Effects of combined waterborne Cd and Cu exposures on ionic composition and plasma cortisol in tilapia, *Oreochromis mossambicus*

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Plasma ions and cortisol levels were studied in immature tilapia for 6 days to a range of sublethal concentrations of Cu (50, 100 and 200 μg Cu l^{-1}), Cd (20, 35 and 70 μg Cd l^{-1}) and to combinations of these metals (50 μg Cu l^{-1} + 20 μg Cd l^{-1}, 100 μg Cu l^{-1} + 35 μg Cd l^{-1} and 200 μg Cu l^{-1} + 70 μg Cd l^{-1}). Our data show that Na and Ca were markedly, although not exclusively, affected by Cu and Cd, respectively. Plasma Na concentrations were most prominently decreased in Cu-exposed fish, with less pronounced effects in Cd-exposed fish. In fish exposed to 70 μg Cd l^{-1}, the plasma Ca concentration was half of the control value. Cu-induced changes of plasma Ca concentrations were less strongly marked. In combined Cu/Cd exposed fish, Na, Ca and Cl concentrations were significantly changed. Important in the present study was the notion that, in combined Cu/Cd exposed fish, the changes in Na and Ca levels could not be explained by synergism of addition of the effects observed in single metal exposed fish. Plasma cortisol levels were increased in Cu-exposed fish, but an increase was not observed in the Cu/Cd co-exposed fish. It is argued that the absence of this cortisol response contributes to an inadequate recovery of ionic disturbances in the Cu/Cd co-exposed fish.

Key words: Cu/Cd interaction; Combination toxicology; Cortisol; Ion composition; Tilapia; Copper; Cadmium; Fish.

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Introduction

Freshwater fish take up most of the ions necessary for growth and ionic homeostasis from the water via the gills (Eddy, 1982). Branchial function is very sensitive to environmental stressors. During exposure to waterborne heavy metals, the active uptake of ions from the water is initially impaired (Laurén and McDonald, 1987; Verbost *et al.*, 1989), leading to disturbances of ionic homeostasis (McDonald *et al.*, 1989; Pratap *et al.*, 1989; McDonald and Wood, 1993). The physiological disturbances caused by Cu and Cd appear to be metal-specific. In several fish species, sublethal exposure to Cu, an essential metal, primarily affects plasma sodium and chloride concentrations (Stagg and Shuttleworth, 1982; Laurén and McDonald, 1985; Reid and McDonald, 1988; McDonald *et al.*, 1989; Muñoz *et al.*, 1991). Impaired ionic regulation was also reflected in whole body ion-composition (Laurén and McDonald, 1987; Sayer *et al.*, 1991). Exposure to Cd, a non-essential metal, however, in most cases affects Ca metabolism (Reid and McDonald, 1988; Fu *et al.*, 1989), but has occasionally been reported to affect the sodium balance (McCarty and Houston, 1976; Giles, 1984).

After some time, fish demonstrate the ability to restore whole body and plasma ion concentrations after ionic disturbances due to sublethal metal exposure (Fu *et al.*, 1989; McDonald *et al.*, 1989). The process of restoring normal ionic regulation, which is interpreted to indicate acclimation, requires energy. Cortisol regulates both energy mobilization and ion homeostasis in fish, and these functions are of particular importance during adaptation to stressors (Mazeaud *et al.*, 1977). Exposure to sublethal concentrations of Cu or Cd has been shown to result in a rise of plasma cortisol levels in several species (Donaldson and Dye, 1975; Fu *et al.*, 1989; Muñoz *et al.*, 1991), which has been interpreted as an adaptive response intended to correct the ionoregulatory and metabolic imbalance (Mazeaud *et al.*, 1977; Haux and Larsson, 1984; Laurén and McDonald, 1986; Muñoz *et al.*, 1991; Perry *et al.*, 1992).

Because of adaptive regulation mechanisms, enabling fish to respond to changed conditions, fish can usually tolerate moderately increased concentrations of one heavy metal (McDonald and Wood, 1993). However, environmental pollution is not limited to one heavy metal, and generally fish are exposed to mixtures of heavy metals. Yet, in spite of the amount of data published on the effects of waterborne exposure of Cu and Cd singly, information on the effects of Cu/Cd mixtures on aquatic organisms is limited and not uniform. In seawater herring, combined Cu/Cd exposure had an additive effect on embryonic survival and hatching success (Westernhagen *et al.*, 1979), and a more-than-additive effect of acutely lethal Cu/Cd concentrations was observed in a study with the freshwater amphipod *Gammarus lacustris* of de-March (1988). In a previous study on metal accumulation (Pelgrom *et al.*, 1994), we observed significant differences in whole body Cd burden in Cu/Cd co-exposed fish compared with fish exposed to Cd only. The question therefore arises, whether fish are able to cope with the effects of combined Cu/Cd exposure. In this study, juvenile tilapia, *Oreochromis mossambicus*, were exposed for 6 days to sublethal concentrations of Cu or Cd and combinations of these metals. To examine the adaptive performance of fish in response to single and combined metal exposures, plasma cortisol levels were related to whole body and plasma ion concentrations of the experimental fish.

Materials and Methods

Fish

Tilapia, *Oreochromis mossambicus*, were obtained from our laboratory stock. Fish were held, from 9 days after hatching, under artificial

freshwater conditions with undetectable Cu and Cd concentrations (detection levels below 0.1 and $0.01 \mu g \, l^{-1}$, respectively). The artificial freshwater consisted of demineralized water supplemented with 1.3 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 0.06 mM KCl and 0.2 mM $MgCl_2$, at pH 7.8. Composition and preparation of the water was based on the European Community (EEC) instructions for artifical water for use in toxicity studies in fish (EEC Directives 84/449/EEC Annex 5 method c1: Acute toxicity for fish). Water was continuously aerated, filtered and refreshed by means of flow-through. The light/dark regime was 12/12 hr and the water temperature 26°C. Fish were fed commercial tropical fish food Tetramin™, 2% dw/ww per day. The Cu and Cd contents of the food were: $9.86 \pm 0.16 \mu g \, Cu \, g^{-1}$ and $0.22 \pm 0.01 \mu g$ $Cd \, g^{-1}$ dry food (means $\pm$ SE; $n = 10$) (Pelgrom *et al.*, 1994).

Experimental design

Three days before the start of the experiment, 11 groups of 15 immature fish (weighing 1–2 grams, 2 months old) were placed randomly in 3.2 l small aquaria (flux chambers) filled with artificial freshwater. During the acclimation period and the metal exposure, fish were fed 2% dw/ww Tetramin™ per day. The experimental design was comparable with that of the whole body flux experiments performed by Pelgrom *et al.* (in press). Briefly, the exposure period started with the connection of each aquarium to reservoirs filled with artificial freshwater with well-defined Cu and Cd concentrations (added as nitrate, Spectrosol, BDH, U.K.). The metal concentrations, randomly distributed over the aquaria, were raised gradually to 50, 100 or $200 \mu g \, l^{-1}$ Cu, 20, 35 or $70 \mu g \, l^{-1}$ Cd or $50 + 20$, $100 + 35$, $200 + 70 \mu g \, l^{-1}$ Cu + Cd. Two groups served as controls. The metal concentrations in the water (monitored at least once daily by means of a flameless atomic absorption spectrometer [AAS, Philips PU 9200] connected with an electrothermal atomizer [Philips PU 9390X]), deviated not more than 5% of the nominal metal concentrations. After 6 days of metal exposure, fish were anaesthetized with phenoxy-ethanol (1:400). The metal exposures had no effect on the time necessary for the fish to become anaesthetized. Seven anaesthetized fish were killed in dry ice/acetone, and whole body Na, Ca and total phosphate (P) concentrations were determined (Pelgrom *et al.*, 1994). After recovery from anaesthesia, the remaining fish were returned to their own aquarium. On the next day, the fish were anaesthetized (phenoxy-ethanol), and blood from the caudal vessels was taken by means of heparinized mini-capillaries (Hirshmann). After centrifugation.

Na, Cl, Ca and cortisol concentrations were determined in the plasma. Also whole body Na, Ca and P concentrations were determined.

Blood plasma measurements

Sodium concentrations in the plasma were determined with a flame-photometric Auto Analyzer (Model IV, Technicon). The Cl concentrations were determined spectrophotometrically via the formation of ferrothiocyanate (O'Brien, 1962). Total plasma Ca concentrations were measured by means of the cresolphthalein complexone method (Sigma Diagnostics). Cortisol levels were determined by Radio Immuno Assay (RIA) as described by Balm et al. (1994).

Whole body measurements

Fish were weighed and lyophilized. After determination of the dry weight, the fish were completely treated in the following way: 1 hr at 40°C after addition of 150 μl 65% HNO_3 (Merck, ultrapur), 1 hr at 75°C after addition of 200 μl HNO_3 and subsequently dried overnight at 110°C. The samples were dissolved in 4 ml 0.1% HNO_3 (final concentration) and stored until ion analyses. Whole body Cl concentrations could not be determined in HNO_3-destructed fish. Whole body Na, Ca and P contents were determined by using an Inductive Coupled Plasma (ICP) atomic emission spectrometer (Plasma IL 200, Thermo Electron U.S.A.).

Statistics

Data are presented as means $\pm$ SE. Differences between groups were tested for significance by Student's t-test for unpaired observations. The two control groups did not differ significantly for all parameters tested, and were therefore pooled for statistical comparisons. Significant differences between metal-exposed and control fish are indicated by asterisks, whereas significant differences between single metal exposed and Cu/Cd co-exposed groups are indicated by circles, with: * or •: $P < 0.05$; ** or ••: $P < 0.02$; *** or •••: $P < 0.01$ and **** or ••••: $P < 0.001$.

Results

Sodium and chloride

The concentrations and ratios of Na and Cl in the plasma of fish exposed for 6 days to sublethal concentrations of Cu, Cd or Cu + Cd are shown in Fig. 1. Single and combined metal exposure to the highest metal concentrations tested, significantly decreased the plasma Na concentration (Fig. 1A). In fish exposed to 200 μg Cu l^{-1} + 70 μg Cd l^{-1}, the decrease in

plasma Na was similar to the effect observed in fish exposed to 200 μ Cu l^{-1} singly. Compared with controls, plasma Cl concentrations were significantly lower in all Cu-exposed groups (Fig. 1B). Decreased plasma Cl concentrations were also observed in fish exposed to 35 and 100 μg Cd l^{-1}, singly and in combination with Cu. In fish exposed to the highest Cu/Cd combination, the plasma Cl concentration was significantly lower than in single Cu or Cd exposed fish.

Na/Cl ratios were increased in fish exposed to 50 and 100, but not 200, μg Cu l^{-1} (Fig. 1C). In Cd-exposed fish, an increase in the Na/Cl ratio was only observed in the 35 μg Cd l^{-1} group. The Na/Cl ratios observed in combined Cu/Cd exposed fish were significantly different from the effects observed in the single metal exposed fish, particularly in fish co-exposed to the highest metal concentrations. In these combined Cu/Cd exposed fish, the Na/Cl ratio was significantly increased, whereas the single metal exposed fish showed no change in the Na/Cl ratio.

Metal induced differences in the whole body Na concentrations were few (Fig. 2). Only in fish exposed to 200 μg Cu l^{-1} and to 200 μg Cu l^{-1} + 70 μg Cd l^{-1} was whole body Na significantly decreased, whereas in fish exposed to 35 μg Cd l^{-1} the Na concentration was increased. At the two higher metal concentrations tested, whole body Na content in combined Cu/Cd exposed fish was comparable with those in fish exposed to Cu, but not to Cd.

Calcium

Changes in plasma Ca concentrations caused by Cu and/or Cd are shown in Fig. 3. Exposure to 50 and 100 μg Cu l^{-1} resulted in a slight, although significant, increase and decrease, respectively, in plasma Ca levels. Cd exposure to 20, 35 and 70 μg Cd l^{-1} resulted in an increase, no effect and decrease of the plasma Ca concentrations, respectively. In fish exposed to 70 μg Cd l^{-1}, plasma Ca levels were even decreased to half of the concentration observed in control fish. Compared with controls, in Cu/Cd co-exposed fish, the plasma Ca concentrations were significantly decreased in all three combinations tested. Plasma Ca levels in co-exposed fish were also significantly different from the levels in Cu (except 200 Cu) or Cd exposed fish, although apparently not in an additive or synergistic way.

The whole body Ca content (Fig. 4) was only affected in fish singly exposed to the highest Cu or Cd concentrations, whereas in combined Cu/Cd exposed fish, no effect was observed. Compared with fish exposed to 20 or 35 μg Cd l^{-1}, whole body Ca levels in the combined Cu/Cd exposed fish were significantly lower and higher, respectively. Metal exposure had no
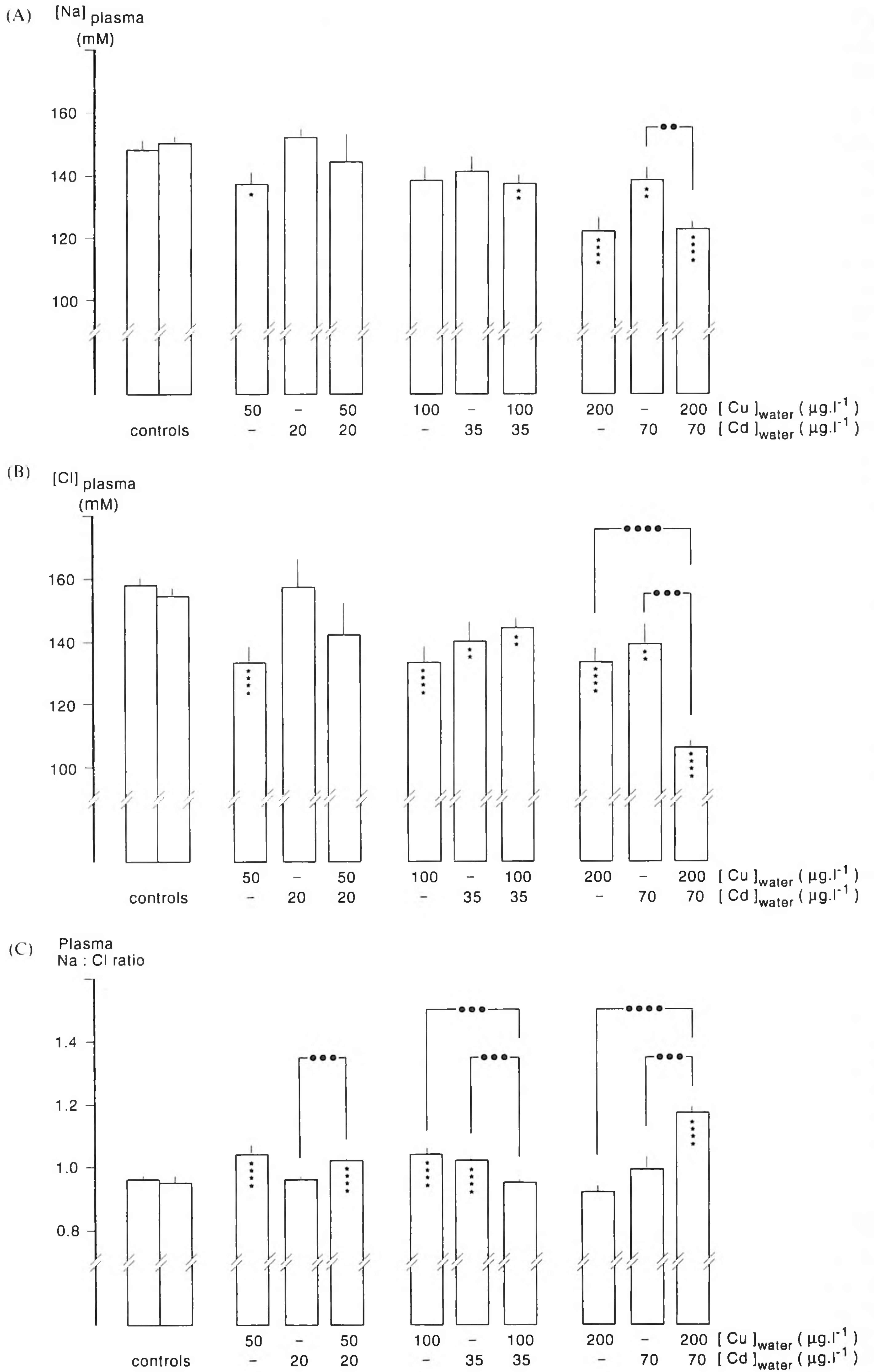
Fig. 1. Concentrations of Na (A), Cl (B) (in mM) and the ratios Na/Cl (C) in plasma of immature tilapia exposed for 6 days to sublethal concentrations of Cu and Cd, singly and in combination. Data are means ± SE (n = 5). Significant differences between control and experiment groups of fish are indicated by asterisks in the bars, whereas significant differences between single and combined metal exposed fish are indicated by closed circles between the bars.
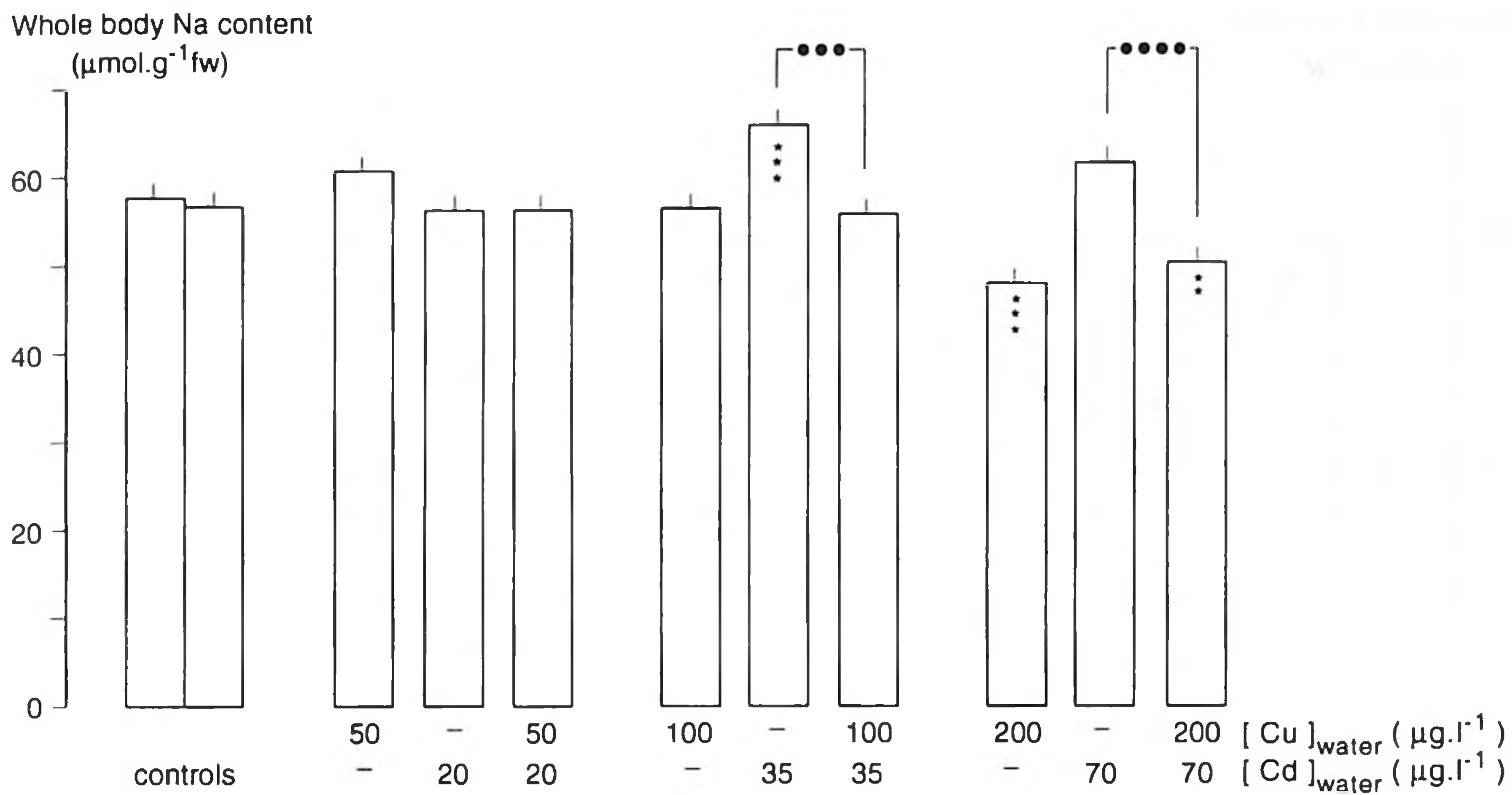
Fig. 2. Whole body Na contents (in μmol per gram fresh weight) of immature tilapia exposed for 6 days to sublethal concentrations of Cu and Cd, singly and in combination. Data are means $\pm$ SE of at least nine fish. Significant differences are indicated as described in Fig. 1.

effect on whole body P content (Table 1), except in fish exposed to 35 μg Cd l^{-1}. Metal-induced changes in the whole body Ca/P ratio (Table 1) were solely observed in fish exposed to 35 μg Cd l^{-1} or 200 μg Cu l^{-1} singly (Table 1).

Cortisol

Plasma cortisol concentrations are shown in Fig. 5. In Cu-exposed fish, but not in Cd-exposed fish, the plasma cortisol levels were increased in relation to the Cu concentration in the water. The Cu-induced increase in plasma cortisol concentration was absent in the corresponding Cu/Cd co-exposed groups of fish.

Discussion

From the results presented in this study, two major conclusions are drawn. Firstly, in many instances, the effects observed on ion composition in combined Cu/Cd exposed fish were significantly different from those observed in single metal exposed fish. Our results indicate that the effects observed during combined Cu/Cd exposure cannot be predicted from the effects observed in single metal exposed fish. Secondly, combined Cu/Cd exposure abolished the Cu-induced increase in plasma cortisol levels.
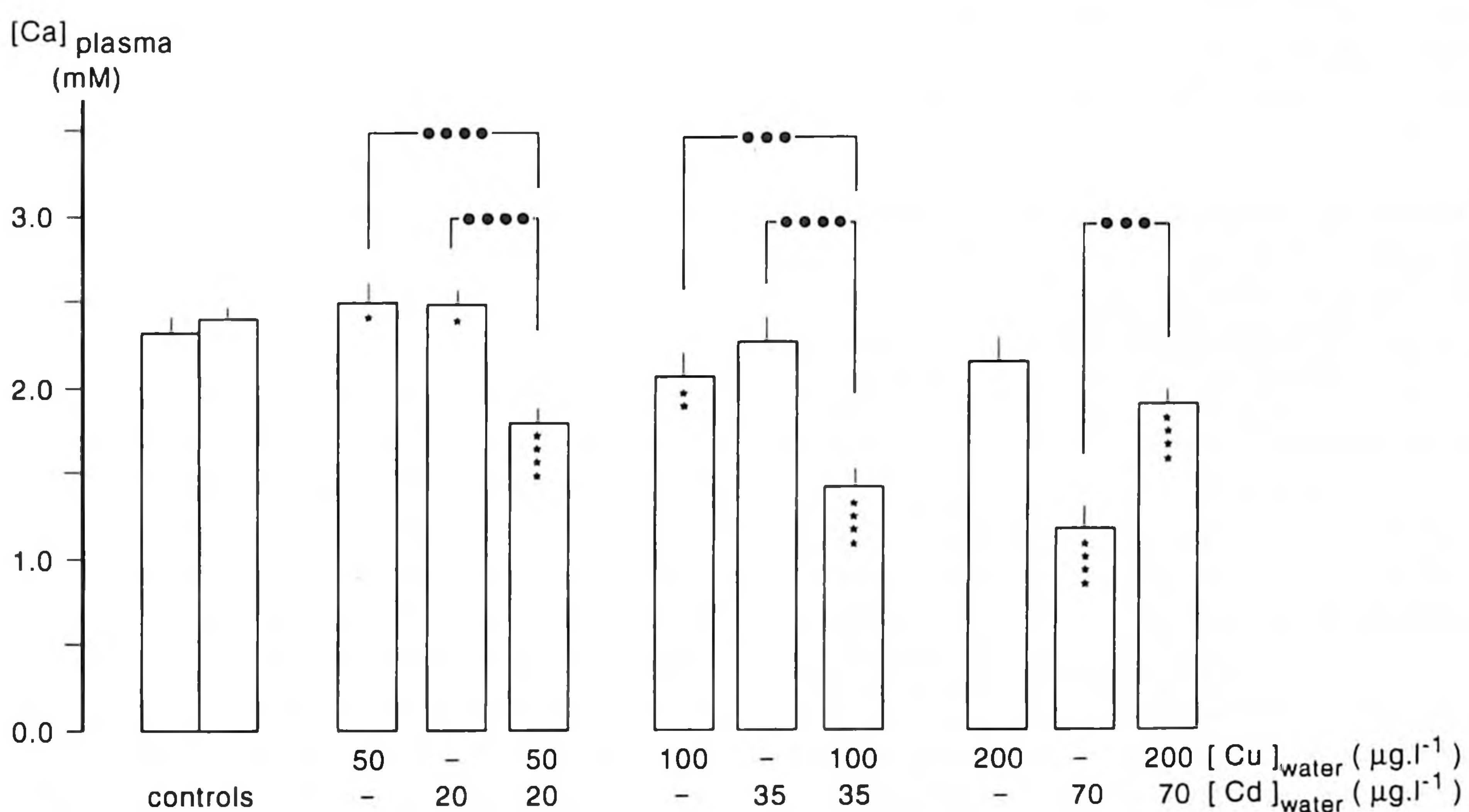

Fig. 3. Total Ca concentrations (in mM) in plasma of fish exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination. Data are means $\pm$ SE ($n = 5$). Significant differences are indicated as described in Fig. 1.
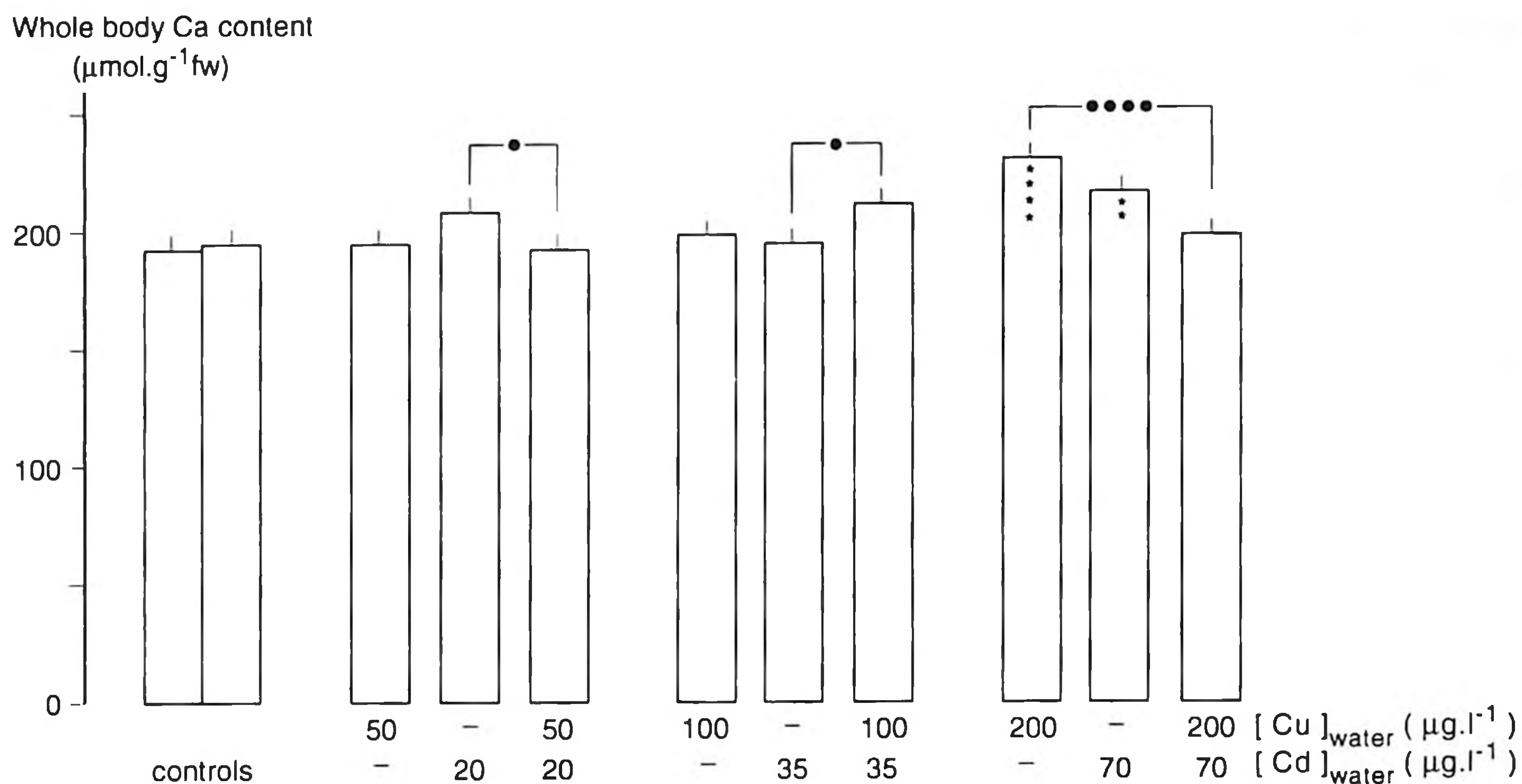
Whole body Ca content
(μmol.g^{-1}fw)

Fig. 4. Whole body Ca contents (in μmol per gram fresh weight) of immature tilapia exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination. Data are means $\pm$ SE of at least nine fish. Significant differences are indicated as described in Fig. 1.

Sodium and chloride

The observed effects of single metal exposure on ionic composition are in line with the previous results of those other investigators, who reported that the Na balance was most prominently affected by Cu, while Cd had a less pronounced effect (McDonald *et al.*, 1989; Pelgrom *et al.*, in press). A Cu-induced decrease in plasma Na concentration has also been reported for flounder (Stagg and Shuttleworth, 1982) and rainbow trout (Laurén and McDonald, 1985; Reid and McDonald, 1988; Muñoz *et al.*, 1991). In a previous study with mature tilapia, we observed a decreased plasma Na concentration after exposure to 200 μg Cu l^{-1} (Pelgrom *et al.*, in press). Upon Cd exposure, either no effect (Smith *et al.*, 1976; Christensen *et al.*, 1972) or, compared with Cu exposure, less pronounced decreases of plasma Na concentrations were

observed (Giles, 1984; Reid and McDonald, 1988; Fu *et al.*, 1989). In our study, not only single exposure to 200 μg Cu l^{-1}, but also co-exposure to 200 μg Cu l^{-1} and 70 μg Cd l^{-1} resulted in a decreased plasma Na concentration. No other data are available concerning the effects of Cu/Cd co-exposure on plasma ion composition in fish, which illustrates the necessity for further study.

The observed decreases of plasma Na levels in fish exposed to 200 μg Cu l^{-1} and 200 μg Cu l^{-1} + 70 μg Cd l^{-1} were also reflected in the whole body Na concentration. Laurén and McDonald (1987) also observed a Cu-induced decrease in whole body Na concentration after 24 hr. The Cu-induced disruption of the Na balance in their fish was reflected in plasma concentrations and in the whole body level. Laurén and McDonald (1986) previously concluded that about 75% of the total body Na content may be considerable as exchangeable. Consequently, major Cu-induced changes in the plasma Na concentration will be readily reflected in the whole body Na concentration.

In general, plasma Na and Cl tend to be similarly affected by waterborne toxicants (McDonald *et al.*, 1989). In our study, exposure to Cu as well as to Cd also resulted in a decreased plasma Cl concentration. Both Cu-induced (McKim *et al.*, 1970; Stagg and Shuttleworth, 1982; Laurén and McDonald, 1985) as well as Cd-induced (Christensen *et al.*, 1972; Giles, 1984) reduction of plasma Cl concentrations have been described. In fish exposed to the two highest combinations of Cu and Cd, plasma Cl levels were also decreased. In fish exposed to a combination of 200 μg Cu l^{-1} + μg Cd l^{-1}, the plasma Cl concentration was even significantly

Table 1. Whole body P contents (in μmol per gram fresh weight) and whole body Ca/P ratios of immature tilapia exposed for 6 days to sublethal Cu and Cd concentrations, singly and in combination

	Whole body P content (μmol g^{-1} fw)	Whole body Ca:P ratio
Controls	222 ± 8	0.92 ± 0.05
	219 ± 7	0.94 ± 0.03
50 Cu	230 ± 5	0.84 ± 0.03
20 Cd	225 ± 5	0.94 ± 0.04
50 Cu + 20 Cd	225 ± 7	0.87 ± 0.03
100 Cu	222 ± 5	0.91 ± 0.04
35 Cd	252 ± 8***	0.81 ± 0.04**
100 Cu + 35 Cd	214 ± 5	0.99 ± 0.03
200 Cu	210 ± 5	1.10 ± 0.02****
70 Cd	231 ± 6	0.93 ± 0.03
200 Cu + 70 Cd	220 ± 7	0.94 ± 0.03

Data area means ± SE of at least nine fish; significant differences are indicated as described in Fig. 1.
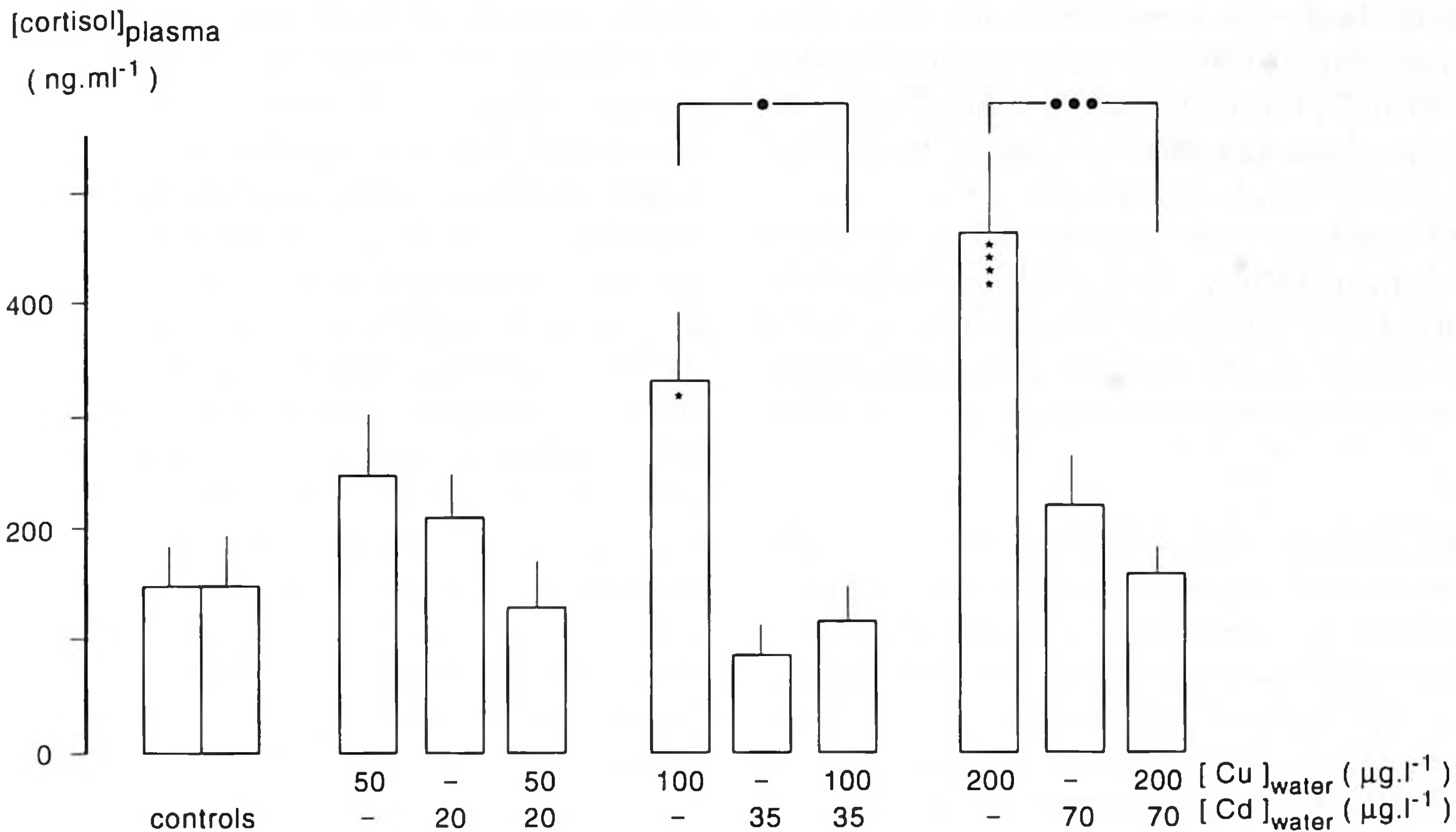
[cortisol]$_{plasma}$

(ng.ml^{-1})

Fig. 5. Plasma cortisol levels (in nonogram per ml) of immature tilapia exposed for 6 days to sublethal concentrations Cu and Cd, singly and in combination. Data are means $\pm$ SE ($n = 5$). Significant differences are indicated as described in Fig. 1.

lower than in Cu or Cd exposed fish, resulting in more-than-addition of the effects of the metals, separately.

As a consequence of their electroneutral coupling (Perry and Laurent, 1993) reduction of Cl uptake is believed to reduce HCO$_3$-excretion, while reduced Na uptake will affect Na$^+$/H$^+$ exchange. Therefore, an imbalance in the plasma Na/Cl ratio has been interpreted to reflect a disturbed acid–base regulation (Perry and Laurent, 1993). In the present study, metal-induced changes of the Na/Cl ratios were observed. In fish exposed to combinations of Cu and Cd, plasma Na/Cl ratios were significantly different from the ratios in single metal exposed fish. Disturbance in the Na/Cl ratio was most obvious in fish co-exposed to 200 μg Cu l^{-1} + 70 μg Cd l^{-1}, whereas in Cu or Cd exposed fish, Na/Cl ratios were not affected. Therefore, the change in Na/Cl ratio in combined Cu/Cd exposed fish cannot be described only by the additive or synergistic action of the metals.

Calcium

In addition to changed plasma Na and Cl concentrations, waterborne metal exposure also affected Ca-homeostasis. In fish exposed to 70 μg Cd l^{-1} plasma Ca concentrations were decreased most prominently to half of the control plasma Ca values. It has been demonstrated in rainbow trout, that Cd-induced effects on Ca apparently result from a specific reduction of Ca^{2+} uptake via the gills (Verbost *et al.*, 1989; Reid and McDonald, 1988). Cu or Cd induced impairment of the Ca balance has also been reported for rainbow trout (Giles, 1984; Reid

and McDonald, 1988), carp (Koyama and Itazawa, 1977) and tilapia (Fu *et al.*, 1989; Pelgrom *et al.*, 1995). Effects of Cu/Cd co-exposure on the ion-homeostasis have not been studied before. In all Cu/Cd co-exposed groups of fish, we observed significantly lower plasma Ca concentrations than in controls. Even more striking was the observation that the decrease of plasma Ca in Cu/Cd co-exposed fish differed from that observed in single metal exposed fish. In the presence of Cu, even low concentrations of Cd reduced plasma Ca levels, whereas this effect was absent in fish exposed to Cd alone. It should be noticed that, unlike other studies on Cd exposure (Giles, 1984; Fu *et al.*, 1989), these effects on plasma Ca concentrations were observed in immature fish.

Changes in the Ca concentration of the plasma were not reflected in the whole body Ca concentration. Reid and McDonald (1988) have demonstrated for rainbow trout, that the Ca turnover, as a percentage of the whole body Ca concentration, is about 100-fold lower than the Na turnover. In teleosts, plasma Ca represents less than 3–6% of the total body Ca (Fleming, 1974). Therefore, even a decrease to half of the control plasma Ca concentration, as observed in the present study, will not be readily reflected in whole body Ca levels. In line with this assumption and with our results, Fu *et al.* (1989) observed no Ca mobilization from bone in hypocalcemic tilapia exposed to waterborne Cd. In a previous study on Cu, we observed in fish exposed to 50 or 200 μg Cu l^{-1} no changes in whole body Ca fluxes (Pelgrom *et al.*, in press). Together with the observed effects on plasma

and whole body Ca concentrations, this illustrates that conclusions on metal-induced effects on Ca should preferably not be based solely on one of the three parameters (whole body flux, plasma Ca or whole body Ca).

Metal exposure also had no effect on whole body P concentration. As a result, in the present study, the Ca/P ratios are not different in metal exposed fish. For Cd exposed fish, comparable results have been reported by Fu *et al.* (1990).

Cortisol

In the present study, plasma cortisol levels were determined to gain insight in their adaptive performance to counteract the metal-induced disturbance of ion homeostasis. Plasma cortisol levels in the control fish were relatively high, although within the range of plasma cortisol levels observed in mature control fish. Data on plasma cortisol levels in young fish are scarce (Pottinger and Mosuwe, 1994) and we cannot, therefore, exclude the possibility that the control cortisol values measured may be characteristic of the developmental stage of the species presently used. We do not assume that they indicate stressful experimental conditions, because the high net sodium fluxes observed in fish kept under these conditions (Pelgrom *et al.*, in press) are reliable indications of well-being in this species (Dharmamba and Maetz, 1972).

Plasma cortisol levels were elevated in fish exposed to Cu, but not to Cd, which confirm observations by Donaldson and Dye (1975) on sockeye salmon and Muñoz *et al.* (1991) on rainbow trout. More important, however, is the absence of a Cu-induced cortisol response in Cu/Cd co-exposed fish, in which Cd co-accumulated with Cu (Pelgrom *et al.*, 1994). Obviously, the cortisol response induced by Cu alone was restrained in the presence of Cd, although Cd itself did not affect plasma cortisol levels. It is unlikely that the whole body Cu concentration is of sole importance for the rise in cortisol, because we previously demonstrated that whole body Cu accumulation did not differ between Cu and Cu/Cd exposed fish (Pelgrom *et al.*, 1994). Cortisol is known to affect osmotic ionic regulation, and is also released in the circulation as part of the stress response of fish (Mazeaud *et al.*, 1977). The absence of a cortisol response might be interpreted to indicate either succesful adaptation or a defect in the stress-response. This latter could result from a direct action of the metals (e.g. metal effects in the central nervous system) or alternatively could be associated with disturbed homeostasis. Obviously, the absence of a cortisol response in our Cu/Cd co-exposed fish cannot be related to successful adaptation given the present observations and our previous results showing altered whole body

water content in both Cu- as well as Cu/Cd co-exposed fish (Pelgrom *et al.*, 1994). In the present study, the plasma ion balance of Cu/Cd co-exposed fish was significantly disturbed after 6 days of metal exposure, even more than in fish exposed to Cu singly. Therefore, we interpret the ionic disturbances in Cu/Cd co-exposed fish as major consequences of the absence of a cortisol response, leading to impairment of the cortisol mediated adaptive responses in these fish. Cortisol is known to induce proliferation of chloride cells (Perry *et al.*, 1992) in the branchial and opercular epithelia of fish. This has been interpreted as a reaction of the fish to compensate for ion-losses (Fu *et al.*, 1989; Muñoz *et al.*, 1991; Perry *et al.*, 1992; Wood, 1992). In addition, cortisol also regulates carbohydrate and protein metabolism in fish (Mazeaud *et al.*, 1977). In a previous study, we observed an increase in the plasma glucose concentration and the number of opercular chloride cell numbers in Cu exposed fish (Pelgrom *et al.*, in press). This increase in plasma glucose concentration and chloride cell numbers was probably mediated by an increase in cortisol release of the head kidney. Pickering and Pottinger (1987) and McMaster *et al.* (1994) observed a suppressed cortisol response in salmonids and white sucker experiencing more than one form of stress or exposed to a complex mixture of contaminants. Our results show that a suppression of a cortisol response during exposure to combinations of environmental stressors may be a more general phenomenon than recognized previously. This demonstrates that plasma cortisol levels are not always a reliable index of environmental stress.

In conclusion, this study shows that effects on ionic composition and plasma cortisol concentrations in combined Cu/Cd exposed fish are not predictable from observations of single metal exposed fish. Since heavy metal contamination of freshwaters usually involves mixtures, our results demonstrate the need for further study on the adverse effects of Cu/Cd combinations on fish.

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