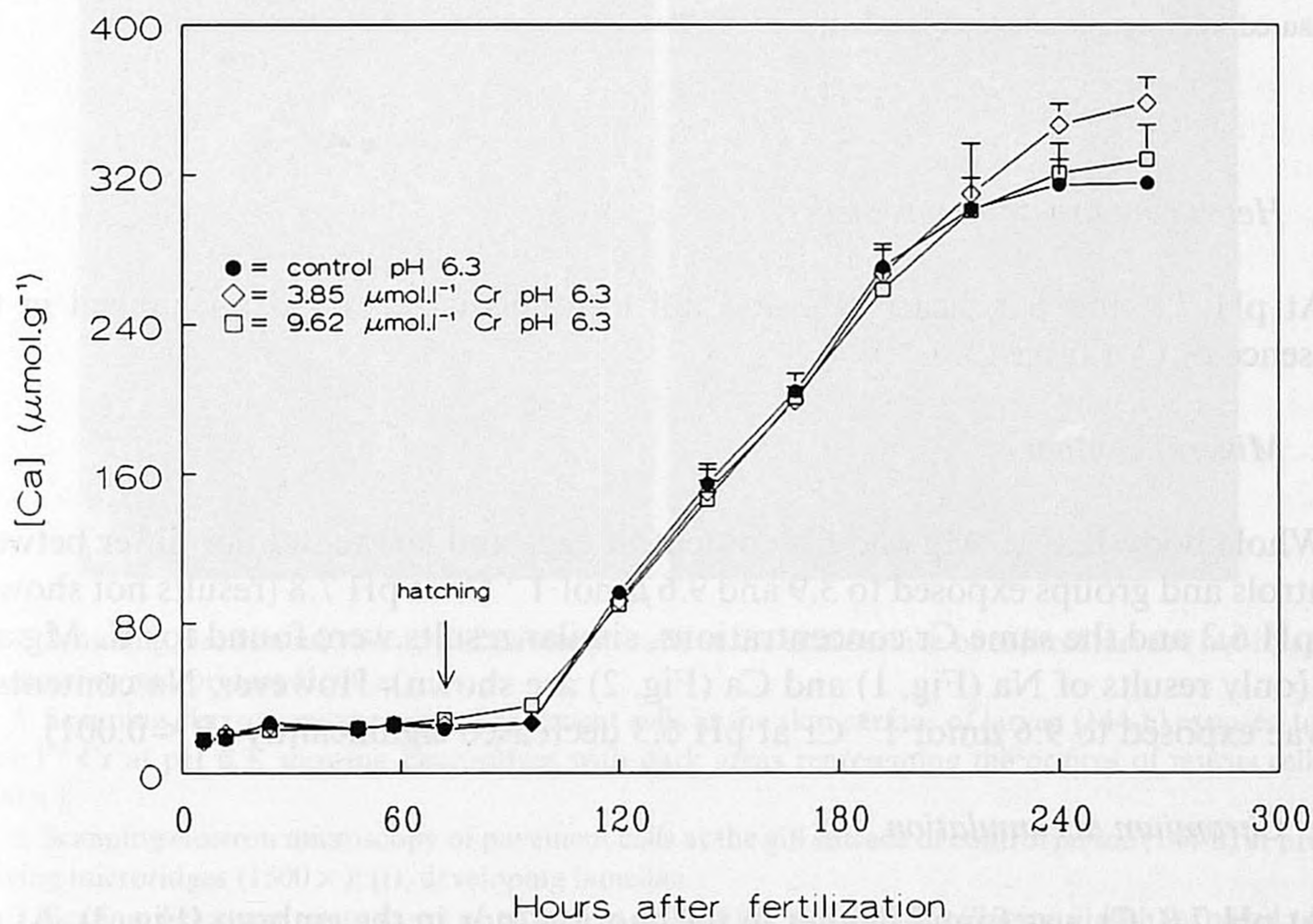


Fig. 2. Whole body Ca concentrations ($\mu\text{mol}\cdot\text{g}^{-1}$ dry weight) of carp eggs and larvae exposed to Cr(VI) at pH 6.3. Data are expressed as means \pm s.e. ($n = 6$).

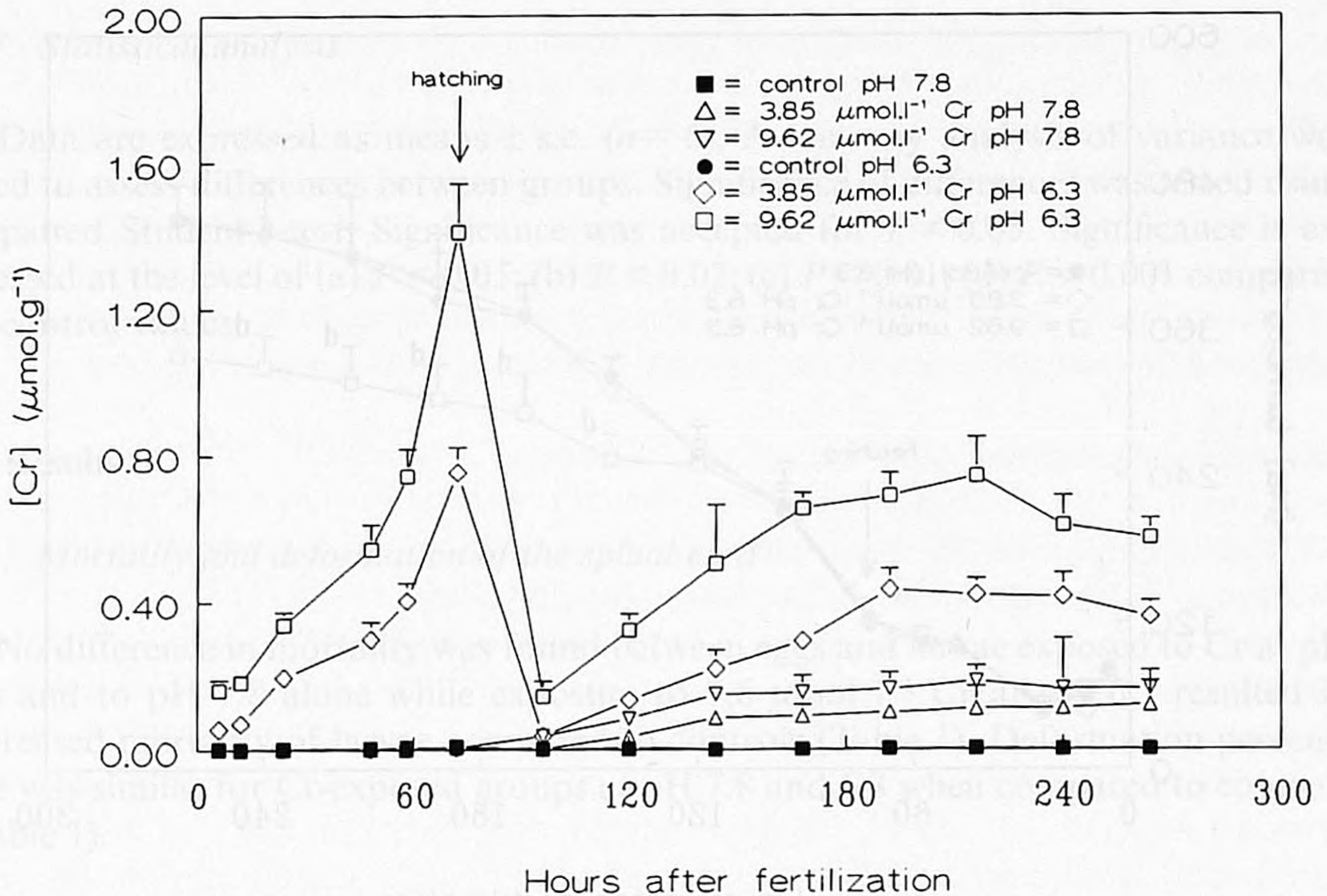


Fig. 3. Concentration of Cr in carp eggs and larvae exposed to Cr at indicated concentrations at pH 7.8 and pH 6.3. Hatching started at 60 h. Data are expressed as means \pm s.e. ($n = 6$); in the eggs exposed to pH 6.3, the Cr concentration was significantly increased after 24 h ($P < 0.001$); the Cr concentrations of eggs exposed at pH 7.8 were indistinguishable from those of the controls; after hatching all Cr concentrations measured were significantly increased after 144 h ($P < 0.02$ at pH 7.8 and $P < 0.001$ at pH 6.3).

3.2. Heart rate and tail movements

At pH 7.8 and 6.3, heart rate and tail movements remained unchanged in the presence of Cr (Table 2).

3.3. Mineral content

Whole body K, Na, Mg and Ca content of eggs and larvae did not differ between controls and groups exposed to 3.9 and 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 7.8 (results not shown). At pH 6.3 and the same Cr concentrations, similar results were found for K, Mg and Ca (only results of Na (Fig. 1) and Ca (Fig. 2) are shown). However, Na contents in larvae exposed to 9.6 $\mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 decreased significantly ($P < 0.001$).

3.4. Chromium accumulation

At pH 7.8, Cr was found neither in the chorion, nor in the embryo (Fig. 3). At pH 6.3, Cr entered the chorion in a concentration-related manner (Fig. 3). After dissection no Cr could be detected in chorions or embryos. However, immediately after

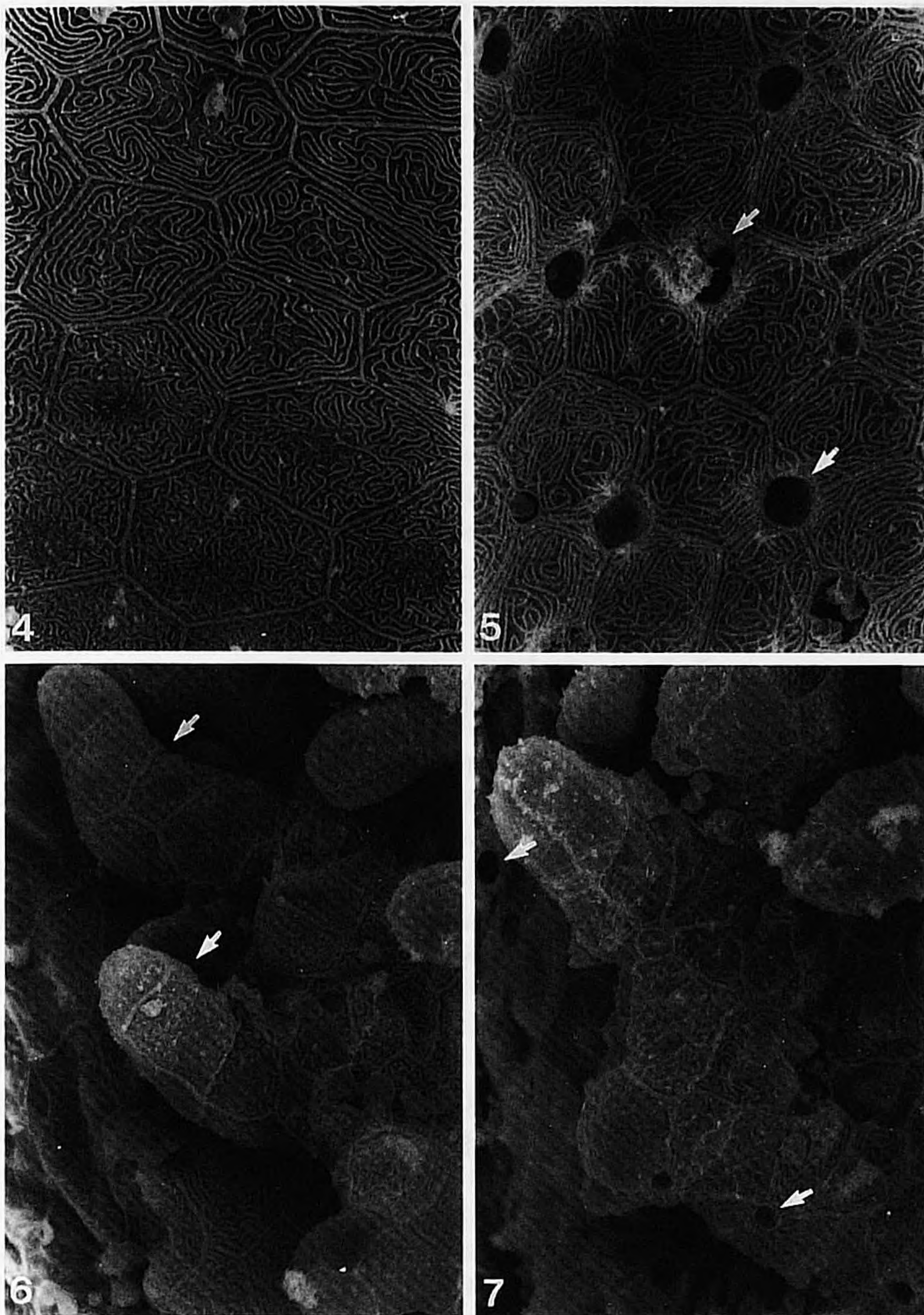


Fig. 4. Scanning electron microscopy of pavement cells at the skin surface of control larvae (144-h) at pH 6.3, showing microridges (1500 ×).

Fig. 5. Scanning electron microscopy of pavement cells at the skin surface of larvae (144-h) exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3, showing microridges with dark areas representing the orifices of mucus cells (↓) (1500 ×).

Fig. 6. Scanning electron microscopy of pavement cells at the gill surface of control larvae (144-h) at pH 6.3 showing microridges (1500 ×); (↓), developing lamellae.

Fig. 7. Scanning electron microscopy of pavement cells at the gill surface of larvae (144-h) exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3, showing microridges and wrinkled gill epithelium with dark areas representing the orifices of mucus cells (↓) (1500 ×).

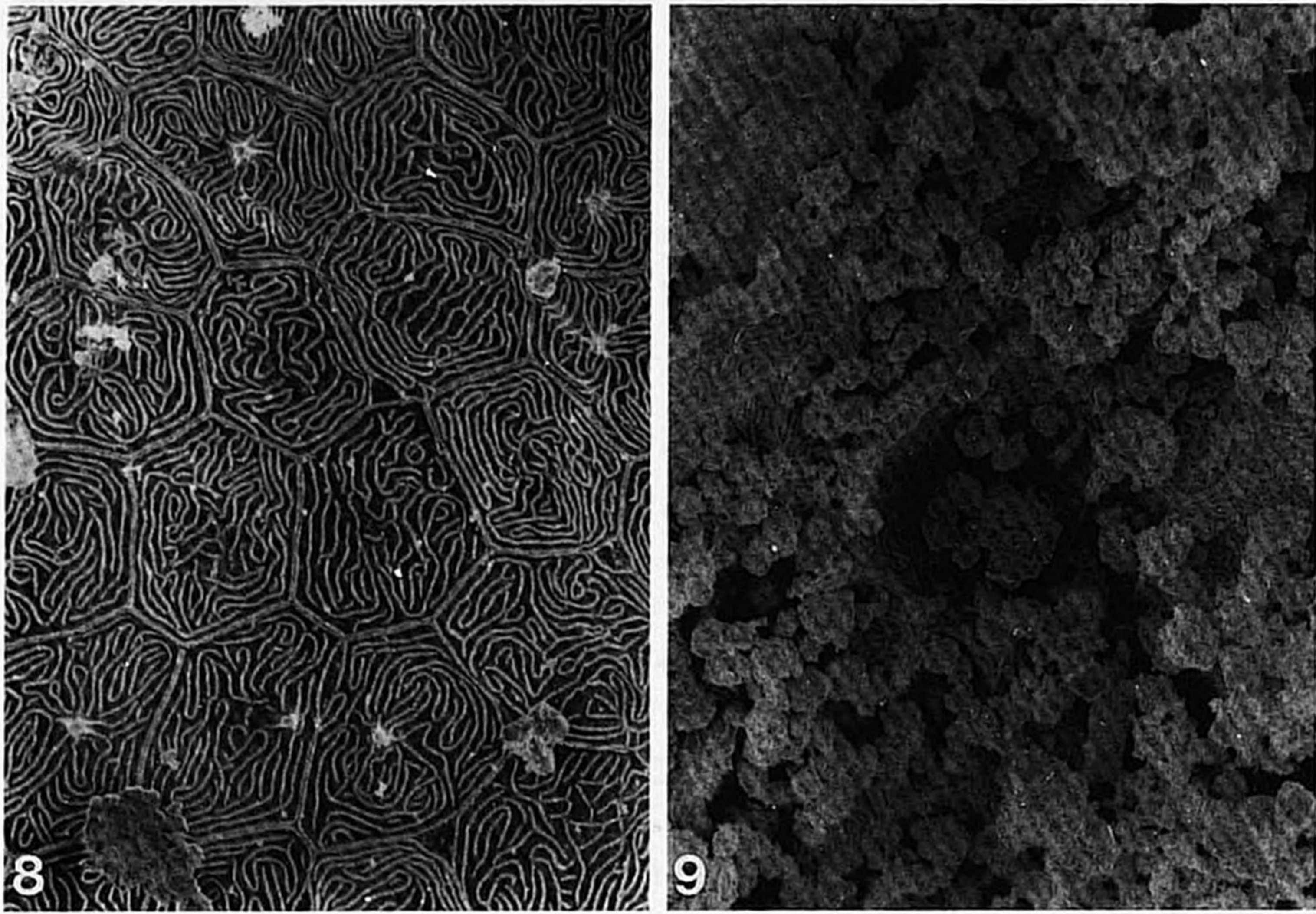


Fig. 8. Scanning electron microscopy of pavement cells at the skin surface of control larvae (240-h) at pH 6.3 showing microridges (1500 ×).

Fig. 9. Scanning electron microscopy of pavement cells at the skin surface of larvae (240-h) exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3, showing microridges with excessive mucus secretion (1500 ×).

hatching, larvae at both pH 7.8 and 6.3 started to accumulate Cr in a concentration-related manner. The difference with the controls was highly significant at pH 6.3 only.

3.5. Hatching success

Hatching success of the eggs exposed to Cr at pH 7.8 and 6.3 (respectively 98.6% and 99.1%) was not significantly different from that of control eggs (98.8%).

3.6. Scanning electron microscopy (SEM) of skin and gills

Scanning electron microscopy pictures of skin and gill from control and Cr-exposed larvae are shown in Figs. 4–9. Skin of 144-h controls (Fig. 4) and Cr-exposed larvae ($9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 7.8; not shown) showed pavement cells with microridges without mucus cells, whereas skin of larvae exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 (Fig. 5) exhibited several actively secreting mucus cells. Similar results were obtained for 240-h-old control larvae (Fig. 8) and larvae exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 7.8. Whereas only remnants of the mucus layers were observed on the skin of Cr-exposed larvae, on the skin of Cr-exposed larvae at pH 6.3 large amounts of mucus were observed (Fig. 9). The skin of larvae exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 was almost completely covered with mucus. Intact branchial epithelium with few mucus cells was seen in the 144-h control larvae (Fig. 6) and in larvae exposed to 9.6

$\mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 7.8. Contrastingly, branchial epithelium of larvae exposed $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.3 (Fig. 7) was wrinkled, whereas mucus cells were common. Observations of the gill epithelium of 240-h-old larvae could not be made because the gills were completely covered by the opercula.

4. Discussion

At water pH 6.3, Cr seriously affected larval development at a concentration which did not show any effect at pH 7.8. Hatching success was not changed, indicating that the chorion and the perivitelline fluid (pvf) form an effective barrier for Cr(VI) during the egg stage. Indeed, the freshly hatched larvae contained a very low content of Cr. This protective effect has earlier been demonstrated for other metals such as Al, Zn, Hg, Cu, Ag and Pb (Eddy and Talbot, 1985; Rombough, 1985; Oyen, 1993; Stouthart et al., 1994). Rombough (1985) suggested that the chorion may act as a cation exchanger, preventing metal ions with high binding affinities (Hg^{2+} , Cu^{2+} and Ag^{+}) from entering the pvf, whereas metals with low binding affinities (Zn^{2+} , Pb^{2+} and Cd^{2+}) easily pass the chorion and accumulate in the pvf according to the Donnan equilibrium. Since Cr was neither found in the chorion, nor in the embryo, it likely accumulated in the pvf. Most of the Cr in biological material is strongly associated with proteins, nucleic acids, and a variety of low-molecular-weight ligands (Strik et al., 1975) and perivitelline compounds such as proteins, gelatins and collagens are able to bind cations, because of their net negative charge (Eddy and Talbot, 1985). This will reduce their toxicity, and could very well explain why almost no Cr was found in the embryos at pH 7.8 and pH 6.3.

During the larval stage Cr accumulated and mortality increased. A remarkable effect was the specific decrease of the Na content in the larvae exposed to $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr(VI) at pH 6.3. The question arises how Cr(VI) exerts this toxic action. Van der Putte et al. (1981a) found that the gills of adult trout concentrated significantly more Cr(VI) at pH 6.5 than at pH 7.8, irrespective of exposure time and concentration. The pH-dependent uptake of Cr(VI) may, therefore be related to the relatively high concentration of HCrO_4^- , which, because of its monovalency (Trama and Benoit, 1960), is more readily taken up by the gill tissue than CrO_4^{2-} . A decrease in pH and therefore an increase in HCrO_4^- concentration is also associated with an increased oxidizing action of Cr(VI) (Sillén and Martell, 1964). This may further explain the greater toxicity of Cr at low pH. We found that Cr at pH 6.3 reduced the Na concentration of the larvae, without affecting their Ca concentration.

After hatching, fish larvae actively accumulate ions from the water, such as Ca^{2+} and Na^{+} , which are required for growth and ionic homeostasis. The likely sites of active ion uptake from the water are the chloride cells in the yolk sac epithelium, skin and the developing gills (Shen and Leatherland, 1978; Hwang and Hirano, 1985; Oyen, 1993). Oyen (1993) found that the first chloride cells appear outside the branchial area, while later on they become restricted to the gills. During the embryonic stage chloride cells are generally concentrated in the skin covering the epicardial region, on the yolk sac and in the tail region of the trunk (Alderdice, 1988). Gill et al.

(1987) observed that Cr caused hypertrophy and hyperplasia of the chloride cells. Branchial uptake of waterborne Ca^{2+} and Na^+ is mediated by chloride cells in a transport process that is passive across the apical membrane and active across the basolateral membrane (Perry and Flik, 1988; Flik et al., 1993). Cr likely interferes with the osmoregulatory processes of gills, kidney and intestine. Indeed, Kuhnert et al. (1976) showed that Na^+/K^+ -ATPase activity of kidney and intestine significantly decreased in adult rainbow trout exposed to $0.2 \mu\text{mol}\cdot\text{l}^{-1}$ Cr. The possibility that in our experiments Cr inhibited the active uptake of Na^+ , by decreasing Na^+/K^+ -ATPase activity appears therefore likely. Because of the specific effect of Cr on the Na concentration of the larvae, it is improbable that this effect was caused by osmotic water uptake or changes in the permeability characteristics of the gills.

Our scanning electron microscopic results showed that especially the skin and gills of the Cr-exposed larvae (144-h) at pH 6.3 exhibited circle-shaped spaces, probably orifices of mucus cells, between the pavement cells of the skin and gills. In addition, the gill epithelium appeared to be wrinkled. Examination of the skin in 240-h-old Cr-exposed larvae at pH 6.3 was impossible because of a thick mucus layer covering the skin. Also no observation could be made of the gills at this stage, because of coverage of the opercula. Gills of Cr-exposed adult *Barbus conchoni* showed wilting of the pillar system of the branchial lamellae, together with separation and disruption of the lamellar epithelium (Gill et al., 1987). This structural change is likely to affect the oxygen uptake by the gill lamellae due to disturbed blood flow and increase in the water-to-blood diffusional distance.

Cr apparently stimulated mucus secretion at low pH. Stimulatory effects of heavy metals on mucus secretion in the gills and skin of fish have been reported by several investigators (Lock and Van Overbeeke, 1981; Eddy and Fraser, 1982; Mallatt, 1985). Although in mucus no specific metal-binding proteins have been isolated so far, the glycoproteins and proteoglycans of the mucus apparently have a high binding capacity for heavy metals (Lock and Van Overbeeke, 1981). Indeed, Pärt and Lock (1983) found that mucus acted as a strong complexing agent for Cd^{2+} and Hg^{2+} . The strong metal complexing capacity of mucus might prevent passage of heavy metals through the epithelial membrane. Also the mucus layer on the gills may increase the water-to-blood diffusional distance (Mallatt, 1985). For example, Ultsch and Gross (1979) found that the increased mucus secretion in the gills of carp in acid water was correlated with reduced oxygen concentrations in the arterial blood of these fish. A decreased rate of oxygen transfer to the blood may have contributed to the increase of larval mortality as observed in our experiments.

In summary, it can be concluded that water pH is a crucial factor determining the toxicity of Cr(VI) for embryonic development of fish. Although $9.6 \mu\text{mol}\cdot\text{l}^{-1}$ Cr(VI) did not affect egg or larval development at pH 7.8, a slight decrease of water pH already caused Cr(VI) to significantly increase larval mortality. Similarly, Van der Putte et al. (1981a,b) found NOEC values of $3.9 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 7.8 but $0.4 \mu\text{mol}\cdot\text{l}^{-1}$ Cr at pH 6.5 for eggs of *Oncorhynchus mykiss* (late eyed stage). Therefore, this increasing effect of acidification of natural waters by human activities in many parts of the world should be taken into account when evaluating the water quality standards for heavy metals such as Cr.

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