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Behavioral Alterations in Juvenile *Cyprinus carpio* (Linnaeus, 1758) Exposed to Sublethal Waterborne Cadmium

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Cadmium (Cd^{2+}) is a biologically nonessential metal that has acquired great toxicological (Waisberg et al. 2003) and ecotoxicological importance (WHO 1992). Anthropogenic activity and natural processes like weathering and erosion continuously remove the metal from its natural deposits and spread it among the different environmental compartments, with the aquatic environment being the most important place for final disposal of the different soluble forms. Cadmium effects are varied, ranging from those involving suborganismic level to those identified as alterations in the ecosystems (Wendelaar Bonga 1997; Wright and Welbourn 1994). Due to the significance of Cd^{2+} contamination in aquatic ecosystems, it is important to evaluate its effects on fish. When fish are exposed to stressors, their physiological, biochemical and behavioral parameters, among others, can be severely modified (Atchison et al. 1987; de la Torre et al. 2000; Espina et al. 2000; Shedd et al. 2001). These alterations may become useful as early biomarkers of exposure.

Changes in fish behavior appear to be among the most sensitive and earliest indicators of environmental stress conditions (Beitinger 1990). Recent investigations have focused on the impact of toxicants, such as Cd^{2+}, on the swimming activity of fish (Eissa et al. 2006). Alterations associated with forage, predator avoidance behavior, reproduction and social hierarchy can also be relevant when considering the potential impact on the fish population (Scott and Sloman 2004). Our objective was to study the alterations in the patterns of *Cyprinus carpio* moves within a 2-dimensional gradient exposed to sublethal Cd^{2+} concentrations. *C. carpio* is a recommended species for measuring and assessing aquatic toxicity.

MATERIALS AND METHODS

Juvenile *C. carpio* (3.5–5.9 g, 4-5 cm total standard length) without previous exposure to contaminants were acquired from a private fish hatchery.
Prior to use, stockfish were held in a glass aquarium supplied with continuously aerated Cl-free freshwater(FW), flowing at a rate of 15ml min^{-1}.

Assays were run at constant temperature (22 ± 2 °C) and photoperiod (12:12 light:dark). The media flow was regulated with the help of a Masterflex™

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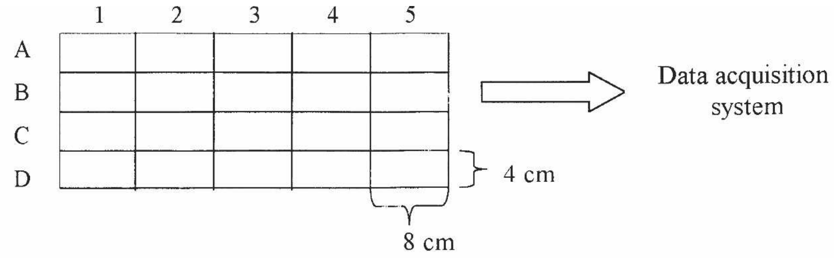

Figure 1. Schematic representation of the experimental device. Lateral section of the aquarium. The letters represent arbitrary horizontal zones; the numerals represent vertical arbitrary zones.

peristaltic pump. Media were thus renewed almost twice a day. In a previous study (Ferrari et al. 2003) it has been shown that under the conditions of the experiments here described the risk of ammonia accumulation is negligible.The experimental device was based on that used by Shirer et al. (1968).

Each glass aquarium containing 12.8 L of media and one randomly selected fish was fitted with infrared (IR) sensors and emitters on the external aquarium wall. When the IR beam was interrupted, the location of the fish was detected. These signals were recorded (once per second) by a computer and analysed using specifically designed software. Simultaneous and continuous real-time recording of fish positions were facilitated by dividing each aquarium into a grid with four horizontal and five vertical zones (Fig 1).

Experiment included three phases. Phase 1, the acclimation period, lasted 7 days while Phase 2 and 3 lasted 4 days each, during which time the animals were successively maintained in FW (Co_e) and in FW + Cd^{2+} (E); FW + Cd^{2+} medium was prepared fresh every day. Besides, a group of fish (n = 9) was simultaneously run that did not come into contact with Cd^{2+} for 15 days: 7 (acclimation period) + 4 (Co1) + 4 (Co2) days in FW.

Two Cd^{2+} concentrations (as $CdCl_2$) (analytical grade, Merck) were used: 0.3 (n = 5) and 0.5 (n = 5) mg L^{-1}. These concentrations were previously shown to be sublethal (de la Torre et al. 2000). FW and FW + Cd^{2+} media were dripped into the aquaria in zone 1.

Fish were fed Hikari Staple fish food daily at a rate of 1% bw. Food was given after recording the activity parameters. Appropriate assays showed that the feed was not contaminated with Cd^{2+}. Environmental conditions were as previously indicated. Daily assessments were carried out on media chemical parameters: pH, hardness and dissolved oxygen. Metal concentrations were analysed on samples acidified with HNO_3 (pH $\leq$ 2) by Atomic Absorption Spectrometry.

In order to avoid variations in locomotive activity due to circadian rhythms (Morgan et al. 1982; Weber and Spieler 1994) swimming parameters were recorded daily for 4 h from 10 am to 2 pm. Based on these parameters the following assessments were performed:

- *swimming activity index* (A_i), calculated from the average number of the total moves at each of the considered periods (4 days) divided by the number of total moves recorded on day *i*, where *i* = experimental day; thus a mean of daily registered A_i data was calculated. The value of A_i =1 denotes no effect while A_i<1 and A_i >1 respectively denote increases or decreases in fish activity. This index avoids the use of the large amounts of total moves.

- *lateral preference*, refers to the number of times the fish appeared in each vertical zone of the aquaria (1 to 5) (Figure 1). Results were expressed as the mean percentage of the daily records in each zone ± SEM. (Table 2).

- *altitudinal preference*, refers to the number of times the fish appeared in each horizontal zone of the aquaria (A, B, C, or D) (Figure 1). Results were expressed as the mean percentage of the daily records in each zone ± SEM. (Table 3).

Normal distribution of all data was checked using the Kolmogorov-Smirnov test. Homogeneity of variances was assessed by Bartlett test. The significance of observed differences was calculated by the analysis of variance test (ANOVA) (Zar 1996). Significance of changes in the swimming activity index was checked out by paired *t* test. The analysis of the changes in the lateral and altitudinal preferences was carried out applying the maximum likelihood test. For all these analyses Instat and Infostat Programs were used. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 indicates the values of the physicochemical water quality parameters recorded in the aquaria. Hardness as well as pH were stable during assays. DO levels are suitable for the species (de la Torre et al. 2000) causing no additional stress on animals. Actual Cd^{2+} concentrations in samples taken during the exposure period remained constant. Cd^{2+} concentration in local FW was always below the detection limit (0.01 mg L^{-1}).

Fish exposed to Cd^{2+} (n = 18) reduced their swimming activity as A_i increased (Fig 2) while no alteration was observed in the unexposed animals (n = 9). This response was identified as *hypoactivity syndrome* by Drummond and Russom (1990). The reductions were statistically significant ($p < 0.001$) in both cases. The higher variability of the responses of fish exposed to 0.5 mg. L^{-1} may be attributed to a more intense responses in their activity during the exposure to the metal. The index calculated in the control fishes (Co_e, Co1, Co2) did not show significant differences in any of the experimental periods.

The statistical analysis of the lateral preference between Co1 and Co2 fish showed no significant differences, with fish found more frequently in positions 1 and 5. The altitudinal preference followed a similar pattern, with the animals found more frequently in zones A and D (Tables 2 and 3).

The exposure of carps to Cd^{2+} did not affect the lateral preference, reflecting the same pattern found in controls. Consideration of the experimental data in overall terms shows that control fish were detected in positions 1 and 5 between 49-53% of the recordings while the rest of the time they were distributed homogeneously

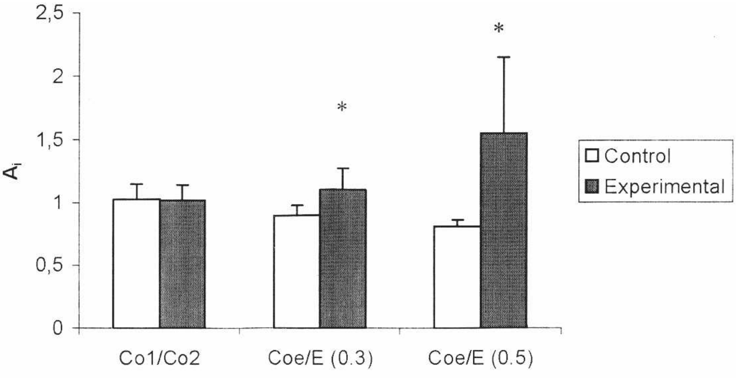

Figure 2. Swimming activity index (A_i) of *Cyprinus carpio*; Co1, Co2, (n= 9), controls in FW for 8 days; Co_e, controls in FW and exposed (E) for 4 days at 0.3 (n = 9) and 0.5 (n = 9) mg Cd L^{-1}. The average value for the period ± SEM is represented. Asterisks indicate significant differences compared to the control values.

among the remaining zones.
The analysis of the altitudinal preferences among fish exposed to Cd^{2+} showed a different pattern depending on the metal concentration. At 0.3 mg L^{-1} animals were found most frequently (75%) in positions A and B. Conversely, in 0.5 mg. L^{-1} fish were mainly distributed in positions A and D (Table 3). Statistical analysis showed significant differences ($p < 0.005$) in the positions of animals exposed to 0.3 mg Cd L^{-1} compared with their controls. Conversely, no differences were observed between those exposed to 0.5 mg Cd^{2+} L^{-1} and their controls. Adaptive behavioural alterations were observed in the swimming activity of the juvenile *Cyprinus carpio* exposed to media containing sublethal Cd^{2+}. Fish reduced their swimming activity. The monitored effects were immediately noticeable.

Table 1: Physicochemical parameters in the exposure media

Parameter	Mean ± SEM
pH	8.41 ± 0.02 (223)
DO (mg L^{-1})	8.25 ± 0.03 (224)
Hardness (mM $CaCO_3$)	0.76 ± 0.01 (192)
Cd^{2+} concentrations (mg L^{-1}):	
Nominal (0.3) – Actual:	0.328 ± 0.011 (20)
Nominal (0.5) – Actual:	0.510 ± 0.002 (20)

(n)

Besides, several authors have reported the high individual variability in the swimming activity of fish in captivity (Kolok et al. 1998; Kolok 1999). It is thus interesting to point out that in our experiments each individual was a control for itself and results were expressed using expressions that represent the average value for different indicative parameters in the daily activity of each period.

Table 2. Lateral preference of *Cyprinus carpio*.

	Co1	Co2	Co_e	E (0.3)	Co_e	E (0.5)
1	25.2 ± 3.0*	26.8 ± 3.3*	32.1 ± 2.9*	23.0 ± 3.2*	29.3 ± 3.2*	29.5 ± 5.2*
2	14.6 ± 2.2	14.5 ± 2.6	17.8 ± 3.1	15.5 ± 1.3	16.4 ± 2.3	15.4 ± 2.4
3	17.6 ± 3.0	19.6 ± 3.4	15.6 ± 4.5	15.6 ± 4.5	17.1 ± 2.3	16.8 ± 3.8
4	14.7 ± 1.7	16.6 ± 2.2	12.9 ± 2.3	12.9 ± 1.7	12.9 ± 1.5	11.2 ± 1.5
5	27.8 ± 3.0*	22.3 ± 2.5*	21.6 ± 2.7*	34.2 ± 3.1*	24.2 ± 3.7*	27.1 ± 3.8*

Co1, Co2, (n= 9) controls in FW for 8 days; controls in FW (Co_e) and exposed (E) to 0.3 (n = 9) and 0.5 (n = 9) mg Cd L^{-1} for 4 days. Vertical zones are indicated by numbers: 1: zone in which solutions drip, 5: further zone. The mean percentage of daily records in each zone at each period ± SEM is represented. Asterisks indicate significant differences among data of each column.

Table 3. Altitudinal preference of *Cyprinus carpio*.

	Co1	Co2	Co_e	E (0.3)	Co_e	E (0.5)
A	26.6 ± 4.3*	28.2 ± 4.1*	30.1 ± 4.5*	41.4 ± 4.0*	28.4 ± 3.1*	35.7 ± 3.1*
B	20.6 ± 3.8	14.9 ± 3.0	25.4 ± 2.2	33.2 ± 3.0*	18.4 ± 2.7	18.6 ± 2.7
C	21.0 ± 4.1	18.5 ± 4.1	15.3 ± 2.7	12.3 ± 2.1	18.7 ± 2.5	14.7 ± 2.5
D	31.8 ± 9.0*	38.3 ± 1.0*	29.1 ± 3.3*	13.1 ± 2.3	34.4 ± 5.0*	30.9 ± 1.1*

Co1, Co2, (n= 9) controls in FW for 8 days; controls in FW (Co_e) and exposed (E) to 0.3 (n = 5) and 0.5 (n = 4) mg Cd L^{-1} for 4 days. Horizontal zones are indicated by letters: A: surface zone , D: bottom zone. The mean percentage of daily records in each zone at each period ± SEM is represented. Asterisks indicate significant differences among data of each column.

The depressive effect of Cd^{2+} on the fish swimming activity (indicated as A_i) was observed at the assayed concentrations. Compared to their respective controls, the activity of fish decreased between 25 and 90 %. The Index in 4-day exposed fish controls was slightly lower than those recorded in 8-day controls. The differences can be interpreted as a consequence of the variability of the fish responses when kept in captivity conditions.

As regards altitudinal preference, results indicated that during the control period fish were located most of the time in the surface and bottom areas of the aquarium. During exposure to 0.3 mg L^{-1} Cd^{2+} they preferred the surface zones of the aquaria. However, at 0.5 mg L^{-1} Cd^{2+} their altitudinal preference pattern was similar to those observed in control groups.

Given that one of the target organs of Cd^{2+} is the gill, the results corresponding to the lowest concentration exposure may be interpreted as physiological compensatory responses associated with a higher demand of O_2, which takes place after damage to epithelial mechanisms of O_2 intake (Espina et al. 2000). It has recently been shown (Ferrari et al. 2005) that gills of juvenile *C. carpio* observed under the scanning electron microscope, exhibited clear signs of alterations in gill morphology after a 6-day incubation in 0.3 mg L^{-1} Cd^{2+}.

It should be mentioned that the swimming activity of fish is a sensitive adaptive behavioral biomarker (Little and Finger 1990) since the changes detected integrate the responses of multidimensional processes (biochemical, cellular and neuronal). Compared to other techniques the used testing system is easy to perform and inexpensive.
Finally, it is important to point out that non-invasive behavioral contaminant-induced responses of fish to polluted water may constitute valuable early warning signals of water quality both in laboratory and field risk assessment programs.

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