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

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Abstract

Carp eggs were exposed immediately after fertilization to Pb concentrations of 0.12 to 0.96 μmol l^{-1} at water pH 7.5 and 5.6. At regular intervals mortality, the incidence of spinal cord deformation, heart rate, body movements, hatching success, and whole body concentration of K, Na, Mg, Ca, and Pb were assessed. At pH 7.5, Pb increased heart rate, and decreased body movements, while at pH 5.6, Pb also reduced hatching success, caused spinal cord deformation, decreased net Ca^{2+} uptake, and increased mortality of the larvae, in a concentration-dependent manner. In controls of pH 5.6 no significant changes of any of the above parameters were observed when compared to controls at pH 7.5.

Key words: Acid stress; Lead; Embryonic development; Carp; *Cyprinus carpio*

1. Introduction

Lead (Pb) is one of the mostly used industrial metals. The anthropogenic release of Pb to the environment is the highest of all heavy metals (Pb > Ag > Mo > Sb > Zn > Cd > As > Cr > Co > Mn > Hg; Salomon and Förstner, 1984). Although the release of Pb into the environment has been greatly reduced in the past decade, e.g., via introduction of Pb-free gasoline, and recycling of Pb, it is still used by fishermen and hunters in large quantities. Indeed, hunting and fishing activities are responsible for 86% of the yearly Pb emission of approximately 375 t into Dutch surface waters (Janus et al., 1991).

In buffered and eutrophic water the bioavailability of Pb is reduced because of complexation with water-borne organic particles (Nriagu, 1979). In contrast, the bioavailability of Pb is strongly enhanced at decreasing water pH (Brown, 1979), increasing the toxicity of Pb for fish.

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However, the effects of this phenomenon on the early developmental stages have received little attention. Eggs and larvae, are considered much more sensitive to heavy metals than adults (Macek et al., 1977; McKim, 1985; Hodson et al., 1979; Mohan et al., 1983; Dave and Xiu, 1991; Sayer et al., 1991). We have therefore examined the effects of varying Pb concentrations at water pH 5.6 in comparison to pH 7.5 on the development of eggs and larvae of the common carp (*Cyprinus carpio*).

2. Materials and methods

In vitro fertilization and incubation of eggs

Carp gametes were obtained after artificial fertilization of carp eggs as described by Oyen et al. (1991). Carp eggs (300–400) placed in petridishes were fertilized by sperm from three different males resulting in three different batches of eggs. Immediately after fertilization different petridishes with the fertilized eggs stuck to the bottom were placed in a 4-l aquarium (five per group; for details, see Oyen et al., 1991; Oyen, 1993), containing the following main ion concentrations in demineralized water (in mmol l^{-1}): 0.06 KCl; 0.40 $NaHCO_3$; 0.20 $MgSO_4$; 0.80 $CaCl_2$). Mortality of eggs and larvae, deformation of larvae and hatching success were examined. In each aquarium one petridish was specifically used to examine mineral content, heart rate and tail movements of the developing embryos.

Eggs were exposed, during a 12 h photoperiod in water (8.7 ppm DOC) of 23°C of pH 7.5 ± 0.05 and pH 5.6 ± 0.07 (controls) and to Pb (as $Pb(NO_3)_2$) in a concentration range of 0.12, 0.24, 0.48 and 0,96 μmol l^{-1}. Water pH was adjusted to 5.6 via gradual addition of 0.01 M sulfuric acid using pH-stat equipment. Constant Pb concentrations were maintained via a flow-through system resulting in a complete turnover of the system's water content after 10 h. Desired Pb concentrations were within 5% of the calculated values as verified by Atomic Absorption Spectrophotometric (AAS) analyses.

Mortality and deformation of the spinal cord

Dead eggs and larvae were counted at 6, 12, 24, 48, 58, 72, 96, 120, 144 and 170 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 deformed larvae (including dead ones) was determined after microscopic examination. Deformed larvae tended to lay on the bottom of the aquaria.

Heart rate and tail movements

Shortly before hatching, heart rate (beats min^{-1}) and rate of tail movements (beats min^{-1}) were determined for at least 20 embryos per group.

Mineral content

To determine dry weight, 125 eggs or larvae from each group were collected at

6, 12, 24, 48, 58, 72, 96, 120, 144, and 170 h after fertilization. To discriminate between the amount of Pb adsorbed to the chorion and the amount of which had entered 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.

Total body K, Na, Mg, Ca and Pb content of eggs, chorions and larvae were calculated on a dry weight basis. Tissues were dissolved for 24 h at 70°C in 65% HNO_3. The K and Na concentrations were measured in a flame photometric Auto Analyzer (Technicon); Mg and Ca were analyzed with Inductively Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo Electron, USA). Pb was determined with an atomic absorption spectrometer (Video II, Thermo Jarrell Ash, USA). All concentrations are expressed as μmol g^{-1} dry weight.

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 egg membrane by the tail. Fully as well as partially hatched larvae were counted.

Statistical analysis

Data are expressed as means ± SE ($n = 6$). A one-way analysis of variance was used to assess differences between groups. Significance of differences was subsequently tested using the 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

Mortality of eggs and larvae and deformation rate

No significant difference in mortality of eggs and larvae was found between the groups exposed to Pb at pH 7.5 and the control group (Table 1). At pH 5.6 egg mortality was not significantly increased by Pb. Exposure to pH 5.6 resulted in concentration-related mortality of larvae compared to the control (Table 1). Deformation percentage was identical for all experimental groups at pH 7.5 (Table 2). However, the Pb-exposed larvae exhibited a deviating swimming pattern compared to control larvae. Most of the Pb exposed larvae tended to lie down on the bottom whereas most of the control larvae were freely swimming (Fig. 1). Significant deformation occurred in all Pb exposed treatments at pH 5.6 (Table 2). A 100% deformation with increasing severity was observed at concentrations of 0.48 and 0.96 μmol l^{-1} Pb. The different types of deformation are shown in Fig. 2.

Heart rate and tail movements

Heart rate and tail movements were not significantly increased at pH 5.6 compared to pH 7.5. In the presence of Pb, a concentration-related increase of heart rate and tail movements was observed (Table 3).

Table 1
Mortality of carp eggs and larvae during exposure to Pb at pH 7.5 and 5.6

[Pb]	Mortality of eggs (%)		Mortality of larvae (%)	
μmol l^{-1}	pH 7.5	pH 5.6	pH 7.5	pH 5.6
n.d.	11.2 ± 3.5	12.0 ± 3.9	3.7 ± 1.4	3.3 ± 1.3
0.12	8.9 ± 2.4	11.6 ± 3.3	2.9 ± 0.5	12.5 ± 4.2^b
0.24	8.8 ± 2.7	9.8 ± 2.5	3.8 ± 2.7	23.5 ± 2.8^d
0.48	9.6 ± 3.1	11.8 ± 2.5	3.5 ± 1.0	33.0 ± 1.2^d
0.96	10.7 ± 4.7	11.0 ± 3.1	3.8 ± 1.3	42.0 ± 1.1^d

Significance is expressed at the level of $P < 0.02$; $^cP < 0.01$; $^dP < 0.001$ compared to control values. Mortality is expressed as percentage of total number of eggs and larvae: means ± S.E. ($n = 6$) are given (n.d., non-detectable: less than $1 \times 10^{-6} \mu$mol l^{-1}).

Table 2
Percentage of deformed larvae during exposure to Pb at pH 7.5 and 5.6

[Pb]	Deformation of larvae (%)	
μmol l^{-1}	pH 7.5	pH 5.6
n.d.	11.7 ± 3.8	12.2 ± 4.1
0.12	13.2 ± 2.3	20.5 ± 1.4^a
0.24	10.6 ± 4.9	26.7 ± 2.4^c
0.48	10.4 ± 3.8	100.0 ± 0.0^d
0.96	13.7 ± 2.8	100.0 ± 0.0^d

Significance is expressed at the level of $^aP < 0.05$; $^cP < 0.01$; $^dP < 0.001$ compared to control values. Deformation is expressed as percentage of the total number of larvae; means ± S.E. ($n = 6$) are given (n.d., non detectable: less than $1 \times 10^{-6} \mu$mol l^{-1}).

Table 3
Heart rate (beats min^{-1}) and the frequency of tail movements (beats min^{-1}) of carp embryos 50 h after fertilization exposed to different ambient Pb levels at pH 7.5 and 5.6

[Pb]	Heart rate		Tail movements	
μmol l^{-1}	pH 7.5	pH 5.6	pH 7.5	pH 5.6
n.d.	130 ± 4	131 ± 2	24 ± 3	23 ± 1
0.12	129 ± 3	147 ± 2^d	27 ± 3	22 ± 3
0.24	129 ± 2	166 ± 3^d	18 ± 2	18 ± 3
0.48	136 ± 3	171 ± 4^d	16 ± 1^b	16 ± 3
0.96	142 ± 3^b	186 ± 4^d	14 ± 3^b	13 ± 1^d

Significance is expressed at the level of $^bP < 0.02$; $^dP < 0.001$ compared to control values. Data are expressed as means ± S.E. ($n = 6$) (n.d., non-detectable: less than $1 \times 10^{-6} \mu$mol l^{-1}).

Mineral content

No differences in mineral content were observed between controls at pH 7.5 and pH 5.6. Also no significant differences were observed in whole body K, Na, Mg and
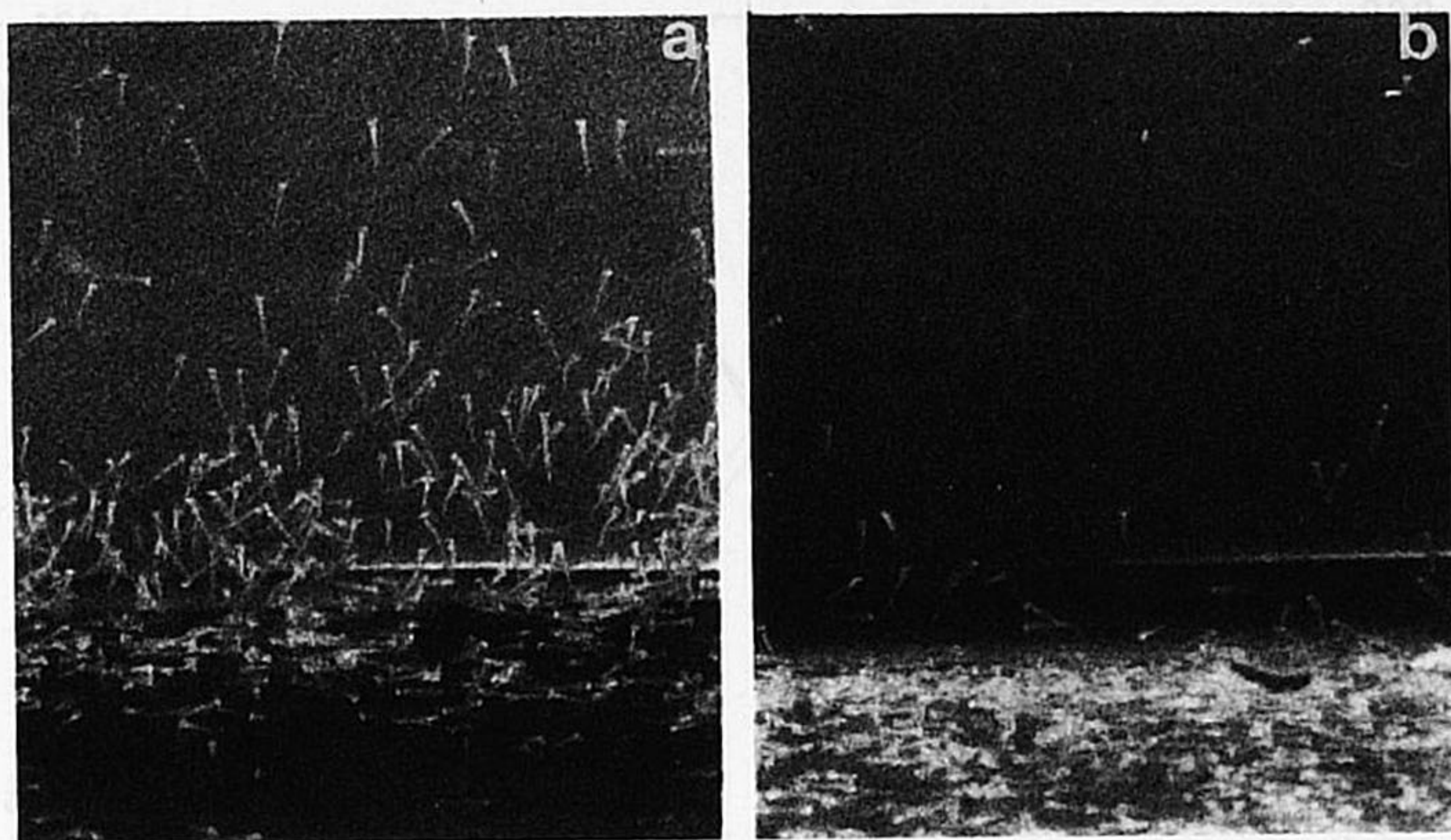
Fig. 1. Representative view of larvae 114 h reared at pH 7.5 (1a) and larvae 144 h exposed to 0.96 μmol l^{-1} Pb at pH 7.5 (1b), showing the difference in swimming activity between both groups.

Ca content of eggs and larvae between controls and groups exposed to 0.12, 0.24, 0.48 and 0.97 μmol l^{-1} Pb at pH 7.5 (Fig. 3a, 3b, 3c and 3d). Similar results were found for K, Na and Mg content in eggs and larvae exposed to the same concentrations of Pb at pH 5.6 (Fig. 4a, 4b and 4c). A significant decrease was observed for the Ca content in larvae exposed to 0.24, 0.48 and 0.97 μmol l^{-1} Pb (Fig. 4d).

Lead accumulation

Pb was recovered from the chorion in a concentration-related fashion (Fig. 5a and 5b). At pH 5.6 more Pb was associated with the chorion than at pH 7.5. Only a small
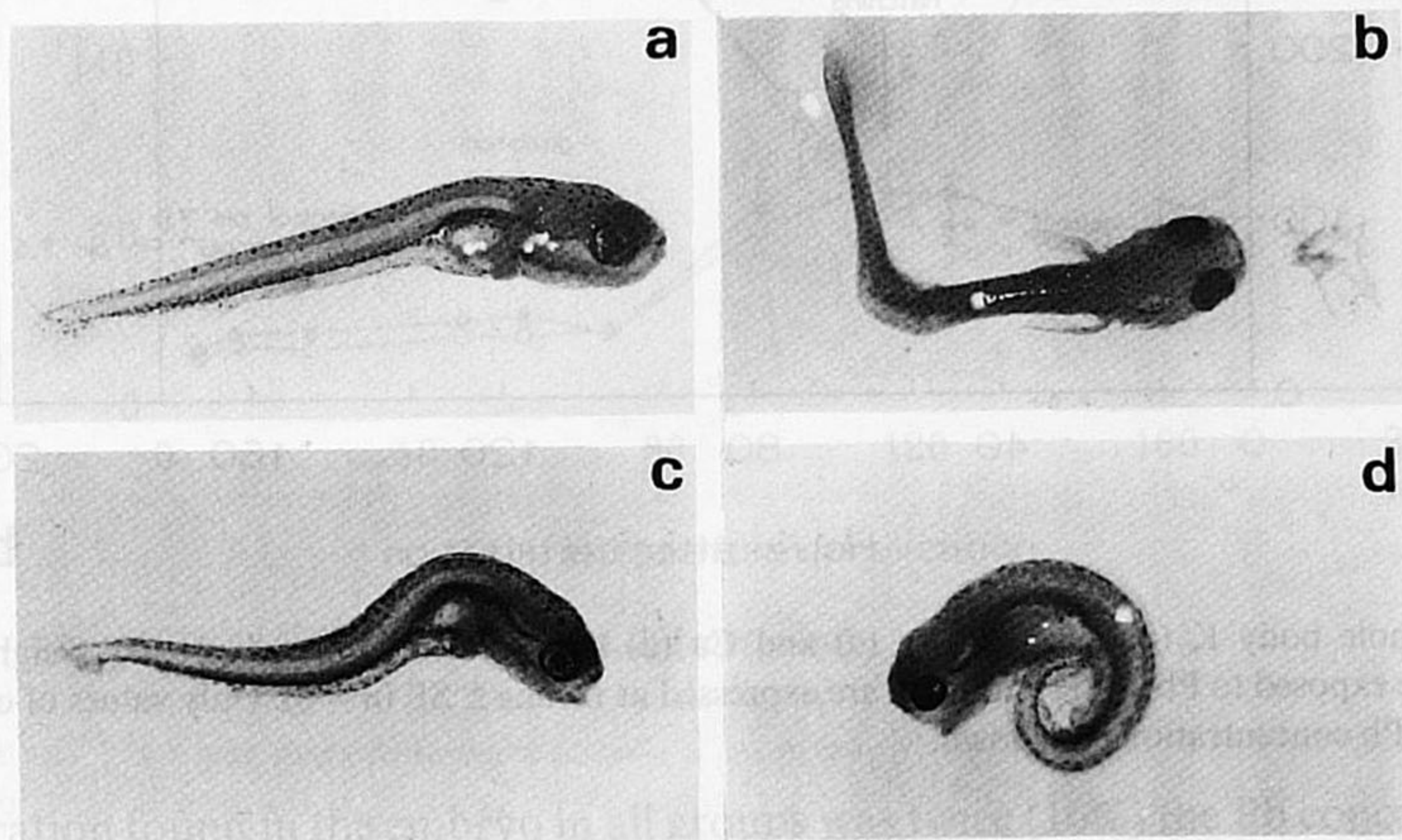
Fig. 2. Deformation of larvae 144 h reared at pH 5.6 and 0.96 μmol l^{-1} Pb. (2a) Normal larvae, pH 5.6, 8×. (2b)–(2d) Deformed larvae, pH 5.6 and 0.96 μmol l^{-1} Pb, 8×.

a   Hours after fertilization
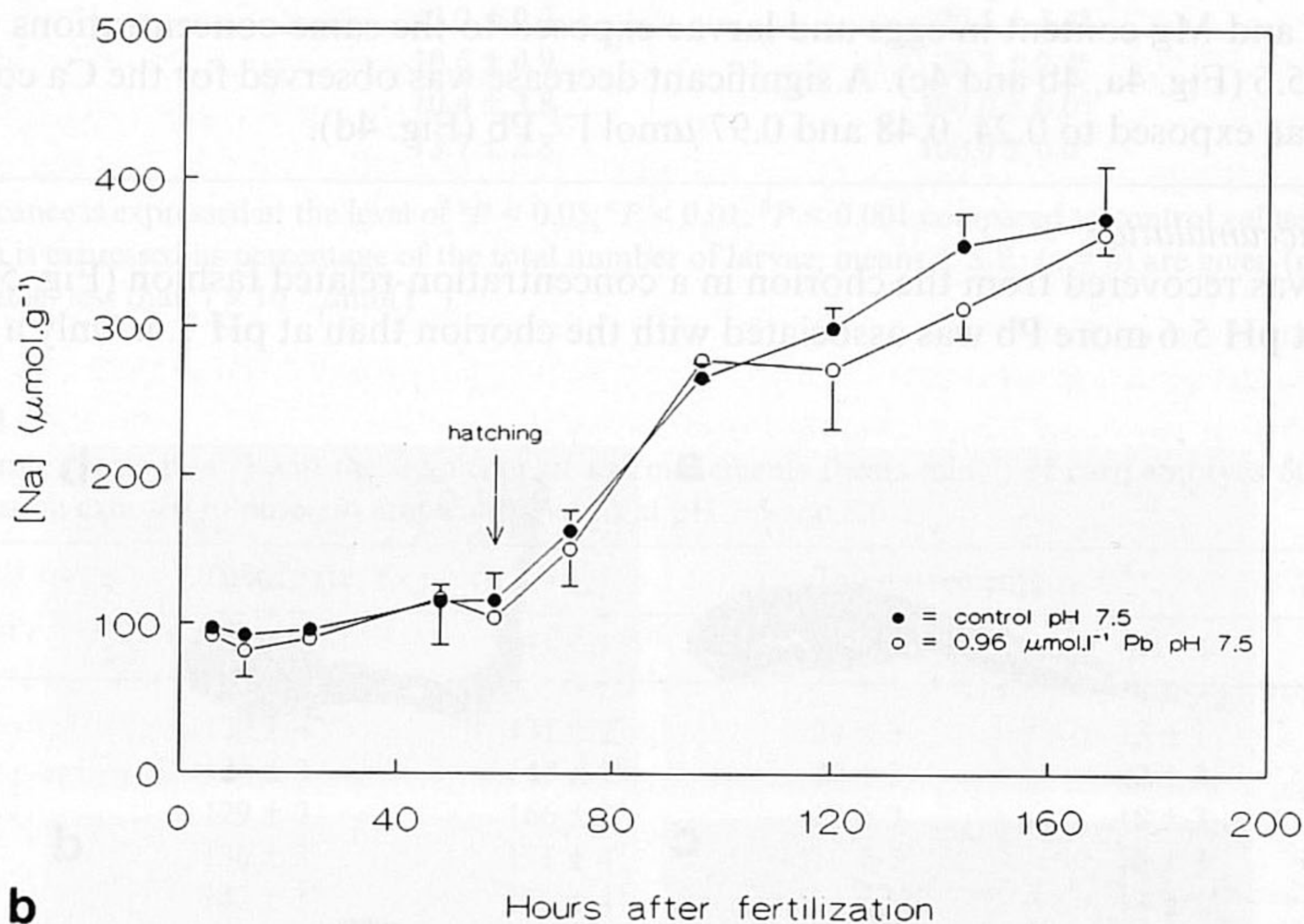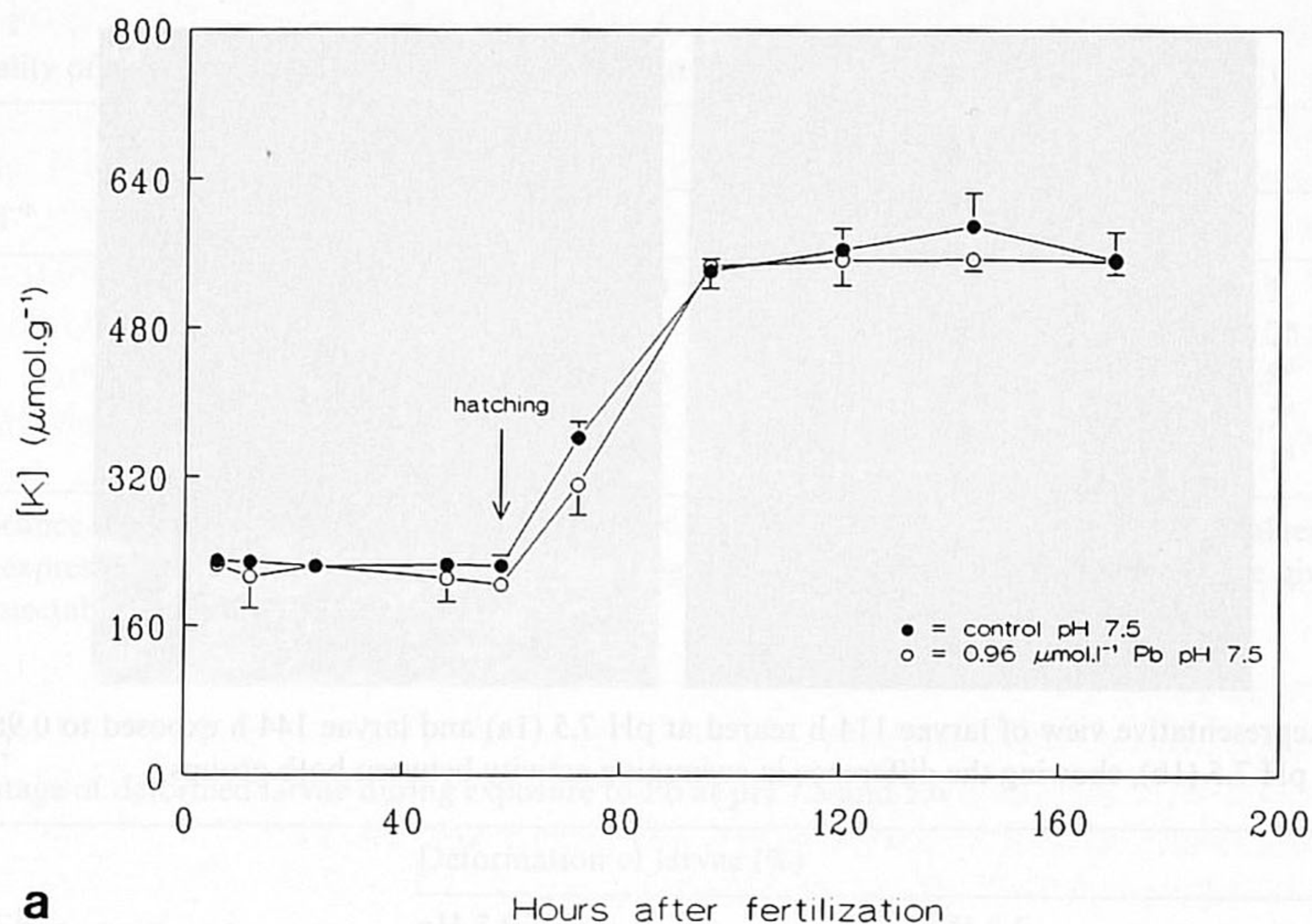

b   Hours after fertilization

Fig. 3. Whole body K (a), Na (b), Mg (c) and Ca (d) concentrations (μmol g^{-1} dry weight) of carp eggs/larvae exposed to Pb at pH 7.5. Data are expressed as means ± SE ($n = 6$). Only values of control and of highest Pb concentration are shown.

amount of Pb entered the eggs and the embryos kept at either pH. At pH 7.5 and pH 5.6 respectively 92% and 84% of the Pb was bound by the chorion. At pH 5.6 the Pb

c

Hours after fertilization
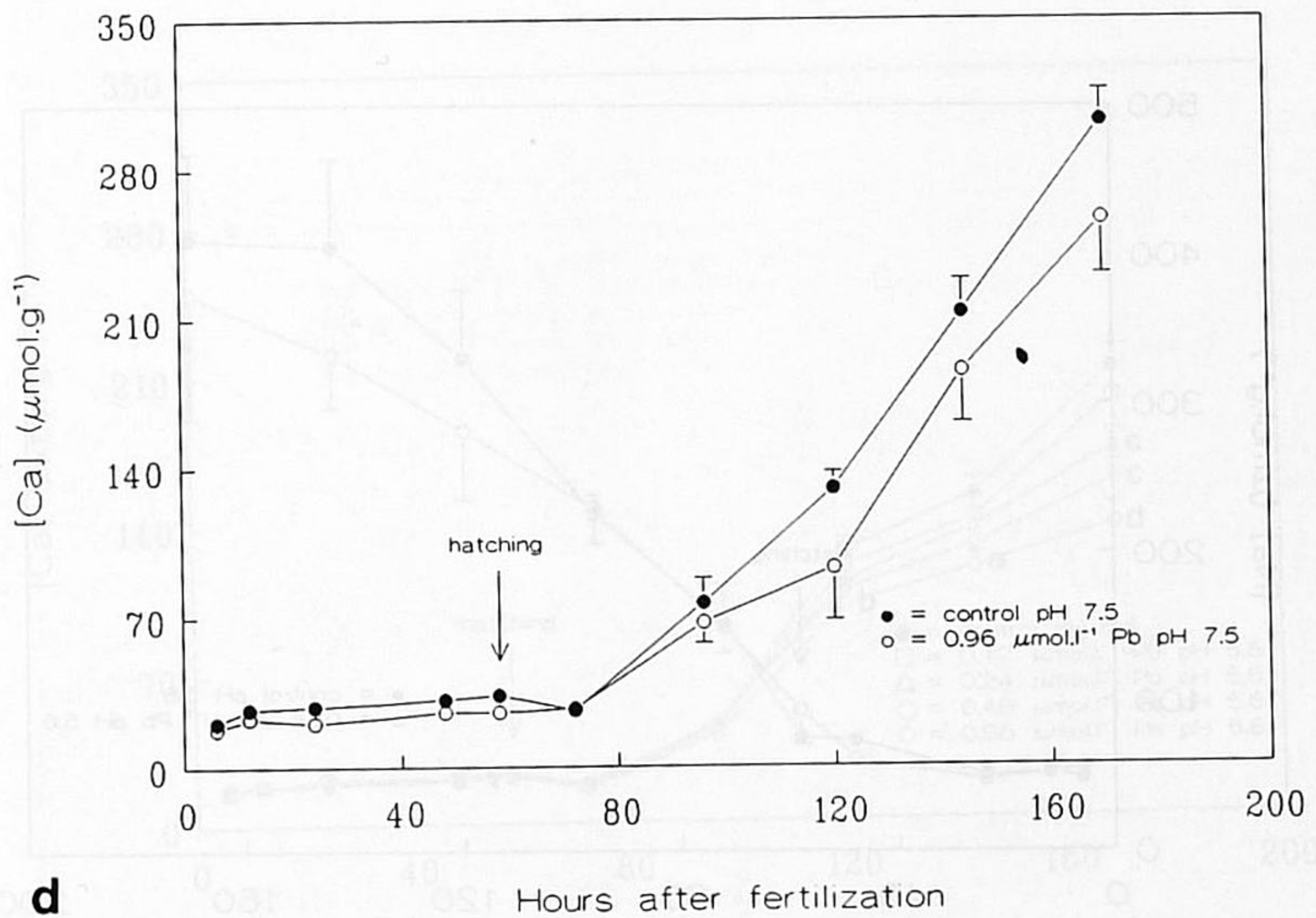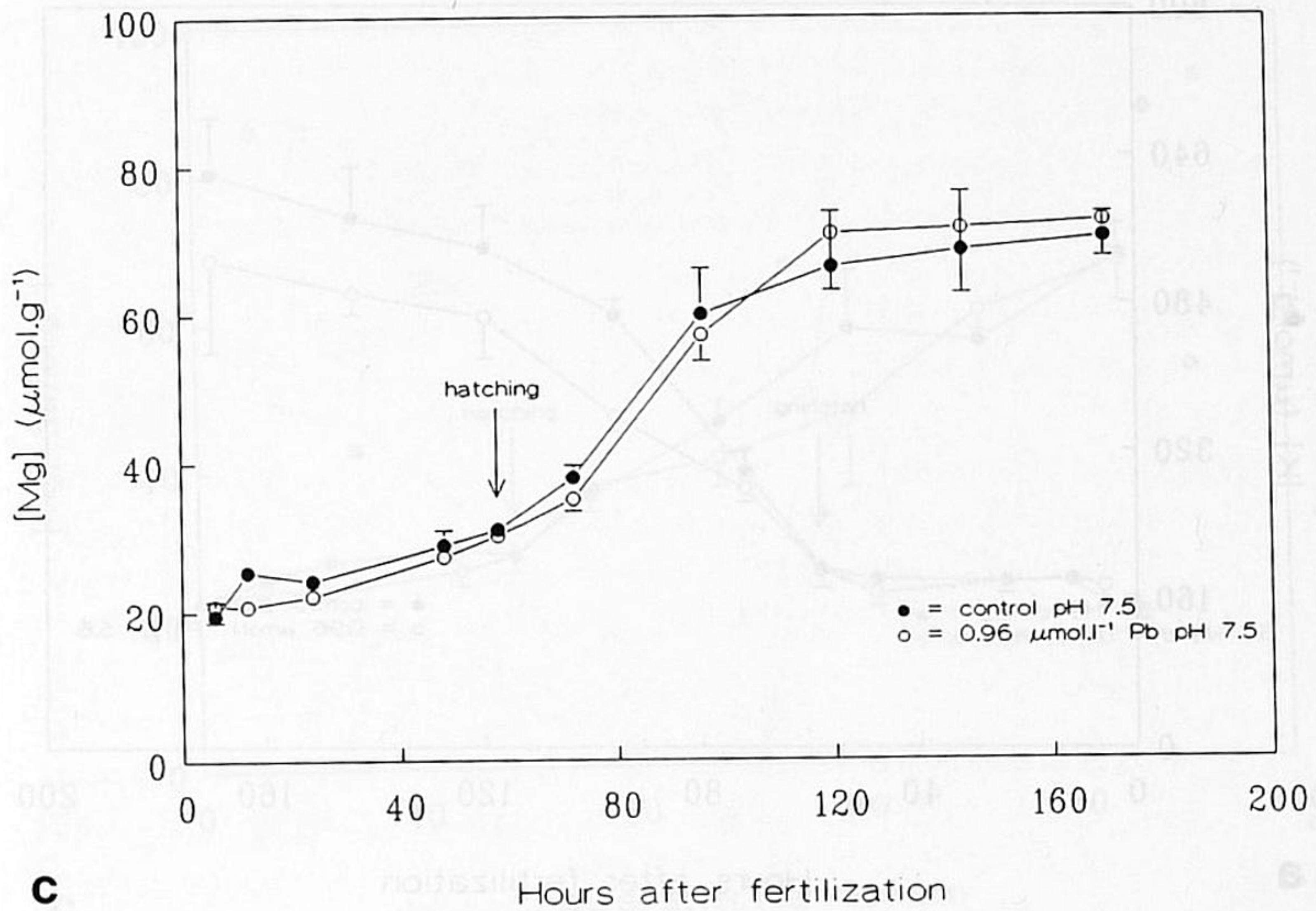

d

Hours after fertilization

Fig. 3. (continued)

concentration found in the embryo in all groups was twice (16%) the Pb concentration of embryos from pH 7.5 (8%). Accumulation of Pb in the embryos was not influenced by rising Pb concentrations. Immediately after hatching, larvae started to accumulate
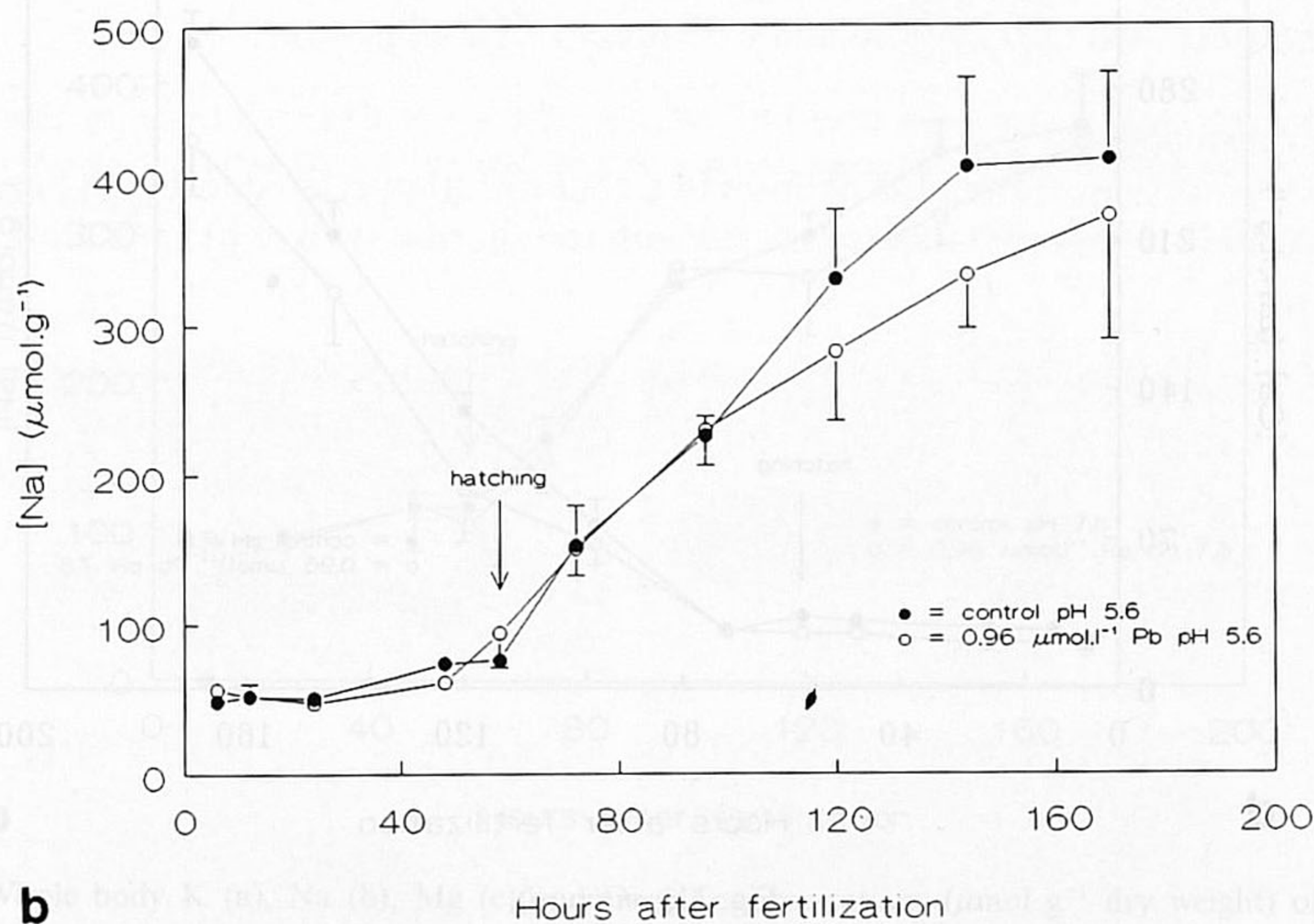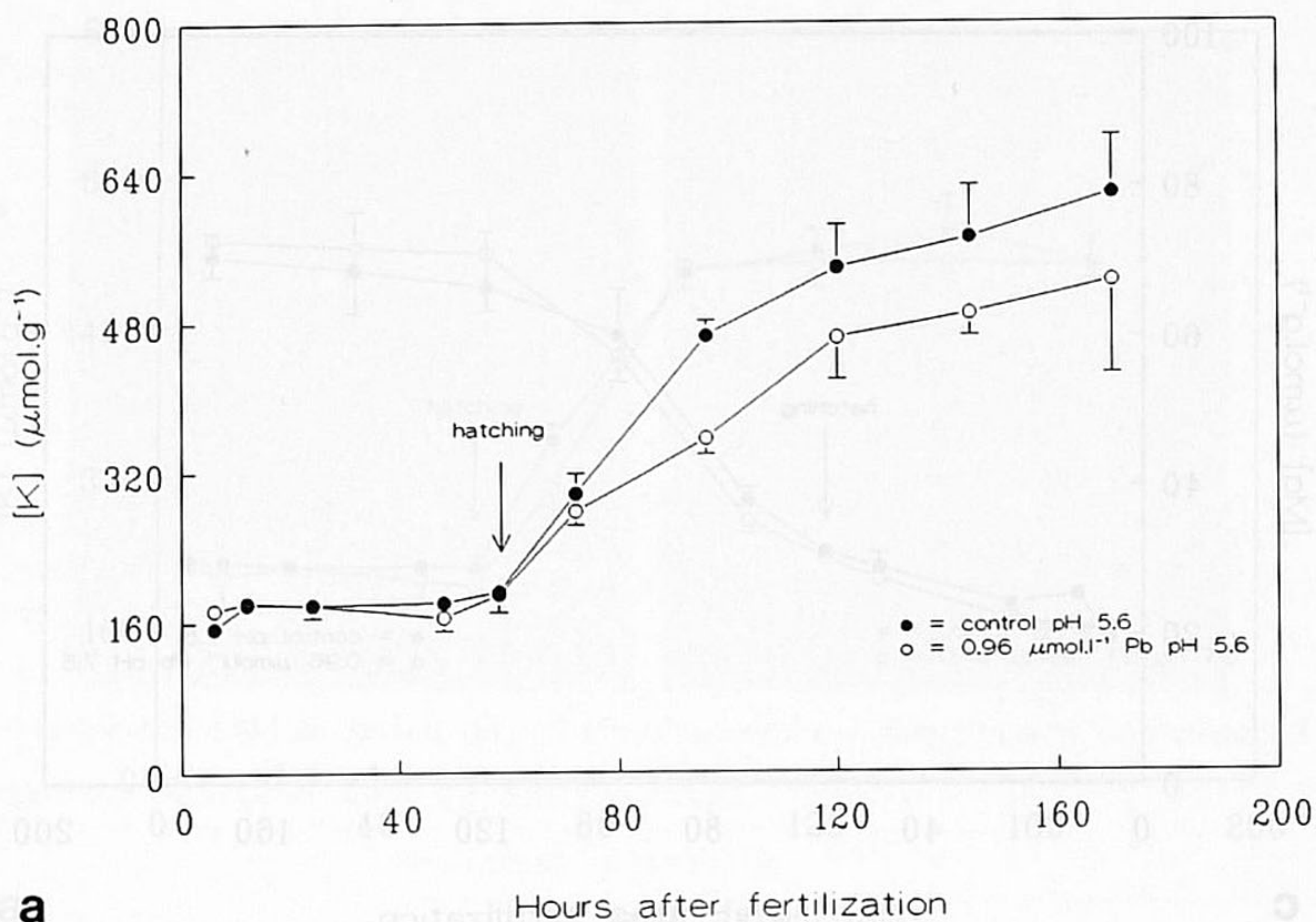
a

Hours after fertilization

b

Hours after fertilization

Fig. 4. Whole body K (4a), Na (4b), Mg (4c) and Ca (4d) concentrations (μmol g^{-1} dry weight) of carp eggs/larvae exposed to Pb at pH 5.6. In Fig. 4d, significance is expressed at the level of (a) 0.05, (b) 0.02, (c) $P < 0.01$ and (d) $P < 0.001$, compared to control values. Data are expressed as means ± SE ($n = 6$). For 4a, 4b and 4c, only values of controls and highest Pb concentration are shown, and no significant differences were observed.
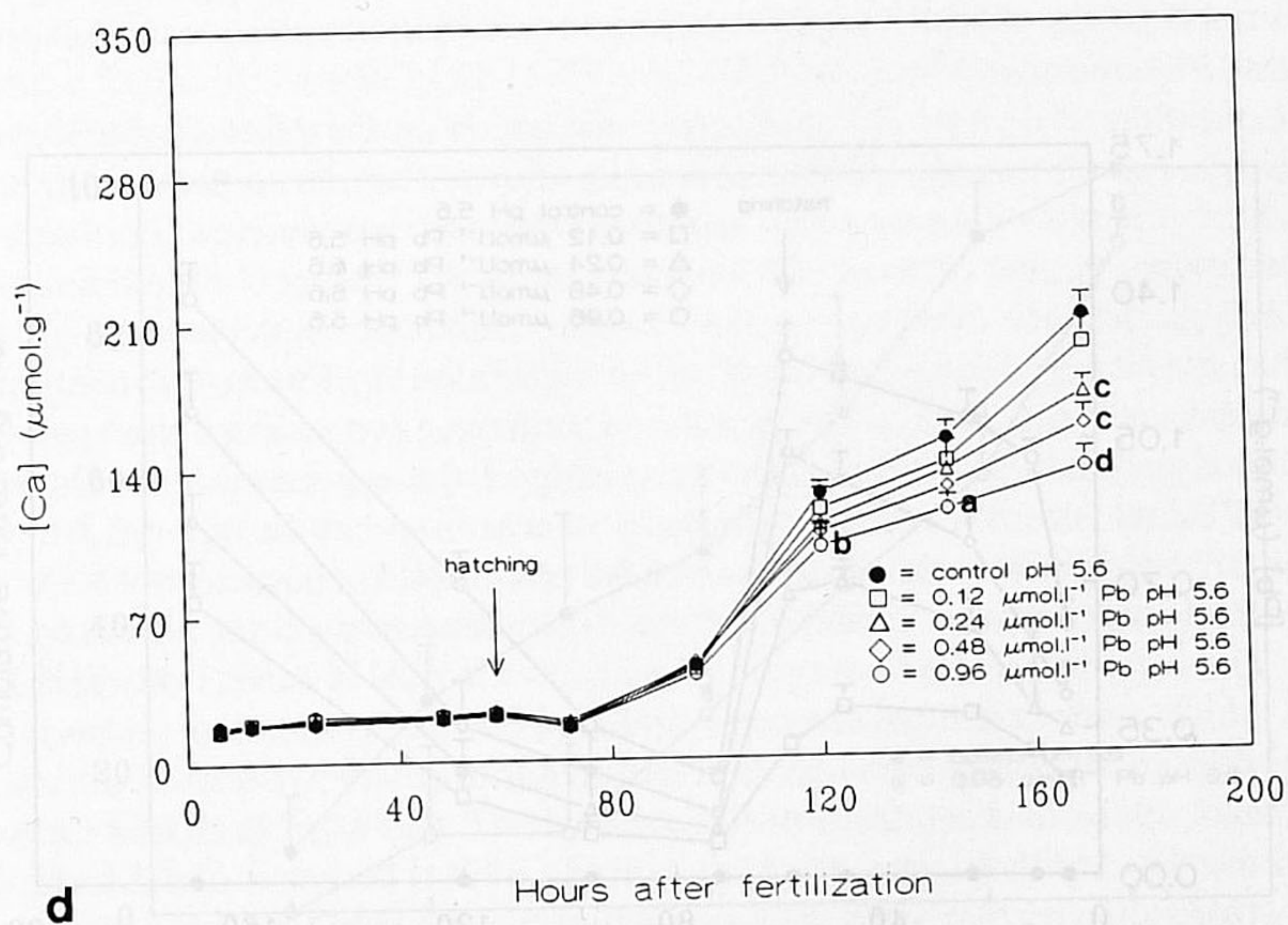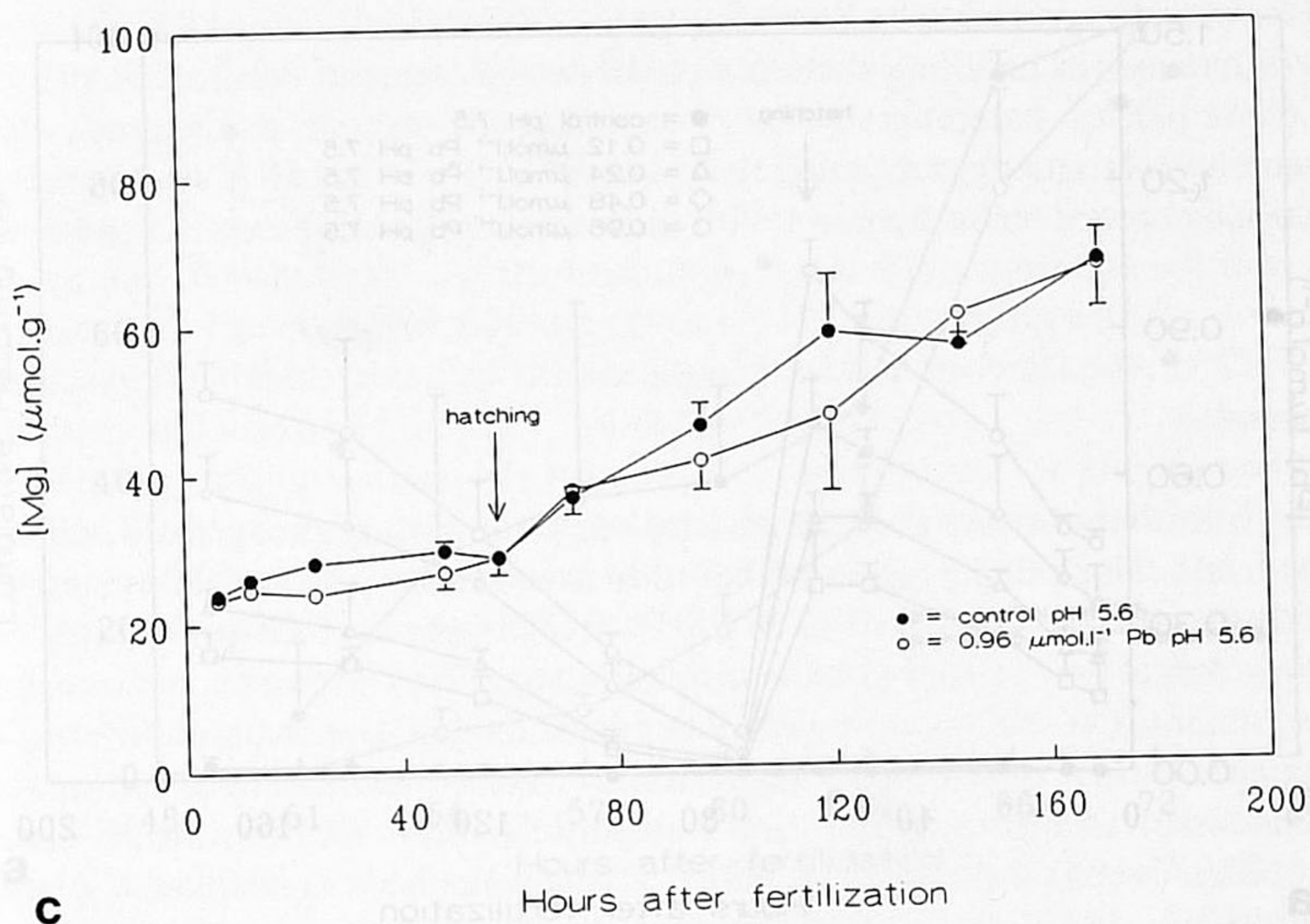
c

Hours after fertilization

d

Fig. 4. (continued)

Pb. The Pb content of the larvae exposed to 0.97 μmol l^{-1} Pb at pH 7.5 was similar to that of the larvae exposed to 0.12 μmol l^{-1} Pb at pH 5.6.
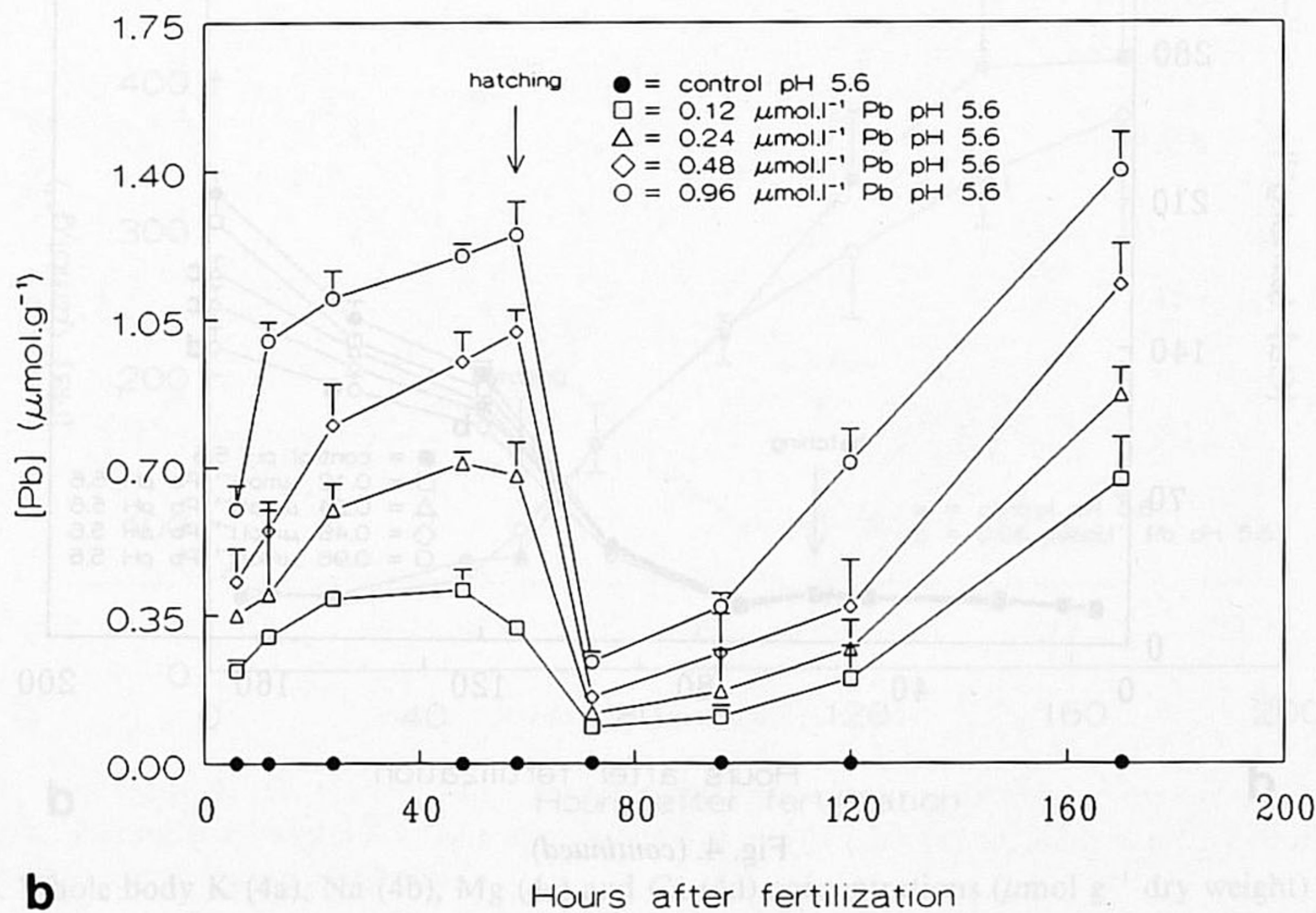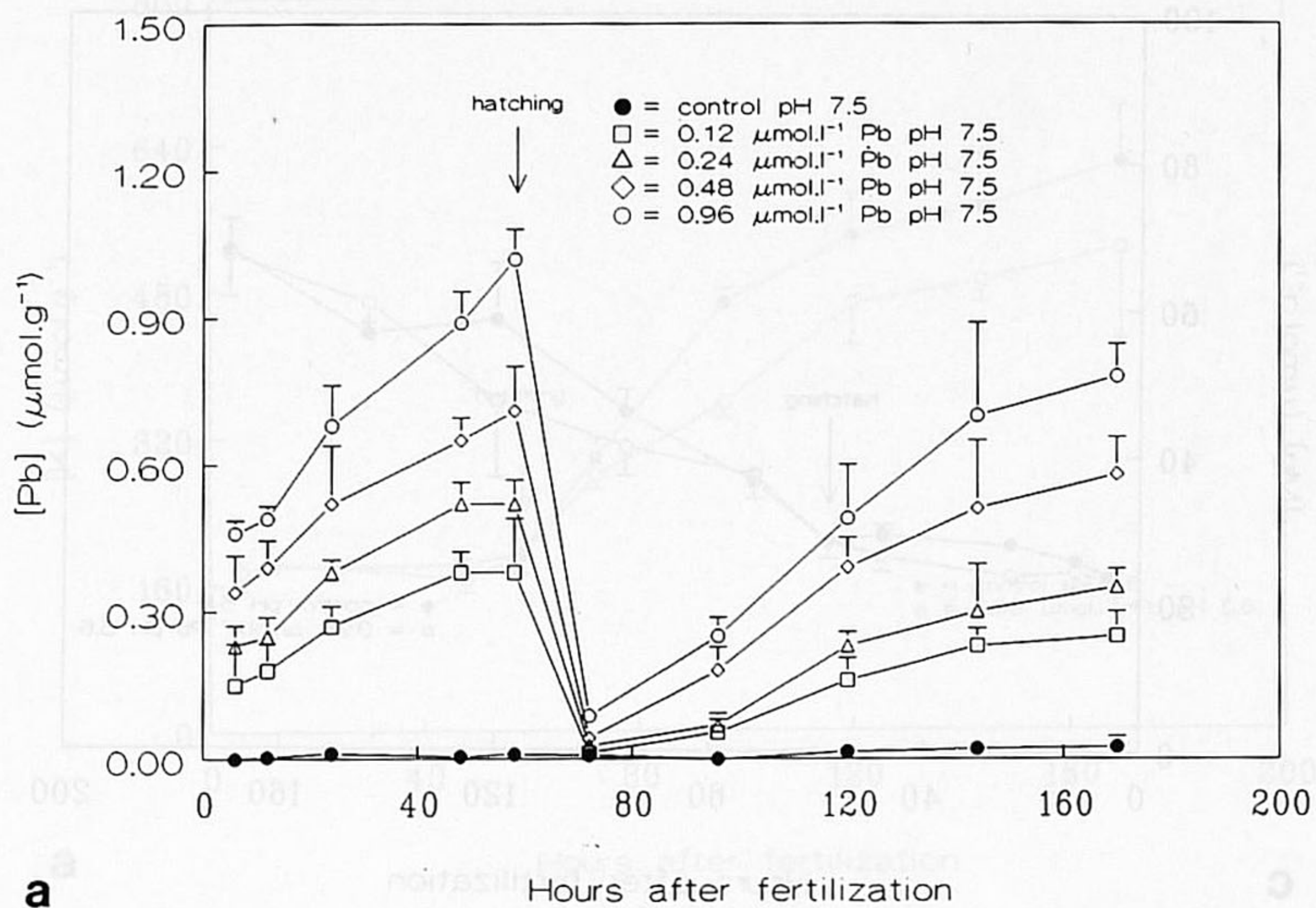
a

b

Fig. 5. Accumulation of Pb by carp eggs and larvae exposed to Pb at indicated concentration at pH 7.5 (5a) and pH 5.6 (5b). All values are significantly different compared to control values. Hatching, took place between 52 and 72 h. Data are expressed as means ± SE ($n = 6$).
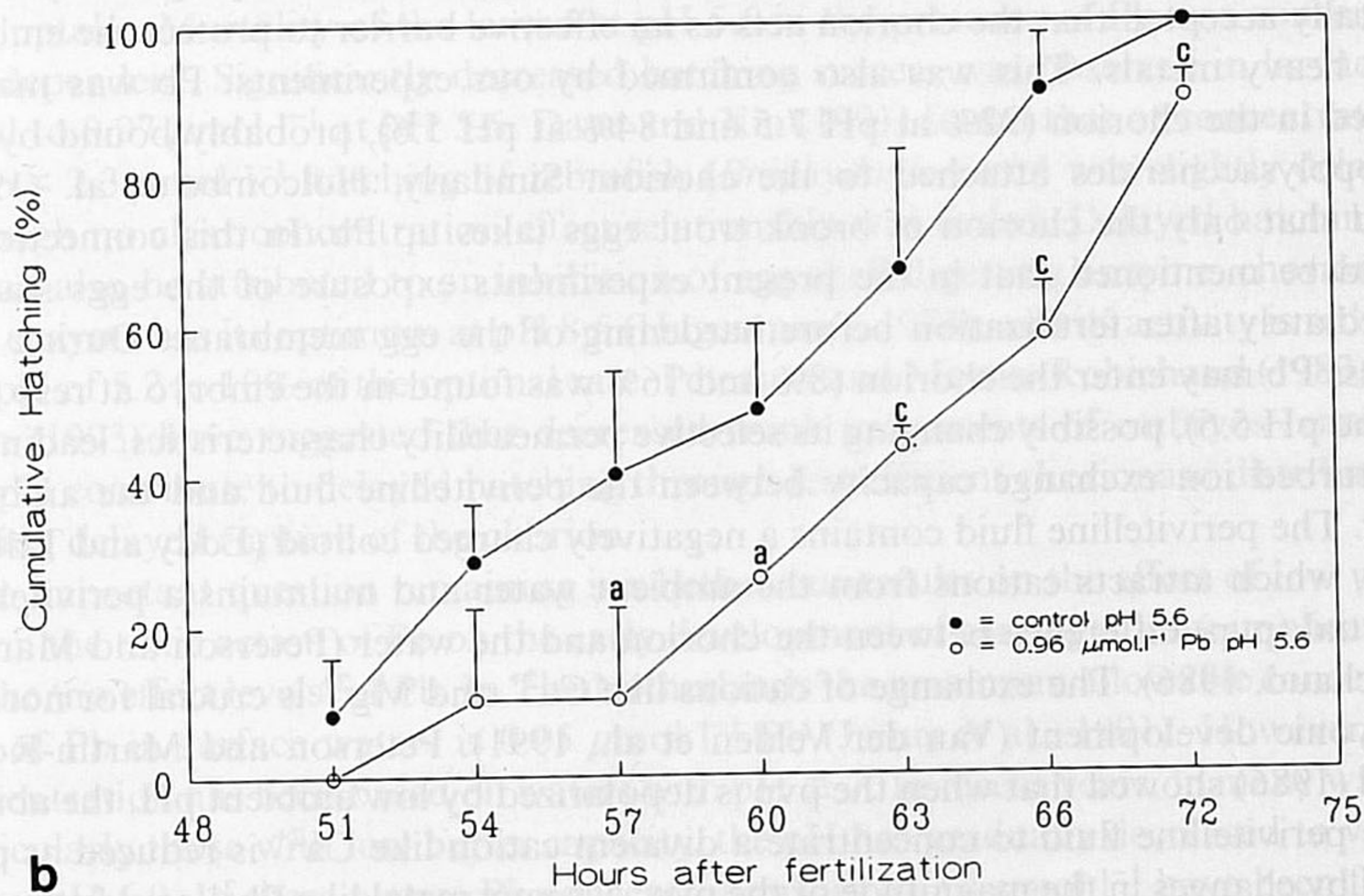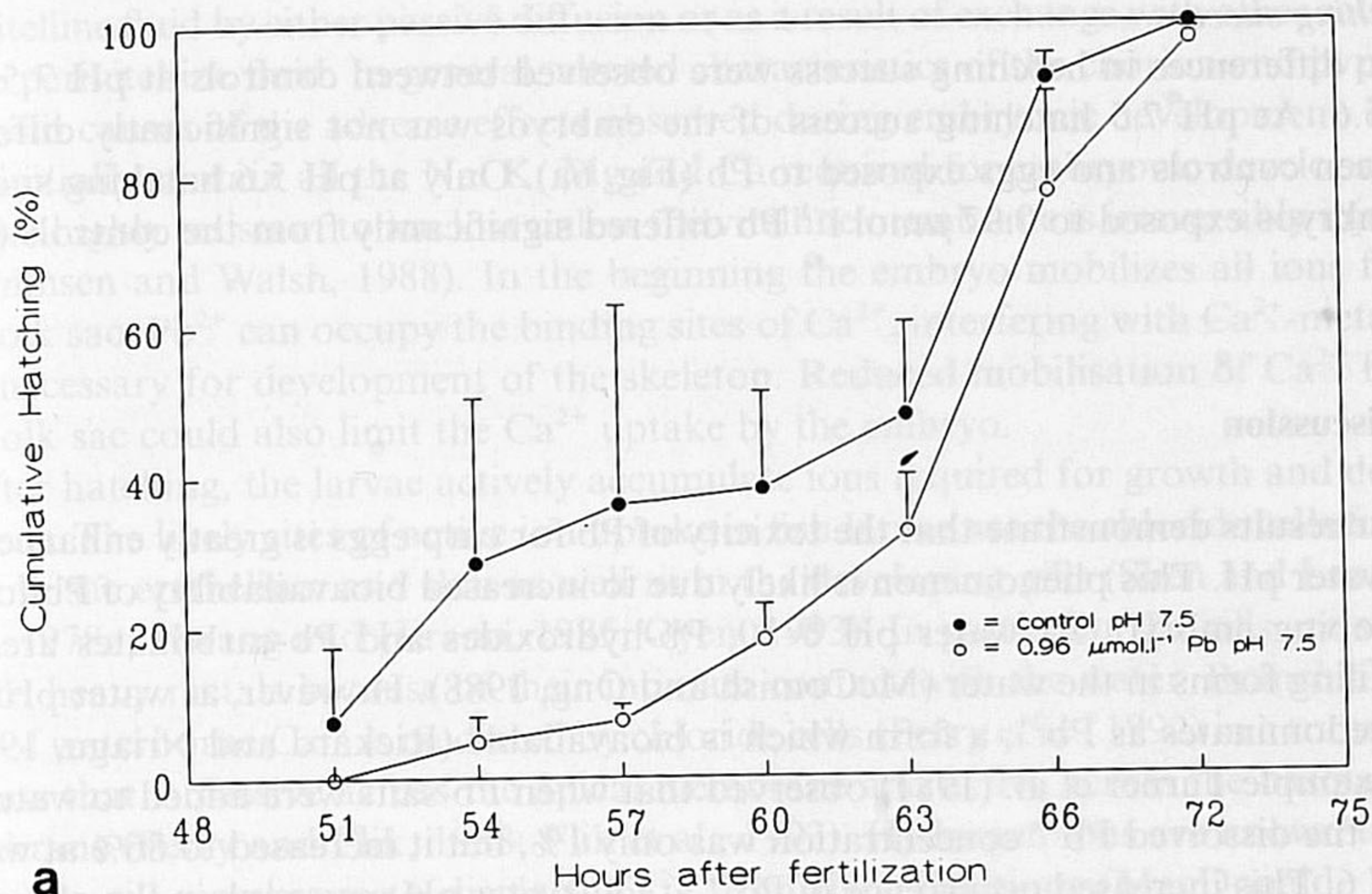
Fig. 6. Cumulative hatching success of carp eggs exposed to Pb at pH 7.5 (6a) and pH 5.6 (6b). In Fig. 6b, significance is expressed at the level of (a) $P < 0.05$ and (c) $P < 0.01$, compared to control values. Data are expressed as means ± SE ($n = 6$). Only values of controls and of highest Pb concentration are shown.

Hatching success

No differences in hatching success were observed between controls at pH 7.5 and pH 5.6. At pH 7.5 hatching success of the embryos was not significantly different between controls and eggs exposed to Pb (Fig. 6a). Only at pH 5.6 hatching success of embryos exposed to 0.97 μmol l^{-1} Pb differed significantly from the controls (Fig. 6b).

4. Discussion

Our results demonstrate that the toxicity of Pb for carp eggs is greatly enhanced at low water pH. This phenomenon is likely due to increased bioavailability of Pb to the developing embryo. At water pH 6-10, Pb-hydroxides and Pb-carbonates are the prevailing forms in the water (McComish and Ong, 1988). However, at water pH < 6 Pb predominates as Pb^{2+}, a form which is bioavailable (Rickard and Nriagu, 1978). For example Turner et al. (1981) observed that when Pb-salts were added to water of pH 9, the dissolved Pb^{2+} concentration was only 1%, but it increased to 86% at water of pH 6. This increased occurrence of Pb^{2+} at low water pH may explain the observed high percentage of embryo's exhibiting a serious deformation of the spinal cord. Our results are in agreement with those of Hodson et al. (1979), who observed necrotic tails (early symptoms indicative of spinal deformities) in rainbow trout exposed to 0.11 μmol l^{-1} Pb at pH 8.2. No deformed embryo's were observed in eggs exposed to the same Pb concentration at pH 7.5.

The question arises how Pb^{2+} exerts its toxic action on the developing embryo. It is generally accepted that the chorion acts as an effective barrier to protect the embryo from heavy metals. This was also confirmed by our experiments: Pb was mainly located in the chorion (92% at pH 7.5 and 84% at pH 5.6), probably bound by the mucopolysaccharides attached to the chorion. Similarly, Holcombe et al. (1976) found that only the chorion of brook trout eggs takes up Pb. In this connection it should be mentioned that in the present experiments exposure of the eggs started immediately after fertilization before hardening of the egg membrane. During this process Pb may enter the chorion (8% and 16% was found in the embryo at resp. pH 7.5 and pH 5.6), possibly changing its selective permeability characteristics, leading to a disturbed ion exchange capacity between the perivitelline fluid and the ambient water. The perivitelline fluid contains a negatively charged colloid (Eddy and Talbot, 1985), which attracts cations from the ambient water and maintains a perivitelline potential (pvp) difference between the chorion and the water (Peterson and Martin-Robichaud, 1986). The exchange of cations like Ca^{2+} and Mg^{2+} is crucial for normal embryonic development (Van der Velden et al., 1991). Peterson and Martin-Robichaud (1986) showed that when the pvp is depolarized by low ambient pH, the ability of the perivitelline fluid to concentrate a divalent cation like Ca^{2+} is reduced as predicted by changes in the magnitude of the pvp. A heavy metal like Pb^{2+} could strongly bind to acidic protein groups in the chorion and the embryo. As a consequence the passive diffusion increases compared to the active process, because of reduced pvp. Concentrations of Pb in the chorion increase the probability of entering the

perivitelline fluid by either passive diffusion or as a result of exchange with other cations in the perivitelline fluid. In general, altered characteristics of the chorion and pvp are the main causes of the adverse effects observed during embryonic development. Fish eggs initially contain all the Na, K, Mg and Ca required for embryonic development and are highly resistant to ion loss unless the vitelline membrane is irreversibly injured (Mommsen and Walsh, 1988). In the beginning the embryo mobilizes all ions from the yolk sac. Pb^{2+} can occupy the binding sites of Ca^{2+}, interfering with Ca^{2+}-metabolism necessary for development of the skeleton. Reduced mobilisation of Ca^{2+} from the yolk sac could also limit the Ca^{2+} uptake by the embryo.

After hatching, the larvae actively accumulate ions required for growth and development. The likely sites of active ion uptake in fish larvae are the chloride cells found on yolk sac epithelium and skin as well as in the developing gills (Shen and Leatherland, 1978a; Hwang and Hirano, 1985; Oyen, 1993). In particular the gills are sensitive to heavy metals because of their intimate contact with the water. Branchial uptake of waterborne Ca^{2+} is mediated by chloride cells (Perry et al., 1992) 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). Hydrogen ions are known to act upon the gill membrane to displace Ca^{2+} from tight junctions (MacDonald, 1983; Marshall, 1985) and block ion-transport channels (MacDonald et al., 1983), thereby decreasing net ion uptake. Similarly, Pb has been shown to interfere with Ca^{2+}-transport mechanisms (Shephard and Simkiss, 1978; Bansal et al., 1985). Reduced net uptake of Ca^{2+} was also found in our experiment at pH 5.6. This was in accordance with results found by Reader et al. (1989) and Sayer et al. (1991) on early life stages of brown trout. Therefore a combination of increased H^+ and Pb^{2+} could block the Ca^{2+} uptake. Mortality of the larvae at pH 5.6 in the presence of Pb was concentration-dependent. Significantly decreased hatching success was observed in larvae exposed to 0.97 μmol l^{-1} at pH 5.6. Dave and Xiu (1991) found that at concentrations of Pb < 2.3 μmol l^{-1} hatching of zebrafish (Brachydanio rerio) was slightly delayed, although no clear concentration-effect relationship was found. Delayed hatching by Pb can also be attributed to an inhibition of egg shell digesting enzyme, chorionase. This enzyme has its optimum at pH 8.5 (Hagenmaier, 1974), and its activity is reduced at a pH of 5.2 to 10% of the optimal rate. Peterson and Martin-Robichaud (1983) and Oyen (1993) have suggested that decreased trunk movements of embryos reared at low pH contribute to delayed hatching through less efficient chorionase distribution, causing delayed rupture of the chorion.

An important question remaining is whether our results on the effect of low water pH on the toxic action of Pb on the early development of carp eggs have implications for the 'no effect levels' of Pb. In The Netherlands the maximum allowable concentration of Pb in surface waters is 0.05 μmol l^{-1} Pb (Janus et al., 1991). However, this concentration has been based on waters with pH $\geq$ 7. In recent years, in many waters, particularly those with low buffer capacity, the pH has gradually decreased to values below pH 6.0. In these waters Pb concentrations up to 2.4 μmol l^{-1} have been found (Janus et al., 1991). The lowest observed effect concentration (LOEC) of Pb in our experiment was 0.12 μmol.l^{-1} at pH 5.6, which is approximately twice the maximal allowable concentration (0.05 μmol.l^{-1} Pb) for surface waters in The Netherlands.

Our studies indicate that a larger safety-margin for Pb in water should seriously be considered.

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