The impact of low concentrations of cadmium on host-monogenean interactions

A thesis presented for the degree of Doctor of Philosophy to the University of Stirling

By

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To Mum, Dad and Jamie

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Declaration

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted for any other degree. All sources of information have been duly acknowledged.

Signed: 

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Abstract

Interpreting data gathered from field studies that investigate the effect of pollution on fish parasites is complicated by the presence of multiple parasite species and mixed pollutant loads. The aim of this study was, therefore, to use controlled experimental studies to assess the impact of a single heavy metal, cadmium, on selected species of the monogenean genera, *Dactylogyrus* Diesing, 1850 and *Gyrodactylus* von Nordmann, 1832 and on their hosts, *Cyprinus carpio* L. and *Poecilia reticulata* (Peters). The first host-monogenean system investigated was the gill fluke *Dactylogyrus extensus* Mueller & Van Cleave, 1932 and its host *C. carpio*; the second system, *Gyrodactylus* - *P. reticulata*, involved individual investigations on *Gyrodactylus turnbulli* Harris, 1986 and *G. bullatarudis* Turnbull, 1956. Independent investigations of parasite biology and host responses were undertaken in order to elucidate host-parasite interactions in the presence of cadmium. The maximum permitted level of cadmium in controlled freshwater sources is set at 5μg/l by EEC Directive 76/464/EEC and experiments were carried out using this concentration and the higher, but environmentally realistic, levels of 20-50μg/l.

For the first time it has been demonstrated that *Dactylogyrus extensus* exposed to 5μg/l cadmium show a subtle enhancement in the *in vitro* rate of oviposition and a statistically significant enhancement in their *in vivo* rate of oviposition after 9-10 days exposure. The effect of cadmium on egg production by *D. extensus* and on selected aspects of the innate immune response of *C. carpio* were investigated in separate experiments. Although run independently of each other, the sample times

of both experiments were the same. It was found that the treatment in which the rate of oviposition by the parasite was greatest, was the treatment, where at the same sample point, the phagocytic activity of *C. carpio* was greatest. Exposure of *D. extensus* to the higher level of 30μg/l cadmium, also enhanced egg production after 9 days, but, 14-30 days exposure resulted in a lower rate of oviposition than that recorded in control parasites, suggesting that cadmium may directly, as well as indirectly, influence the reproductive biology of this parasite species.

The hatching of *D. extensus* eggs was similarly affected by cadmium exposure, with those produced and incubated in 5μg/l cadmium demonstrating a statistically significant more rapid hatch rate than the controls, while those produced and incubated at the higher concentration (30μg/l) showed a statistically significant slower rate of hatch than the controls. Exposure of adult *D. extensus* to cadmium concentrations ranging from 5 to 3,400μg/l *in vitro* resulted in an enhancement of survival above the controls. Only at 13,100μg/l cadmium was there a statistically significant reduction in survival and *in vitro* egg production.

Atomic adsorption spectrometry demonstrated that *D. extensus* is a net accumulator of cadmium, with the concentration of cadmium accumulated by the parasite, increasing with increasing exposure concentrations. The degree of cadmium accumulation in *C. carpio* organs was in the order of gills > kidney > liver > muscle=spleen. Even at 5μg/l, the level of cadmium detected in the muscle (ca. 0.2μg/g) exceeded the permitted level in food fish (0.05 mg/kg (μg/g), after only 9 days exposure.

Abstract

The haematological and immunological responses of *C. carpio* exposed to 5µg/l cadmium showed only subtle differences from the control fish, with these differences being most marked in the early stages of the trial (days 9-14). By the end of the trial there was little difference in the responses of these fish from the controls, suggesting that carp can adapt to low-level cadmium exposure. Cadmium at 50µg/l resulted in exaggerated alterations to the immune responses of carp, with statistically higher cortisol levels, phagocytic activity of kidney phagocytes, granulocyte number and significant lymphopenia, at days 6 to 14 of the trial. Several of these changes are indicative of a typical stress response. The respiratory burst and phagocytosis of kidney phagocytes in *C. carpio* appear to be linked to the level of cortisol, with both factors increasing as cortisol levels fall, suggesting that the effects of cadmium on the immune system may be mediated to some extent via the production of corticosteroids.

Cadmium at concentrations below the permitted maximum caused statistically significant increases in the population size of both *Gyrodactylus bullatarudis* Turnbull, 1956 and *G. turnbulli* Harris, 1986 on *P. reticulata*. Statistically significant differences in the population size of *G. turnbulli* were also recorded between male and female *P. reticulata*. Exposing *P. reticulata* to 5µg/l cadmium resulted in little difference in the respiratory burst of kidney phagocytes and the production of myeloperoxidase when compared to the same parameters in control fish. The phagocytic activity of these fish was, however, consistently elevated above the controls. Exposure to 20µg/l cadmium resulted in a further enhancement of both phagocytosis and respiratory burst in *P. reticulata*. Unlike, *C. carpio*, no adaptation to cadmium exposure was observed in *P. reticulata* within a 30-day period. During

the trial, subtle differences in the innate immune parameters of male and female guppies also emerged. None of the three innate immune parameters investigated, *i.e.* phagocytic activity, respiratory burst and myeloperoxidase production, appeared to correlate to the observed differences in the population growth of *G. turnbulli.*

The finding that cadmium at levels below its permitted maximum can significantly increase numbers of *Gyrodactylus* spp. to the detriment of *P. reticulata*, suggests that the water quality guidelines, with regard to the permitted concentration of this metal, should be reviewed.

This study has provided the first information on the effects of low concentrations of cadmium on selected monogenean parasites and their hosts and has been the first to attempt to elucidate host-parasite interactions in the presence of this metal. The study has provided a range of interesting findings and has offered several avenues for further investigations into a complex field of research that is still in its infancy.

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Chapter 1

General introduction

1.1. Background

1.1.1. Cadmium

Cadmium is released continually into the aquatic environment by natural processes, such as the weathering of rocks. The quantity of cadmium released into water from this source is, however, minimal compared to the amount generated from anthropogenic sources. These sources, along with a wide range of the environmental aspects of cadmium, were reviewed comprehensively by the World Health Organisation Report (1992; Environmental Criteria 135).

Cadmium has five main industrial applications, these being protective plating on steel, stabilisers for PVC, pigments in plastics and glass, electrode material in nickel-cadmium batteries and as a component of various alloys (Wilson, 1988 cited by WHO report, 1992). The WHO report (1992) stated that a major source of the cadmium released into the aquatic environment is, however, from non-ferrous mines, with contamination arising from mine drainage water, overflow from tailings ponds and rainwater run-off from general mine areas. Large quantities of cadmium are also generated as a by-product in the production of phosphate fertilisers.

Wittman (1981) reported that cadmium has become widely recognised as a harmful toxicant due to its ability to concentrate up the food chain, thereby reaching humans where it can produce both chronic and acute ailments. Indeed, Flick, Kraybill & Dimitroff (1971), reviewing the toxic effects of cadmium, commented on the

phenomenon in the late 1960s, where people living alongside the Jintsu River in Japan suffered from a cadmium-induced condition that became known as "Itai, Itai" (Japanese for "Ouch, Ouch") disease and resulted in hundreds of deaths. This disease affected mainly women and resulted in extremely brittle bones. These authors stated that the source of this cadmium was an extinct, non-ferrous metal mine that had operated in the 1800s. Hiatt & Huff (1975) reviewed the harmful effects of cadmium to humans, and these include toxicological effects on the kidneys, blood, skeleton, testes, lungs and liver, as well as carcinogenesis, teratogenesis and mutagenesis. Due to the harmful nature of cadmium, it is categorised as a List 1 substance in the Dangerous Substances Directive (76/464/EEC and Daughter Directives). Every dangerous substance has an Environmental Quality Standard (EQS), which must not be exceeded in controlled watercourses. The Environment Agency (2003) stated "*the dangerous substance is not believed to be detrimental to aquatic life at any concentration below its EQS limit*". The EQS for cadmium in freshwaters is currently 5μg/l. In the last ten years, several freshwater systems in the south west of England have failed the Dangerous Substance Directive for cadmium (Environment Agency data 1992-2002). For the purposes of confidentiality, the locations of these failures were not disclosed; however, concentrations as high as 600μg/l cadmium were recorded at freshwater sites immediately downstream of discharge sites. Aquatic systems are particularly sensitive to metal pollution due to the structure of their food chains. Biomass in aquatic systems is considerably lower than that of terrestrial systems. However, Förstner & Wittman (1981) noted that, as this small biomass occurs in a greater number of trophic levels than on land, this leads to an enhanced accumulation of pollutants. As trace quantities of toxic metals are able exert a negative influence on

plant, animal and human life, it is essential that the levels of these pollutants are monitored to avoid serious damage to the aquatic ecosystem. In Scotland the concentrations of dangerous substances are measured and regulated by the Scottish Environmental Protection Agency (SEPA).

1.1.2. Monogenean parasites

Monogeneans belong to the Phylum Platyhelminthes and are found on most groups of fishes. Cribb, Chisholm & Bray (2002) noted, however, that the greatest diversity of monogeneans is on the bony fishes with only eight families being regularly recorded on the chondrichthyans. These same authors stated how the genera *Gyrodactylus* von Nordmann, 1832 and *Dactylogyrus* Diesing, 1850 comprise at least one-third of the 3000-4000 monogenean species that have been described to date. Dactylogyrids are oviparous, with eggs giving rise to a free-swimming oncomiracidium, while gyrodactylids are viviparous and give birth to live young that attach directly to their host. Of the 1883 papers published on the Monogenea between 1984 and 2001, Cribb *et al.* (2002) noted that 21% were on *Gyrodactylus* and 15% on *Dactylogyrus*. Despite such interest in these groups, there are to date no experimental studies that have investigated the impact of individual heavy metals on these parasites and their hosts. Records of heavy metal-induced alterations on the population size of these genera do exist (Kuperman, 1992; Zharikova, 1993; El-Naggar, Hagras, Ogawa, Hussien & El-Naggar, 2000) but result from field studies where there were either mixed parasite infections, a variety of pollutants or no investigations of host immunity which make it hard to determine the exact cause of these population changes.

1.1.3. Parasites and pollution

The relationship between pollutants, including heavy metals, and outbreaks of diseases in fish has been of interest since the early seventies, when a review by Snieszko (1974) highlighted the coincidences of disease outbreaks with stress caused by pollution, temperature, sewage and eutrophication. Several reviews specifically relating to parasitic diseases of fish and pollution have since been written (see Khan & Thulin, 1991; Poulin, 1992; Overstreet, 1993; MacKenzie, Williams, Williams, McVicar & Siddall, 1995; Lafferty, 1997; Landsberg, Blakesley, Reese, McRae & Forstchen, 1998).

In 1987, Möller reviewed the effects of pollution on parasite communities and highlighted three ways that pollution can impact on host-parasite systems. Firstly, aquatic pollution can affect the parasite fauna of a definitive host directly by acting on the free-living parasite stages or on ectoparasites attached to a host, and secondly by acting indirectly on the intermediate host populations. Poulin (1992) considered that invertebrates acting as intermediate hosts for parasites, particularly planktonic crustaceans, are highly susceptible to almost any pollutant. Heavy losses of these invertebrates, as a result of pollution, are then reflected in similar reductions in the parasite population. Thirdly, pollution can affect the host directly, altering their susceptibility to parasitic infection (Möller, 1987; Poulin, 1992; Hoole, 1997). Hoole (1997) reviewed the effects of pollutants on the immune responses of fish and discussed the implications that any changes may have for their helminth parasites.

Most studies specifically focusing on parasites and heavy metals have been interested in the potential use of endoparasites as bio-indicators of pollution due to their ability to accumulate heavy metals to levels greater than those recorded in their hosts. The majority of these studies have been carried out by Sures and co-workers (Sures, Taraschewski & Jackwerth, 1994a,b,c; Sures & Taraschewski, 1995; Siddall & Sures, 1998; Sures & Siddall, 2001; Zimmerman, Sures & Taraschewski, 1999; Sures, 2001, 2002). Sures (2001) noted that parasitic bio-indicators can be subdivided into two distinct groups, the "effect indicators" and "accumulation indicators". The latter group comprise the acanthocephalans and cestodes, both with a strong accumulation capacity. In comparison to sedentary bio-indicator species, such as the freshwater mussel *Dreissena polymorpha* (Pallas, 1771) which can detect small-scale changes in pollution within a particular region, Sures, Taraschewski & Rydlo (1997) suggested that fish parasites, having a mobile host, would be able to provide information on the host's average exposure to metals within its entire natural range. The other advantages of using acanthocephalan parasites as bio-indicators were also summarised by Sures (2001). Due to the enormous accumulation capacity of acanthocephalans, Sures postulated that these parasites could detect exceedingly low concentrations of metals in water, and that the ratio of metal accumulation in host muscle and in the parasites would provide information on the duration of the exposure to metals. For instance, as acanthocephalans accumulate metals more readily than their hosts, then a high ratio, *i.e.* high accumulation of metal in the parasites and low accumulation in the host muscle, would suggest that the period of metal exposure had been short, while high metal levels in both host and parasite would indicate a longer period of exposure (Sures, 2001).

"Effect indicators" can provide information about the biotic and abiotic state of the environment through changes in their abundance and distribution. Ectoparasites act as particularly useful "effect indicators" because they are in direct contact with the host and the surrounding environment, and so respond readily to environmental perturbations. The sensitivity of monogenean communities to various pollutants has been assessed, with extensive investigations on the effect of pulp and paper mill effluent pollution on *Dactylogyrus* spp. (Koskivaara & Valtonen, 1992; Bagge & Valtonen, 1996; Siddall, Koskivaara & Valtonen, 1997; Valtonen, Holmes & Koskivaara, 1997) and the effect of oil pollution on populations of *Gyrodactylus* spp. (Khan & Kiceniuk, 1988; Marcogliese, Nagler & Cyr, 1998; Moles & Wade, 2001). However, Poulin (1992) stated that, while it is important to know what changes to parasite communities will be caused by pollutants, there are no general rules that can be applied to all situations and thus the use of population changes, as a bio-indicator of pollution, remains uncertain.

Kennedy (1997), Lafferty (1997) and Overstreet (1997) have all expressed concern about the use of fish parasites as indicators of pollution. Indeed, Kennedy (1997) highlighted a number of difficulties associated with their use as indicators of environmental change. These problems include: finding a direct causal link between pollutants and parasite community changes; the need for large sample sizes to assess parasite abundance and the need to know whether parasites are being affected directly or indirectly by changes in the host's immune status and/or metabolism. In view of the lack of definite correlation between parasite populations and communities and pollutants, it was suggested by Kennedy (1997) that parasites cannot be used as "effect indicators" of specific environmental events.

Poulin (1992) also commented on the difficulties involved with quantifying the effects of an individual pollutant on parasite populations from field studies, and how observed differences in parasite number between polluted and unpolluted sites could be attributed to differences in the abiotic and biotic factors between the two areas. In light of the lack of information directly correlating observed changes in parasite population and community structure with pollution, it was considered appropriate that the current trials would, through controlled, experimental studies, attempt to link a specific pollutant (cadmium) to changes in the population dynamics and biology of two species of monogenean genera (*Dactylogyrus* and *Gyrodactylus*), and to elucidate host-parasite interactions in the presence of this metal. While this study was not concerned with using monogeneans and their hosts as bio-indicators of cadmium pollution, the results from such a study could be used by others to help debate the usefulness of parasite systems as bio-indicators.

1.2. Objectives of the present study

As this represents the first possible study to investigate the effects of heavy metals on host-monogenean interactions, there was no previous work with which to compare the findings from this trial. It was, therefore, decided that the selected heavy metal should be one that has been used in ecotoxicology studies on a range of invertebrate and fish species and is still of great environmental concern. For this reason cadmium was chosen. The maximum permissible level (EQS) of cadmium, 5μg/l, was chosen to gain baseline data on the host-parasite interactions, followed by studies assessing the impact of the environmentally realistic concentrations of 20-50μg/l.

Interpreting results gathered from field studies investigating the effect of pollution on parasite communities is made more difficult by the presence of more than one parasite species. Indeed, Overstreet (1997) stated that interrelationships between parasites could alter either the number of individuals or species on fish, further complicating parasite-pollution studies. To avoid these complications from masking the true effect of cadmium on monogenean biology, individual monogenean-host systems were used in this study. The first system comprised the oviparous gill parasite *Dactylogyrus extensus* Mueller & Van Cleave, 1932 and its host *Cyprinus carpio* L., and the second, the study of the viviparous monogeneans of the genus *Gyrodactylus* von Nordmann, 1832 and its host *Poecilia reticulata* (Peters, 1859). For the latter, the effects of cadmium on both *Gyrodactylus turnbulli* Harris, 1986 and *G. bullatarudis* Turnbull, 1956 populations were studied. By using representatives from the genera *Dactylogyrus* and *Gyrodactylus*, the impact of cadmium on both oviparous and viviparous reproduction could be assessed. While there is limited information concerning the biology of *D. extensus* (Bauer, 1959; Bauer & Nikolskaia, 1954; Prost, 1963; Turgut, 1997), information regarding the population dynamics of *G. turnbulli* is much more widely available (Scott, 1982; Scott & Anderson, 1984; Scott & Robinson, 1984; Madhavi & Anderson, 1985; Richards & Chubb, 1998). The greater range of literature relating to *G. turnbulli* may be attributed to the ease with which it can be cultured under experimental conditions. It should be noted here that Scott's papers claimed to detail the population dynamics of *G. bullatarudis*. However, Harris (1986a) later identified this species, from specimens deposited at The Natural History Museum, London, as *G. turnbulli*.

As previously stated, pollutants can impact on monogenean parasites directly, or indirectly, by altering the immune responses of the hosts. Thus, for this study, independent investigations of the effect of cadmium on parasite biology and on host responses were carried out. The toxicity of cadmium can be affected by various factors, including pH and water hardness, and thus all trials were carried out in controlled, experimental conditions, with water quality parameters being monitored throughout.

To understand the impact of low concentrations of cadmium on the biology of *D. extensus*, the effect of various concentrations of cadmium on *in vitro* egg production and survival of this monogenean will be assessed, as will the *in vivo* egg production. The innate immune response of fish is, according to Secombes & Fletcher (1992), more important in disease resistance than the specific immune system. This study will, therefore, investigate several aspects of the innate immune response of *C. carpio*, exposed to different concentrations of cadmium over an extended period and attempt to relate the results to the biology of *D. extensus*. The host responses that are to be measured in the present study have been selected based on existing literature regarding heavy metals and the immune and haematological responses of fish (Sövényi & Szakolczai, 1993; Hutchinson & Manning, 1995; Zeilkoff, Bowser, Squibb & Frenkel, 1995; Anderson, 1996; Rice, Kergosien & Marshall Adams, 1996; Witeska, 1998; Sanchez-Dardon, Voccia, Hontela, Chilmonczyk, Dunier, Boermans, Blakley & Fournier, 1999; Bols, Brubacher, Ganassin & Lee, 2001). These parameters include phagocytosis and respiratory burst by kidney phagocytes and both total and differential blood counts. Hoole (1997) stated that it is not known whether pollutants cause immunological alterations to fish directly, or whether the

changes are caused by corticosteroids produced in a form of stress response. In view of this, the cortisol concentration of *C. carpio* will also be monitored.

The population dynamics of *G. bullatarudis* and *G. turnbulli* exposed to low cadmium concentrations will also be determined in the current study and, as for the *D. extensus*-carp system, will be linked to selected immune parameters in their host *P. reticulata.*

In taking the approach of independently investigating monogenean biology and host responses, it is hoped that the effects of low concentrations of cadmium on monogenean-host interactions can be elucidated and the direct cause-effect responses recorded.

Chapter 2

General materials and methods

The materials and methods routinely used throughout the experimental study are described below. Materials and methods specific to individual experiments will be included in the relevant chapters.

2.1. Fish

2.1.1. Common carp (*Cyprinus carpio* L.)

Common carp *C. carpio*, (length 5.0-7.5 cm) used during the course of these experiments were procured from the same fish farm in the south of England to standardise the experimental subjects as far as possible. For the purposes of confidentiality the identity of the farm will not be disclosed. On arrival, 10 fish were killed and subjected to parasitological examination. Skin scrapes were taken, gills were excised and examined and squashes were made of the internal organs. The fish were then quarantined for 2 weeks to allow them to acclimate to their new conditions. If necessary, carp were treated with an effective chemotheraputant for any unwanted parasites that were identified during the routine screening. All carp were found to be naturally infected with the gill parasite *D. extensus*. The species of dactylogyrid present on the fish was identified by removing the parasitic worms from the gills and mounting them in ammonium picrate glycerine. The hamuli and the reproductive sclerite of each specimen was then examined under ×40 to ×100 magnification and compared to the descriptions and drawings of Gusev (1985) to confirm the species. No other species of *Dactylogyrus* were observed at any time during the experiments.

Between 250 and 300 carp were transported from the fish farm to the Institute of Aquaculture, University of Stirling, in a single plastic bag placed inside a polystyrene box. With a transportation time of often greater than 12 h there were inevitably some mortalities which were screened for parasites and other diseases on their arrival. The screening of these mortalities was in addition to screening 10 carp selected from the stock at random, as described above. On most occasions fish mortalities were attributed to heavy infestations of *Chilodonella* sp. (ciliated protozoan), which generally appeared a week after the fish arrived and may have exploited the fishes stress during transportation and acclimation to new conditions. Heavy infections were treated with 50ppm of chloramine-T for 45 min in well-aerated tanks. The treatment was repeated again 3 days after the first to ensure that the infection was successfully treated. After treatments a further 5 fish were examined and the number of dactylogyrids present on the gills counted and compared to the numbers found on fish prior to treatment. A dose of chloramine-T was chosen so that it did not affect the numbers of *D. extensus* present on the fish, but, did remove the *Chilodonella* sp. infection.

All trials were carried out in the tropical aquarium facility of the Institute of Aquaculture. The fish were moved to this facility after the 2-week quarantine period and were acclimated to the system for 7 days prior to the start of any experiment.

Fish were fed to satiation daily with a commercial pelleted feed (brand name withheld for reasons of confidentiality; see Chapter 5 section 5.4).

2.1.2. Guppies (*Poecilia reticulata* Peters)

Several suppliers of guppies were sourced for a disease/parasite free stock but none were found. Several different stockists thus had to be used throughout the course of the trials. However, the fish used in each trial were always single batches of fish from one supplier. On arrival male and female fish were separated and placed in 20 litre plastic, static tanks heated to 21-23°C with Visi-therm aquarium heaters (100W) and aerated using a 4" air stone. Fifty percent of the water in each tank was replaced daily using a source of fresh de-chlorinated water heated to 21-23°C. A sub-sample (n=5) of the fish and any mortalities that occurred in the first three days after arrival were examined for parasitic infection. In most instances, the fish were infected with *Gyrodactylus* sp. and were, therefore, treated with 100ppm formalin to eradicate the infection. The exact treatment regimes carried out on guppies used for each experiment are detailed in the relevant chapters. Formalin treatments, lasting 45 min, were carried out in well-aerated static tanks. Once treated, the fish were moved to new tanks containing fresh water and were monitored over the next few hours to ensure that they had not been adversely affected by the treatment process. No formalin treatments were carried out until the fish had recovered from transportation stress which was usually 3 + days after their arrival. After formalin treatment, a further 3-5 guppies were examined for parasitic infection. Acclimation periods for each batch of experimental guppies are detailed in the relevant chapters.

Several batches of fish arrived infected with the ciliated protozoan *Tetrahymena* sp. and were treated with the same dose of formalin in an attempt to eliminate the infection. However, due to the invasive nature of the infection, the treatments

proved to be futile and the entire batch of fish was rejected on the advice of the in-house veterinarians.

Guppies were fed twice daily with Aquarian Tropical Fish Flake®. The fish were moved to the experimental system at least 7 days prior to the start of any experiment to acclimate to the new conditions. Male and female fish were only mixed in each tank 3 days prior to the start of the trials.

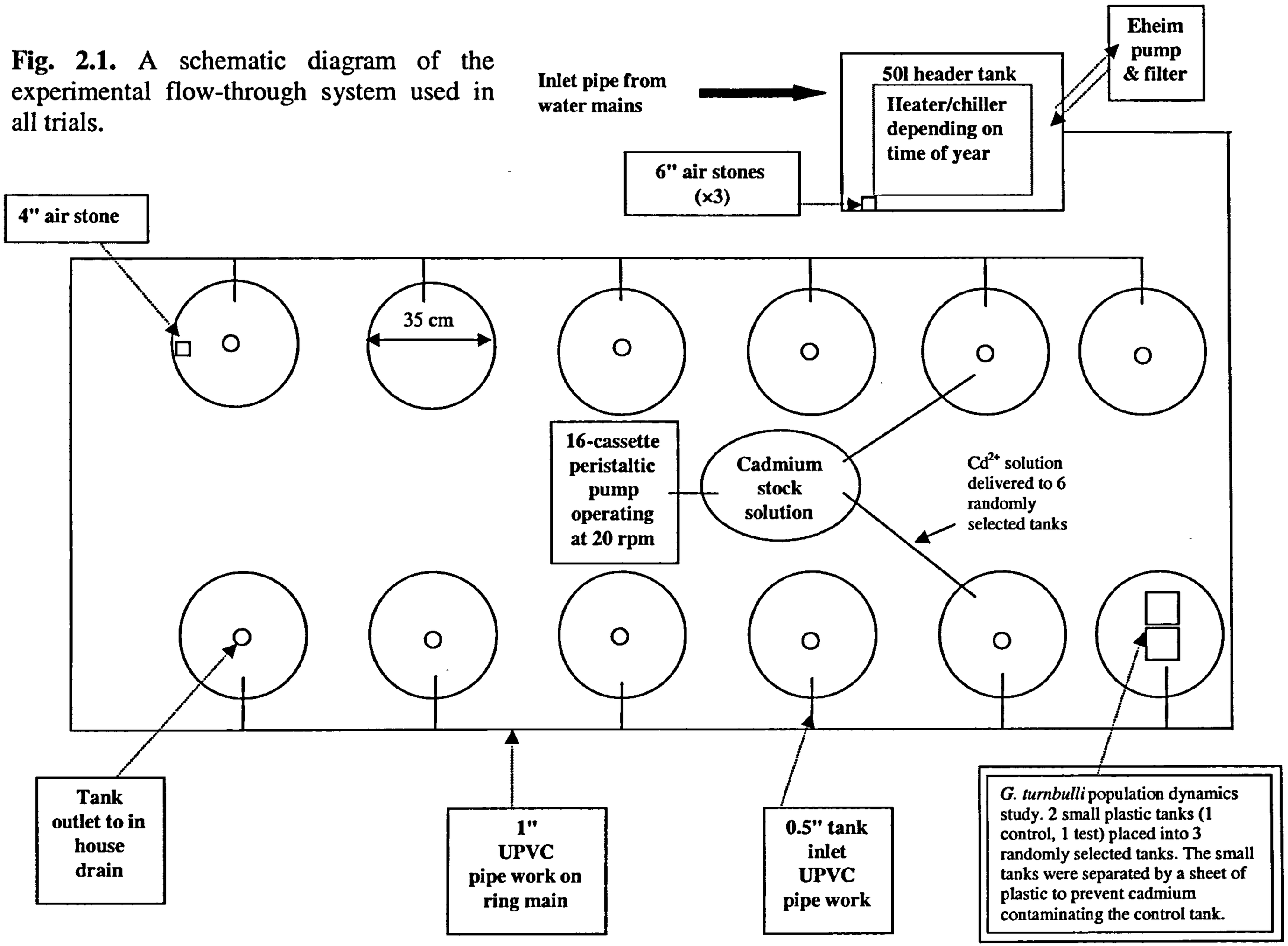
2.2. The experimental system

A flow-through system of 12, polyethylene tanks was built in the tropical aquarium facility of the Institute of Aquaculture. A galvanised steel framework was built, upon which the 12 tanks were set up in 2 rows of 6 as shown in the schematic diagram (see Fig. 2.1).

Water flowed continuously, through 1" UPVC pipework, from a 50 litre plastic header tank situated on a platform 2 m above the tanks. UPVC pipe work was used in place of PVC pipe work, as cadmium is used as a stabiliser in PVC and use of this plastic could potentially have contaminated the incoming water to the tanks. Water was obtained directly from the mains supply and was vigorously aerated in the header tank using three, 6" air stones to remove any chlorine present. A 2 litre Eheim pump and filter, packed with activated charcoal, was set up in the header tank to remove any organic matter entering the system and to further dechlorinate the water.

Each experimental tank measured 35 × 28 cm internally. Tanks were filled to a level of 20 cm giving an average volume in each tank of 22.8 litres. Each tank was

Fig. 2.1. A schematic diagram of the experimental flow-through system used in all trials.



individually fed with water from the header tank and was drained by a 0.5" central outflow pipe that emptied into a 25 litre collection tank connected by UPVC pipe work to an open drain.

Aeration in each tank was effected using 4" air stones. The airflow was adjusted to a level that did not cause distress to the fish or impede their swimming. To prevent the fish from being drawn towards the outflow pipe, a 3 mm, stiff, plastic mesh was attached around each outflow pipe and secured in place with an elastic band. The mesh was washed thoroughly each day to remove any build-up of organic waste ensuring that the water flow was unrestricted and the water quality optimal.

For the trials using carp, the outsides of each tank were covered with black plastic ensuring that the incoming light was unidirectional and standardised between the tanks. A light meter was used to verify that there was no large-scale variation in the amount of light reaching each tank. For all experiments using the guppies, the black plastic was removed from the tanks to replicate the conditions in the aquarists and holding facilities, thus minimising the stress associated with moving the guppies to the flow-through system.

Six test (cadmium-added) and 6 control (untreated) tanks were chosen at random from the 12 tanks in the system and were labelled accordingly. These tanks were maintained as controls or tests throughout all the trials to prevent contamination of control fish and parasites with the test solution of cadmium, however, their position could be moved to suit the randomised pattern of each experiment.

Water flow rates to the tanks were set using individual taps attached to each tank. Flow rates were maintained at 100 ml/min unless otherwise stated. Due to the need to keep the metal levels in the tanks constant, water flow rates were checked daily by means of a measuring cylinder to calculate the flow rate/min. Separate vessels were used for measuring the flow rates in the control and test tanks to avoid contaminating control tanks with cadmium.

The temperature of the water in each tank was maintained at ca. 16°C for the carp-*D. extensus* trials. In summer, the incoming water to the tanks was heated by the ambient air temperature of the aquarium (ca. 27°C), raising the temperature in the tanks to ca. 20°C. To regulate this it was necessary to use a Hakke EK20 water cooler fitted to the header tank. In winter, however, the air temperature of the aquarium was not sufficient to raise the 4°C incoming water to the desired 16°C level. Therefore, it was necessary to use a 3kW Howden immersion heater fitted to the header tank to obtain the desired temperature. The guppy-gyrodactylid trials were run at higher temperatures of 21-22°C. In the static tank trial, the ambient air temperature maintained the temperature of the tank water at the desired level. However, in the flow through trial it was necessary to use the immersion heater to maintain the desired temperature.

2.3. Cadmium dosing system

A 16-cassette Watson-Marlow peristaltic pump (20 rpm) was used to deliver the cadmium sulphate stock solution to the individual experimental tanks.

Both the manifold and the connecting tubing were made from Marprene® and were obtained from Watson-Marlow manufacturers. Prior to purchase, small samples of Marprene® tubing were immersed in cadmium solution (5μg/l) and left in sealed conical flasks in the warm water aquarium for 48 h. After 48 h the pieces of tubing were removed from the flask and checked for any signs of damage, such as perishing or splitting. This test was used to determine that neither the metal solution nor the running temperature of the aquarium would compromise the physical properties of the tubing.

The manifold tubing had a bore size of 0.64 mm which, when combined with the speed of the pump (20 rpm), should have delivered the cadmium solution at a rate of 0.20 ml/min. On testing, however, an average delivery rate of 0.33 ml/min was recorded, and this rate was used when calculating the concentration of each stock solution of cadmium.

The manifold tubing was positioned in the individual cassettes of the pump, so that they lay between the rollers of the pump and the cassette, such that, when the pump was turned on, the tubing would be pressed between the cassette and the rollers at regular intervals. To both ends of the manifold tubing, flexible tubing was connected using small plastic connectors. One end of the tubing was fed into the reservoir of cadmium sulphate stock solution, while the other end was fed into an experimental tank (see Fig. 2.1). The tubing in each tank was positioned next to the water inlet pipe to ensure that the cadmium stock solution would be thoroughly mixed as it entered the tank. The aeration and the movement of the fish in the tank

also ensured constant, suitable mixing of the cadmium solution throughout each tank.

2.3.1. Cadmium source

The cadmium sulphate stock solution was made up in distilled water using cadmium sulphate powder ($3CdSO_4.\ 8H_20$)(BDH Chemicals Ltd). Ten litres of stock solution was made up at a time and stored in a sealed plastic bucket. Small holes were drilled into one side of the bucket just below the lid. Connecting tubing was fed through these holes, ensuring that, in the event of the bucket being accidentally overturned, there would be minimal spillage of the stock solution. The concentration of the stock solution for each experiment was adjusted to compensate for the desired nominal concentration of cadmium, the water flow rate and the delivery rate of the stock solution via the pump and tubing.

The volume of each tank was calculated and, prior to turning the pump on, each tank was made up to the correct cadmium concentration. One litre of a 5,000μg/l cadmium sulphate solution was made and, depending on the desired concentration of cadmium, an appropriate aliquot of this added to the tanks to give a concentration equating to 5μg/l. Once added, the pump was switched on and the experiment was started.

2.4. Monitoring the concentration of cadmium

The nominal concentration of cadmium (5, 20, 30 or 50μg/l) was maintained as accurately as possible by regulating the concentration of metal in the stock solution, the flow rate of incoming water and the delivery rate of the peristaltic pump.

Samples of water (100 ml) were taken from each tank at regular intervals and stored in acid washed (10% nitric acid solution) Nalgene® bottles. Each sample was then acidified with 1% concentrated nitric acid (BDH Chemicals) (*i.e.* 1 ml conc. HNO_3/100 ml of water) and kept in the refrigerator until a time when they could be analysed.

The same Nalgene® bottles were used throughout these series of experiments and were acid washed between sample collections. To ensure that the acid washing was effective and that there was no contamination of new samples with cadmium from previous trials, a small experiment was run. Ten bottles (5 previously used for control samples and 5 for test samples (5μg/l)) were placed in acid wash for 24 h. After this time they were removed, rinsed 3 times in distilled water and then filled with 100 ml of nanopure water. Each sample was then fixed with 1% nitric acid and analysed. In all cases the cadmium in each sample was so low that it was below the detection limits of the graphite furnace atomic adsorption spectrometer, indicating that contamination of samples by this route was unlikely.

Cadmium concentrations in the water samples were analysed at 222.8nm on a Unicam 939 QZAA Spectrometer fitted with an FS90 Furnace Autosampler, with a detection limit of 0.5μg/l. Aliquots of 1 ml were taken from each water sample and placed in sample cups on the carousel of the atomic adsorption spectrometer, with up to 60 samples being analysed at one time.

Throughout all experiments the actual cadmium concentrations deviated from the nominal concentrations despite careful calculation and control. Several factors may

have been responsible for these deviations in concentration. Firstly, the amount of cadmium sulphate powder required to make up the stock solutions was very small, ranging from 0.07 g for 10 litres of 5μg/l stock solution to a maximum of only 0.42 g for 10 litres of 30μg/l stock solution. Due to these small quantities, any experimental error in weighing would have magnified errors in the final concentration of the stock solution. Secondly, during the experiments there were difficulties in setting constant water flow rates to each tank. Despite checking and adjusting the flow rates each day, there were often increases or decreases in the rate by the following day caused by changes to mains water pressure. Again, these alterations could have led to the cadmium concentrations deviating from the nominal levels. Future experiments should be run in a system where water pressure changes are minimal, giving more consistent flow rates.

2.5. Monitoring water quality parameters

Water quality within each experimental tank was monitored regularly throughout each experiment. Temperature and flow rates were monitored daily, while alkalinity, water hardness and pH were monitored approximately every 10 days. If the trial was short, then these parameters were assessed more frequently. The methods for determining these water quality parameters are given in Appendix 1.

Chapter 3

The effect of cadmium on the reproductive biology and survival of *Dactylogyrus extensus*

3.1. Introduction

Some species of dactylogyrid are often the cause of large-scale fry mortality on carp farms (Prost, 1963). These species include *Dactylogyrus vastator* Nybelin, 1924, *D. anchoratus* (Dujardin, 1845) and *D. extensus* Mueller & Van Cleave, 1932 (Prost 1963). Prost (1963) considered *D. vastator* to be the most pathogenic dactylogyrid, resulting in it being the subject of many investigations from the early twenties to the sixties, including those by Nybelin (1924), Layman (1948), Izjumova (1956) and Paperna (1963, 1964). However, studies pertaining to the biology of *D. extensus* are more scarce, with the majority of available information coming from Russian studies carried out in the late 1950s by Bauer (1959) and Bauer & Nikolskaia (1954). The findings of these authors are discussed by Prost (1963), who investigated the development and pathogenicity of both *D. extensus* and *D. anchoratus* parasitising breeding carp. While Prost's study on *D. anchoratus* was comprehensive, incorporating egg production and development studies with an investigation of the development of the parasite from egg to adult, the study on *D. extensus* was less comprehensive, lacking any information about egg production. No further studies on the biology of *D. extensus* appear to have been carried out until an unpublished MSc project at the University of Stirling by Turgut (1997).

3.1.1. Reproductive biology

3.1.1.1. Egg production by dactylogyrids

Kearn (1986) comprehensively reviewed all aspects of egg production, development and hatching in monogenean parasites. As with all dactylogyrids, *D. extensus* is an oviparous hermaphrodite, producing operculate eggs with a proteinaceous tanned egg-shell and a small "appendage" or stalk present at one end. A general description of egg assembly in all monogeneans was also provided by Kearn (1986). A single oöcyte and large numbers of vitelline cells are brought together in the oötype, where vitelline droplets, found in the cytoplasm of the vitelline cells, coalesce around the package and form the eggshell. Kearn (1986) reported that the proteinaceous shell appears white in colour but progressively turns darker as a result of an enzymatic tanning process involving phenolic substances. In the current study, the enzymatic tanning process appears to continue once the eggs of *D. extensus* are released from the parasite, with eggs changing from a light brown to a dark brown colour over several hours.

The rate of oviposition by dactylogyrids has been recorded to increase with increasing temperature, although at temperatures at the upper end of each species range, egg production often falls (Prost, 1963; Paperna, 1963; Molnár, 1971; Kashkovskii, 1982 cited by Kearn, 1986; Turgut, 1997). Several studies have investigated dactylogyrid egg production, with Paperna (1963) investigating that of *D. vastator*, Prost (1963) that of *D. anchoratus*, Molnár (1971) that of *D. lamellatus* Achmerow, 1952 and Turgut (1997) that of *D. extensus* and *D. difformis* (Wagener, 1857)/*difformoides* (Gläser et Gussev, 1971) (morphologically indistinguishable).

At 28°C, Paperna (1963) recorded an average *in vivo* oviposition rate per individual *D. vastator* of 29 eggs/24 h. The same author found that at the lower temperature of 12°C, *in vivo* egg production fell to 1.5 eggs/24 h. Interestingly, Izjumova (1956, cited by Paperna, 1963) found that the *in vivo* rate of oviposition of *D. vastator* at 12°C was greater than that recorded by Paperna at the same temperature, being 4.5 eggs/24 h. The difference in rate of oviposition between these two trials, despite being run at the same temperature, suggests inherent variability in the egg production of dactylogyrids due to adaptation to local conditions. Temperature-related increases in *in vivo* egg production have also been reported by Prost (1963) and Molnár (1971), with *D. anchoratus* producing 0.05 eggs/parasite/24 h at 5°C and 3.87 eggs/parasite/24 h at 23°C and *D. lamellatus* producing 2.5 eggs/parasite/24 h at 12°C, increasing to 15 eggs/parasite/24 h at 28°C.

In a review of the reproduction of and host-location by parasitic platyhelminthes, Whittington (1997) commented on the difference in rate of oviposition between parasites maintained *in vivo* and those maintained *in vitro*. Both Whittington (1997) and Bauer (1959, cited by Paperna, 1963) found that *in vitro* egg production in monogeneans is greater than *in vivo* production. However, Whittington (1997) reported that the longer the parasite was off the host the lower the rate of oviposition became, suggesting that as the parasite becomes starved it is unable to resource egg production. The recent work by Turgut (1997), investigating both the *in vivo* and the *in vitro* egg production by *D. extensus*, also recorded a difference between the two methods of egg production, with *in vivo* egg production by *D. extensus* at 10°C and 17°C being 0.4 and 1.4 eggs/parasite/h, compared to the higher rate of *in vitro* oviposition of 4.0 and 7.0 eggs/parasite/h, respectively.

3.1.1.2. Egg production and heavy metals

There are no data available on the effect of heavy metals or other pollutants on the reproductive biology of dactylogyrids. The only record of metal-induced alterations to parasite reproduction has been reported by Riggs & Esch (1987) and Riggs, Lemly & Esch (1987) in a fish cestode, *Bothriocephalus acheilognathi* Yamaguti, 1934 (*B. gowkongensis* Yeh, 1955), exposed to selenium. These authors found that gravid parasites from control sites shed a greater number of eggs than the parasites taken from selenium-polluted areas. However, intensive studies of this parasite species in different hosts concluded that, despite having an important impact on the biological characteristics of the parasites, selenium pollution is not as important a factor in governing growth and fecundity as the host species (Riggs & Esch, 1987; Riggs *et al.*, 1987).

Information on the impact of heavy metals on the fecundity of free-living organisms is, however, much more widely available than that concerning parasites. Gomot (1998) found that the number of eggs produced by a freshwater snail, *Lymnaea stagnalis* L., decreased with increasing cadmium concentration, ceasing at a concentration of 400μg/l. Similarly, Davies, Singhai & Wicklum (1995) recorded a negative correlation between the number of ova and spermatozoa per unit biomass in the leech *Nephelopsis obscura* Verrill, 1872 (Erpobdellidae) exposed to increasing cadmium concentrations from 0 to 580μg/l over a 24-day exposure period. A sharp decline in the fecundity of female F_1 progeny of the common housefly, *Musca domestica* L., produced by adults fed on a diet containing either chromium, cobalt, lead, cadmium, aluminium or mercury, has also been observed

by Raina, Pawar & Sharma (2001). These authors also recorded a reduction in the egg hatchability of *M. domestica*.

3.1.1.3. Egg development and hatching in dactylogyrids

Egg development in dactylogyrids can be affected by abiotic factors, such as temperature (Paperna, 1963; Prost, 1963; Turgut, 1997). For example, Paperna (1963) found that the rate of egg development in *D. vastator* was linear with temperature up to 28°C. At 37°C, however, he found that all egg development ceased, a phenomenon also recorded at 5°C. Similarly, the eggs of *D. lamellatus*, *D. anchoratus* and *D. extensus* have shown a reduction in development time with increasing temperature (Molnár, 1971; Prost, 1963; Turgut, 1997), and, at incubation temperatures close to zero, eggs of all three species ceased to develop (Prost, 1963; Molnár, 1971). The optimum temperature for egg development varies, depending on the parasite species, but is defined as that at which the greatest percentage of eggs hatch. Interestingly, two different optimum temperatures have been defined for *D. extensus*. Prost (1963) recorded an optimum temperature of 16-17°C, while Turgut (1997) recorded a temperature of 25°C. The differences in these optimum temperatures could be attributed to a range of factors, including host and parasite source and age, acclimation temperatures and the health status of both the parasite population and the hosts.

The exact method by which dactylogyrid oncomiracidia hatch from their eggs is unknown; however, a variety of hypotheses can be suggested based on investigations of other monogenean species. The most detailed study of egg hatching in monogeneans is that by Kearn (1975) on the capsalid *Entobdella soleae*

(van Beneden & Hesse, 1864) and it is possible that similar hatching mechanisms could be applied to the dactylogyrids. The work by Kearn (1975) found that the head glands of *E. soleae* secrete a proteolytic hatching fluid that dissolves the opercular cement, allowing the oncomiracidia to hatch. It has been suggested by Prost (1963) that dactylogyrids dislodge the operculum of the egg through muscular exertions. However, Kearn (1986) stated that, while muscle exertion may play a part in hatching, it is unlikely that, without prior pre-weakening of the operculum, the oncomiracidia would be strong enough to break the opercular seal. Cone (1979) suggested that just prior to the hatching of *Urocleidus adspectus* Mueller, 1936, the opercular cement is softened, possibly by the release of hatching fluid into the space between the larva and the shell. After dislodging the operculum, two fluid-filled sacs, similar to those seen in the eggs of the digenean *Fasciola hepatica* L., were seen to expand in the egg, possibly aiding the expulsion of the larvae from the egg (Cone, 1979). It is thus possible that one of these methods of hatching may apply to dactylogyrids, but, until detailed studies specific to this genus are carried out, the exact mechanism remains unknown.

3.1.1.4. Egg development, hatching and heavy metals

To date there is limited information regarding the effect of heavy metals on the development and hatching of parasite eggs. However, cadmium and zinc at 1,000-10,000μg/l have been recorded to inhibit the hatching of miracidia of the digenean *Schistosoma mansoni* Sambon, 1907 (Morley, Crane & Lewis, 2001b) and the effect of several heavy metals on encystment and excystment of the metacercariae of the digeneans *Notocotylus attenuatus* (Rudolphi, 1809) and *Parorchis acanthus* (Chamberlin & Ivie, 1942) have also been thoroughly investigated (Evans, 1982b;

Morley, Crane & Lewis, 2001a). Evans (1982b) found that exposure to copper and zinc (10ppm) significantly impaired the encystment of *N. attenuatus* cercariae, a phenomenon also recorded by Morley *et al.* (2001a) on exposure of *P. acanthus* cercariae to 25,000-50,000μg/l cadmium and zinc. Of note, however, is that the *in vitro* excystment of *P. acanthus* was not affected by exposure to any concentration of either cadmium or zinc when administered individually, but was significantly impaired when exposed to mixtures of cadmium and zinc. The impairment of excystment only occurred if cercariae were exposed to the metals while encysting; fully-formed cysts were unaffected by metal exposure, suggesting that the cyst can protect against all but the highest concentrations of cadmium and zinc (Morley *et al.*, 2001a).

As with the effect of heavy metals on egg production, information relating to free-living invertebrates and heavy metals is more extensive than the available literature for parasites. Rayms-Keller, Olson, McGaw, Oray, Carlson & Beaty (1998) found that immersing the embryos of a mosquito, *Aedes aegypti* L., in 5μg/l cadmium or 32μg/l copper inhibited embryonic development. Removing the heavy metals in part reversed the arrested development and this phenomenon was also observed by Rafiee, Matthews, Bagshaw & MacRae (1986) in the crustacean *Artemia* exposed to cadmium. The hatch success of eggs produced by the gastropod *Lymnea stagnalis* L. was also reduced to only 0.4% by exposure to 200μg/l cadmium and at the lower concentrations of 25 to 100μg/l cadmium, Gomot (1998) recorded a 5-15 day delay in the hatching of the eggs when compared to the controls.

In contrast to the above studies, cadmium increased the hatching of eggs of acridid grasshoppers (Insecta, Caelifera) when compared to the controls (ca. 60%), with the percentage hatching reaching 90.1% at 20μg/g cadmium (Devkota & Schmidt, 1999). A similar increase in hatching rate was also recorded by Amin, Rodriguez, Hernando, Comoglio, Lopez & Medesani (1998) in eggs of the southern king crab, *Lithodes santolla* (Molina) (Decapoda, Anomura), exposed to cadmium at both 0.2 and 2 mg/l. Both concentrations of cadmium produced a statistically higher hatching rate ($P<0.05$) than the controls (Amin *et al.*,1998).

3.1.2. Survival of dactylogyrids.

3.1.2.1. *In vitro* survival of dactylogyrid adults and oncomiracidia

The only figure available for the *in vitro* survival of adult dactylogyrids comes from Prost's (1963) observations on *D. anchoratus*. Prost recorded survival times of 30-50 h, although some individuals were able to survive for 75 h.

Oncomiracidia from different dactylogyrid species have shown varying survival times depending on the water temperature to which they are exposed. Paperna (1963) recorded the survival of *D. vastator* oncomiracidia to be 3-12 h at 28°C, although some did survive for 24-36 h. At 17-20°C and 10-14°C, Izjumova (1956) recorded a survival time of 10-12 h and 16-18 h, respectively, in *D. vastator* oncomiracidia. However, Turgut (1997) found that oncomiracidia of *D. extensus* survived for generally fewer than 48 h at 17°C, although some individuals were found alive after 78 h. At 25°C, she recorded a reduction in the survival to between 9-36 h. Oncomiracidia of *D. difformis/difformoides* lived twice as long at 17°C (24 h) compared to those maintained at 10°C (12 h) (Turgut, 1997).

3.1.2.2. *In vitro* survival of parasites and heavy metals

The effect of heavy metals on digenean survival has been recorded in several species, including *Schistosoma mansoni*, *Cryptocotyle lingua* Creplin, 1825, *Diplostomum spathaceum* (Rudolphi, 1819) and *Diplostomum* sp. (see Morley, Crane & Lewis, 2001b,c; Cross, Irwin & Fitzpatrick, 2001; Pietrock, Marcogliese & McLaughlin, 2002; Pietrock, Marcogliese, Meinelt & McLaughlin, 2002). There is no similar information available for dactylogyrids.

Morley *et al.* (2001c) recorded impaired survival of *D. spathaceum* cercariae when exposed to increasing concentrations of cadmium and zinc. However, at a number of low metal concentrations (10μg/l cadmium; 0.1 and 10μg/l zinc), enhanced survival of metal-exposed cercariae compared to the controls was recorded. A similar metal-induced enhancement of survival was also observed by the same authors in *S. mansoni* miracidia exposed to both cadmium and zinc (Morley *et al.*, 2001b). Mercury, chromium and mixed heavy metal pollution (copper, zinc, nickel, lead, manganese and iron) have also been documented as causing a reduction in the survival time of cercariae of *D. spathaceum* (see Pietrock *et al.*, 2002a,b) and *C. lingua* (see Cross *et al.*, 2001).

3.1.3. Aims of the present study

Little appears to be known about the responses of monogeneans in the presence of cadmium. The following chapter, therefore, presents a study on the effect of cadmium on the reproductive biology of *Dactylogyrus extensus*. The maximum permissible level of cadmium (5μg/l) was chosen to gain baseline data, followed by an increase in concentration to 30μg/l cadmium in later experiments. The aim of this

chapter is to investigate how cadmium affects the reproductive biology and survival of *D. extensus* by looking at egg production (both *in vitro* and *in vivo*), egg hatching and the survival of both adult parasites and oncomiracidia.

3.2. Materials and methods

3.2.1. Fish

The fish used in this study were common carp *C. carpio* L. These were maintained in the experimental flow-through system as described in section 2.1.1. and Figure 2.1 of the general materials and methods (Chapter 2). The targeted temperature for all egg production experiments was 16°C.

3.2.2. Sampling procedure

3.2.2.1. Experiment 1 - The effect of the maximum permissible level of cadmium (5µg/l) on *in vitro* egg production and hatching of *D. extensus* at days 9, 29 and 31 post-start of the trial.

Six tanks were selected at random from the flow-through system and designated as control tanks, and the remaining 6 tanks were designated as test tanks, *i.e.* for cadmium-exposures. Fourteen carp were randomly allocated to each of the 12 tanks and were acclimated to the experimental system for 7 days prior to the start of the trial.

A sampling schedule was planned for 10, 30 and 60 days, so that several generations of *D. extensus* could be generated and monitored. These times were chosen based on findings of Bauer & Nikolskaia (1954, cited by Prost, 1963) that at 17-19°C the post-embryonic development of *D. extensus* lasts 11 days. At each sample point, 2 tanks of each treatment were selected at random and sampled in their entirety, which took 2 days. Thus the proposed day 30 sample was begun at day 29 and due to very low parasite burdens on these carp, all remaining tanks were sampled at day 31 post-start of the trial.

The procedures for determining egg production and egg hatching are given under section 3.2.3.

3.2.2.2. Experiment 2 - The effect of the maximum permissible level of cadmium (5µg/l) on the *in vitro* egg production and hatching of *D. extensus* at days 14, 21 and 30 post-start of the trial.

The only difference between Experiments 1 and 2 was the time of the samples. Experiment 1 looked at *in vitro* egg production at days 9, 29 and 31, while Experiment 2 looked at *in vitro* egg production at days 14, 21 and 30.

Three control and 3 test tanks were used in this experiment and each tank was stocked with 25 carp. Due to heavy carp mortalities in the first batch of carp, caused by infections of *Chilodonella* sp., a second batch of fish was bought. Two tanks (C1/T1) were stocked with the first batch and a trial using these was commenced 2 weeks prior to the other 4 tanks (C2, C3, T2, T3) that were stocked with the second batch.

Two weeks after the start of the experiment, 10 fish from each tank were sampled and processed as described below. Due to deteriorating fish health, the remaining carp from C1 and T1 were sampled at 21 days rather than 30 days after the start of the trial. The remaining carp from tanks C2, C3, T2, T3 were sampled at day 30.

The procedures for determining egg production and egg hatching are given under section 3.2.3. Some parasites from this experiment were used for *in vitro* survival trials (see section 3.2.5.1.).

3.2.2.3. Experiment 3 - The effect of the maximum permissible level of cadmium (5µg/l) on both the *in vitro* and the *in vivo* egg production and on egg hatching in *D. extensus.*

Two control and 2 test tanks were each stocked with 10 carp. After 9 days, 4 fish were removed from each tank and were used for *in vivo* egg production trials as detailed below in section 3.2.4. The remaining 6 carp were left in the tanks until day 10 when they were processed for *in vitro* egg production as below. Due to the high numbers of parasites with high egg production being recorded on the fish in Experiment 1 at day 10, this time point was chosen as a suitable day for the trial in Experiment 3 to be terminated.

The procedures for determining egg production and egg hatching are given under sections 3.2.3 and 3.2.4.

3.2.2.4. Experiment 4 - The effect of 5 and 30µg/l cadmium on *in vitro* egg production and hatching in *D. extensus.*

Nine experimental tanks were chosen at random and from these 3 were designated as controls, 3 as 5µg/l cadmium tanks and 3 as 30µg/l cadmium tanks. Each tank was stocked with 30 carp. At 10, 14, 21 and 30 days post-start of the trial, 6 fish were sampled from each tank and processed for the measurement of *in vitro* egg production as described in section 3.2.3. Some parasites from this trial were used for *in vitro* survival trials (see section 3.2.5.2.).

3.2.3. *In vitro* egg production and hatching

At chosen sample times in each experiment, the *in vitro* egg production of control and cadmium-exposed *D. extensus* was assessed. At each time point, carp were killed by concussion followed by severing the spinal nerve-cord and pithing the brain. Both sets of gills were excised and placed in individual 3 cm Petri dishes that were labelled with their treatment and tank number. The gills were observed under a dissecting microscope (Olympus SZ30) at ×4 magnification and the number of both adult and juvenile parasites present on each gill was recorded. Adult parasites were classified as those with dark vitelline follicles present, while juveniles were typically opaque and lacked vitelline development.

Multi-well plates (5 ml wells, 12 wells/plate) were filled with water from either the control or test tanks and were labelled accordingly. Single parasites were carefully dissected from the gills, ensuring that each was left attached to a small piece of gill filament, and then placed in individual wells of the corresponding multi-well plates using a Pasteur pipette. The plates were then placed in an incubator (Gallenkamp cooled incubator) set at ca. 16°C and were kept in the dark for 4 h. The total egg production over this 4 h period was determined. Preliminary observations confirmed the findings of Turgut (1997) that the *in vitro* egg production of *D. extensus* was greatest in the first 3 h, with production falling at 4 h and generally ceasing after 5 h. For this reason all *in vitro* egg production trials were conducted over a 4 h period only. After 4 h, the parasites were removed from the wells and were either mounted in ammonium picrate glycerine to confirm that infections were of only a single species or were analysed to determine their cadmium content (see Chapter 5). The

well plates were returned to the incubator and screened daily using the dissecting microscope.

The day after the eggs were laid, the number in each well was counted. Leaving the counting to the following day ensured that the eggs had become enzymatically tanned and were dark brown and thus clearly visible. The number of undeveloped eggs was noted after the last developed egg had hatched. Undeveloped eggs remained a dark brown colour over the duration of the hatching experiment, while some eggs that had developed failed to hatch and it was apparent, through lack of movement, that the oncomiracidium in these eggs was dead. Every 2 days, half of the water in each well (ca. 2 ml) was removed and replaced from a fresh source using a Pasteur pipette. Fresh water was taken from the control or cadmium-exposed tanks as appropriate.

3.2.4. *In vivo* egg production

Four carp were removed from each tank (see 3.2.2.3) and placed individually into small, lidded, aerated, plastic aquaria (internal dimensions of 21.1 × 13.4cm) containing 2 litres of control or 5μg/l cadmium water.

Three large tanks (internal dimensions 70 × 52 × 45cm) were filled to a depth of 4 cm (ca. 14.6 litres) and heated to 17°C using 100W Visi-therm aquarium heaters. Two of these large tanks held 5 randomly allocated small aquaria each, while the third tank held 6 small aquaria.

After 24 h the first egg counts were made in each of the 16 small tanks. The carp were removed from the tanks and placed in a bucket containing control or test water accordingly. The water in the tanks was allowed to settle for ca. 10-15 min before the bottom of the tank was siphoned using flexible plastic tubing. This ensured that any eggs that had been disturbed from the tank bottom by the action of netting the fish would fall back to the bottom of the tank and be included in the count. Each tank was siphoned several times and the sediment collected into a 1 litre beaker. Two litres of fresh control or test water was then put into each tank and the fish returned. The above procedure was repeated for a further 2 days, after which time the fish were killed, the gills excised and the number of parasites present on each fish counted.

The sediment collected each day was screened using a dissecting microscope and the number of eggs present counted. Any dead parasites found in the sediment were also recorded. Four multi-well plates, 1 for each tank, were filled with water from the corresponding source. Batches of eggs were placed in each well of the corresponding plate and their development monitored over time. The total hatch success from each treatment was determined once the last developed egg had hatched.

3.2.5. *In vitro* survival of adult dactylogyrids and oncomiracidia

These experiments investigated the *in vitro* survival of *D. extensus* adults after both chronic and acute exposure to cadmium. Chronic exposure refers to those parasites that had been exposed *in vivo* to cadmium for a period of time between 9 and 30 days (see Experiments 2 and 4, sections 3.2.2.2 and 3.2.2.4.). Acute exposure refers

to *D. extensus* that had not been experimentally exposed to cadmium prior to the survival trial.

3.2.5.1. Experiment 2 - *In vitro* survival of adult dactylogyrids and oncomiracidia after chronic exposure to 5µg/l cadmium

Dactylogyrids collected from control carp and from the carp exposed to 5µg/l cadmium in Experiment 2 (see section 3.2.2.2) were taken at days 14 and 30 post-start of the trial and used to assess their *in vitro* survival. Multiwell plates were filled with either control water or test water (nominal 5µg/l) taken from the experimental tanks. A stock solution (1 litre) of 5000µg/l cadmium sulphate was made using control water from the experimental system and diluted to give a nominal concentration of 30µg/l cadmium. At both time points (14 & 30 days) control worm survival was assessed in control water, 5 and 30µg/l cadmium water. Similarly, test worm survival was assessed in the water from which they had been removed (nominal 5µg/l cadmium), control water and the 30µg/l cadmium water.

Four dactylogyrids from each tank were placed in individual wells of each of the 3 multi-well plates containing the 3 different concentrations of cadmium (0, 5 and 30µg/l). The plates were placed in an incubator set at 16°C and the parasites were monitored every hour for the first 3 h and then every 2 h thereafter for a total of 15 h. The dactylogyrids were observed under a dissecting microscope and considered live if they were seen moving or if they moved when gently stimulated with a blunt-ended seeker.

Control and test eggs produced in the *in vitro* egg production trial (section 3.2.2.2) were monitored daily for hatching. Individual oncomiracidia that hatched from the eggs produced at 21 and 30 days were removed from the multi-well plate using a Pasteur pipette and placed in a new well in a new plate containing water of a corresponding cadmium concentration, *i.e.* control or 5μg/l cadmium water. The plates containing the oncomiracidia were then kept in an incubator set to 16°C (the same temperature as the water into which they hatched) and were monitored daily using a dissecting microscope. Oncomiracidia were considered live if they were actively swimming or if, when floating and touched with a fine seeker, they swam off using a rapid corkscrew motion. Due to the difficulty in observing the larvae, it was often necessary to gently swirl the water in the wells by moving the well plates back and forth. The number of days for which the oncomiracidia survived was noted.

3.2.5.2. Experiment 4 - *In vitro* survival of adult dactylogyrids after chronic exposure to 5 and 30μg/l cadmium

A minimum of 2 and a maximum of 12 dactylogyrids from each tank in Experiment 4 (see section 3.2.2.4.) were used to assess the survival of control, 5 and 30μg/l cadmium-exposed dactylogyrids. A stock of artificial soft water (A.S.T.M) (hardness 40ppm $CaCO_3$) was made and stored at 4°C. From this artificial water, 1 litre stock solutions of 5 and 30μg/l cadmium were made and stored at 4°C. Before use, these stock solutions were placed in an incubator set to the temperature of the experimental tanks to ensure that the parasites were not exposed to changes in temperature, which could affect their survival. Dactylogyrids from the experimental system were removed at 9 and 30 days post-start of the trial and were placed in

multi-well plates containing artificial water of the corresponding cadmium concentration. Survival of the day 9 dactylogyrids was monitored for 45 h and the day 30 dactylogyrids were monitored for 60 h. For the first 3 h, survival was monitored hourly, after which time assessments were made every 5 h.

Oncomiracidia hatched from eggs produced in the *in vitro* egg production trial (section 3.2.2.4) were placed in individual wells of multi-well plates containing artificial water of the corresponding cadmium concentration, *i.e.* 0, 5 or 30μg/l cadmium. Oncomiracidia hatched from eggs produced at day 30 only were monitored. These larvae were monitored in the same way as in 3.2.5.1.

3.2.5.3 *In vitro* survival of adult dactylogyrids on acute exposure to cadmium

Dactylogyrids were taken from non-experimental stock carp for the following trial. A 15,000μg/l cadmium sulphate stock solution was made in artificial (A.S.T.M) soft water and diluted to give stock solutions of 5000, 500, 250 and 50μg/l cadmium.

A minimum of 18 and a maximum of 30 worms were used to monitor survival in artificial water at 0, 50, 250, 500, 5,000 and 15,000μg/l cadmium. Survival of the dactylogyrids was monitored for 75 h as detailed in 3.2.5.1. The number of eggs produced by dactylogyrids in each concentration was recorded.

3.2.6. Statistical analysis

Due to the small sample sizes and the large amount of variation in egg production of *D. extensus*, the egg production data were not normally distributed. In light of this, non-parametric tests (Kruskal-Wallis; Mann-Whitney U) were used to compare the median egg production values of parasites from individual tanks in all treatments.

Egg production data from replicate tanks within each treatment were then pooled and the Mood's median test applied. To determine if any statistical differences were apparent between the means of each treatment, the pooled data were analysed using a bootstrap 2-sample t-test. Both the Mood's median test and the bootstrap 2-sample t-test were calculated using the statistics package Quantitative Parasitology 2.0. Budapest (see Rozsa, Reiczigel & Majores, 2000).

3.3. Results

3.3.1. Water quality

The water quality parameters (mean ± S.E.) measured in each of the 4 *D. extensus* biology experiments are presented below in Table 3.1.

Table 3.1. Summary of the water quality data from all 4 *D. extensus* biology experiments.

Experiment 1	**Controls**	**Tests (5μg/l)**	
Cadmium concentration (μg/l) ± S.E. (Range) (n = 36)	0.11 ± 0.01 (0.01 - 0.212)	8.07 ± 0.32 (5.36 - 11.6)	
Temperature (°C) (n = 144)	16.14 ± 0.06	16.03 ± 0.03	
pH (n = 18)	6.38 ± 0.01	6.41 ± 0.01	
Alkalinity (meq/l) (n = 30)	0.03 ± 0.002	0.03 ± 0.0008	
Hardness (ppm $CaCO_3$) (n = 24)	26.25 ± 0.16	24.64 ± 0.18	
Experiment 2	**Controls**	**Tests (5μg/l)**	
Cadmium concentration (μg/l) ± S.E. (Range) (n = 39)	0.06 ± 0.01 (nd - 0.165)	7.45 ± 0.48 (4.67 - 11.95)	
Temperature (°C) (n = 75)	16.62 ± 0.09	16.55 ± 0.08	
pH (n = 15)	6.82 ± 0.02	6.83 ± 0.02	
Alkalinity (meq/l) (n = 15)	0.03 ± 0.0009	0.03 ± 0.001	
Hardness (ppm $CaCO_3$) (n = 21)	24.52 ± 0.71	24.77 ± 0.74	
Experiment 3	**Controls**	**Tests (5μg/l)**	
Cadmium concentration (μg/l) ± S.E. (Range) (n = 14)	0.16 ± 0.05 (nd - 0.38)	4.15 ± 0.12 (3.6 - 4.6)	
Temperature (°C) (n = 22)	16.34 ± 0.03	16.38 ± 0.03	
pH (n = 8)	6.81 ± 0.06	6.79 ± 0.04	
Alkalinity (meq/l) (n = 8)	0.03 ± 0.0009	0.03 ± 0.0007	
Hardness (ppm $CaCO_3$) (n = 8)	27.00 ± 0.42	26.88 ± 0.30	
Experiment 4	**Controls**	**Tests (5μg/l)**	**Tests (30μg/l)**
Cadmium concentration (μg/l) ± S.E. (Range)	0.28 ± 0.06 (nd - 0.68) (n = 17)	6.99 ± 0.21 (4.92 - 9.70) (n = 22)	29.17 ± 0.75 (22.56 - 36.09) (n = 20)
Temperature (°C) (n = 44)	16.78 ± 0.06	16.66 ± 0.07	16.71 ± 0.05
pH (n = 12)	7.29 ± 0.03	7.30 ± 0.03	7.30 ± 0.03
Alkalinity (meq/l) (n = 12)	0.02 ± 0.04	0.02 ± 0.05	0.02 ± 0.04
Hardness (ppm $CaCO_3$) (n = 20)	25.52 ± 0.41	25.60 ± 0.48	25.14 ± 0.64

The concentrations of cadmium in the control tanks ranged from undetectable levels (nd), *i.e.* below the detection limit of the graphite furnace AAS, to 0.378μg/l. Concentrations of cadmium in the test tanks deviated from the nominal

concentrations of 5 and 30μg/l, ranging from 3.6-11.95μg/l for the lower level and 22.56-36.09μg/l for the higher level. The lowest mean cadmium concentration (nominal 5μg/l) was observed in Experiment 3 and the highest in Experiment 1 (4.15μg/l and 8.07μg/l, respectively). Except in Experiment 4, where tank T3 (5μg/l cadmium) differed significantly from the other two 5μg/l tanks (One-way ANOVA, $P = 0.004$), there were no statistical differences in cadmium concentration within each treatment. No other water quality parameter differed significantly between any tank in any of the 4 experiments.

3.3.2. Egg production

All egg production values presented are the mean number of eggs/parasite/4 h, unless stated otherwise.

3.3.2.1. Experiment 1 - The effect of the maximum permissible level of cadmium (5μg/l) on the *in vitro* egg production of *D. extensus* at days 9, 29 and 31 post-start of trial.

Adult *D. extensus* were removed from carp maintained in control conditions and from those exposed to 5μg/l cadmium for 9, 29 and 31 days and the *in vitro* rate of oviposition of the 2 groups determined and presented in Table 3.2.

At day 9 of the trial, *D. extensus* in both cadmium-exposed tanks produced a greater number of eggs than the control dactylogyrids, 3.88 ± 0.8 eggs/parasite/4 h and 4.33 ± 0.94, compared to 1.96 ± 0.58 and 3.13 ± 0.8, in the control tanks. Similarly, the percentage of egg-producing parasites was also greater in the cadmium-exposed tanks compared to the controls. Statistically, however, the difference in the median

rate of oviposition between the control and cadmium-exposed *D. extensus* was non-significant (see Tables 3.10-3.11). Interestingly, in all 4 experiments cadmium-exposed *D. extensus* were found to produce more eggs than the control parasites at the early stages of each trial (ca. days 9-14).

At day 29, the pooled mean egg production value was statistically greater in the cadmium-exposed *D. extensus* compared to the controls (P = 0.025), producing 8.0 ± 1.5 eggs/parasite/4 h compared to 3.3 ± 1.2 eggs/parasite/4 h, respectively (Tables 3.2, 3.10-3.11).

After 31 days exposure to 5μg/l cadmium, the egg production of the cadmium-exposed *D. extensus* was lower than that of the control parasites, 1.18 ± 0.69 and 1.71 ± 0.74. However, when the non-egg-producing parasites were removed from the data-set, the cadmium-exposed parasites were observed to produce a greater number of eggs than the controls 5.2 ± 2.4 eggs/egg-producing parasite/4 h and 3.0 ± 1.1 eggs/egg-producing parasite/4 h. Neither difference was, however, found to be statistically significant (Tables 3.10-3.11).

Over time, the percentage of egg-producing *D. extensus* that were exposed to cadmium decreased from ca. 70% at day 9 to ca. 25% at day 31. The mean number of parasites found on carp from both the control and cadmium-exposed tanks was also observed to decrease in a time-dependent manner, falling from 20.8 ± 4.3 and 22.6 ± 4.1 at day 9 to 2.4 ± 0.1 and 1.9 ± 0.7 at day 31, respectively (Table 3.2).

Table 3.2. Experiment 1 - Summarised data from the *in vitro* egg production trial comparing *D. extensus* in 0 and 5µg/l cadmium at 9, 29 and 31 days post-start of the trial.

Day 9	**C1**	**C2**	**T1**	**T2**	**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	10	9	10	9	19	19
Total no. parasites	245	139	223	206	384	429
Mean no. parasites/fish	24.5 ± 7.11	15.4 ± 3.13	22.3 ± 5.86	22.9 ± 6.07	20.8 ± 4.34	22.6 ± 4.10
Total no. eggs/4 h	75	47	93	104	122	197
No. parasites used for egg production	24	24	24	24	48	48
Percentage of egg-producing adults	62.5	41.7	70.8	70.8	52.1	70.8
Mean no. eggs/parasite/4 h	3.1 ± 0.80	2.0 ± 0.58	3.9 ± 0.80	4.3 ± 0.94	2.54 ± 0.49	4.10 ± 0.61
Range of egg no.	0-13	0-9	0-12	0-18	0 - 13	0 - 18
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	4.52 ± 0.66	5.79 ± 0.67
Day 29	**C3**	**C4**	**T3**	**T4**	**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	6	6	4	8	12	12
Total no. parasites	12	22	27	20	34	47
Mean no. parasites/fish	2.0 ± 1.9	5.5 ± 1.28	6.8 ± 3.45	2.5 ± 0.57	2.8 ± 0.1	3.9 ± 1.26
Total no. eggs/4 h	5	5	25	31	10	56
No. parasites used for egg production	5	6	12	7	11	19
Percentage of egg-producing adults	33.3	33.3	25.0	57.1	33.3	41.1
Mean no. eggs/parasite/4 h	1.0 ± 1.00	0.8 ± 1.00	2.1 ± 1.35	4.4 ± 1.63	0.91 ± 0.55	2.95 ± 1.05
Range	0-5	0-4	0-15	0-10	0 - 5	0 - 15
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	3.33 ± 1.20	8.00 ± 1.50
Day 31*	**C5**	**C6**	**T5**	**T6**	**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	6	8	6	6	14	12
Total no. parasites	17	17	40	10	34	50
Mean no. parasites/fish	2.8 ± 1.45	1.6 ± 0.49	6.7 ± 1.67	1.7 ± 0.56	2.36 ± 0.68	1.92 ± 0.7
Total no. eggs/4 h	9	15	13	13	24	26
No. parasites used for egg production	9	5	12	10	14	22
Percentage of egg-producing adults	55.5	60.0	25.0	20.0	57.8	22.5
Mean no. eggs/parasite/4 h	1.0 ± 0.53	3.0 ± 1.84	1.1 ± 0.87	1.3 ± 1.19	1.71 ± 0.74	1.18 ± 0.69
Range	0-5	0-10	0-10	0-12	0 - 10	0 - 12
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	3.0 ± 1.12	5.2 ± 2.40

* Dwindling parasite numbers necessitated processing of all fish from tanks C5/T5, C6/T6 on this day.

3.3.2.2. Experiment 2 - The effect of the maximum permissible level of cadmium (5µg/l) on the *in vitro* egg production of *D. extensus* at days 14, 21 and 30 post-start of trial.

In Experiment 2, the *in vitro* rate of oviposition by control and cadmium-exposed *D. extensus* was determined at days 14, 21 and 30. Data were collected at day 21 from tanks C1 and T1, rather than at day 30, due to deteriorating fish health.

As in the early stages of Experiment 1 (day 9), cadmium-exposed *D. extensus* produced a greater number of eggs than the controls at day 14 of the trial, 3.21 ± 0.47 and 2.97 ± 0.58, respectively. However, when the mean egg production was calculated from only those parasites that produced eggs, (*i.e.* excluding the non egg-producing individuals), the controls were found to have the greatest rate of oviposition, 4.59 ± 0.87 eggs/egg-producing parasite/4 h compared to 3.75 ± 0.58 eggs/egg-producing parasite/4 h in the cadmium-exposed tanks. The pattern of greater egg production by the control *D. extensus* was also observed at day 21 of the trial, being twice as great by the controls as by the cadmium-exposed parasites (5.0 ± 1.85 and 2.4 ± 1.39, respectively). At day 30, however, cadmium-exposed parasites produced a greater number of eggs than the controls, 5.67 ± 0.47 and 5.42 ± 0.23, respectively. As with day 14 data, when the egg production was adjusted to account only for those parasites that produced eggs, the control values were greater than those from the test population, 5.91 ± 0.23 eggs/egg-producing parasite/4h and 5.67 ± 0.47 eggs/egg-producing parasite/4h, respectively. None of the differences in egg production between treatments were found to be statistically

Table 3.3. Experiment 2 - Summarised data from *in vitro* egg production trial comparing *D. extensus* in 0 and 5µg/l cadmium at 14, 21 and 30 days post- start of the trial.

Day 14	**C1**	**C2**	**C3**	**T1**	**T2**	**T3**	**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	10	7	8	10	8	8	25	26
Total no. parasites	141	191	200	145	154	251	532	550
Mean no. parasites/fish	14.1 ± 2.24	27.3 ± 4.52	25.0 ± 4.14	14.5 ± 3.42	19.3 ± 3.23	31.4 ± 5.83	21.79 ± 2.40	21.15 ± 2.73
Total no. eggs/ 4 h	39	39	17	45	31	30	95	106
No. parasites used for egg production	14	9	9	15	9	9	32	33
Percentage of egg-producing adults	57.1	100	66.7	80.0	77.8	88.9	74.6	82.2
Mean no. eggs/parasite/4 h	2.8 ± 2.92	4.3 ± 1.30	1.9 ± 0.72	3.0 ± 0.79	3.4 ± 0.93	3.3 ± 0.88	2.97 ± 0.58	3.21 ± 0.47
Range	0 - 12	0 - 5	0 - 4	0 - 6	0 - 5	0 - 4	0 - 12	0 - 6
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	×	×	4.59 ± 0.87	3.75 ± 0.58
Day 21*	**C1**	**T1**					**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	10	15					10	15
Total no. parasites	63	54					63	54
Mean no. parasites/fish	6.3 ± 0.94	3.6 ± 0.84					6.3 ± 0.94	3.6 ± 0.84
Total no. eggs/4 h	40	22					40	22
No. parasites used for egg production	8	9					8	9
Percentage of egg-producing adults	75.0	55.6					75.0	55.6
Mean no. eggs/parasite/4 h	5.0 ± 1.85	2.4 ± 1.39					5.0 ± 1.85	2.4 ± 1.39
Range	0 - 13	0 - 7					0 - 13	0 - 7
Mean no. eggs/egg-producing parasite/4 h	6.7 ± 2.04	4.4 ± 2.18					6.7 ± 2.04	4.4 ± 2.18
Day 30	**C2**	**C3**	**T2**	**T3**			**Pooled controls**	**Pooled 5µg/l cadmium**
No. fish	5	6	6	5			11	11
Total no. parasites	181	120	137	113			301	250
Mean no. parasites/fish	36.2 ± 8.99	20.0 ± 3.53	22.8 ± 8.67	22.6 ± 4.88			28.1 ± 8.1	22.7 ± 0.1
Total no. eggs/4 h	37	28	28	40			65	68
No. parasites used for egg production	6	6	6	6			12	12
Percentage of egg-producing adults	100	83.3	100	100			91.7	100
Mean no. eggs/parasite/4 h	6.2 ± 2.32	4.7 ± 1.74	4.7 ± 1.31	6.7 ± 1.82			5.42 ± 0.23	5.67 ± 0.47
Range	0 - 7	0 - 7	0 - 5	0 - 5			0 - 7	0 - 5
Mean no. eggs/egg-producing parasite /4 h	×	×	×	×			5.91 ± 0.23	5.67 ± 0.47

* Deteriorating fish health necessitated processing of all C1/T1 fish on this day.

significant (Tables 3.3, 3.10-3.11). It should be noted that the number of eggs produced by *D. extensus* in both treatments, increased over time.

Unlike in Experiment 1, there was little difference in the number of parasites on carp in tanks C2/T2 and C3/T3 at the beginning and end of the trial. Likewise, the percentage of egg-producing parasites remained high in all tanks throughout the trial (> 56%).

3.3.2.3. Experiment 3 - The effect of the maximum permissible level of cadmium on both the *in vitro* and the *in vivo* egg production in *D. extensus* at 10 days post-start of the trial.

In Experiment 3, both the *in vivo* and *in vitro* egg production by *D. extensus* maintained in control conditions and exposed to 5μg/l cadmium was assessed. The *in vivo* rate of oviposition was determined at days 9, 10 and 11 of the trial and the *in vitro* egg production at day 10 of the trial only. The results from both these trials can be found in Tables 3.4 and 3.5.

As in Experiment 1, both *in vivo* and *in vitro* egg production were greater in the cadmium-exposed *D. extensus* compared to their control counterparts. *In vitro* egg production (pooled) by cadmium-exposed parasites was 8.21 ± 1.26 compared to 6.21 ± 0.74 by the controls (Table 3.4). *In vivo* egg production in tank T1 at day 9 was twice as great (0.16 eggs/parasite/4 h) as that in control tank C1 at the same time point (0.08 eggs/parasite/4 h), while there was a 7.5-fold greater egg production in T2 compared to C2 (0.24 and 0.03 eggs/parasite/4 h). The number of eggs (pooled within treatments) produced by these cadmium-exposed parasites was

Table 3.4. Experiment 3 - Summarised data from the *in vitro* egg production trial comparing *D. extensus* in 0 and 5µg/l cadmium at 10 days post-start of the trial.

Day 10	C1	C2	T1	T2	Pooled controls	Pooled 5µg/l cadmium
Total no. eggs/4 h	85	64	28	201	149	229
No. parasites used for egg production	12	12	4	24	24	28
Percentage of egg-producing adults	100	83.3	100	95.8	91.5	97.9
Mean no. eggs/parasite/4 h	7.1 ± 1.09	5.3 ± 1.10	7.0 ± 2.48	8.4 ± 1.74	6.21 ± 0.78	8.21 ± 1.26
Range	2 -15	0 - 12	1 - 13	0 - 23	0 - 15	0 - 23
Mean no. eggs/egg-producing worm/4 h	×	×	×	×	6.77 ± 0.74	8.32 ± 1.31

Table 3.5. Experiment 3 - Summarised data from *in vivo* egg production trial comparing *D. extensus* in 0 and 5µg/l cadmium at 9, 10 and 11 days post-start of the trial.

Tank	Days post-start	No. fish	Total no. parasites	Mean no. parasites	Mean no. eggs/parasite /h	Mean no. eggs/parasite /4 h
C1	9	4	48	12.0 ± 4.6	0.02 ± 0.004	0.08
	10				0.13 ± 0.03	0.52
	11				0.34 ± 0.12	1.36
C2	9	4	25	6.3 ± 1.31	0.008 ± 0.005	0.03
	10				0.06 ± 0.01	0.24
	11				0.21 ± 0.006	0.84
T1	9	3	9	3.0 ± 1.15	0.04 ± 0.02	0.16
	10				0.15 ± 0.08	0.60
	11				0.63 ± 0.33	2.52
T2	9	4	65	16.3 ± 9.69	0.06 ± 0.01	0.24
	10				0.15 ± 0.05	0.60
	11				0.60 ± 0.29	2.40
Pooled controls	All days pooled	8	73	×	0.13 ± 0.03	0.52
Pooled 5µg/l cadmium	All days pooled	7	74	×	0.27 ± 0.08	1.08

statistically greater than the controls as shown by the Mood's median test and the bootstrap 2-sample t-test (P = 0.021 and 0.026, respectively) (Tables 3.10-3.11).

From day 9 to day 11, the *in vivo* egg production was seen to increase in all tanks in both treatments. Egg production by the parasites *in vitro* was greater than that when they were still attached to the host (*in vivo*).

3.3.2.4. Experiment 4 - The effect of 5 and 30μg/l cadmium on the *in vitro* egg production in *D. extensus*.

Experiment 4 investigated egg production by *D. extensus* in 2 different concentrations of cadmium, 5 and 30μg/l, at 9, 14, 21 and 30 days post-start of the trial. The results from this experiment are presented in Tables 3.6-3.9.

As has been seen in 2 of the previous experiments (Expts 1 & 3) discussed thus far, the egg production by cadmium-exposed *D. extensus* at day 9 was greater than the controls and increased with increasing cadmium concentration. Thus, the pooled mean number of eggs produced at day 9, by control parasites in a 4 h period, was 2.96 ± 0.57 compared to 4.17 ± 0.87 and 4.41 ± 0.88 by the 5 and 30μg/l cadmium-exposed parasites, respectively (Table 3.6). The increase in the rate of oviposition with increasing cadmium concentration was even more apparent on removing all non-egg-producing parasites from the calculations [3.89 (0μg/l), 5.76 (5μg/l) and 6.25 (30μg/l) eggs/parasite/4 h, respectively]. As was seen in Experiment 2, the number of eggs produced by *D. extensus* in all 3 treatments increased over time to 6.19 ± 1.52 in the controls and 6.45 ± 2.14 and 5.29 ± 1.14 (pooled values) in the 5 and 30μg/l cadmium-exposed parasites, respectively, at day 30. From day 14 to the end of the trial, control egg production was greater than that by *D. extensus* exposed

to 30μg/l cadmium (Tables 3.7-3.9). Indeed, at day 30, the egg production by control parasites (egg producing parasites only) was statistically greater than that of the 30μg/l parasites (egg producing parasites only) (Table 3.11). A similar pattern was seen in the 5μg/l cadmium-exposed parasites, with a lower rate of egg production than the controls from day 14 to day 21 (Tables 3.7-3.8). At day 30 the mean number of eggs produced by the 5μg/l parasites was, however, slightly greater than both the controls and the 30μg/l cadmium-exposed parasites, 6.45 ± 2.14 (5μg/l), 6.19 ± 1.52 (0μg/l) and 5.29 ± 1.00 (30μg/l) eggs/parasite/4 h, respectively (Table 3.8).

There appears to be no consistent pattern in the percentage of egg-producing *D. extensus* between the treatments in Experiment 4. However, the percentage of egg-producing adults never fell below 50% in any tank, except for test tank 1 (T1, 5μg/l cadmium) at day 30, where the percentage was zero. However, this can be attributed to there only being 1 parasite available for use in the egg production trial at this time (Table 3.9).

As in Experiment 1, the number of parasites that were being recovered from carp in all treatments decreased over the duration of Experiment 4.

Table 3.6. Experiment 4. Day 9 - Summarised data from the *in vitro* egg production trial comparing egg production by *D. extensus* maintained in 0, 5 and 30µg/l cadmium.

Day 9	Controls			5µg/l cadmium			30µg/l cadmium			Pooled		
	C1	C2	C3	T1	T2	T3	T1	T2	T3	Controls	5µg/l	30µg/l
No. fish	7	6	6	7	7	7	6	8	7	19	21	21
Total no. parasites	9	18	8	8	17	8	11	13	19	35	33	43
Mean no. parasites/fish	1.3 ± 0.57	3.0 ± 1.13	1.3 ± 0.61	1.1 ± 0.26	2.4 ± 0.48	1.1 ± 0.40	1.8 ± 0.60	1.6 ± 0.48	2.7 ± 1.08	2.37 ± 0.55	1.63 ± 0.28	1.89 ± 0.48
Total no. eggs/4 h	9	49	16	6	62	28	36	8	106	74	96	152
No. parasites used for egg production	5	12	8	3	12	8	10	8	16	25	23	34
Percentage of egg-producing adults	50.0	91.7	75.0	75.0	75.0	62.5	80.0	75.0	81.0	72.2	70.8	78.7
Mean no. eggs/parasite/4 h	1.8 ± 1.11	4.1 ± 0.92	2.0 ± 0.71	2.0 ± 1.15	5.2 ± 1.40	3.5 ± 1.28	3.6 ± 1.14	1.0 ± 0.57	6.6 ± 1.51	2.96 ± 0.57	4.17 ± 0.87	4.41 ± 0.88
Range	0-5	0-10	0-6	0-4	0-14	0-9	0- 12	0-4	0-18	0 - 10	0 -14	0 -18
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	×	×	×	×	×	3.89 ± 0.61	5.76 ± 0.91	6.25 ± 1.03

Table 3.7. Experiment 4. Day 14 - Summarised data from the *in vitro* egg production trial comparing egg production by *D. extensus* maintained in 0, 5 and 30µg/l cadmium.

Day 14	Controls			5µg/l cadmium			30µg/lcadmium			Pooled		
	C1	C2	C3	T1	T2	T3	T1	T2	T3	Controls	5µg/l	30µg/l
No. fish	8	8	8	8	7	6	6	8	8	24	21	22
Total no. parasites	10	7	13	4	7	10	11	11	14	30	21	26
Mean no. parasites/fish	1.3 ± 0.38	0.9 ± 0.3	1.6 ± 1.22	0.5 ± 0.19	1.0 ± 0.38	1.7 ± 0.8	1.8 ± 0.54	1.4 ± 0.52	1.8 ± 0.8	1.25 ± 0.44	1.0 ± 0.28	1.64 ± 0.36
Total no. eggs/4 h	43	37	69	13	27	35	33	41	66	149	75	140
No. parasites used for egg production	10	7	11	4	6	9	8	9	14	28	19	31
Percentage of egg-producing adults	60.0	71.4	90.0	100	83.3	55.6	75.0	56.6	71.4	73.8	79.6	67.7
Mean no. eggs/parasite/4 h	4.3 ± 1.75	5.3 ± 2.02	6.3 ± 1.45	3.3 ± 0.75	4.5 ± 1.61	3.9 ± 1.37	4.1 ± 1.55	4.6 ± 1.71	4.7 ± 1.29	5.25 ± 0.93	4.10 ± 0.88	4.52 ± 0.84
Range	0 -17	0-13	0-14	1-4	0-11	0-10	0-13	0-14	0-16	0 - 17	0 - 11	0 - 16
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	×	×	×	×	×	7.10 ± 1.02	5.36 ± 0.80	6.67 ± 0.91

Table 3.8. Experiment 4. Day 21 - Summarised data from the *in vitro* egg production trial comparing egg production by *D. extensus* maintained in 0, 5 and 30µg/l cadmium.

Day 21	Controls			5µg/l cadmium			30µg/l cadmium			Pooled		
	C1	C2	C3	T1	T2	T3	T1	T2	T3	Controls	5µg/l	30µg/l
No. fish	8	7	7	7	7	7	5	6	6	22	21	19
Total no. parasites	3	6	6	11	5	8	6	12	7	15	24	25
Mean no. parasites/fish	0.4 ± 0.36	0.9 ± 0.26	0.9 ± 0.26	1.6 ± 0.92	0.7 ± 0.36	1.2 ± 0.52	1.2 ± 0.58	2.0 ± 0.68	1.2 ± 0.83	0.68 ± 0.15	1.19 ± 0.36	1.47 ± 0.40
Total no. eggs/4 h	18	36	24	71	24	58	29	70	29	78	153	128
No. parasites used for egg production	3	6	5	11	5	9	6	13	6	14	25	25
Percentage of egg-producing adults	66.7	83.3	60.0	100	100	87.5	83.3	92.3	66.7	70	95.8	80.8
Mean no. eggs/parasite/4 h	6.0 ± 3.79	6.0 ± 2.39	4.8 ± 2.75	6.5 ± 1.24	4.8 ± 1.50	6.4 ± 1.93	4.8 ± 2.10	5.4 ± 1.50	4.8 ± 1.99	6.73 ± 1.87	6.12 ± 0.91	5.12 ± 1.00
Range	0-13	0-13	0-12	1-14	2-10	0-17	0-14	0-18	0-12	0 - 13	0 - 17	0 - 18
Mean no. eggs/egg-producing parasite/4h	×	×	×	×	×	×	×	×	×	8.67 ± 1.55	6.65 ± 0.91	6.10 ± 1.07

Table 3.9. Experiment 4. Day 30 - Summarised data from the *in vitro* egg production trial comparing egg production by *D. extensus* maintained in 0, 5 and 30µg/l cadmium.

Day 30	Controls			5µg/l cadmium			30µg/l cadmium			Pooled		
	C1	C2	C3	T1	T2	T3	T1	T2	T3	Controls	5µg/l	30µg/l
No. fish	8	5	8	7	7	7	8	8	8	21	21	24
Total no. parasites	7	4	8	1	8	6	6	4	5	19	15	15
Mean no. parasites/fish	0.9 ± 0.55	0.8 ± 0.37	1.0 ± 0.50	0.1	1.1 ± 0.55	0.9 ± 0.46	0.7 ± 0.42	0.5 ± 0.30	0.6 ± 0.26	0.76 ± 0.23	0.71 ± 0.25	0.63 ± 0.19
Total no. eggs/4 h	52	33	25	0	56	15	25	12	37	109	71	74
No. parasites used for egg production	7	4	5	1	8	2	5	4	5	16	11	14
Percentage of egg-producing adults	71.4	75.0	60.0	0	62.5	50.0	80.0	100	100	68.8	37.5	93.3
Mean no. eggs/parasite/4 h	7.4 ± 2.93	8.3 ± 4.33	5.0 ± 2.14	×	7.0 ± 2.49	7.5 ± 7.5	5.0 ± 1.67	3.0 ± 1.22	7.4 ± 2.42	6.19 ± 1.52	6.45 ± 2.14	5.29 ± 1.14
Range	0 -17	0 -18	0 -10	×	0-18	0-15	0-10	1-6	1-15	0 - 18	0 - 18	0 - 15
Mean no. eggs/egg-producing parasite/4 h	×	×	×	×	×	×	×	×	×	10.0 ± 1.81	11.83 ± 2.02	5.69 ± 1.15

Table 3.10. Statistical summary of egg production by *D. extensus* in 0 and 5μg/l cadmium in Experiments 1-3 and exposed to 0, 5 and 30μg/l cadmium in Experiment 4. Experiment 3 incorporated a study of both *in vivo* and *in vitro* egg production. Figures that are statistically significant are in bold.

Experiment	Day of sample	Kruskal - Wallis P		Mood's median test P
1	9	0.140		1.000
	29	0.349		0.061
	31	0.268		1.000
2	14	0.586		0.386
	21	0.699		0.567
	30	0.182		1.000
3 *In vivo* egg production	9	0.158		**0.021**
	10	0.284		0.160
	11	0.420		0.168
3 *In vitro* egg production	10	0.461		0.246
Experiment	**Day of sample**	**Kruskal - Wallis P**	**Comparisons**	**Mood's median test P**
4	9	0.135	Control *vs* 5μg/l	0.182
			Control *vs* 30μg/l	0.543
			5μg/l *vs* 30μg/l	0.748
	14	0.961	Control *vs* 5μg/l	0.296
			Control *vs* 30μg/l	0.058
			5μg/l *vs* 30μg/l	0.491
	21	0.987	Control *vs* 5μg/l	0.433
			Control *vs* 30μg/l	0.123
			5μg/l *vs* 30μg/l	0.548
	30	0.980	Control *vs* 5μg/l	0.335
			Control *vs* 30μg/l	0.414
			5μg/l *vs* 30μg/l	0.487

Table 3.11. Bootstrap 2-sample t-test analysis of egg production by *D. extensus* in each treatment. Data are pooled between replicates of the same treatment. Figures that are statistically significant are in bold.

Experiment	Day of sample	Bootstrap 2-sample t-test	
		All *D. extensus* P	Egg-producing *D. extensus* only P
1	9	0.071	0.343
	29	0.133	**0.025**
	31	0.619	0.414
2	14	0.772	0.772
	21	0.596	0.594
	30	0.669	0.774
3 *In vivo* egg production	9	**0.026**	**0.048**
	10	0.155	0.301
3 *In vitro* egg production	11	0.154	0.100
	10	0.157	0.190

Experiment	Day of sample	Bootstrap 2-sample t-test *					
		All *D. extensus*			Egg-producing *D. extensus* only		
		A	B	C	A	B	C
4	9	0.062	0.174	0.861	0.256	0.063	0.859
	14	0.230	0.707	0.731	0.379	0.142	0.416
	21	0.869	0.707	0.686	0.220	0.142	0.454
	30	0.917	0.636	0.743	0.296	**0.010**	0.535

*For Experiment 4, egg production of the controls was compared to (A) the 5μg/l cadmium exposed parasites and (B) the 30μg/l cadmium-exposed parasites, (C) is the comparison of egg production by the 2 cadmium-exposed groups.

3.3.3. Egg hatching

In all 4 of the current experiments the hatch rate and hatch success of the eggs produced by *D. extensus* in control conditions and after exposure to cadmium were recorded. The development time of the eggs was recorded as the number of days taken to hatch. For presentational purposes the development time has been recorded as the time taken for fixed percentages of the eggs to hatch, *i.e.* 25, 50, 75, 95 and 100%, and the data are presented in Tables 3.12-3.15. The time taken for 95% of the eggs to hatch has been included, as it was thought that the time taken for 100% of the eggs to hatch would incorporate the slow developing eggs and these may distort the true picture of hatching. The percentage hatch has been calculated from only those eggs that hatched, *i.e.* excluding all undeveloped eggs from the calculations. The overall percentage hatch success has also been included and this has been calculated from all the eggs laid, including the undeveloped proportion.

The percentage of eggs hatching over time in each tank of each treatment is presented graphically in Figures 3.1-3.7. The percentage hatch is calculated from only those eggs that hatched, *i.e.* excluding the undeveloped eggs in each tank.

The rate of egg hatching of the cadmium-exposed eggs was determined by the Kaplan-Meier estimator and was compared (log-rank test) with the corresponding rates of the controls. The Kaplan-Meier estimator was used on hatch data pooled between replicates of the same treatment and incorporates only the eggs that hatched, again, undeveloped eggs were excluded from the analysis. The results from Kaplan-Meier survival analysis are presented in Tables 3.16 and 3.17.

3.3.3.1. Experiment 1 - The effect of the maximum permissible level of cadmium (5μg/l) on the hatching of oncomiracidia from eggs produced at days 9, 29 and 31 post-start of the trial.

In Experiment 1, the eggs laid by control and cadmium-exposed dactylogyrids at days 9, 29 and 31 were monitored daily and their hatch rate and success calculated and presented in Table 3.12. Eggs laid by cadmium-exposed *D. extensus* at day 9 tended to hatch more quickly, with 75% hatching, on average, 4 days earlier than the controls (Table 3.12). However, it should be noted that, despite the more rapid hatching of the cadmium-exposed eggs, the longest time taken for 95% and 100% of eggs to hatch was 28 and 36 days, respectively, in tank T2 (5μg/l cadmium-exposed). When the data from each treatment were pooled and subjected to Kaplan-Meier analysis, the hatch rate of the 2 treatments differed statistically, P = 0.0001. The median hatch time of the control eggs was 26 days compared to 19 days for the cadmium-exposed eggs (Table 3.16).

Interestingly, 100% of the eggs laid at days 29 and 31 by parasites from both treatments hatched, in general, much earlier than those that had been laid at day 9 of the trial. Generally, at these time points, 95% of eggs produced by cadmium-exposed *D. extensus* hatched more quickly than the same percentage of control eggs. Kaplan-Meier analysis showed that the hatch rate of the day 29 cadmium-exposed eggs was statistically quicker than that of the control eggs produced at the same time point, with a median hatch rate of 7 days compared to 10 days in the controls (P = 0.0008) (Table 3.16).

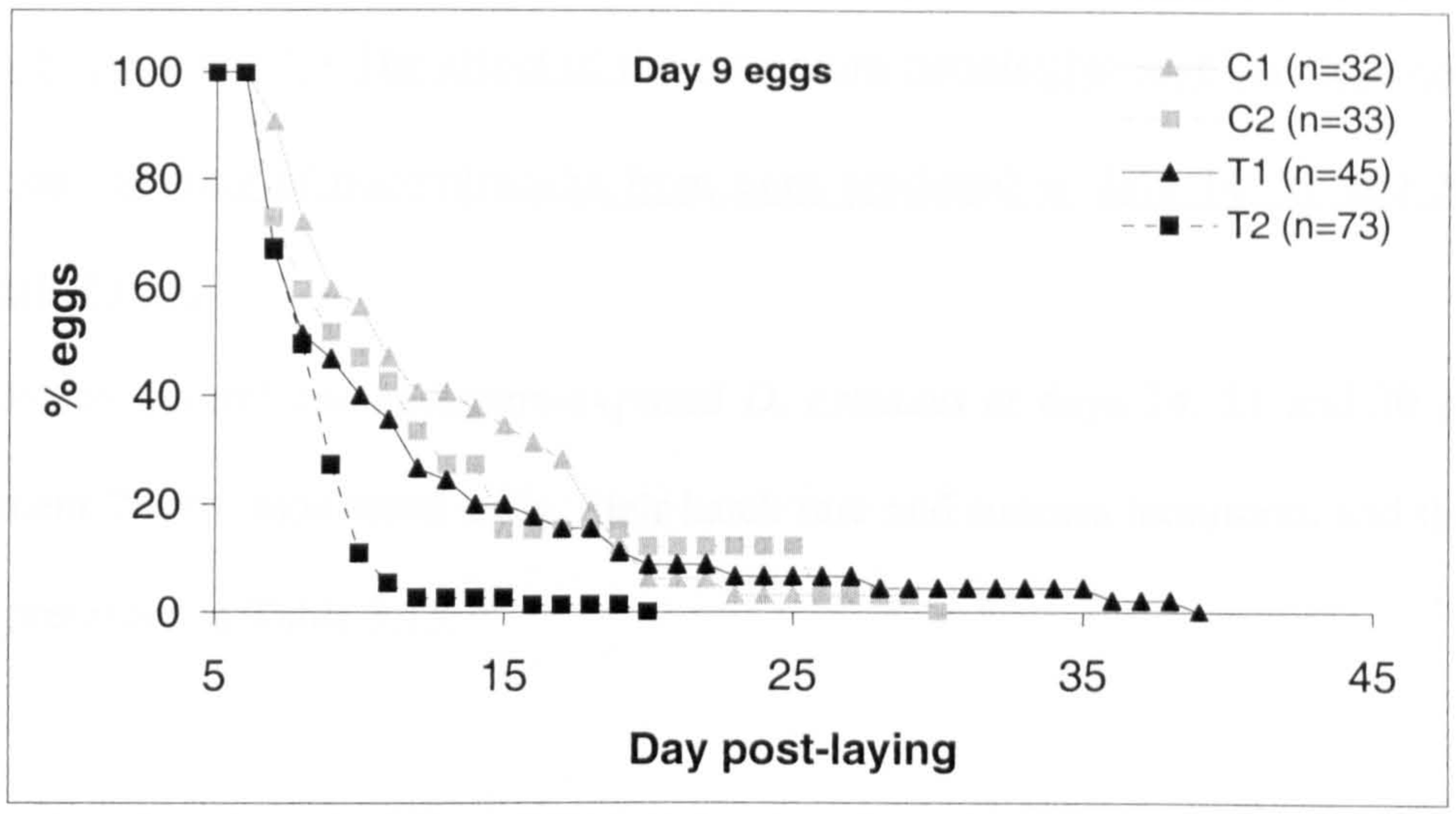
The percentage hatch success of eggs showed no clear consistencies between replicates of the same treatment. For example, both the lowest (24%) and highest (84.6%) hatch success were recorded from cadmium-exposed eggs, in tank T3 at day 29 and tank T5 at day 31, respectively.

Table 3.12. Experiment 1- Summary of egg hatching data from eggs laid by control and 5μg/l cadmium-exposed *D. extensus* at 9, 29 and 31 days post-start of trial. Percentages are calculated from the number of eggs that hatched only. The number given in parentheses is the total number of eggs hatching in each tank.

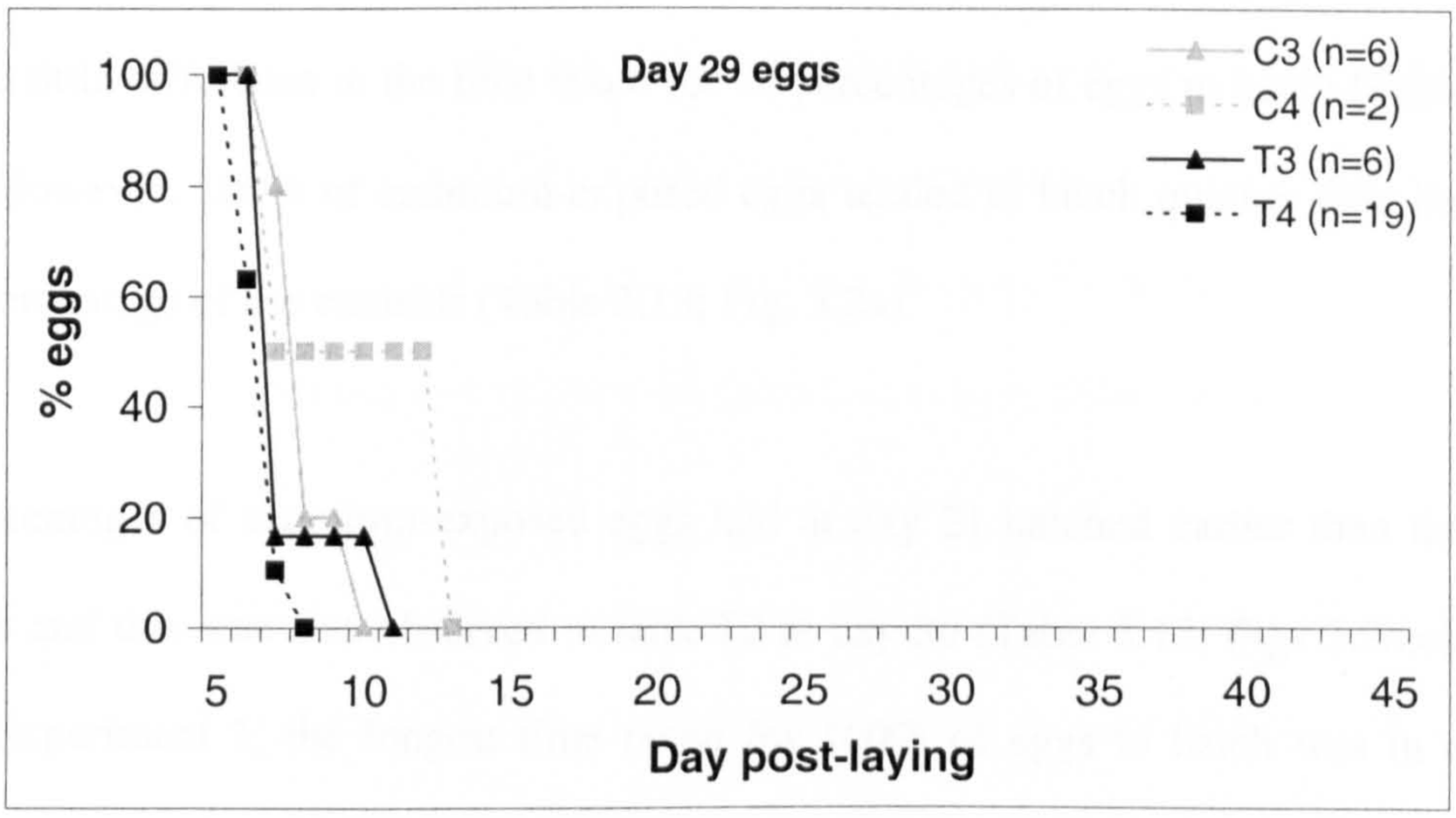
Day 9		**Day to % hatch**					**% Hatch success**
		25	**50**	**75**	**95**	**100**	
C1	**(32)**	8	11	18	20	30	42.7
C2	**(33)**	7	10	15	26	30	76.7
T1	**(45)**	7	9	13	28	39	47.9
T2	**(73)**	7	8	10	10	20	71.6
Day 29		**Day to % hatch**					**% Hatch success**
		25	**50**	**75**	**95**	**100**	
C3	**(6)**	8	8	8	8	10	62.5
C4	**(2)**	7	7	13	13	13	40.0
T3	**(6)**	7	7	7	11	11	24.0
T4	**(19)**	6	7	7	8	8	61.3
Day 31*		**Day to % hatch**					**% Hatch success**
		25	**50**	**75**	**95**	**100**	
C5	**(3)**	10	10	10	18	18	33.3
C6	**(8)**	6	6	6	7	7	53.3
T5	**(11)**	6	7	8	8	10	84.6
T6	**(3)**	6	6	7	10	10	23.0

* Dwindling parasite number necessitated processing of all C5/T5, C6/T6 fish on this day.

a)



b)



c)

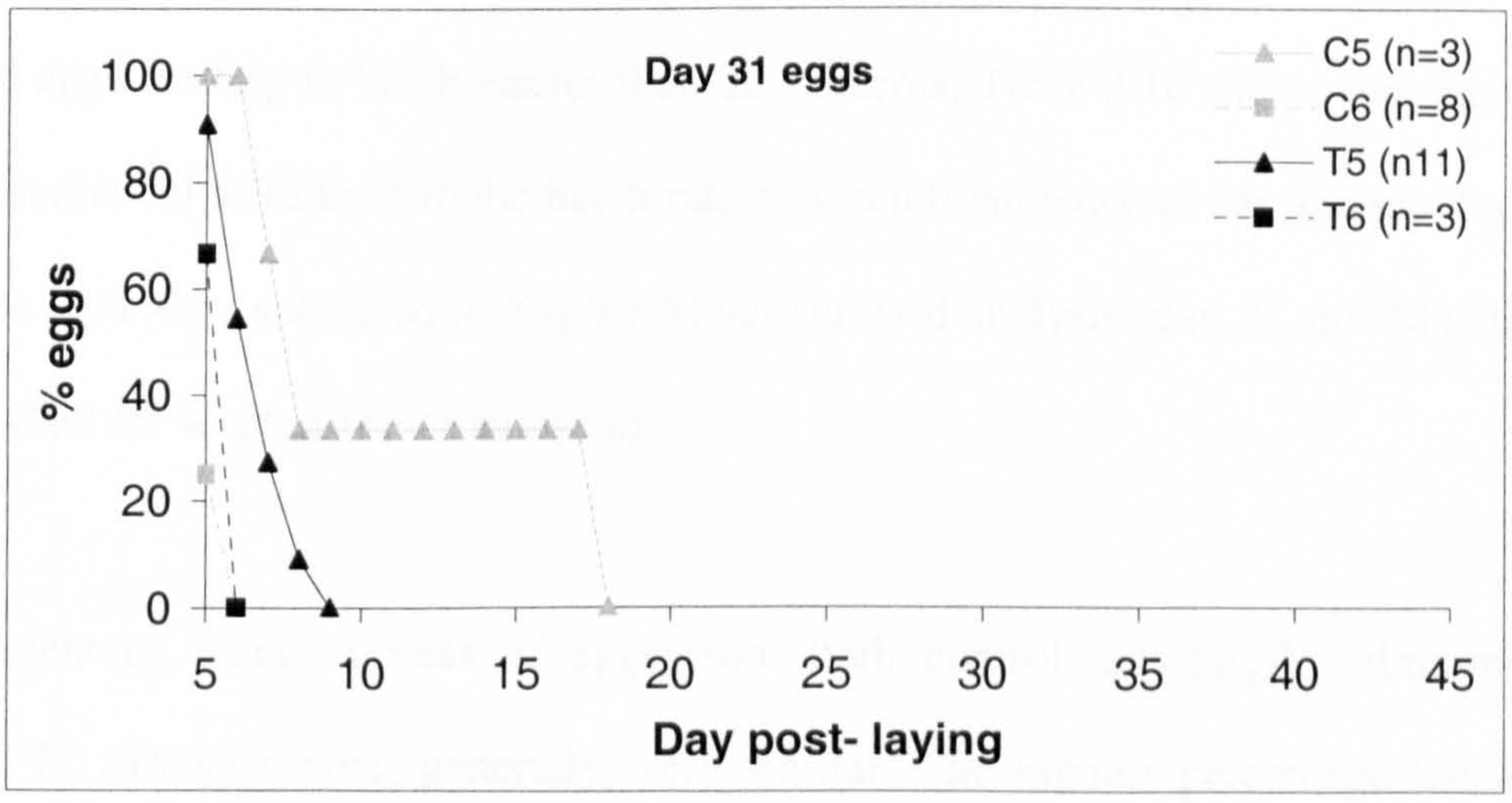


Fig. 3.1. Experiment 1 - The percentage of control and 5μg/l cadmium eggs, laid at (a) 9, (b) 29 and (c) 31 days post-start of the trial, hatching on each day post-laying. (n=total number of eggs hatching)

3.3.3.2. Experiment 2 - The effect of the maximum permissible level of cadmium (5μg/l) on hatching of oncomiracidia from eggs produced at days 14, 21 and 30 post-start of trial.

Eggs laid by control and cadmium-exposed *D. extensus* at days 14, 21 and 30 of Experiment 2 were monitored daily, their hatch rate and success monitored and the results presented in Table 3.13.

Eggs produced at day 14 of Experiment 2, by dactylogyrids from both populations, showed little difference in the time taken for all percentages of eggs to hatch (Table 3.13). However, 100% of cadmium-exposed eggs tended to hatch quicker than the same percentage of the controls (Table 3.13; Fig. 3.2a).

All percentages of cadmium-exposed eggs laid at day 21 hatched earlier than the controls and this was also observed in tank T2 at day 30 (Table 3.13; Figs 3.2b,c). As in Experiment 1, the longest time taken for 100% of eggs to hatch was in a cadmium-exposed tank, T3 (eggs produced at day 30). Despite the cadmium-exposed eggs tending to hatch earlier than the controls, Table 3.16 shows that there was no statistical difference in the hatch rate between treatments, at any sample day, when the data were subjected to Kaplan-Meier survival analysis (day 21 and 30 data were pooled for Kaplan-Meier analysis).

The percentage hatch success of eggs from both control and 5μg/l cadmium-exposed *D. extensus* were, generally, very similar. The highest percentage hatch success was 100% and was recorded in control tank C3 at day 14, while the lowest value was 35.6%, recorded in test tank T1 at day 21 (Table 3.13).

3.3.3.3. Experiment 3 - The effect of the maximum permissible level of cadmium (5µg/l) on the hatching of oncomiracidia from eggs produced at day 10 post-start of the trial.

In Experiment 3 the *in vitro* hatch rate and success of eggs laid after 10 days exposure to cadmium was ascertained, as well as the hatch success of eggs laid *in vivo* after 11 days exposure to 5µg/l cadmium. The results are shown in Table 3.14 and Figure 3.3.

As has been seen in the results from the other experiments discussed so far, the percentage hatch success of dactylogyrid eggs laid *in vitro* appears to be unaffected by exposure to 5µg/l cadmium. The hatch success of eggs from both treatments differed little between those laid *in vivo* and those laid *in vitro* (Table 3.14).

Inconsistencies in the hatch rate of eggs laid *in vitro* in both treatments make it difficult to determine a clear pattern in the hatch response in Experiment 3 (Table 3.14; Fig. 3.3). However, the hatch rate of the 2 treatments differed statistically (P = 0.0268) with cadmium-exposed eggs having a median hatch time of 15 days compared to 20 days in the control eggs (Table 3.16).

Table 3.13. Experiment 2 - Summary of egg hatching data from eggs laid by control and 5μg/l cadmium-exposed *D. extensus* at 14, 21 and 30 days post-start of trial. Percentages are calculated from the number of eggs that hatched only. The number given in parentheses is the total number of eggs hatching in each tank.

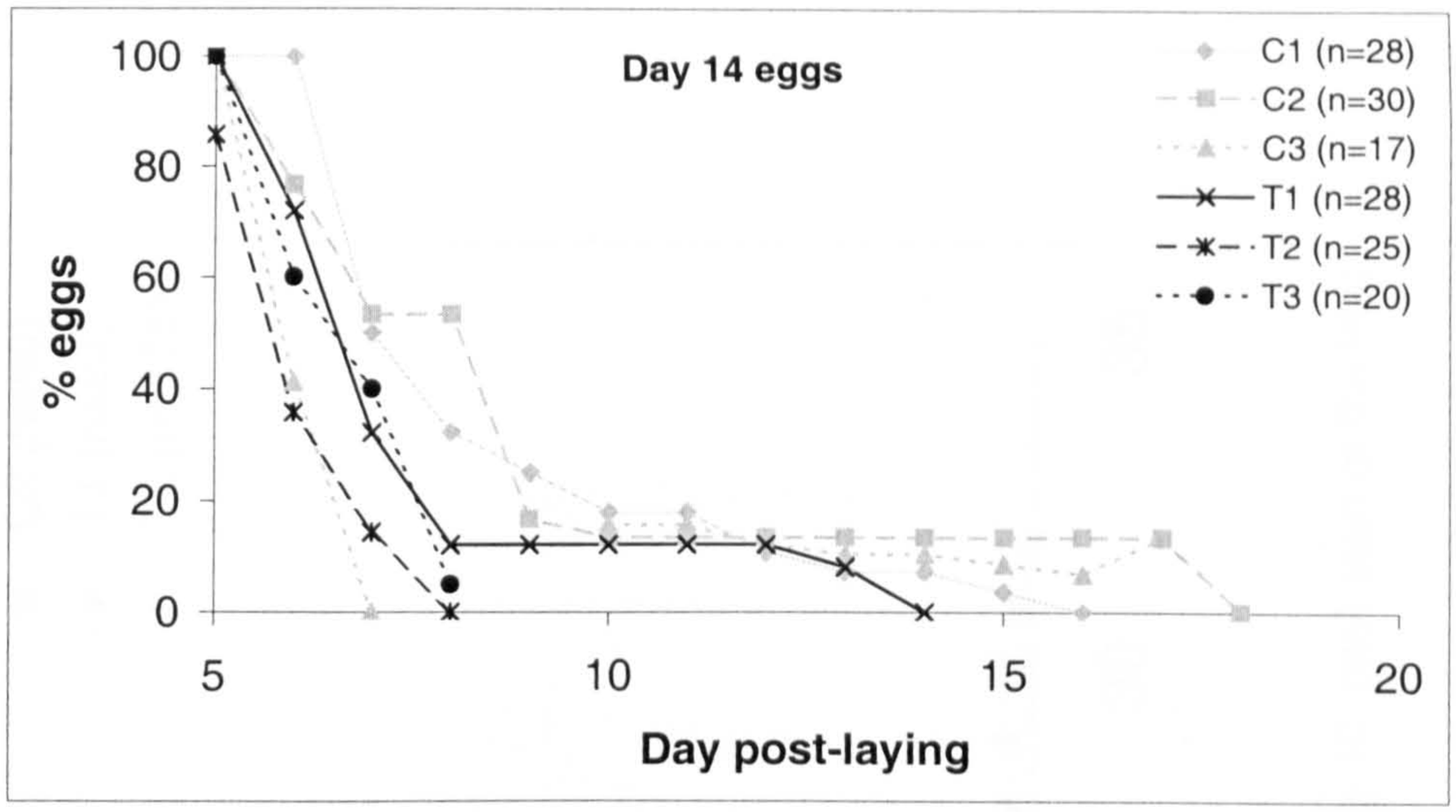
Day 14		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
C1	**(28)**	7	7	9	15	16	71.8
C2	**(30)**	7	8	8	10	18	71.4
C3	**(17)**	6	6	7	7	7	100
T1	**(28)**	6	6	8	14	14	62.2
T2	**(25)**	6	7	7	8	9	80.6
T3	**(20)**	6	7	8	8	8	66.6
Day 21*		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
C1	**(22)**	6	7	9	12	14	56.4
T1	**(16)**	6	6	7	7	8	35.6
Day 30		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
C2	**(22)**	6	7	8	9	14	59.5
C3	**(20)**	6	6	8	12	14	82.3
T2	**(16)**	5	6	6	7	9	57.1
T3	**(30)**	6	6	7	19	25	73.3

* Deteriorating fish health necessitated processing of all C1/T1 fish on this day.

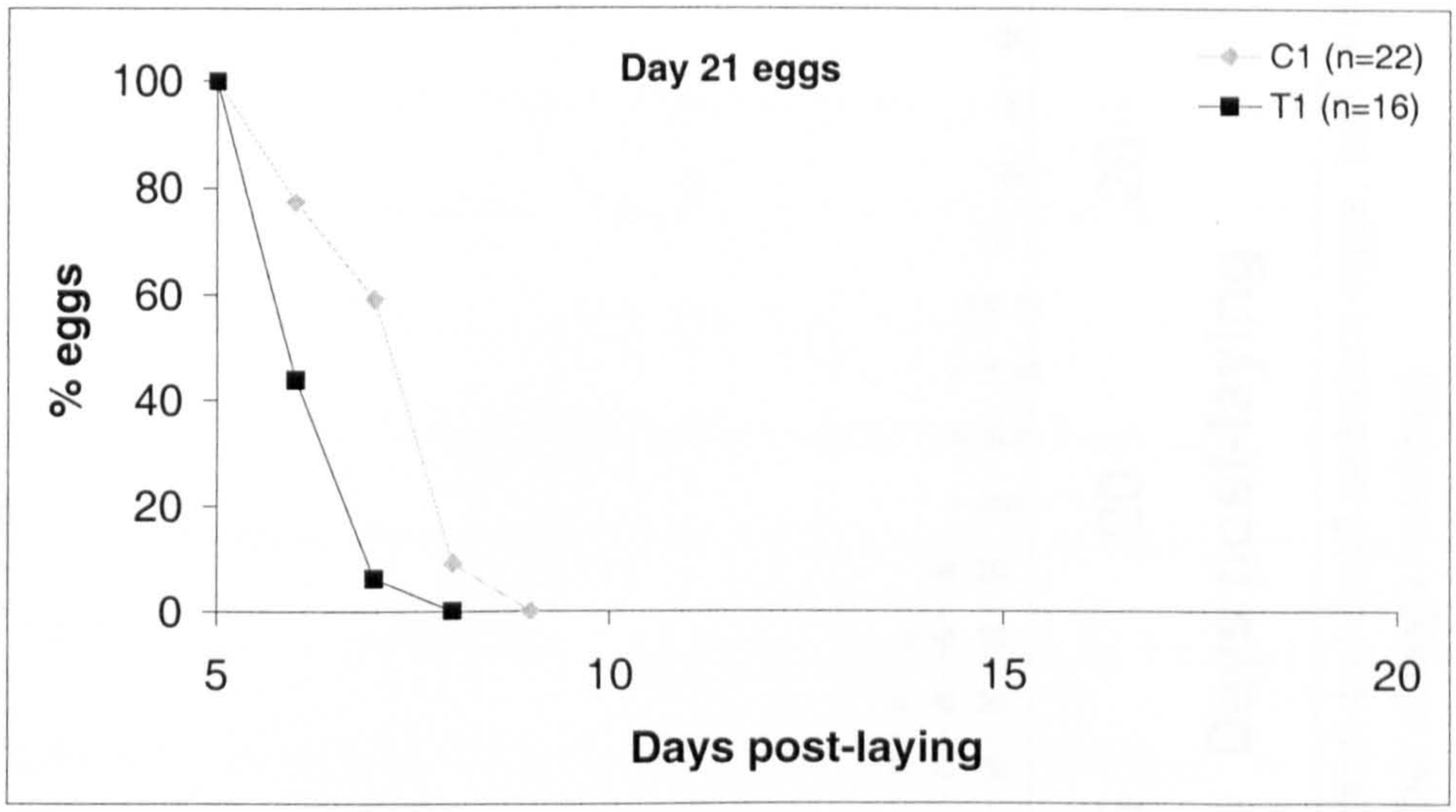
Table 3.14. Experiment 3 - Summary of *in vitro* egg hatching data from eggs laid by control and 5μg/l cadmium-exposed *D. extensus* at 10 days post-start of trial. Percentages are calculated from the number of eggs that hatched only. The number given in parentheses is the total number of eggs hatching in each tank. The *in vivo* hatch success from eggs laid at 11 days post-start of trial is also shown.

Day 10		**Day to % hatch**					***In vitro* % hatch success**	***In vivo* % hatch success**
		25	**50**	**75**	**95**	**100**		
C1	**(68)**	7	7	10	24	32	80.0	84.7
C2	**(60)**	7	7	7	9	23	93.8	96.3
T1	**(23)**	6	6	10	20	22	82.1	79.4
T2	**(175)**	6	7	9	15	31	87.1	90.4

a)



b)



c)

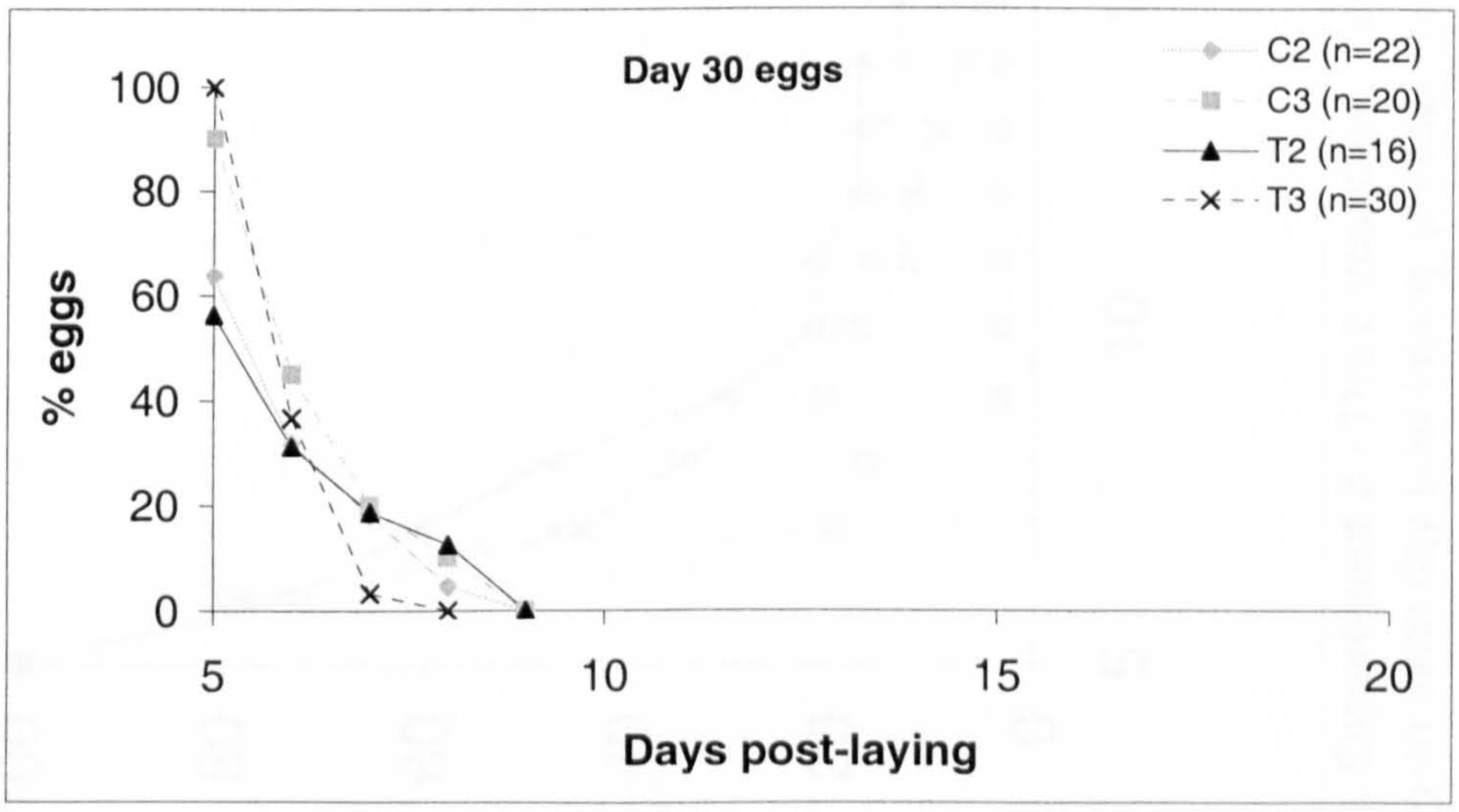


Fig. 3.2. Experiment 2 - The percentage of control and 5μg/l cadmium eggs, laid at (a) 14, (b) 21 and (c) 30 days post-start of the trial, hatching on each day post-laying. (n=total number of eggs hatching)

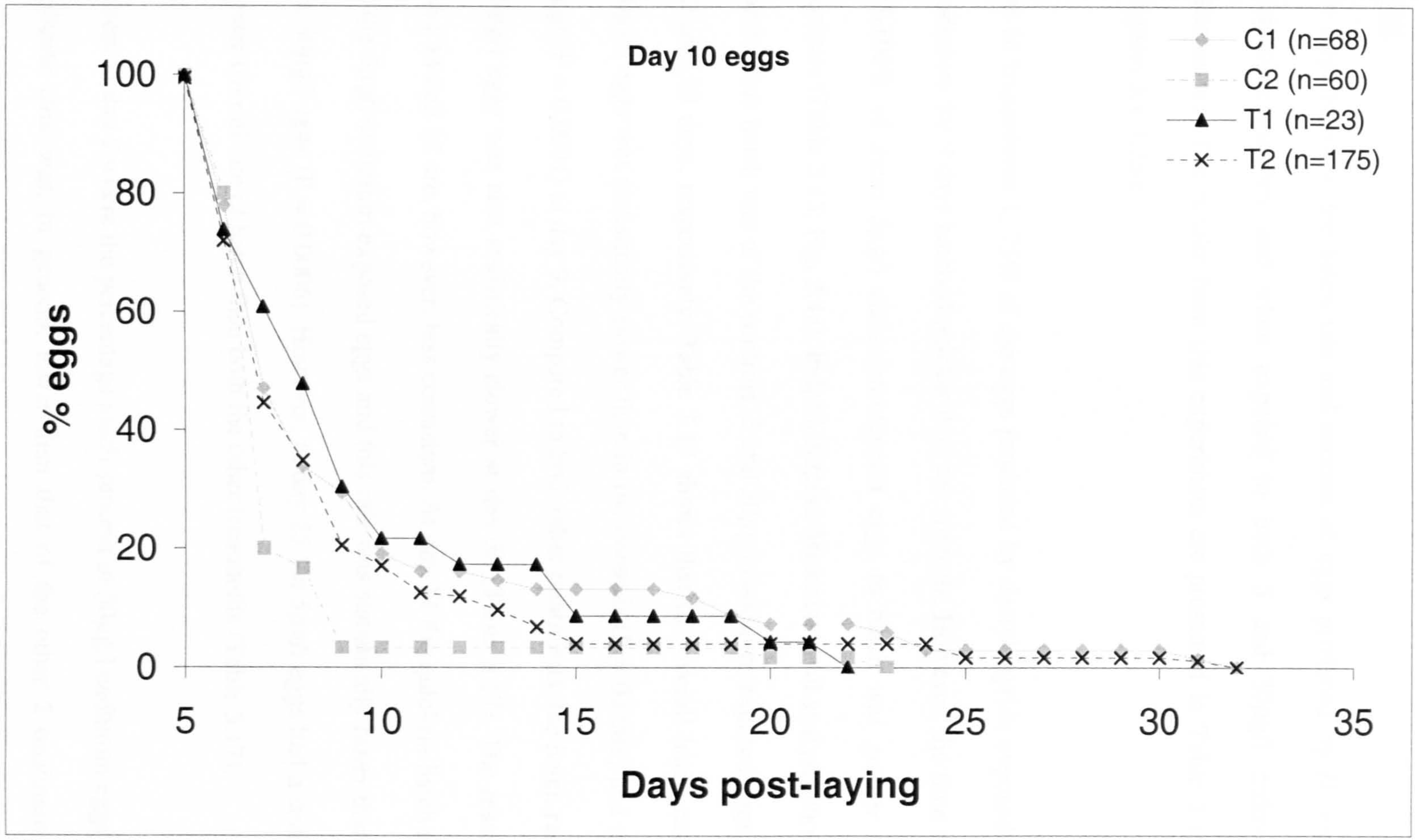


Fig. 3.3. Experiment 3 - The percentage of control and 5μg/l cadmium eggs, laid at day 10 post-start of the trial, hatching on each day post-laying. (n=total number of eggs hatching)

3.3.3.4. Experiment 4 - The effect of both 5 and 30μg/l cadmium on the hatching of oncomiracidia from eggs produced at day 9, 14, 21 and 30 days post-start of the trial.

In Experiment 4, the hatch rate and success of eggs produced by *D. extensus* in control conditions and when exposed to both 5 and 30μg/l cadmium was determined. The results from this experiment are presented in Table 3.15 and in Figures 3.4-3.7a-c.

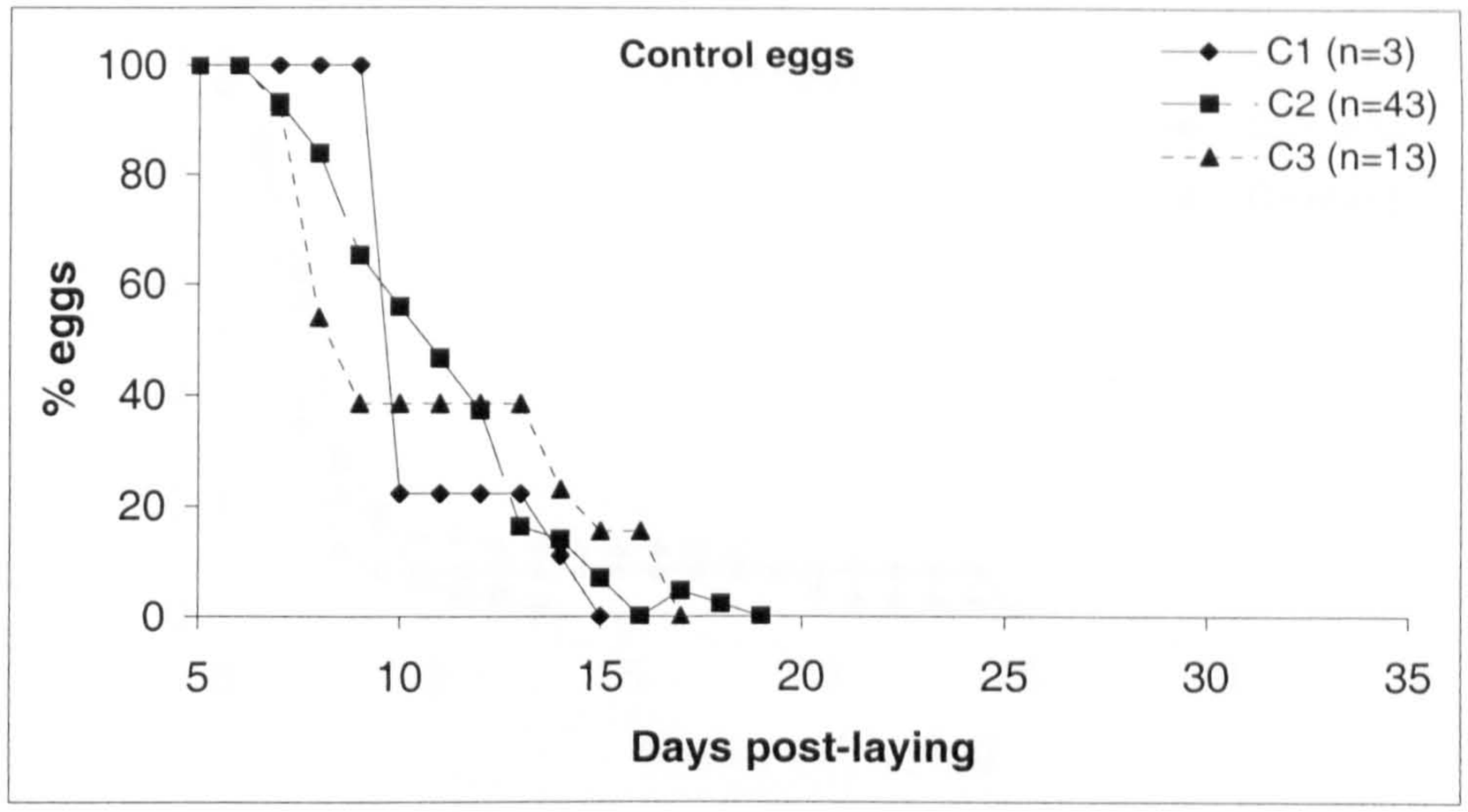
As in Experiment 1, 75% of the eggs produced by dactylogyrids exposed to 5μg/l cadmium for 9 days hatched quicker than the controls. However, the time taken for 95-100% of these 5μg/l cadmium-exposed eggs to hatch was greater than the controls (Table 3.15; Fig. 3.4a). Indeed, Kaplan-Meier survival analysis showed that the median hatch rate of the controls, 5 and 30μg/l cadmium-exposed eggs was 16, 22 and 29 days, respectively. Table 3.17 shows that the overall hatch rate of the 30μg/l eggs was statistically slower than in the controls ($P = 0.0001$) and the 5μg/l eggs ($P = 0.0001$) at day 9. Compared to both other treatments, the hatch rate of the 30μg/l eggs was also statistically slower at day 30 (Table 3.17). The results from days 14 and 21 are, however, less consistent. At day 14 the quickest hatch rate was in the 5μg/l cadmium-exposed eggs and this rate was statistically faster than that of the 30μg/l eggs ($P = 0.0006$). However, at day 21 the 5μg/l eggs had a statistically slower overall rate of hatch than both the other treatments (Table 3.17).

Except at day 9 where the percentage hatch success of 30μg/l cadmium eggs in each replicate tank was, in general, lower than that of the other 2 treatments, there appears to be little consistency in the hatch success of eggs within treatments.

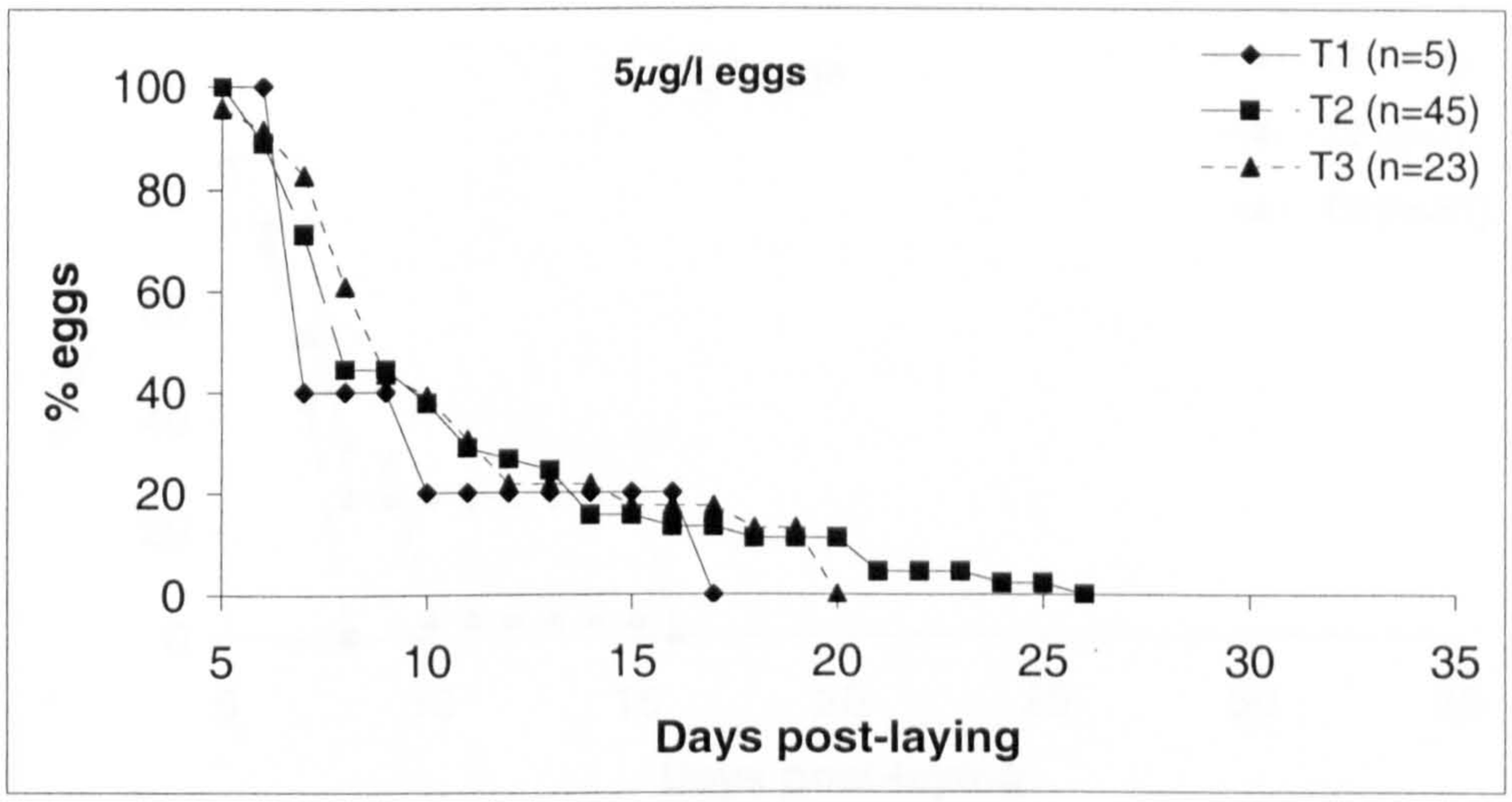
Table 3.15. Experiment 4 - Summary of egg hatching data from eggs laid by control and 5 and 30µg/l cadmium-exposed *D. extensus* at 9, 14, 21 and 30 days post-start of trial. Percentages are calculated from the number of eggs that hatched only. The number given in parentheses is the total number of eggs hatching in each tank.

Day 9		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
Controls	**C1 (3)**	10	14	14	15	15	33.3
	C2(43)	9	11	13	16	19	87.8
	C3(13)	8	9	15	17	17	81.3
5µg/l cadmium	**T1 (5)**	7	7	10	17	17	83.3
	T2 (45)	7	8	11	22	26	72.6
	T3 (23)	8	9	12	20	20	82.1
30µg/l cadmium	**T1 (21)**	7	8	9	15	16	58.3
	T2 (4)	7	8	8	8	8	66.7
	T3 (62)	8	15	21	34	37	58.5
Day 14		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
Controls	**C1 (27)**	7	7	8	25	25	62.8
	C2 (33)	7	8	9	10	13	89.2
	C3 (43)	7	8	8	20	25	62.3
5µg/l cadmium	**T1 (4)**	5	8	8	16	16	30.8
	T2 (7)	6	7	8	8	8	25.9
	T3 (31)	6	8	10	10	16	88.6
30µg/l cadmium	**T1 (20)**	7	7	9	14	17	60.6
	T2 (35)	7	8	9	9	30	85.4
	T3 (37)	7	8	9	19	32	56.1
Day 21		**Day to % hatch**					**% hatch Success**
		25	**50**	**75**	**95**	**100**	
Controls	**C1 (8)**	6	6	8	10	10	44.4
	C2 (43)	6	6	7	9	13	100
	C3 (22)	6	7	7	8	8	91.7
5µg/l cadmium	**T1 (55)**	6	6	8	13	17	77.5
	T2 (22)	7	7	10	11	11	91.7
	T3 (47)	6	7	7	10	10	81.0
30µg/l cadmium	**T1 (26)**	6	7	7	10	10	89.7
	T2 (50)	6	6	7	9	11	71.4
	T3 (29)	7	7	8	11	11	100
Day 30		**Day to % hatch**					**% hatch success**
		25	**50**	**75**	**95**	**100**	
Controls	**C1 (33)**	6	6	7	9	17	63.5
	C2 (26)	6	6	6	7	8	78.8
	C3 (20)	6	7	8	9	9	80.0
5µg/l cadmium	**T1 (0)**	×	×	×	×	×	×
	T2 (20)	6	6	7	7	8	35.7
	T3 (13)	6	6	6	8	9	86.7
30µg/l cadmium	**T1 (14)**	6	7	8	9	10	93.3
	T2 (9)	7	7	7	10	10	75.0
	T3 (33)	6	7	8	9	10	89.2

a)



b)



c)

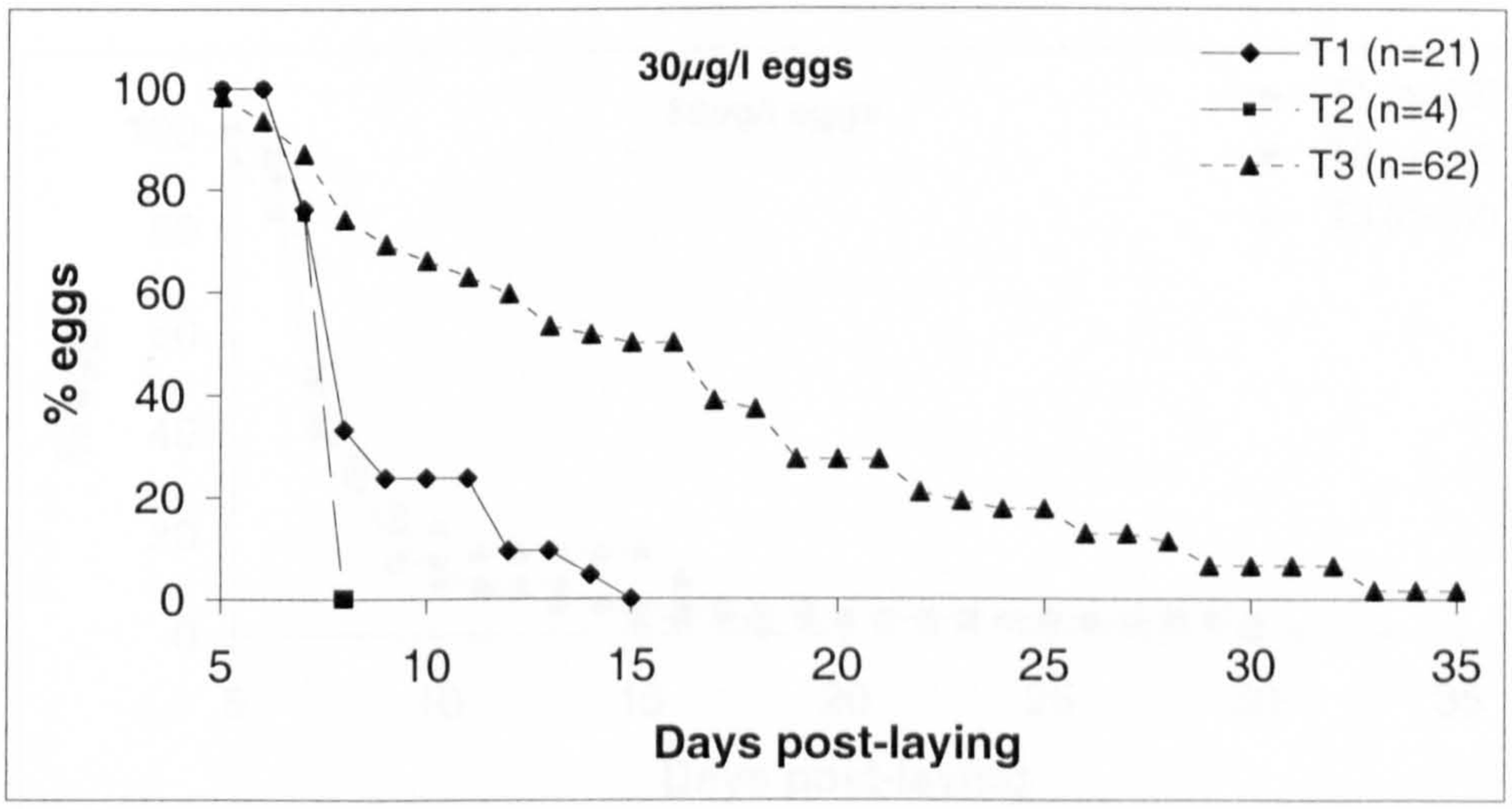
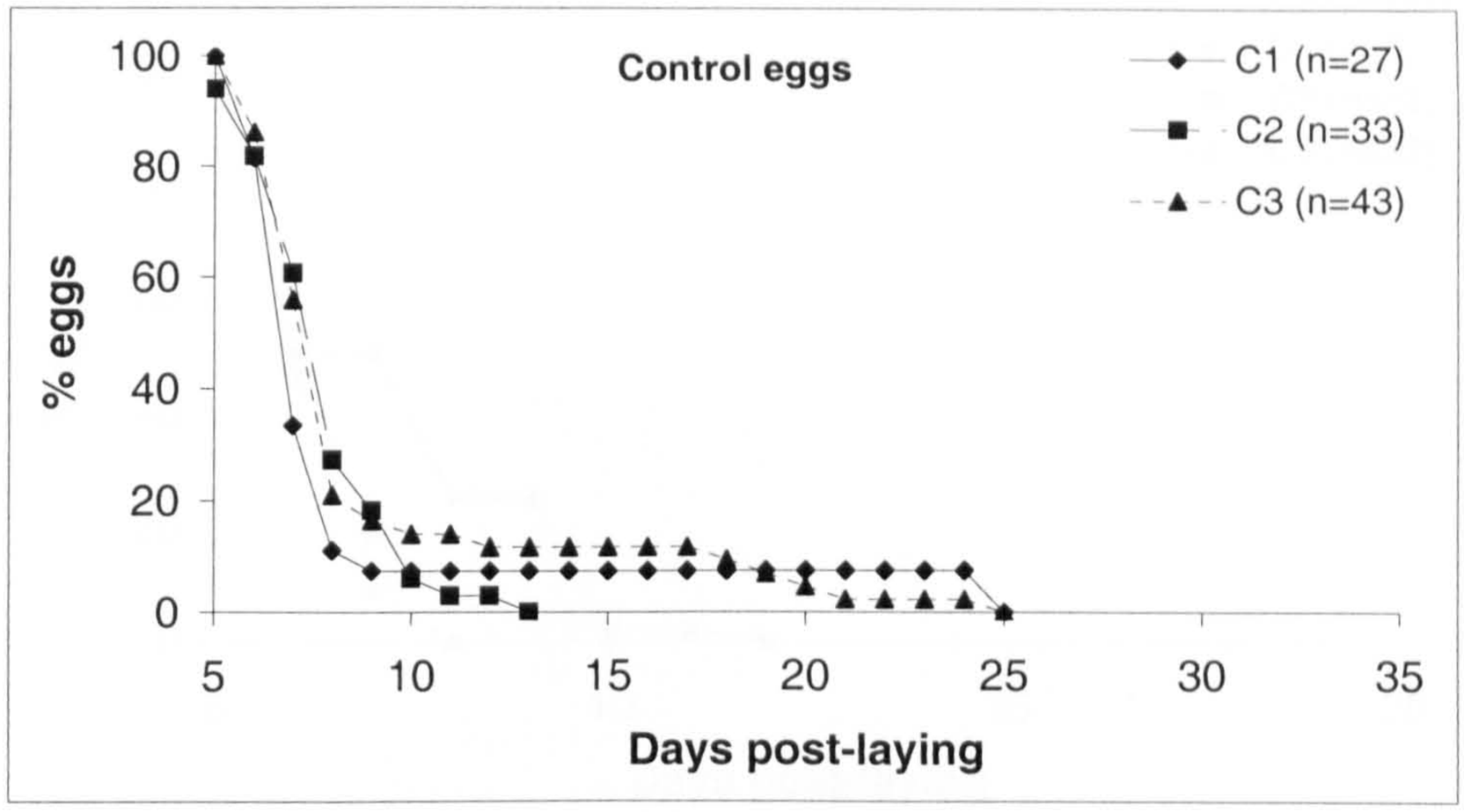
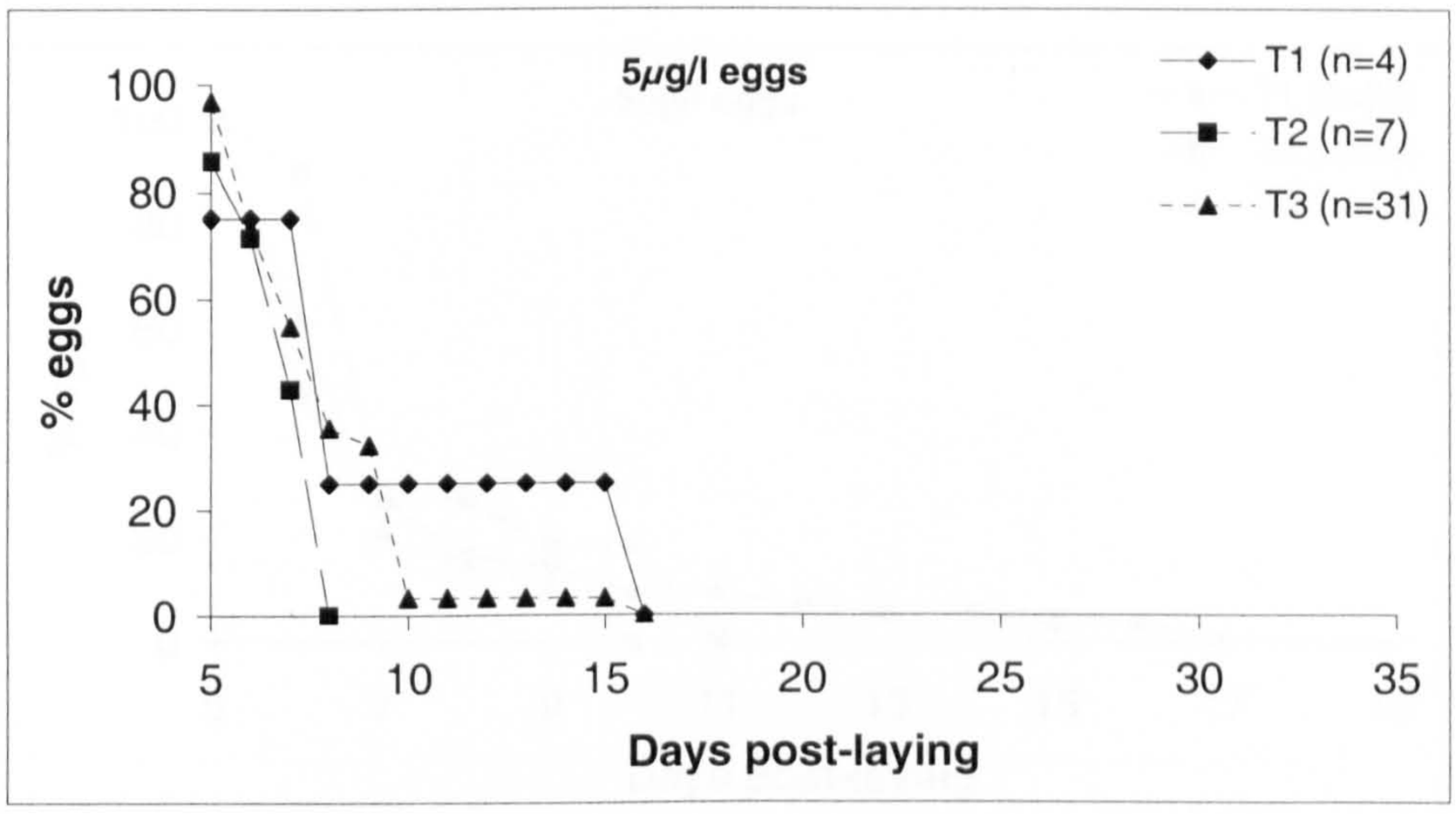


Fig. 3.4. Experiment 4, Day 9 - The percentage of (a) control, (b) 5µg/l and (c) 30µg/l eggs produced at day 9, hatching on each day post-laying.
(n = total of number of eggs hatching).

a)



b)



c)

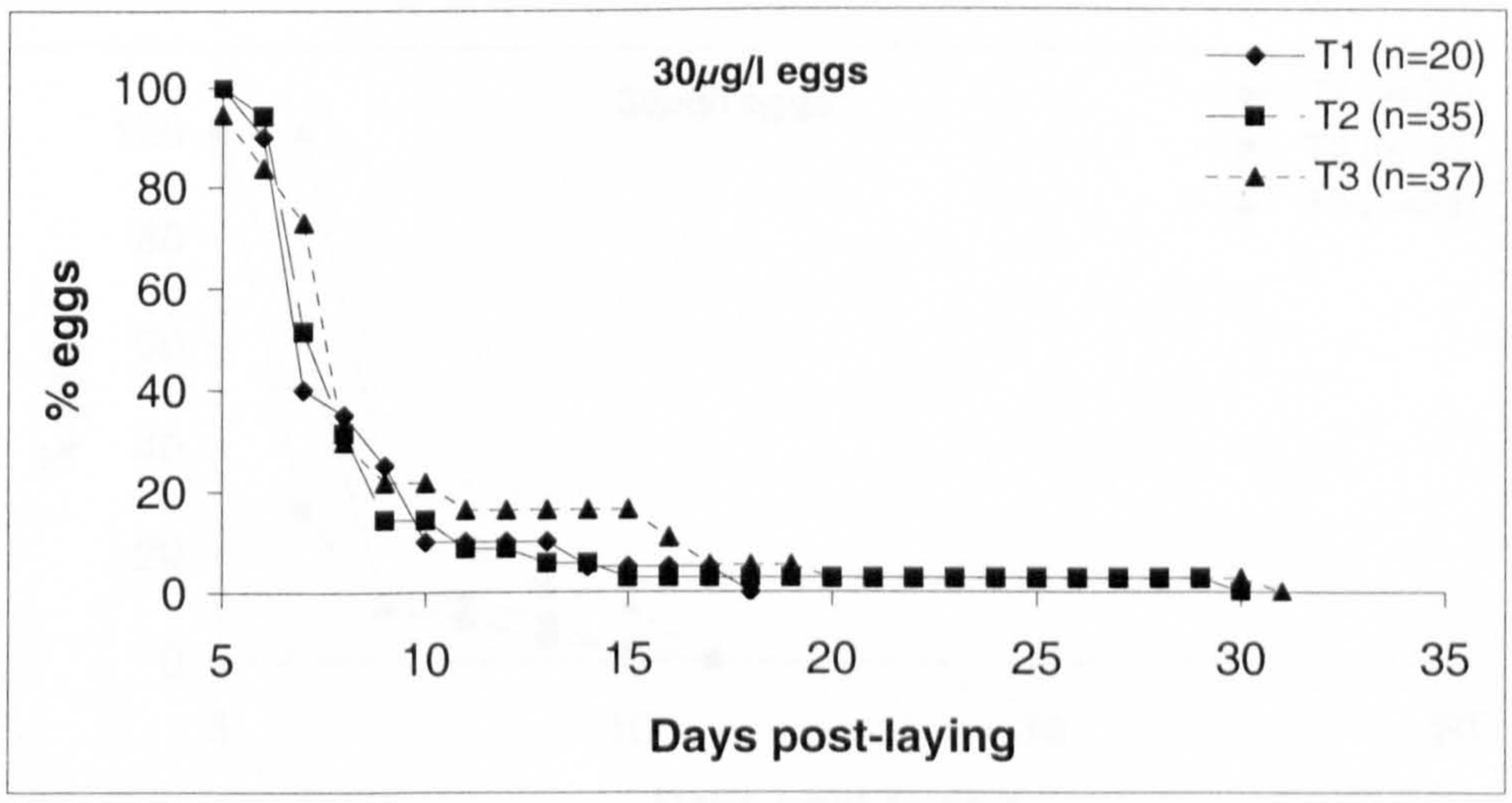
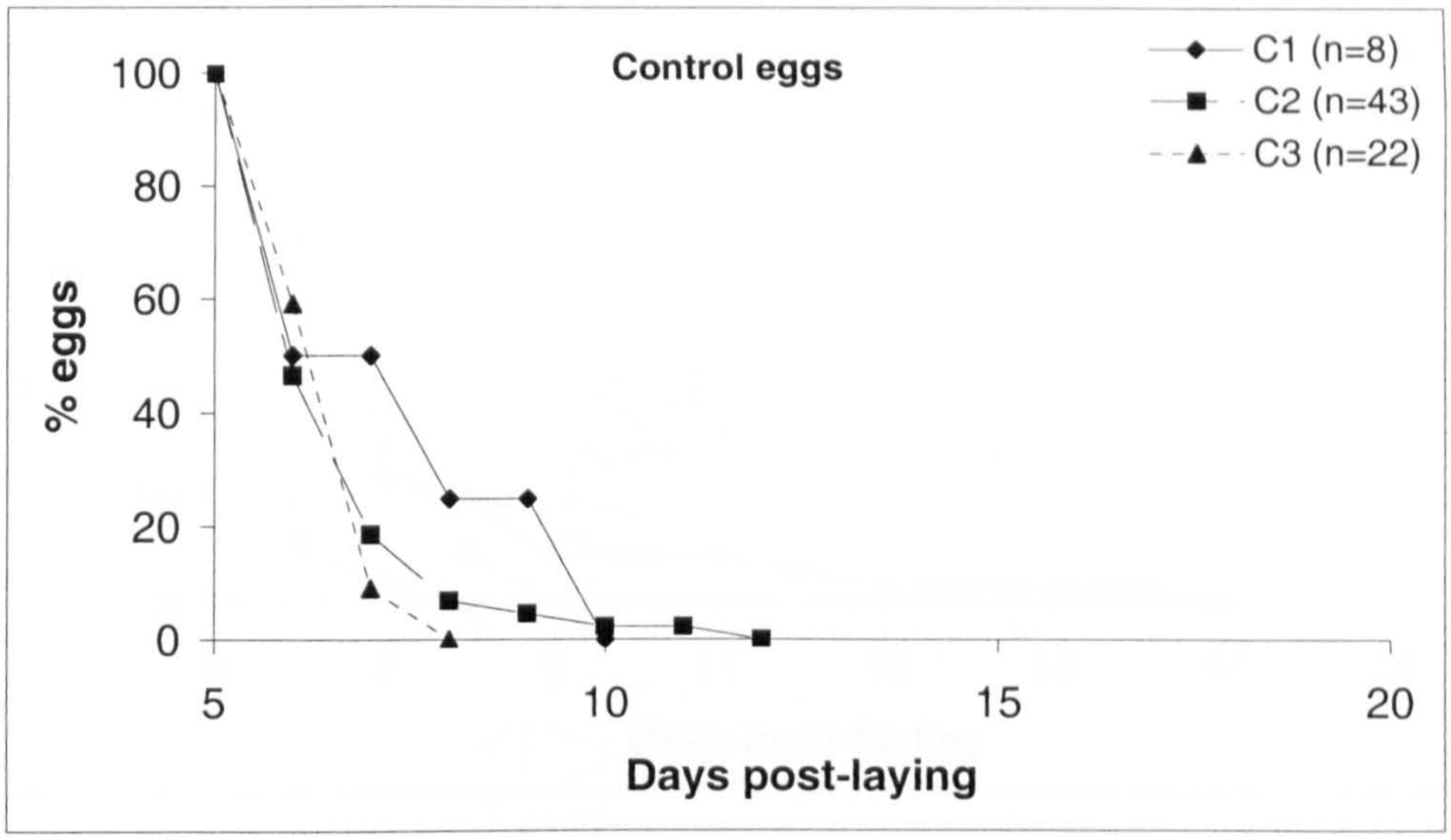
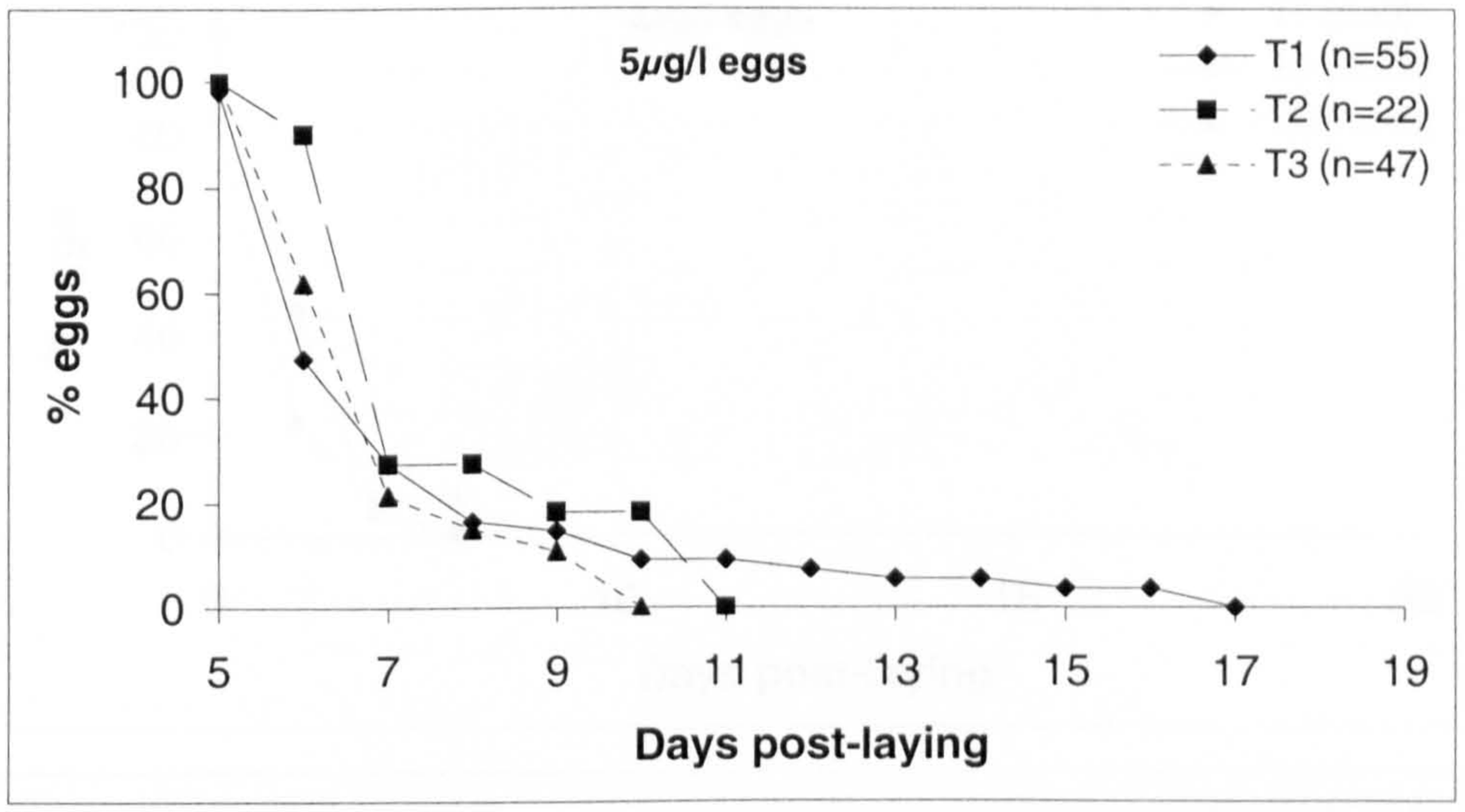


Fig. 3.5. Experiment 4, Day 14 - The percentage of (a) control, (b) 5µg/l and (c) 30µg/l eggs produced at day 14, hatching on each day post-laying.
(n = total number of eggs hatching).

a)



b)



c)

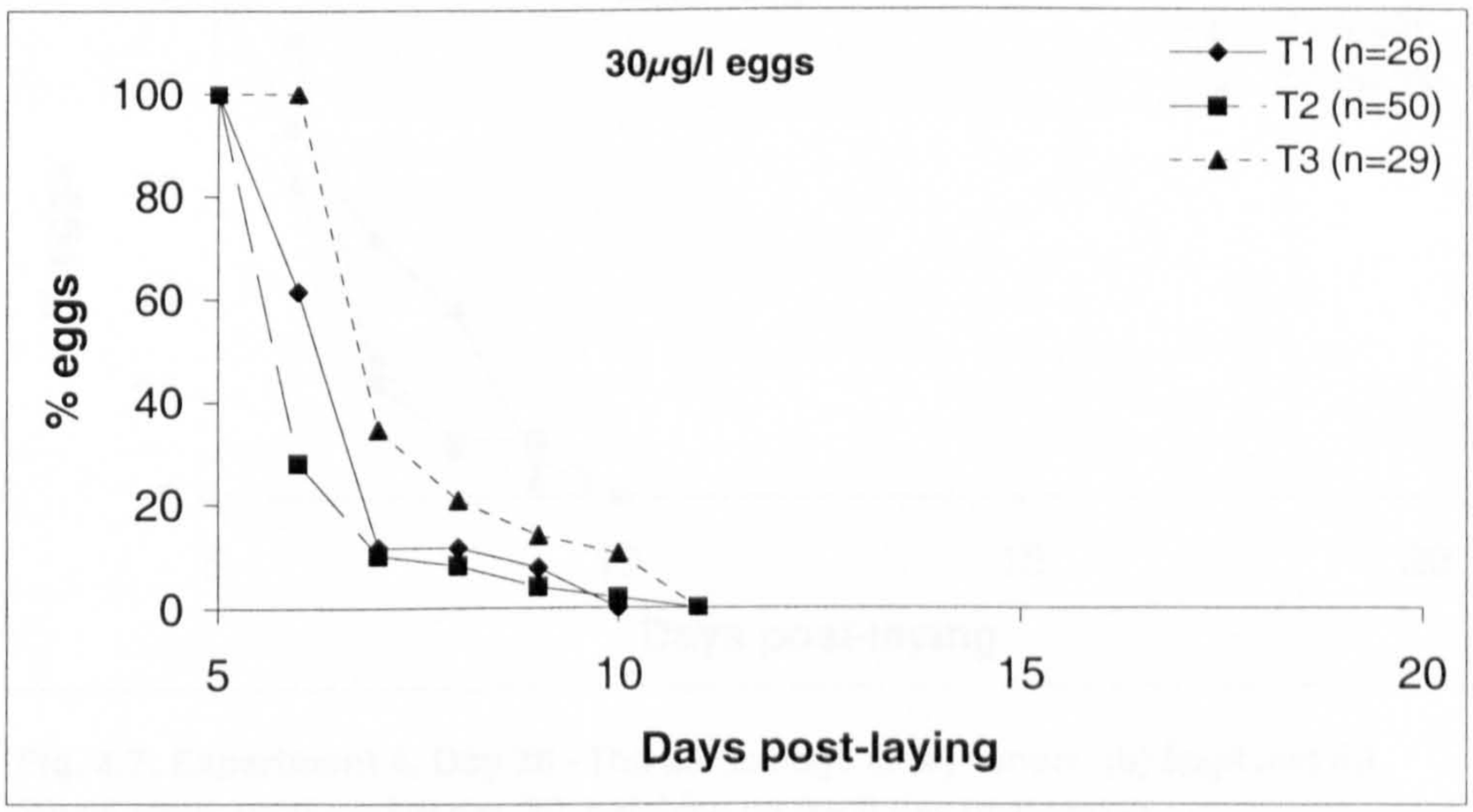
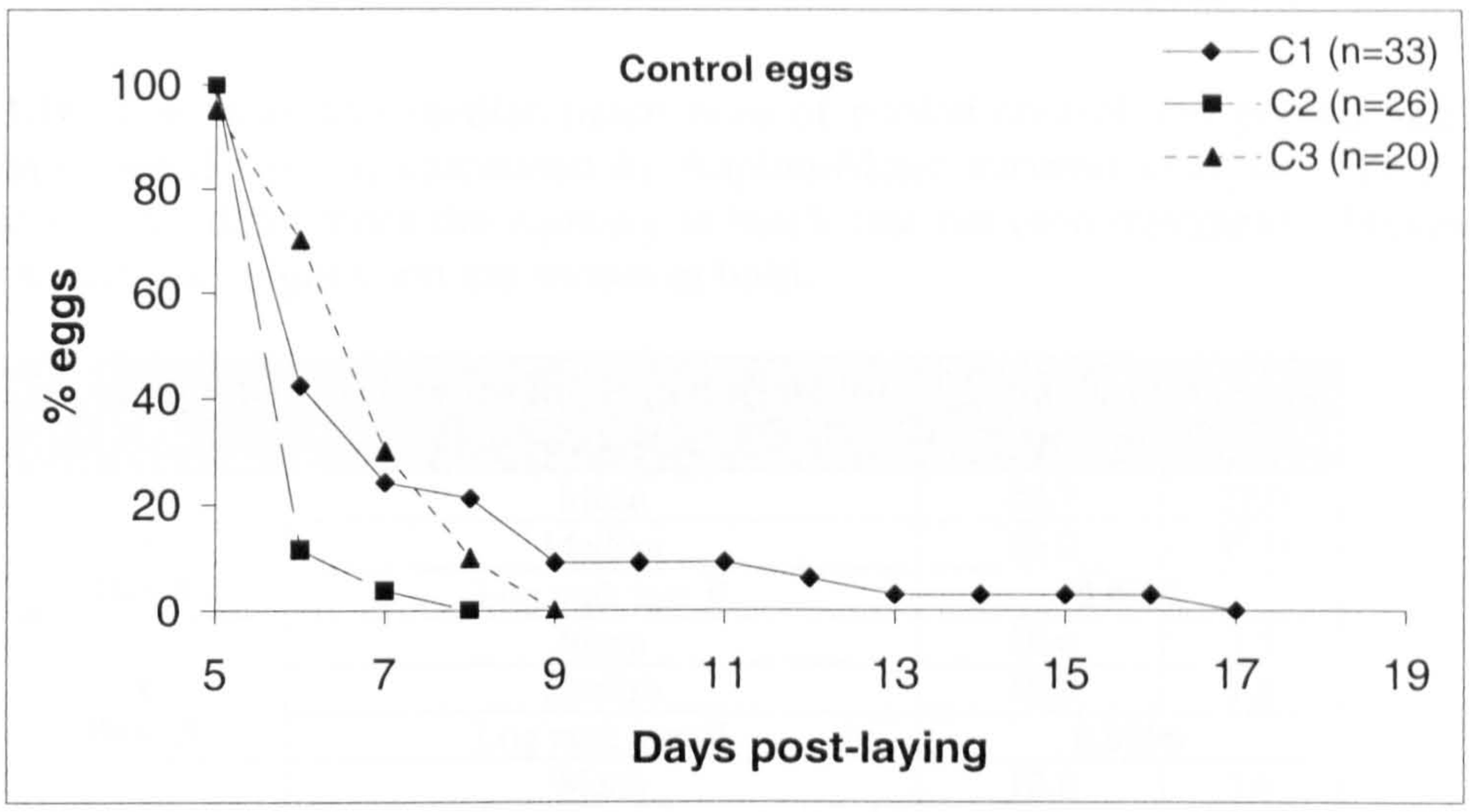
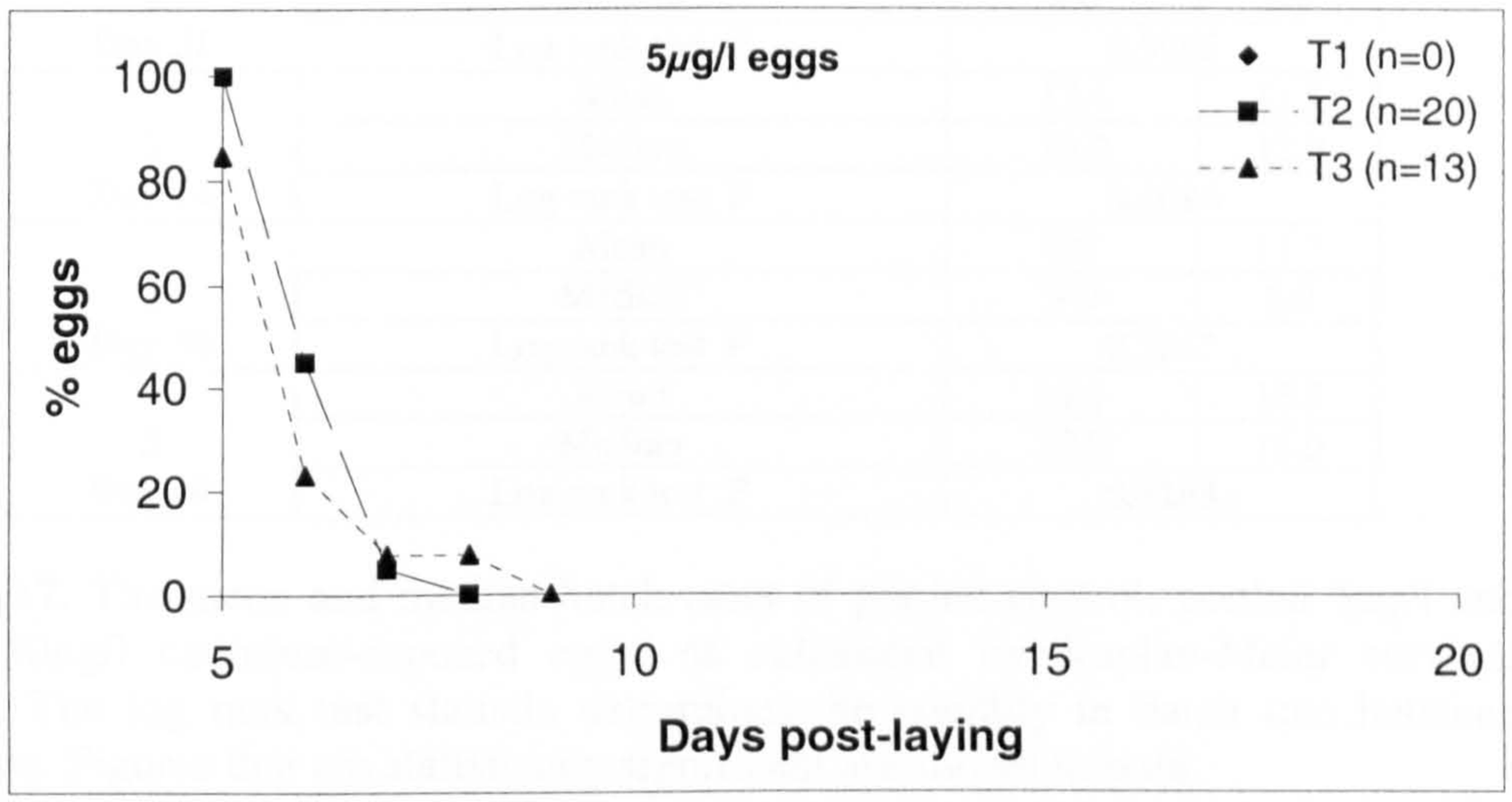


Fig. 3.6. Experiment 4, Day 21 - The percentage of (a) control, (b) 5µg/l and (c) 30µg/l eggs produced at day 21, hatching on each day post-laying.
(n = total number of eggs hatching).

a)



b)



c)

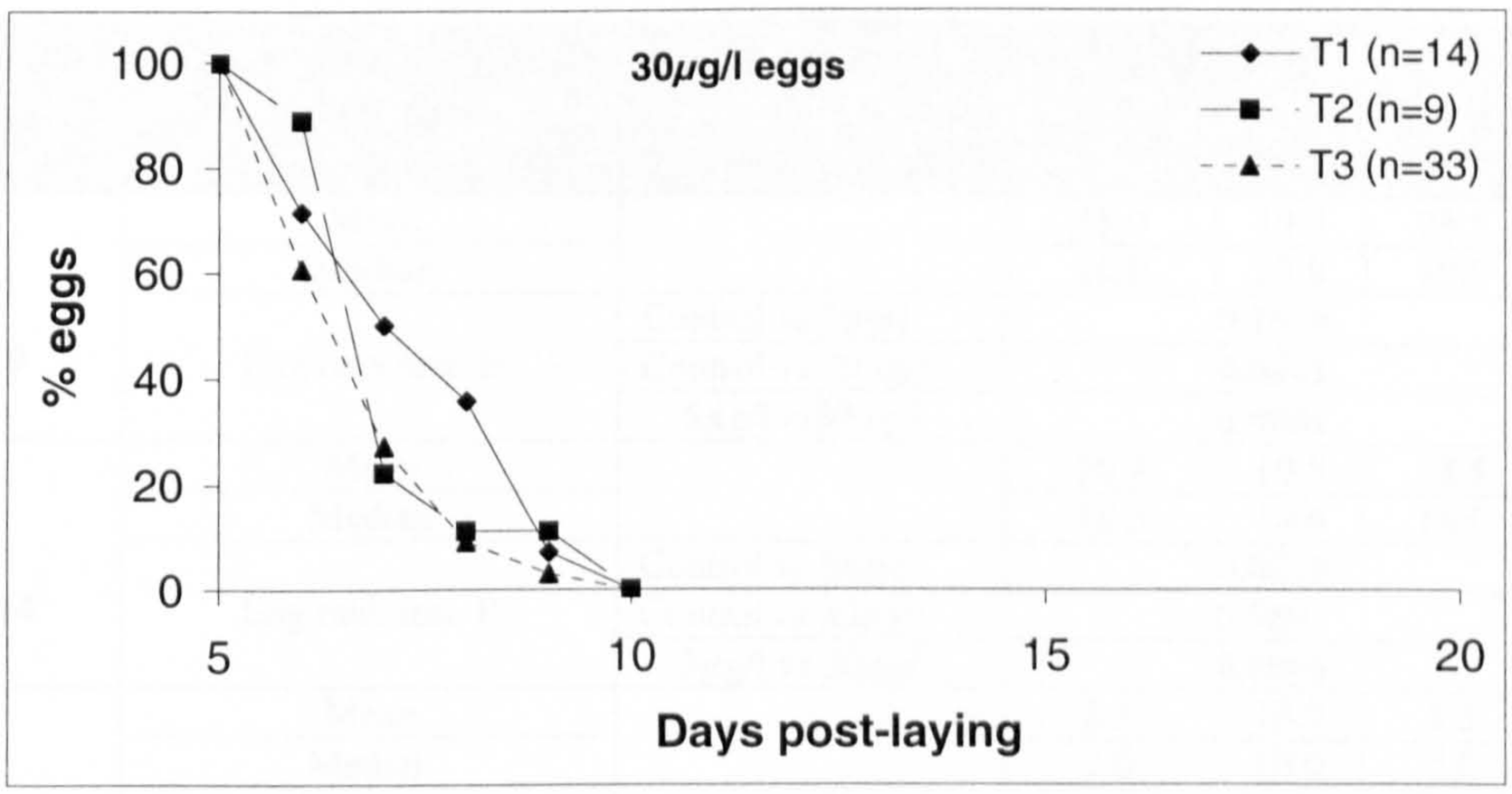


Fig. 3.7. Experiment 4, Day 30 - The percentage of (a) control, (b) 5µg/l and (c) 30µg/l eggs, produced at day 30, hatching on each day post-laying.
(n = total number of eggs hatching).

Table 3.16. The mean and median hatch rates of pooled control and pooled 5μg/l cadmium-exposed eggs as calculated by Kaplan-Meier survival analysis. The log rank test statistic determines the equality in hatch rate between treatments. Figures that are statistically significant are shown in bold.

Experiment	Mean and median hatch rate Log rank test (P)	Hatch rate in days	
		Control	5μg/l
1 Day 9	Mean	22.7	22.0
	Median	26.0	19.0
	Log rank test P	**0.0001**	
1 Day 29	Mean	10.0	7.5
	Median	10.0	7.0
	Log rank test P	**0.0008**	
1 Day 31	Mean	10.8	7.6
	Median	8.0	8.0
	Log rank test P	0.5965	
2 Day 14	Mean	12.4	11.5
	Median	13.0	12.0
	Log rank test P	0.1096	
2 Day 30	Mean	9.8	11.7
	Median	9.0	8.0
	Log rank test P	0.2267	
3 Day 10	Mean	18.5	16.5
	Median	20.0	15.0
	Log rank test P	**0.0268**	

Table 3.17. The mean and median hatch rates of pooled control, pooled 5μg/l and pooled 30μg/l cadmium-exposed eggs, as calculated by Kaplan-Meier survival analysis. The log rank test statistic determines the equality in hatch rate between treatments. Figures that are statistically significant are shown in bold.

Experiment	Mean and median hatch rate Log rank test (P)		Hatch rate in days		
			Control	5μg/l	30μg/l
4 Day 9	Mean		15.0	19.9	28.5
	Median		16.0	22.0	29.0
	Log rank test P	Control *vs* 5μg/l	0.1658		
		Control *vs* 30μg/l	**0.0001**		
		5μg/l *vs*30μg/l	**0.0001**		
4 Day 14	Mean		16.4	10.5	18.5
	Median		18.0	10.0	18.0
	Log rank test P	Control *vs* 5μg/l	0.0818		
		Control *vs* 30μg/l	0.0697		
		5μg/l *vs* 30μg/l	**0.0006**		
4 Day 21	Mean		8.3	10.9	8.3
	Median		7.0	10.0	7.0
	Log rank test P	Control *vs* 5μg/l	**0.0001**		
		Control *vs* 30μg/l	0.3049		
		5μg/l *vs* 30μg/l	**0.0010**		
4 Day 30	Mean		8.9	7.8	9.0
	Median		7.0	8.0	9.0
	Log rank test P	Control *vs* 5μg/l	0.1462		
		Control *vs* 30μg/l	**0.0001**		
		5μg/l *vs* 30μg/l	**0.0001**		

3.3.4. Survival of adult *D. extensus*

3.3.4.1. Experiment 2 - *In vitro* survival of adult dactylogyrids after chronic exposure to 5μg/l cadmium

Tables 3.18 and 3.19 show the mean survival time of *D. extensus* maintained in 0 and 5μg/l cadmium for 14 and 30 days. Survival was assessed in the water from which the parasites were removed, in water from the opposite treatment and in 30μg/l cadmium (actual concentration 19.98μg/ at day 14 and 35.67μg/l at day 30).

Of particular interest is the greater longevity of control parasites (at both day 14 and 30) and those exposed to 5μg/l cadmium for 14 days, when their survival was assessed in 5μg/l cadmium (actual concentration 7.45μg/l), compared to their survival in other media. Survival times did not, however, show any statistically significant differences between treatments (P = 0.052) (Tables 3.18-3.19).

The survival time of control parasites removed from carp after 30-days in the experimental conditions was greater in control water and in 5μg/l cadmium than it had been at day 14, but lower in the 30μg/l solution than previously (Tables 3.18-3.19). By contrast, the survival of the 5μg/l cadmium-exposed parasites was reduced at day 30 in all solutions, compared to the day 14 values, except in 30μg/l cadmium, *i.e.* 9.5 ± 1.25 h at day 14 and 13.4 ± 0.24 h at day 30.

Table 3.18. Experiment 2 - Day 14. Mean survival time (h) of control and 5µg/l cadmium-exposed *D. extensus* in various media. n = 4 in all cases. Actual cadmium concentrations are given in parentheses below the nominal values.

Control tanks	In control water (0.06µg/l)	In 5µg/l cadmium (7.45µg/l)	In 30g/l cadmium (19.98µg/l)	Statistical significance (Kruskal-Wallis)
C1	9.6 ± 1.33	10.4 ± 1.47	12.2 ± 1.25	
C2	10.4 ± 1.29	11.4 ± 1.03	7.5 ± 1.57	
C3	6.2 ± 0.92	8.2 ± 1.74	10.0 ± 1.26	
Pooled controls	8.7 ± 0.80	10.0 ± 0.85	9.9 ± 0.89	
Cadmium-exposed tanks	**In control water (0.06µg/l)**	**In 5µg/l cadmium (7.45µg/l)**	**In 30µg/l cadmium (19.98µg/l)**	P = 0.05
T1	11.2 ± 1.71	12.0 ± 1.38	10.8 ± 2.52	
T2	12.4 ± 0.6	12.4 ± 0.6	9.1 ± 2.12	
T3	7.6 ± 1.33	8.4 ± 1.6	8.6 ± 2.23	
Pooled tests	10.4 ± 0.88	10.9 ± 0.83	9.5 ± 1.25	

Table 3.19. Experiment 2 - Day 30. Mean survival time (h) of control and 5µg/l cadmium-exposed *D. extensus* in various media. *D. extensus* pooled from C1-C3 and from T1-T3 due to low parasite burdens on fish. Actual cadmium concentrations are given in parentheses below the nominal values. (n = 4).

Tank	In control water (0.06µg/l)	In 5µg/l cadmium (7.45µg/l)	In 30µg/l cadmium (35.67µg/l)	Statistical significance (One-way ANOVA)
C1/C3	12.2 ± 1.59	13.5 ± 1.19	7.0 ± 1.67	
Tank	**In control water (0.06µg/l)**	**In 5µg/l cadmium (7.45µg/l)**	**In 30µg/l cadmium (35.67µg/l)**	P = 0.32
T1/T3	6.0 ± 3.14	8.2 ± 2.62	13.4 ± 0.24	

3.3.4.2. Experiment 4 - *In vitro* survival of adult dactylogyrids after chronic exposure to 5 and 30μg/l cadmium

The nominal concentrations of cadmium for this trial were 5 and 30μg/l. The actual concentrations of cadmium in the stock solutions made from artificial water were 18.8 and 30.62μg/l for day 9 and 11.52 and 36.30μg/l for day 30, respectively.

As seen in Experiment 2, 5μg/l cadmium-exposed *D. extensus* removed from carp at day 9 of the trial and incubated for 45 h in water of similar cadmium concentration to that from which they had been removed, exhibited, in general, a greater mean survival than the parasites in the other 2 treatments (Table 3.20). When the survival data were pooled from replicates in the same treatment, they resulted in a mean survival time of 21.5 ± 2.37 h for the 5μg/l parasites compared to 18.4 ± 2.25 and 19.4 ± 3.65 h for the control and 30μg/l cadmium-exposed parasites, respectively. At day 30, the mean survival time of control parasites was the lowest of all 3 treatments, 23.4 ± 3.17 h. The survival times of the 2 cadmium-exposed groups were very similar, with 5μg/l cadmium-exposed parasites surviving for 24.4 ± 1.94 h and 30μg/l cadmium-exposed parasites surviving for 27.6 ± 1.88 h. Kruskal-Wallis tests showed that there were no statistically significant differences in the survival of the parasites between treatments at either day 9 ($P = 0.05$) or at day 30 ($P = 0.19$).

In Table 3.20, the figure given in parentheses is the percentage of the parasites in each treatment that were still alive at the end of the 45 h observation period at day 9, and at the end of the 60 h observation period at day 30. Surprisingly, at day 30 the

Table 3.20. Experiment 4 - Mean survival time (h) of control and 5 and 30μg/l cadmium-exposed *D. extensus* in ASTM water. Survival times are calculated from the time to death of the first 50% of parasites in each tank. Percentages represent the percentage of parasites that were still alive at the end of the 45 h observation period at day 9 and at the end of the 60 h observation period at day 30. The actual cadmium concentrations of the ASTM stock solutions are given in parentheses below the nominal values. (n=number of parasites used from each tank).

Tank		Mean survival time (h)	
		Day 9	Day 30
Controls (0.04μg/l)	C1	29.0 ± 5.0 (n=4) (50%)	22.0 ± 0.0 (n=6) (0%)
	C2	13.7 ± 2.5 (n=9) (25%)	15.0 ± 0.0 (n=4) (0%)
	C3	20.3 ± 2.1 (n=4) (50%)	34.0 ± 5.0 (n=4) (0%)
Pooled controls		18.4 ± 2.25	23.4 ± 3.17
5μg/l cadmium (Day 9 = 18.88 μg/l) (Day 30 = 11.52μg/l)	T1	26.0 ± 0.0 (n=3) (33.3%)	29.0 ± 0.0 (n=1) (0%)
	T2	15.5 ± 2.6 (n=11) (27%)	25.0 ± 2.0 (n=6) (0%)
	T3	30.7 ± 3.3 (n=6) (50%)	33.0 ± 0.0 (n=2) (0%)
Pooled 5μg/l cadmium		21.5 ± 2.67	27.4 ± 1.94
30μg/l cadmium (Day 9 = 30.62 μg/l) (Day 30 = 36.30μg/l)	T1	27.8 ± 5.8 (n=10) (30%)	28.3 ± 1.7 (n=5) (33%)
	T2	15.0 ± 7.09 (n=6) (50%)	24.0 ± 8.0 (n=4) (25%)
	T3	14.5 ± 2.7 (n=11) (27%)	29.3 ± 1.67 (n=6) (0%)
Pooled 30μg/l cadmium		19.4 ± 3.7	27.63 ± 1.88
Statistical significance (Kruskal-Wallis test)		**P = 0.05**	P = 0.19

only tanks where parasites were still found alive at the end of the 60 h observation period were the 30μg/l cadmium-exposed tanks.

3.3.4.3. *In vitro* survival of adult dactylogyrids after acute exposure to cadmium

The *D. extensus* used for this trial had been removed from stock carp and had been previously unexposed to cadmium. The cadmium concentrations in parentheses in Table 3.21 are the actual cadmium concentrations recorded in the stock solutions used for this trial.

The survival time of parasites in 15,000μg/l was 16.08 ± 1.45 h and was statistically lower than the survival of parasites in all other concentrations of cadmium (P = 0.001). The mean survival time of parasites in cadmium concentrations ranging from 0 to 5000μg/l did not differ statistically from one another. Interestingly, the lowest recorded survival was in parasites immersed in control water (43.48 ± 2.35 h), while the greatest longevity was recorded from the parasites immersed in 5,000μg/l cadmium (54.50 ± 3.29 h).

At the end of the survival trial, the number of eggs that had been produced by the dactylogyrids in each cadmium concentration was counted and the results are presented in Table 3.22. Parasites exposed to 50μg/l cadmium produced a statistically greater mean number of eggs (7.75 ± 1.39 eggs) than the parasites exposed to 15,000μg/l (2.81 ± 0.96) (P = 0.025). Egg production by parasites exposed to 5,000μg/l cadmium was also low (3.25 ± 0.89) compared to egg production by parasites in all other cadmium concentrations, which ranged from 5.30 ± 1.06 in 500μg/l to 5.75 ± 1.13 eggs in 250μg/l cadmium.

Table 3.21. Acute survival. Mean survival time (h) of previously unexposed *D. extensus* in a range of cadmium concentrations from 0 to 15,000μg/l. The actual cadmium concentrations are given in parentheses below the nominal values.

Tank	Control (0.04μg/l)	50μg/l (77μg/l)	250μg/l (284μg/l)	500μg/l (611μg/l)	5000μg/l (3408μg/l)	15,000μg/l (13, 100μg/l)
Mean survival time (h)	43.48 ± 2.35	51.10 ± 3.29	44.26 ± 3.97	48.30 ± 3.30	54.50 ± 3.29	16.08 ± 1.45
% worms alive after 75 h	3.3	5.0	0	0	0	0
n	30	20	19	20	18	26
Statistical significance (Kruskal - Wallis)	**P = 0.0001** Significant difference in survival of worms in 15,000ug/l cadmium compared to all other concentrations					

Table 3.22. Acute survival. Mean number of eggs produced by previously unexposed *D. extensus* in range of cadmium concentrations from 0 to 15,000μg/l over a 75 h period.

Treatment	Mean no. eggs produced	Statistical significance
Controls	5.68 ± 1.29	**Kruskal -Wallis test** ***P = 0.025**
50μg/l cadmium*	7.75 ± 1.39*	
250μg/l cadmium	5.75 ± 1.13	
500μg/l cadmium	5.30 ± 1.06	
5,000μg/l cadmium	3.25 ± 0.89	
15, 000μg/l cadmium*	2.81 ± 0.96*	

* Denote statistically significant differences between the means ($P < 0.05$).

3.3.5. *In vitro* survival of oncomiracidia

3.3.5.1. Experiment 2 - *In vitro* survival of oncomiracidia hatched from eggs produced in control conditions and in 5µg/l cadmium

The survival time of oncomiracidia hatched from eggs collected in the *in vitro* egg production trials in Experiment 2 (see section 3.2.2.2) was ascertained and the results are presented in Table 3.23.

In Experiment 2, the survival of oncomiracidia did not differ statistically between treatments at either 21 or 30 days post-start of the trial ($P = 0.70$ and $P = 0.53$, respectively). At day 21, the mean survival times of control and 5µg/l cadmium-exposed oncomiracidia were 8.3 ± 0.3 and 7.7 ± 0.33 days, and at day 30 these values were 7.8 ± 0.92 and 8.1 ± 0.70 days, respectively.

3.3.5.2. Experiment 4 - *In vitro* survival of oncomiracidia hatched from eggs produced in control conditions and 5 and 30µg/l cadmium

The survival times (day) of oncomiracidia hatched from eggs produced by parasites from all 3 treatments, after 30-days exposure to experimental conditions in Experiment 4 (see section 3.2.2.4), are presented in Table 3.24. The survival of oncomiracidia was not statistically different between any treatment ($P = 0.062$). Of particular note, as seen with the adult parasites, is that the oncomiracidia hatched from eggs produced by parasites exposed to 5µg/l cadmium survived longer than those hatched from control and 30µg/l cadmium-exposed eggs.

Table 3.23. Experiment 2 - Mean number of days for control and 5μg/l cadmium-exposed oncomiracidia, hatched from day 21 and day 30 eggs, to survive.

Day	Control tanks	Mean no. days surviving	5μg/l cadmium tanks	Mean no. days surviving	Statistical Significance
21	**C2**	8.3 ± 0.3 (n=10)	**T2**	7.67 ± 0.33 (n=3)	Unpaired t-test P = 0.70
30	**C3**	7.8 ± 0.92 (n=5)	**T3**	8.09 ± 0.70 (n=11)	Kruskal-Wallis P = 0.53

Table 3.24. Experiment 4 - Mean number of days for control and 5 and 30μg/l cadmium-exposed oncomiracidia, hatched from day 30 eggs, to survive. Actual cadmium concentrations are given in parentheses below the nominal values.

Treatment and tank		Mean no. days surviving	
Controls (0.04μg/l)	**C1**	4.75 ± 0.77	(n=8)
	C2	4.00 ± 0.53	(n=9)
	C3	2.75 ± 0.25	(n=4)
5μg/l cadmium (11.52μg/l)	**T1**	5.00	(n=1)
	T2	5.33 ± 1.45	(n=3)
	T3	5.00 ± 0.71	(n=4)
30μg/l cadmium (36.30μg/l)	**T1**	4.00	(n=1)
	T2	4.25 ± 0.25	(n=4)
	T3	4.17 ± 0.16	(n=6)
Statistical significance (Kruskal-Wallis)	**All tanks compared**	P = 0.062	

3.4. Discussion

The aim of this chapter was to determine the effect of cadmium at its permitted level, 5μg/l, and at the equally environmentally realistic level of 30μg/l, on the reproductive biology and survival of the monogenean gill parasite *Dactylogyrus extensus*.

Before discussing the impact of cadmium on the rate of oviposition by *D. extensus*, the lack of recruitment during the current trials should be addressed. Bauer & Nikolskaia (1954, cited by Prost, 1963) found that at water temperatures of 17-19°C, the post-embryonic development of *D. extensus* lasted 11 days. The current trials, run at 16-17°C, had been expected to last for 60 days, allowing for several generations of *D. extensus* to develop and be monitored. However, by day 30 of each experiment, it was apparent that the numbers of *D. extensus* on both control and cadmium-exposed carp were very low, resulting in the termination of the experiments at this time. The lack of recruitment is confusing given that oncomiracidia from both treatments were observed to hatch and survive for several days (ca. 5-8 days) *in vitro*. As the larvae were able to survive *in vitro*, this suggests that the conditions in the experimental tanks were not conducive to recruitment. To encourage recruitment of the oncomiracidia, two precautionary measures were implemented in each experiment. Firstly, the flow rate of the incoming water to each tank was maintained at only 100 ml/min. At this flow rate the water trickled slowly into each tank, minimising water turbulence and ensuring that oncomiracidia were retained in the tanks rather than being flushed from them by strong water currents. It had originally been decided to surround the outflow pipe in each tank with a fine mesh to help retain the oncomiracidia in the system. However, the small

size of the mesh required (< 100μm) meant that water flow from the tanks was restricted; in the cadmium-exposed tanks, this would have resulted in a build up of cadmium beyond the concentration required and, consequently, the use of this mesh was disregarded. The second measure implemented was to cover the sides of the experimental tanks with black plastic, leaving the lids uncovered thus creating a unidirectional light source. *D. extensus* oncomiracidia, as with many monogenean larvae, have four pigment-shielded eye-spots (Whittington, Chisholm & Rohde, 2000). Llewellyn (1963), reviewing Bychowsky's (1957) work on the behaviour of larval monogeneans, reported that dactylogyrid oncomiracidia have two behavioural phases; an early positively phototactic swimming phase followed by a negatively phototactic phase during which the larvae attaches to the host. It was expected, therefore, that, during the photonegative phase, *D. extensus* oncomiracidia would have moved to and remained near the bottom of the tank, bringing them in close contact with their carp hosts. However, as neither of these preventative mechanisms encouraged recruitment, it is possible that the stocking density of the tanks was too low (ca. 10-30 carp), making it more difficult for the oncomiracidia to come into contact with a host. Increasing the stocking density to encourage recruitment would, however, require a greater water flow rate into each tank to maintain the health of the carp, and problems with retaining oncomiracidia in the tanks may, therefore, still prove to be an issue.

The principal aim of this chapter was to determine how cadmium exposure would affect the rate of oviposition by *D. extensus*. It had been hoped that the egg production of successive generations of *D. extensus* could be determined, thus, indicating whether each new generation was becoming more tolerant to cadmium-

exposure. Unfortunately, due to the lack of recruitment in each trial, this was not possible. Nonetheless, egg production by adult *D. extensus* did appear to be affected by exposure to cadmium, with several consistent trends emerging.

Of particular interest is that cadmium-exposed *D. extensus*, in the early stages of all four experiments (ca. day 9/10), produced a greater number of eggs, both *in vitro* and *in vivo*, than their control counterparts. Interestingly, only the *in vivo* egg production of the 5μg/l cadmium-exposed parasites was statistically greater than the controls. However, over the four experiments there were eleven *in vitro* egg production sample points; 64% of these showed that cadmium-exposed *D. extensus* produced more eggs than the control parasites.

The increase in egg production by dactylogyrids exposed to cadmium can be attributed to two possible factors. Firstly, cadmium may have been directly affecting the parasites. Flick, Kraybill & Dimitroff, (1971) have stated that cadmium has a strong affinity for binding to proteins. It is possible, therefore, that cadmium became incorporated in to the proteinaceous shells of the dactylogyrid eggs and that the production and release of more eggs by the cadmium-exposed dactylogyrids reduced the amount of cadmium they had to detoxify and/or sequester, thus lowering the toxicity of this metal. Burger (1994) found that avian eggshells provide a mechanism for the excretion of heavy metals for the roseate tern *Sterna dougallii* (Mont.) and the herring gull *Larus argentatus*, and it is possible that *D. extensus* use a similar method to remove cadmium from the body. It had been hoped to determine the amount of cadmium incorporated into the eggshells of *D. extensus* eggs through X-ray elemental analysis. Unfortunately, two problems were encountered. The first

problem arose in trying to maintain the integrity of the egg when it was placed on the carbon disc required by this technique. Once on the disc, it was extremely hard to locate an egg and, once located, it was found that the egg had burst on drying. This would have made it very difficult to ascertain whether any cadmium detected was part of the eggshell or had come from leakage of the egg contents. Producing thin sections through an egg and analysing these with X-ray elemental analysis was also considered. However, the second problem encountered was that the X-ray elemental equipment has an optimal detection limit at concentrations of cadmium greater than 500ppm (*pers. comm.*, J. Spratt, The Natural History Museum, London), considerably more than could possibly be incorporated into a single monogenean egg. Further work is needed to identify an appropriate method which would allow this hypothesis to be investigated. For now, however, it is unlikely that this interpretation completely explains the greater production of eggs by cadmium-exposed *D. extensus*, as it would be expected that the 30μg/l cadmium-exposed parasites would have produced consistently more eggs than the 5μg/l group. However, this was only true of days 9 and 14 in Experiment 4. That the egg production of the parasites exposed to the higher cadmium concentration (30μg/l) was less than that of the parasites exposed to 5μg/l cadmium from day 21 onwards, suggests that cadmium may accumulate in the parasites over time and impair reproductive output. The ability of *D. extensus* to accumulate cadmium will be explored in Chapter 5.

The second hypothesis to explain the greater egg production by cadmium-exposed *D. extensus* is that this metal may affect some aspects of the host immune response, resulting in an indirect effect of the metal on the parasite and its reproductive

biology. This hypothesis is particularly likely given that the *in vivo* egg production at day 9 was found to be statistically different between the treatments, and that between days 9-11 of the *in vivo* trial the production of eggs by parasites in both treatments increased. To obtain the dactylogyrid eggs for counting in the *in vivo* trial, the carp had to be netted from their tanks daily while the bottom of each tank was siphoned. Jacobs (1995) found that stressing carp, infected with *Dactylogyrus* sp., by netting them out of their tanks for 30 seconds three times daily, resulted in the infection levels of these carp increasing over time, compared to the non-stressed control group, whose levels of infection fell after 21 days. Thus, during the *in vivo* egg production trial, the stress caused by the netting and moving of the carp may have caused alterations to the host immune responses, which resulted in the parasites producing a greater number of eggs over time. The added stress of cadmium-exposure on the carp could thus explain the increased rate of oviposition in the *D. extensus* exposed to 5μg/l cadmium compared to the controls. Aspects of the carp immune response will be explored in the subsequent chapter (Chapter 4 - The effect of cadmium on selected aspects of the innate immune response of carp) in an attempt to elucidate how host responses may be influencing parasite biology.

In both Experiments 2 and 4, the rate of *in vitro* egg production by the control and the cadmium-exposed *D. extensus* increased at each subsequent sample. As the experimental conditions remained the same throughout each experiment this finding also suggests that host responses may play a role in dactylogyrid egg production. It also indicates that, where the control parasites were recorded as producing more eggs than the cadmium-exposed parasites, this was a function of increased

oviposition by the controls rather than a suppression of oviposition in the cadmium-exposed individuals.

The general increased rate of oviposition by *D. extensus* exposed to cadmium, as demonstrated by these current trials, is in contrast to the findings of other authors on a range of free-living invertebrates. As there have been no previous studies investigating the effect of heavy metals on the reproductive biology of monogenean fish parasites, the findings from these current experiments can only be discussed in relation to data available on these free-living invertebrates. For example, Davies *et al.* (1995) exposed the leech *Nephelopsis obscura* to varying concentrations of cadmium (0-580μg/l) for 24 days and found a reduction in the number of ova and spermatozoa per unit biomass with increasing concentrations of cadmium. Similarly, Gomot (1998) found that exposing the freshwater snail *Lymnea stagnalis* to 0 to 400μg/l cadmium for seven weeks resulted in a reduction in egg production with increasing concentrations of this metal and complete inhibition of egg production at 400μg/l. The different effects of cadmium on free-living invertebrates and on *D. extensus* suggest that cadmium is influencing the two groups in different ways. The suppression of egg production in the invertebrates noted above must be due to the direct effect of the cadmium on these organisms due to their free-living status. However, unlike the trials noted above, at the majority of time points in the current trial, 5μg/l cadmium-exposed parasites produced more eggs than the controls, suggesting that host responses may indeed be playing a part in the rate of oviposition of *D. extensus* and suggesting a dynamic interaction between parasite and host. However, it should be noted that previously unexposed *D. extensus* did

show a reduced rate of oviposition when exposed to more than 5000μg/l cadmium, suggesting that at these high levels, cadmium directly impacts on the parasites.

Despite the relatively consistent trend for cadmium-exposed *D. extensus* to produce more eggs than the controls, only at two time points (Day 29, Experiment 1 *in vitro* production and Day 9, Experiment 3 *in vivo* production) were the differences between the groups statistically significant. The lack of statistical differences should be addressed and can be attributed to several possible factors. The first factor is that the cadmium concentrations of 5 and 30μg/l were too low to elicit a strong response from either the parasites directly or from their hosts. During the acute survival trial, the longevity of *D. extensus* that had previously been unexposed to cadmium was assessed in cadmium concentrations ranging from 0 to 13,100μg/l and the mean number of eggs they produced in each concentration recorded. Only on incubation of *D. extensus* in the highest cadmium concentration (13,100μg/l) was a statistically significant reduction in egg production recorded. The statistically lower rate of egg production was in comparison to the parasites exposed to 77μg/l, which, interestingly, produced a greater number of eggs than the controls. These findings suggest that *D. extensus* is very tolerant of high levels of cadmium and that much higher levels than those used in the current trials (Experiments 1-4) are needed to significantly affect its reproductive output. A second hypothesis for the lack of statistical differences, particularly in the *in vitro* egg production trials, is the heterogeneity in the rate of oviposition displayed by parasites from the same tanks and treatments. The heterogeneous results may have masked the true effect of cadmium on dactylogyrid egg production. It was hoped that, by selecting only adult *D. extensus* for the egg production trials, the individual parasites would have been

of approximately the same age and maturity. However, Kearn (1986) has stated that the only way to achieve parasites at the same stage of maturity is to experimentally infect fish with oncomiracidia. However, this would require experiments to be run on a much smaller scale than the present studies and would not reflect the natural conditions of this host-parasite system. Larger sample sizes may have overcome this heterogeneity, however, due to problems with recruitment and the need for parasites for a range of procedures, obtaining larger samples in these trials was not possible.

From the results of these egg production trials it can be seen that at about days 9-10 appears to be a critical period for *D. extensus* exposed to cadmium, with egg production by these parasites being greater than the controls in all experiments. The consistent trend for elevated rates of oviposition by the cadmium-exposed parasites is of great interest, despite the lack of statistical difference between the treatments in all experiments. It would seem that 5μg/l cadmium enhances egg production, while exposure to 30μg/l cadmium, possibly due to its greater toxicity, begins to detrimentally affect reproduction over time. It is, therefore, possible that small, low-level, cadmium-induced increases in egg production by dactylogyrids may be enough to increase the infection levels and potentially increase mortalities.

The eggs that had been produced in the egg production trials were monitored and the time taken for each to hatch recorded in order to determine how cadmium would impact on both hatch success and rate. As with the egg production trials, egg hatching trials were carried out at 16-17°C, which, according to Prost (1963), is the optimum temperature for *D. extensus* egg development. All hatching times were calculated using only the percentage of eggs that hatched; undeveloped eggs were

not included in the calculations. Two types of undeveloped egg became apparent during these trials. The first was the type that, once enzymatically tanned, never developed any further and no larvae formed. These formed the bulk of the undeveloped eggs during the trials. The second type were eggs that completed larval development but the larva subsequently died and never hatched. These larvae then appeared to degenerate within the eggshell. This second type has been recorded by Paperna (1963) for *D. vastator* and, although he monitored them for a year, they failed to hatch. For the purpose of these experiments, no distinction was made between the types of undeveloped eggs, and they were classed together and excluded from calculations.

There are several records of cadmium inhibiting or arresting egg hatching in various invertebrate species, including *Artemia* (10μM), the mosquito *Aedes aegypti* (5 mg/l), the freshwater snail *Lymnea stagnalis* (400μg/l) and the calanoid copepods *Acartia hudsonica* Pinhey, 1926 and *A. tonsa* Dana, 1848 (10μM) (see Rafiee *et al.*, 1986; Gomot, 1998; Rayms-Keller *et al.*, 1998; Hook & Fisher, 2001). Inhibition of hatching has also been reported in several species of digenean (Evans, 1982a, b; Abd Allah, Wanas & Thompson, 1997; Morley *et al.*, 2001a, b). The hatching of the 30μg/l cadmium-produced *D. extensus* eggs was similarly inhibited in comparison to the control and the 5μg/l cadmium-exposed eggs. Surprisingly, however, the eggs laid and hatched in 5μg/l cadmium showed evidence of a statistically quicker hatch rate than the control eggs. The reasons for these eggs tending to hatch more quickly than the controls are not clear, but similar results have also been recorded by Devkota & Schmidt (1999) in acridid grasshoppers exposed to 2-100μg/g cadmium and in the southern king crab *Lithodes santolla*

exposed to 0.2 mg/l and 2 mg/l cadmium by Amin *et al.* (1998). The explanation given for the early hatching of *L. santolla* zoea, despite their not being fully formed, was an escape response to metal injury. It was suggested that hatching early as a swimming larvae would allow the zoea to avoid the cadmium stressor (Amin *et al.*, 1998). However, as a similar response was not displayed in the hatching of the 30μg/l cadmium-exposed *D. extensus* eggs, this hypothesis may not be applicable to *D. extensus* oncomiracidia. A second possible explanation for the early hatching of 5μg/l cadmium-exposed eggs could be that cadmium at this concentration weakens the opercular cement, making it easier for the oncomiracidia to hatch. It is possible that cadmium may be able to directly soften the opercular cement, enabling the oncomiracidia to hatch more easily. Likewise, cadmium may interfere with the enzymatic tanning process of the eggshells, resulting in their not hardening completely and making it easier for the oncomiracidia to hatch. A final hypothesis for the induction of the earlier hatching of dactylogyrid eggs exposed to 5μg/l cadmium could be attributed to hormesis. Hormesis (derived from the Greek word hormaein, meaning to excite) is the stimulation of any system by low doses of an agent such as heavy metals (Sander, Jenkins, Sunda & Costlow, 1983; Luckey, 2000). High doses of the same agent, however, can result in detrimental effects (Luckey, 2000). Sander *et al.* (1983) reported that hormesis was responsible for the increased weight of crab larvae (hormetic growth enhancement) exposed to copper. Hormetic growth enhancement in the 5μg/l cadmium-exposed oncomiracidia may have made it easier for the larvae to hatch from the eggs, thus resulting in the observed earlier hatch rate. Despite the extensive use of hormesis as an explanation for a variety of observed effects, the biological basis for this phenomenon has never been satisfactorily explained.

The most likely hypothesis for the 5μg/l cadmium-exposed eggs hatching earlier is, however, the interference of cadmium with the enzyme systems involved in the tanning process. Electron microscope studies of eggs could be used to show any weaknesses in the eggshells in order to determine if cadmium at 5μg/l does influence the tanning process in comparison to the controls. The reason for the slower rate of hatch in the 30μg/l eggs can probably be attributed to those eggs that went unhatched for over 30 days, only to hatch successfully after that time. This was particularly evident in eggs produced and hatched in 30μg/l cadmium. When dactylogyrids are producing eggs *in vitro*, the eggs laid last tend to be much smaller than the others (*pers. obs;* Paperna, 1963). It is, therefore, possible that the production of these smaller eggs was greatest by the 30μg/l cadmium-exposed *D. extensus* due to the added stress of the cadmium exposure and that the delay in hatching was attributed to the longer development and poorer viability of these eggs. However, it should be noted that there is at present no evidence to suggest that smaller eggs take longer to hatch. Thus, future experiments should determine the size of eggs produced in each treatment, at both the beginning and end of the egg laying period in order to determine whether cadmium is increasing the production of these small eggs, and the time to hatch of these eggs should also be monitored.

Fish and parasites are exposed to anthropogenic sources of heavy metals in two distinct ways. Chronic exposure refers to continuous exposure to relatively low concentrations of metals and comes from non-point sources, such as agricultural and urban run-off. Acute exposure refers to infrequent exposure to much higher concentrations and results from discharges from manufacturing plants or from hazardous waste disposal sites. Because of the different exposure methods host-

parasite systems may be exposed to in the wild, it was decided that survival of *D. extensus* exposed to chronic and acute levels of cadmium be assessed.

Except for Experiment 2, the survival of *D. extensus* was assessed in artificial water. Experiment 2 was run as a preliminary trial, with the survival of the parasites being assessed in water taken from the experimental tank system. By using tank water there were possible inconsistencies in water quality between treatments, particularly with regard to the concentration of calcium ions, Ca^{2+}. Cadmium is known to enter some invertebrates through active transport pumps for calcium (Rainbow, 1996) and, consequently, different concentrations of calcium ions in the water may affect the toxicity of cadmium to the parasites, potentially affecting their survival time. The results of all trials carried out in artificial water only will be discussed, as the water quality was standard throughout (8.1-8.4 ppm Ca^{2+}) and the results are considered to be more representative than those from Experiment 2.

The most interesting feature of note from the chronic survival trials was the general trend for *D. extensus* exposed to cadmium to survive longer off the host than the controls. Morley *et al.* (2001b,c) recorded a similar enhancement in the survival of the cercariae of the digeneans *Schistosoma mansoni* and *Diplostomum spathaceum* when exposed to 10μg/l cadmium. The enhanced survival of these digeneans was attributed to cadmium acting as an enzyme inhibitor and inhibiting the enzymes involved in glycogen utilisation, which, coupled with lowered cercarial activity, reduced the utilisation of the finite glycogen reserves (Morley *et al.*, 2001c). However, in contrast to the cercarial stages of digeneans, adult dactylogyrids are not free-living and they are, therefore, not dependent on a limited glycogen store for

their survival. Cadmium may, however, slow down the metabolism of *D. extensus* by inhibiting the enzymes involved with various metabolic reactions, resulting in their prolonged survival compared to the controls. However, detailed studies on the metabolic systems of monogeneans need to be undertaken before it can be determined which metabolic reactions might potentially be affected by cadmium exposure. It would be of great interest in subsequent experiments to maintain and culture successive generations of *D. extensus* to determine the survival and egg production of each generation in order to ascertain whether a cadmium tolerant population is being selected for over time. This was planned in this study but, as explained previously, population decline exceeded the anticipated 3 generations in the 30 day trial period.

In contrast to the results of the current trials, most records of the effect of heavy metals on the survival of parasites have resulted in a reduction in survival. This has been noted in a wide range of parasite species, from the cercariae of *Cryptocotyle lingua, Schistosoma mansoni, Echinoparyphium recurvatum* Linstow, 1873 and *Notocotylus attenuatus* to the larval nematode *Nippostrongylus brasiliensis* Travassos, 1914 (Mecham & Holliman, 1975; Asch & Dresden, 1977; Holliman & Esham, 1977; Evans, 1982a, b; Badawy & Al-Sharkawi, 1997; Gadmoska & Zakrzewska, 1997; Cross *et al.*, 2001; Pietrock *et al.*, 2002a,b).

The lack of statistical difference in survival in the current trials can be attributed to the small sample sizes obtained for each trial. Unfortunately, the requirement for specimens for a broad range of trials meant that the overall number used in each survival trial had to be reduced. Future experiments should attempt to use greater

numbers of parasites for the survival trials to improve the accuracy of the results. However, the cadmium concentrations that were used for the chronic survival trials may just have been too low to significantly affect the survival of *D. extensus*. This is the most likely hypothesis, given that the acute survival trial showed that *D. extensus* are extremely tolerant to high concentrations of cadmium, being able to tolerate concentrations of 13,100μg/l for an average of 16.08 ± 1.45 h.

Problems with collecting, moving and locating oncomiracidia meant that the sample sizes for the larval survival trials were also small. This may have biased the results and they may not be as representative as desired. As in the adult parasites, there was a slightly enhanced survival of the oncomiracidia in 5μg/l cadmium compared to the controls. Llewellyn (1963) stated that, in most monogenean oncomiracidia, the gut contains lipid food reserves. As with the adult parasites, it is possible that cadmium disrupts the enzyme systems concerned with metabolising these lipid reserves, resulting in these oncomiracidia surviving longer than the controls.

In conclusion, consistent, subtle trends in the data suggest that low levels of cadmium can affect the reproductive biology and survival of both *D. extensus* adults and oncomiracidia. With regard to the adult parasites, it is possible that cadmium is causing the effects directly and/or indirectly via the host responses. The following chapter will investigate the impact of cadmium on common carp in order to try and determine any host-parasite interactions in the presence of this metal.

Chapter 4

The effect of cadmium on selected aspects of the innate immune response of carp

4.1. Introduction

Fish, being in direct contact with their surroundings, are susceptible to changes in water quality, and the presence of heavy metals has been shown to alter their immune functions and thus their resistance to disease-causing agents (O'Neill, 1981; Anderson, 1996). Several authors have investigated the effect of heavy metals on the fish immune system, including Thuvander (1989), Zelikoff *et al.* (1995), Sanchez-Dardon *et al.* (1999) and Kotsanis, Iliopoulou-Georgudaki & Kapata-Zoumbos (2000). A comprehensive review by Hoole (1997) has summarised the impact of heavy metals on the immune responses of fish and has considered how these changes may affect helminth parasites.

The innate (non-specific) immune response of fish comprises physical barriers, such as the skin and mucus, as well as phagocytes (macrophages, monocytes and granulocytes) and natural killer cells. In teleosts, the innate immune system is particularly important with regard to disease resistance, perhaps more so than the specific immune system (Secombes & Fletcher, 1992; Hutchinson & Manning, 1995). Woo (1992) also considered that the specific immune response was less important in fish, as it is slow to develop due to their low body temperature. The specific immune responses act as a second line of defence against invading pathogens, acting against specific antigens and having a memory component. Several studies have investigated the effect of heavy metals on aspects of the

adaptive immune response, including those by O'Neill (1981), Thuvander (1989), Mohan (1990) and Khangarot, Rathore & Tripathi (1999). More emphasis has, however, been placed on the effect of heavy metals and other pollutants on aspects of the non-specific immune response. The following is an overview of the information available on the effect of cadmium on some aspects of the innate immune response and haematological parameters of fish.

4.1.1. Fish phagocytes

A range of phagocytic leucocytes have been described in teleosts, including granulocytes, neutrophils and macrophages (Secombes & Fletcher, 1992). These phagocytes have many roles and are a particularly important component of the innate immune system. As well as phagocytosing a wide range of inert and antigenic particles, Secombes & Fletcher (1992) state that these cells have potent bactericidal and larvacidal properties via both intra- and extracellular killing mechanisms. These phagocytic cells also act as accessory cells, with macrophages being known to initiate many responses, as well as controlling a range of ongoing reactions by releasing immunomodulatory molecules, such as the cytokine interleukin-1 (IL-1) and a range of eicosanoids. Macrophages are also known to have antigen-presenting abilities in fish as well as being capable of spontaneous cytotoxicity (Secombes & Fletcher, 1992; Secombes, 1996).

4.1.1.1. Phagocytosis

Phagocytosis occurs when a particle attaches to the membrane of the phagocyte. The attachment of these particles is enhanced by opsonins, such as complement, antibodies and C-reactive protein (CRP) (Secombes & Fletcher, 1992; Secombes,

1996; Bols *et al.*, 2001; Paul, Mandal & Mandal, 1998). Opsonins are involved in mediating the ligand-receptor interactions between the phagocyte and the adhering cell (Secombes & Fletcher, 1992).

The phagocytic activity of leucocytes has shown varied responses with regards to heavy metal exposure, depending on the fish species and the type and concentration of metal used. Information relating only to cadmium will be detailed below, although the effect of other heavy metals and pollutants on aspects of the immune response, including phagocytosis, have been detailed by Weeks, Warinner, Mason & McGinnis (1986), Weeks & Warinner, (1986), Muhvich, Jones, Kane, Anderson & Reimscheussel (1995), Dethloff, Schlenk, Khan & Bailey (1999), Lacroix, Fournier, Lebeuf, Nagler & Cyr (2001) and Shariff, Jayawardena, Yusoff & Subasinghe (2001).

The effect of cadmium on phagocytosis has shown varied responses, depending on the concentration, the exposure period and the fish species used. Thuvander (1989) found that exposing *Oncorhynchus mykiss* (Walbaum) to 3.6μg/l cadmium for 12 weeks resulted in no statistical difference in the proportion of phagocytic cells compared to the controls. However, despite the lack of statistical differences, at weeks 2, 5 and 9, the proportion of phagocytic cells in the cadmium-exposed fish was slightly lower than the controls, while after 12 weeks the proportion of these cells was twice as great as the controls.

Interestingly, conflicting results were obtained in two separate studies on *O. mykiss* exposed to low concentrations of cadmium. Zelikoff *et al.* (1995) exposed the fish

to 2μg/l cadmium for 30 days and found that by day 8 the phagocytic activity of these fish was significantly elevated above the controls. Further samples taken at days 17 and 30 saw a fall in the phagocytic activity of the cadmium-exposed *O. mykiss*, although the activity remained consistently higher than the controls (Zelikoff *et al.*, 1995). A similar enhancement in phagocytic activity was also recorded in *Cyprinus carpio* after 96 hours exposure to 5 mg/l cadmium (Sövényi & Szakolczai, 1993), in *O. mykiss* exposed to 2μg/l cadmium for 17 days (Enane, Bowser, Frenkel, Squibb & Zelikoff, 1991) and in the pulmonary macrophages of rats after aerosol exposure to 1.5 mg/m^3 of cadmium (Greenspan & Morrow, 1984). However, exposure of rats to cadmium at 5 mg/m^3 resulted in a suppression of this activity when compared to the controls (Greenspan & Morrow, 1984).

In contrast to the findings of Zelikoff *et al.* (1995), a further study, carried out by Sanchez-Dardon *et al.* (1999), exposed *O. mykiss* to 1 and 5μg/l cadmium and found that phagocytosis was significantly inhibited by both these concentrations. Neither of these two studies detailed the water quality parameters that the fish were exposed to, and it is possible that differences in water hardness between the two experiments affected the toxicity of the cadmium, thus affecting its impact on the phagocytes.

4.1.1.2. Phagocyte respiratory burst

Phagocytes are capable of killing a variety of pathogens by both oxygen-dependent and oxygen-independent means. With small invading microorganisms, the killing is usually intracellular, but extracellular killing also occurs in the case of parasite larvae (see Whyte, Chappell & Secombes, 1989). On phagocytosis, there is increased oxygen uptake by the cell and the oxygen is reduced to the superoxide

anion (O_2^-). The reduction is carried out by the enzyme NADPH oxidase that is found in the membrane of phagocytes. The O_2^- anion is then converted into a range of reactive oxygen species (ROS) such as hydrogen peroxide and hypochlorous acid, which act as powerful microbicidal agents (Secombes & Fletcher; 1992, Secombes, 1996; Bols *et al.*, 2001). The respiratory burst can be measured *in vitro* by stimulation of phagocytes with synthetic agonists, such as phorbol-12-myristate-13-acetate (PMA), or with pathogen derived agents such as whole bacteria.

Both Zelikoff *et al.* (1995) and Sanchez-Dardon *et al.* (1999) found that low level cadmium exposure significantly reduced the respiratory burst activity of phagocytic cells in *O. mykiss*. Zelikoff *et al.* (1995) found that the spontaneous respiratory burst, *i.e.* unstimulated by PMA, increased significantly above the controls after 8 and 17 days exposure to cadmium, before showing a significant fall below control values at day 30 of the trial. It was suggested that the fall in spontaneous respiratory burst activity at day 30 may result in the inability of the *O. mykiss* to carry out normal defence processes (Zelikoff *et al.*, 1995). The marine fish *Limanda limanda* (L.), exposed to 1.3, 2.7 and 5.5 mg/l of cadmium for 9 weeks, also showed a significantly reduced respiratory burst compared to the controls, and this reduction was still significant in the fish exposed to 1.3 and 2.7 mg/l after a three week depuration period (Hutchinson & Manning, 1995). The *L. limanda* exposed to 5.5 mg/l also continued to display a reduced respiratory burst compared to the controls after depuration, but this difference was found to be non-significant, which is not unexpected as the results at this time were based on data from one fish only (Hutchinson & Manning, 1995).

In contrast to the studies detailed above, Enane *et al.* (1991) exposed *O. mykiss* to 2μg/l cadmium for 17 days and recorded an enhanced production of both the superoxide anion and hydrogen peroxide. Zelikoff, Wang, Islam, Flescher & Twerdok (1996, reviewed by Zelikoff, 1997), found that medaka *Oryzias latipes* Temminck & Schlegel exposed to 6 and 60μg/l cadmium showed enhanced intracellular and extracellular production of superoxide. While this macrophage function was found to be altered by exposure to these cadmium concentrations, she recorded no changes in the haematocrit, leucocrit and immunoglobulin levels.

4.1.2. Haematological parameters

Tort & Torres (1988) stated that a study of the haematological variables of fish is important due to their relationship with defence mechanisms (leucocyte values), respiration (haemoglobin concentration) and energetics (metabolite levels). Coupled with the fact that blood parameters respond quickly to environmental alterations and are relatively easy to measure, they are an important factor to monitor with regard to cadmium exposure. Information concerning haematocrit levels in fish exposed to cadmium is conflicting and varies with both fish species and the concentration of cadmium to which they are exposed. High levels of cadmium (25 mg/l and 17-25 mg/l) resulted in a decrease in haematocrit level in both the dogfish *Scyliorhinus canicula* (L.) (Tort & Torres, 1988) and the tilapia *Tilapia zillii* (Gervais) (Ghazaly, 1992), a phenomenon also observed when *C. carpio* were exposed to the much lower level of 50μg/l cadmium (Mohan, 1990). Mohan (1990) found that the haematocrit level of carp exposed to 50μg/l cadmium was lower than the controls at all time points in the 30 day trial except days 1 and 30. In contrast to the findings of Mohan (1990), Witeska (1998) found that exposing *C. carpio* to 10 mg/l cadmium

resulted in elevated haematocrit levels compared to the controls. In all the trials discussed above, the white blood cell counts were reported to be lower in the cadmium-exposed fish compared to those in the controls (Tort & Torres, 1988; Mohan, 1990; Ghazaly, 1991; Witeska, 1998), and Tort & Torres (1988) and Witeska (1998) found that the erythrocyte number increased on exposure to cadmium. From a study by Kotsanis *et al.* (2000), it would appear that, unlike adult fish, embryonic exposure to cadmium does not predispose *O. mykiss* to altered haematological parameters as they develop. It should be noted that haematological alterations can also be triggered by a variety of stressors, including handling stress (Ellis, 1981; Wedemeyer & McLeay, 1981; Ellsaesser & Clem, 1986).

4.1.3. Cortisol levels

Hoole (1997) stated that the effects of heavy metals on the immune responses of fish may be mediated directly or via changes in the levels of corticosteroids, resulting as part of a general stress response. Schreck (1996) reviewed the impact of cortisol, the major corticosteroid produced by teleosts, and detailed the impact that this hormone can have on some aspects of the immune response.

Mohan (1990) found that exposing *C. carpio* to 50μg/l cadmium resulted in a significant elevation in plasma cortisol level above control values in the early stages of his trial. However, the response was transitory, with the levels decreasing from 28ng/ml at day 1 to 16ng/ml at day 5 of the trial, before remaining relatively consistent for the remainder of the 30-day trial. The transitory return of elevated cortisol to control levels after long-term exposure to cadmium (10μg/l for 35 days) has also been observed in *Oreochromis mossambicus* (Peters) indicating the ability

of this fish to adapt to low cadmium concentrations (Pratap & Wendelaar Bonga, 1990). A similar elevation of plasma cortisol was observed in the American eel *Anguilla rostrata* Lesueur exposed for 16 weeks to 150μg/l cadmium, and it was suggested that prolonged hypercortisolemia might affect the eel's immune system (Gill, Leitner, Porta & Epple, 1993). An interesting study by Ricard, Daniel, Anderson & Hontela (1998) showed that adult *Oncorhynchus mykiss*, exposed to 10μg/l cadmium for 30 days in water with a hardness of 130 mg/l $CaCO_3$, not only had cortisol levels that were elevated above the controls but that were also elevated above those of adult fish exposed to 25μg/l cadmium for the same time period. In contrast, a study by Pelgrom, Lock, Blam & Wendelaar Bonga (1995) that found that exposing immature *Oreochromis mossambicus* (2 months old) to 20, 35 and 70μg/l cadmium did not result in any elevation of the cortisol concentration above the controls, while exposure to copper did cause an increase in the cortisol level of these fish.

4.1.4. Aims of the present study

The primary objective of this chapter was to determine whether exposure of common carp to cadmium, both at its maximum permissible level (5μg/l) and at the environmentally realistic level of 50μg/l, could elicit an alteration in one or more of the innate immune system parameters. The results from this chapter will be considered with those from Chapter 3 (The effect of cadmium on the reproductive biology and survival of *Dactylogyrus extensus*) in order to begin to elucidate host-parasite interactions in the presence of cadmium.

4.2. Materials and methods

4.2.1. Fish

One hundred and thirty common carp (8-12 cm) were held in an 80 litre flow-through tank for 5 months, at an ambient temperature of 4-9°C, and fed daily on a commercial pelleted feed. These fish, which had been bought to use for a *Dactylogyrus extensus* biology trial, were found to harbour very low levels of the parasite and were not used and were thus held as stockfish. Two weeks prior to the start of the trial, 5 fish were randomly selected from the tank and were thoroughly examined for parasite infection. Skin scrapes and gill examinations showed no evidence of parasite infection and, coupled with the absence of any mortalities in the population over a 3-month period, the population was considered to be disease free.

A month prior to the start of the trial, 3 Visi-Therm (100W) aquarium heaters were introduced to the holding tank and the temperature was brought slowly to 16°C. Two weeks prior to the start of the trial the fish were transferred to the flow-through system in the warm water aquarium of the Institute of Aquaculture. The temperature of the water in these aquaria was also set at 16-17°C. Six tanks were chosen at random from the 12 available tanks and 20 fish were placed into each. The fish were fed daily and left to acclimate to their new conditions for 2 weeks before the trial was started.

4.2.2. Cadmium concentrations

Two tanks of fish were maintained as controls during the trial, 2 were exposed to 5μg/l and 2 to 50μg/l cadmium.

4.2.3. Sampling regime

At 9, 14, 21 and 29 days post-start of the trial, 3 fish were taken from each tank and were processed, as detailed in section 4.2.4, for a range of immunological assays. The sample times were chosen to coincide with the data obtained on the reproductive biology of *Dactylogyrus extensus* (Chapter 3 - The effect of cadmium on the reproductive biology and survival of *Dactylogyrus extensus*) at the same time points.

4.2.4. Immunological assays

4.2.4.1. Removal of blood

Fish were killed by anaesthetic overdose and percussive stunning and were then bled from the caudal vein using heparinized 1 ml syringes fitted with 25 gauge needles. The blood was transferred to labelled 1.5 ml Eppendorfs and kept on ice until it could be processed. Each fish was then put in an individual labelled bag and placed on ice until it could be processed further.

4.2.4.2. Haematocrit (packed cell volume)

The blood samples were removed from the ice and shaken gently to ensure thorough mixing. A heparinised microcapillary tube was inserted into the blood sample and was filled by capillary action. One end of the tube was sealed with Critoseal (Hawksley and Sons Ltd, UK) and the tube placed in a Hawksley micro-haematocrit centrifuge for 3 min. Haematocrit levels were determined using a micro-haematocrit reader and were expressed as the percentage packed red cell volume in relation to the whole blood volume.

4.2.4.3. Total blood counts

The total erythrocyte and leucocyte counts were made on a Neubaeur's counting chamber (haemocytometer) by diluting 20μl of whole blood in 4 ml of Natt Herrick's staining solution (1:200 dilution). The stain was created by the mixing of sodium chloride (3.88 g), sodium sulphate (2.50 g), sodium phosphate (1.74 g), potassium phosphate (0.25 g), formalin (37%, 7.50 ml) and methyl violet (0.10 g) (see Noga, 2000). The Natt Herrick's stain, stained leucocytes violet making them easy to distinguish from the red blood cells. Total blood counts were expressed as the number of cells per ml following the methods of Klontz (1994).

4.2.4.4. Differential blood counts

A drop of blood was smeared across a glass microscope slide using the edge of a second slide. The slides were allowed to dry at room temperature and were then stained in a 3-step process using Stain Quick (Raymond A. Lamb Ltd). Slides were viewed at ×100, 100 cells per slide were counted in random fields and the number and type of leucocytes (granulocytes, lymphocytes and thrombocytes) present recorded.

4.2.4.5. Phagocyte isolation

Due to the small size of the fish, the entire kidney was dissected out in an aseptic environment. The kidney was disaggregated through a 100μm nylon mesh into a 3 cm Petri dish containing 5 ml L-15 (Leibovitiz) media (Sigma) at room temperature and 10μl of cell grade heparin (Sigma). The mesh was removed and scraped against the edge of the Petri dish to ensure that all phagocytic cells were retained in the media. The kidney cell suspension was then transferred to a labelled bijou and kept

on ice until all the fish had been processed. As the whole kidney of the carp was used, it is not possible to say that the cell suspension consisted exclusively of macrophages as other phagocytic cells, such as neutrophils, may have been present and thus the term kidney phagocytes will be used in place of macrophages.

4.2.4.6. Phagocytosis by kidney phagocytes

Glass microscope slides were labelled with fish, tank and treatment details and were then dipped in 100% ethanol and allowed to air dry. Two wax circles were drawn, one above the other, on each slide with a PAP pen. To prevent the slides drying out, they were placed into large Petri dishes, acting as humidity chambers, with moistened filter paper in the base. To each circle, 100μl of kidney cell suspension from each carp was pipetted and the slides were then left at room temperature for 1 h.

Just prior to the completion of the hour of incubation, a solution of Baker's yeast and L15 (5 mg yeast/ml) was made in a plastic universal and shaken until all the yeast had dissolved. Each slide was then removed from the Petri dish and both circles washed 3 times with room temperature L-15 media. Washing removed any cells that had not adhered to the slides. The top circle on each slide was then filled with 100μl of L-15 media only and the bottom circle with 100μl of the yeast mixture. The slides were incubated at room temperature for 1 h.

After 1 h, both circles on each slide were rinsed 3 times with L-15. The adhered cells were then fixed onto the slides by addition of 100% methanol for 3 min. The methanol was then tapped off the slides onto absorbent tissue and the slides allowed

to air dry. Once dried the slides were stained in Stain Quick as for the blood smears (see section 4.2.4.4).

Slides were viewed at ×100, 200 cells were counted and the number of yeast cells engulfed by the macrophages noted.

The phagocytic index (PI) and the phagocytic ratio (PR) were then calculated as follows:

$$PI = \frac{\text{No. of yeast cells in N macrophages}}{\text{No. of macrophages}}$$

$$PR = \frac{\text{No. of macrophages with 1 or more yeast cells}}{\text{No. of macrophages}} \times 100$$

4.2.4.7. Phagocyte respiratory burst

Each row of a 96 well sterile multi-well plate was allocated to an individual fish and labelled accordingly. Into 8 wells on each row of the plate, 100 μl of kidney cell suspension from the same fish was added. The plates were left at room temperature for 1 h to allow the macrophages to adhere to the bottom of the wells. During the hour-long incubation the solutions needed for the next step of the procedure were made. Into 2 universals 10 ml of L-15 were added. One nitro blue tetrazolium tablet (NBT) (Sigma) was added to each tube (1 mg NBT/ml L-15) and shaken vigorously until the tablet had dissolved completely. Phorbol 12-myristate 13-acetate (PMA) (Sigma) was then added to one universal (1 μl/ml) as a cell stimulant.

After 1 h, the cell suspension in all wells was removed using a multichannel pipette. Each well was washed 3 times with L-15, ensuring that no residual L-15 remained in the wells. NBT solution (100μl) was then added to 3 wells in each row, NBT and PMA (100μl) into 3 further wells and lysis buffer (100 ml distilled water, 0.0021g citric acid, 1 ml tween, 0.05g crystal violet) (100μl) added to the remaining 2 wells. The plates were left at room temperature for 1 h during which time the soluble yellow NBT was reduced to a blue formazan precipitate. After the hour, the NBT and NBT/PMA solutions were removed from the wells using a multichannel pipette. The lysis buffer was left in the wells. After removing all the NBT solutions from the wells, 100μl of 100% methanol was added and left for 3 min. Each well was then washed 3 times with 70% methanol and allowed to air dry.

Once dried, 120μl of 2 M potassium hydroxide (KOH) solution and 140μl of dimethyl sulphoxide (DMSO) (Sigma) were added to each well (except the wells containing lysis buffer). The 2 solutions were mixed together using a multichannel pipette and the formazan precipitate solubilised, resulting in a pale blue colour. The addition of these 2 chemicals was carried out in a fume hood. The absorbance of each well was then read at 610nm using an ELISA plate reader.

In order to calculate the respiratory burst, the number of macrophages in the cell suspension from each fish was determined. Addition of the lysis buffer to the wells resulted in the release of nuclei from the macrophages. The nuclei were stained dark blue/purple by the crystal violet in the buffer. A small amount of the lysis buffer solution from each of the 2 wells was placed on a Neubauer haemocytometer and

the number of nuclei present in the 4 large corner squares (composed of 16 squares each) were counted.

The respiratory burst (OD 610nm) was then calculated:

$$\frac{\text{Mean absorbance (nm)/fish}}{\text{No. of adhering cells/well } (10^5)}$$

4.2.4.8. Cortisol levels

Concentrations of plasma cortisol were determined with the use of a radioimmunoassay adapted from Pickering, Pottinger & Sumpter (1987). The full methodology can be found in Appendix 2. The cortisol assay was carried out by a member of the university registered to use radioisotopes.

4.2.5. Statistical analysis

Due to the small sample size in each tank (n=3), data from both tanks within each treatment were pooled for statistical analysis. Non-parametric Kruskal-Wallis tests (plus Dunn's comparisons) or Mann-Whitney U tests were used to analyse the data.

4.3. Results

4. 3. 1. Water quality

Table 4.1. A summary of the water quality data from each tank in the trial.

Parameter	Controls		5μg/l cadmium		50μg/l cadmium	
	C1	C2	T1	T2	T1	T2
Cadmium concentration (μg/l)	0.35 ± 0.11	0.44 ± 0.12	7.10 ± 0.41	6.96 ± 0.32	51.18 ± 3.32	50.62 ± 1.67
Temperature (°C)	16.12 ± 0.11	16.18 ± 0.11	16.25 ± 0.11	16.10 ± 0.12	16.14 ± 0.09	16.25 ± 0.08
pH	7.01 ± 0.05	7.08 ± 0.05	7.05 ± 0.04	7.06 ± 0.04	6.87 ± 0.09	6.93 ± 0.14
Alkalinity (meq/l)	0.22 ± 0.07	0.23 ± 0.15	0.23 ± 0.10	0.21 ± 0.06	0.18 ± 0.17	0.19 ± 0.16
Hardness (ppm $CaCO_3$)	24.91 ± 0.53	24.82 ± 0.67	25.27 ± 0.94	25.01 ± 2.04	26.00 ± 0.77	26.13 ± 1.13

In the current trial there were no within-treatment statistical differences in cadmium concentration ($P > 0.05$) and no statistical differences in temperature, pH, alkalinity and water hardness between any tank ($P > 0.05$).

4.3.2. Fish health

Carp exposed to 5μg/l cadmium showed no deviation from normal health throughout the 30-day trial. However, by day 6 of the trial, several individuals in both of the 50μg/l tanks were showing signs of abnormal behaviour and hyperactivity, interspersed with periods of lying motionless on the bottom of the tank. Several more fish from day 6 onwards exhibited this behaviour. All fish displaying this behaviour were sacrificed by overdose in anaesthetic. These fish were then processed for a range of immunological assays, the results of which are presented in sections 4.4.3 to 4.4.7. No carp from any other treatment were sampled at day 6 and due to the early removal of some of these carp from the 50μg/l cadmium tanks there were no fish remaining at the end of the trial and thus no

immunological data from these fish at day 29. It is worth noting that there appeared to be heterogeneity in the response of the 50μg/l cadmium-exposed carp to metal exposure, as some individuals in these tanks did not display any abnormal behaviour during the course of the trial.

4.3.3. Total blood counts

Total blood counts [leucocytes (10^3/ml) and erythrocytes (10^6/ml)] were determined in control carp and in carp exposed to both 5 and 50μg/l cadmium at 14, 21 and 29 days of the trial and the results are presented in Figures 4.1-4.4. Day 6 and day 9 data have been omitted due to unsatisfactory data collection resulting from poor staining of the cells by the Natt Herrick's solution. For further samples a fresh solution of the stain was created.

Figure 4.1 shows the leucocyte counts from carp in each tank of each treatment. It is hard to determine a pattern in the leucocyte response of carp exposed to 50μg/l cadmium over time, as, due to a lack of fish in the first replicate [50μg/l (1)], data were only collected at day 14. However, in the second replicate [50μg/l (2)], the number of leucocytes increased from 7.3 ± 2.9 at day 14 to 22 ± 9.2 (10^3)/ml at day 21. From Figure 4.3, it can be seen that the number of erythrocytes also increased in this tank between from 0.62 ± 0.1 (10^6) at day 14 to 0.82 ± 0.2 (10^6) by day 21.

Carp exposed to 5μg/l cadmium showed the most consistent pattern in both replicate tanks, with the number of leucocytes and the number of erythrocytes increasing over time (Figs 4.1, 4.3). Control carp showed less consistency in the number of these cell types in each tank over time (Figs 4.1, 4.3). However, when the data are

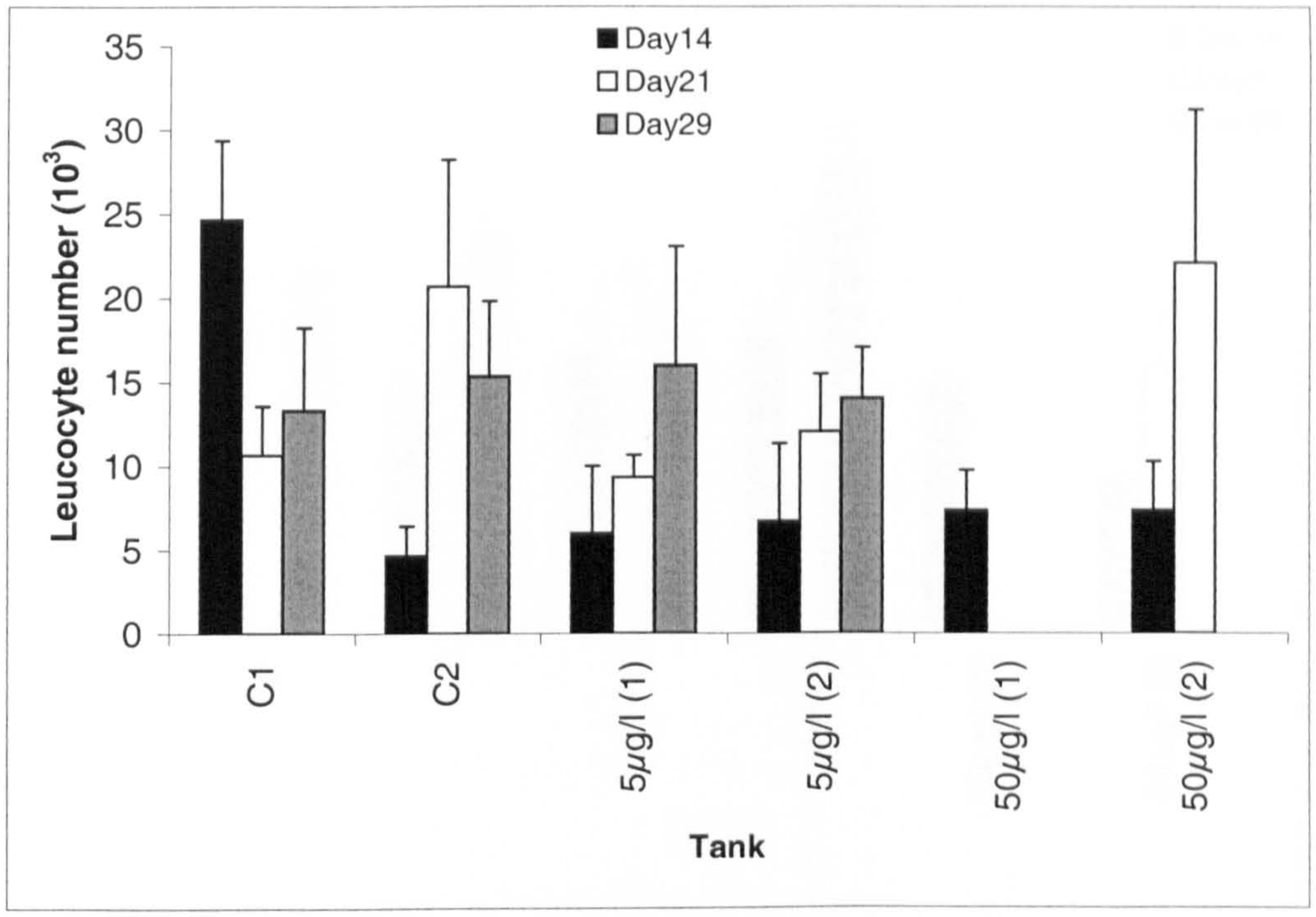


Fig. 4.1. The total (mean±S.E.; n=3) number of leucocytes (10^3)/ml of blood in control carp and in carp exposed to 5 and 50μg/l cadmium at 14, 21 and 29 days post-start of the trial. Data for 2 replicate tanks/treatment are presented.

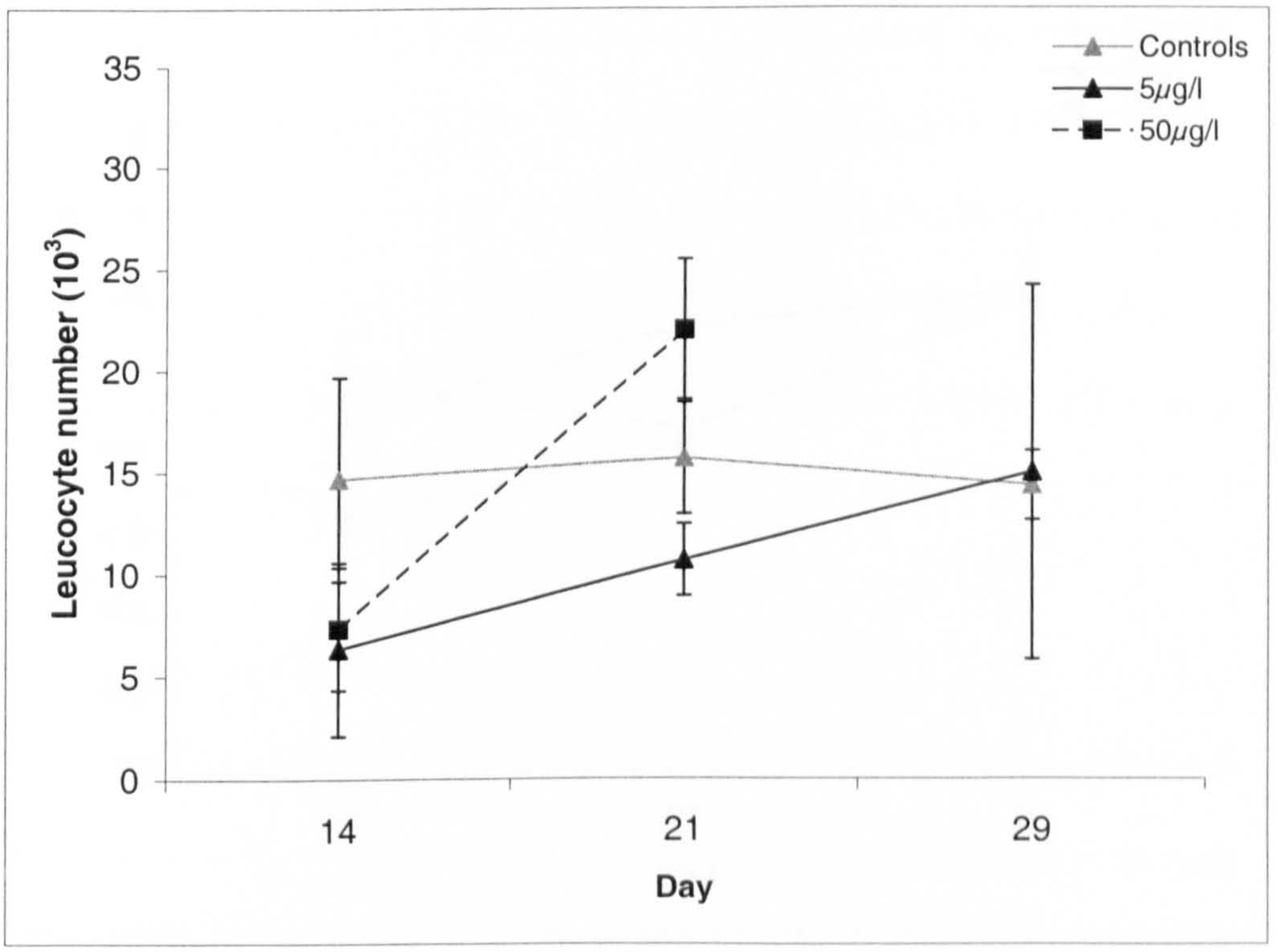


Fig. 4.2. The pooled total (mean±S.E.; n=6) number of leucocytes (10^3)/ml of blood in control carp and in carp exposed to 5 and 50μg/l cadmium for 14, 21 and 29 days.

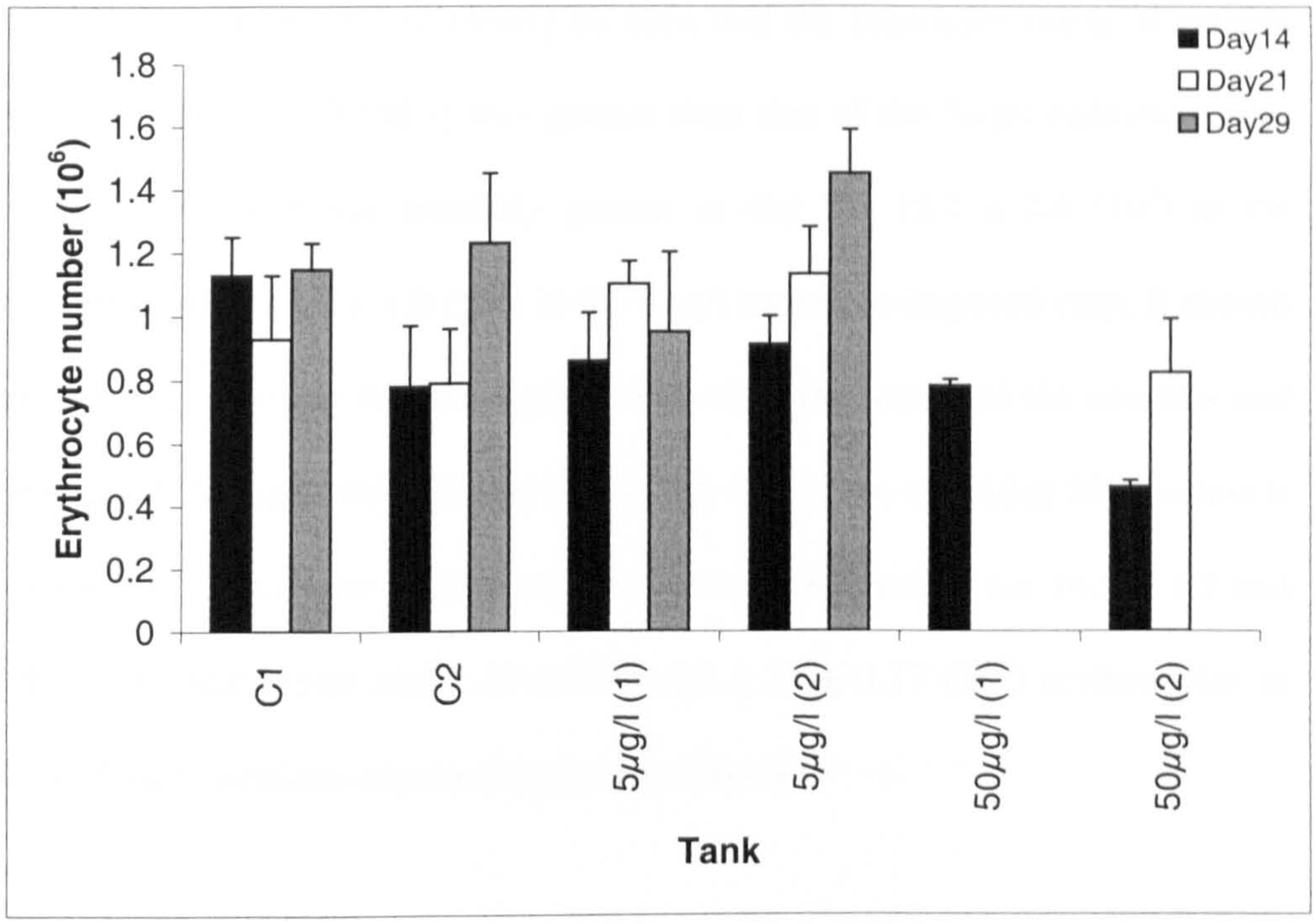


Fig. 4.3. The total (mean±S.E.; n=3) number of erythrocytes (10^6)/ml of blood in control carp and in carp exposed to 5 and 50μg/l cadmium at 14, 21 and 29 days post-start of the trial. Data for 2 replicate tanks/treatment are presented.

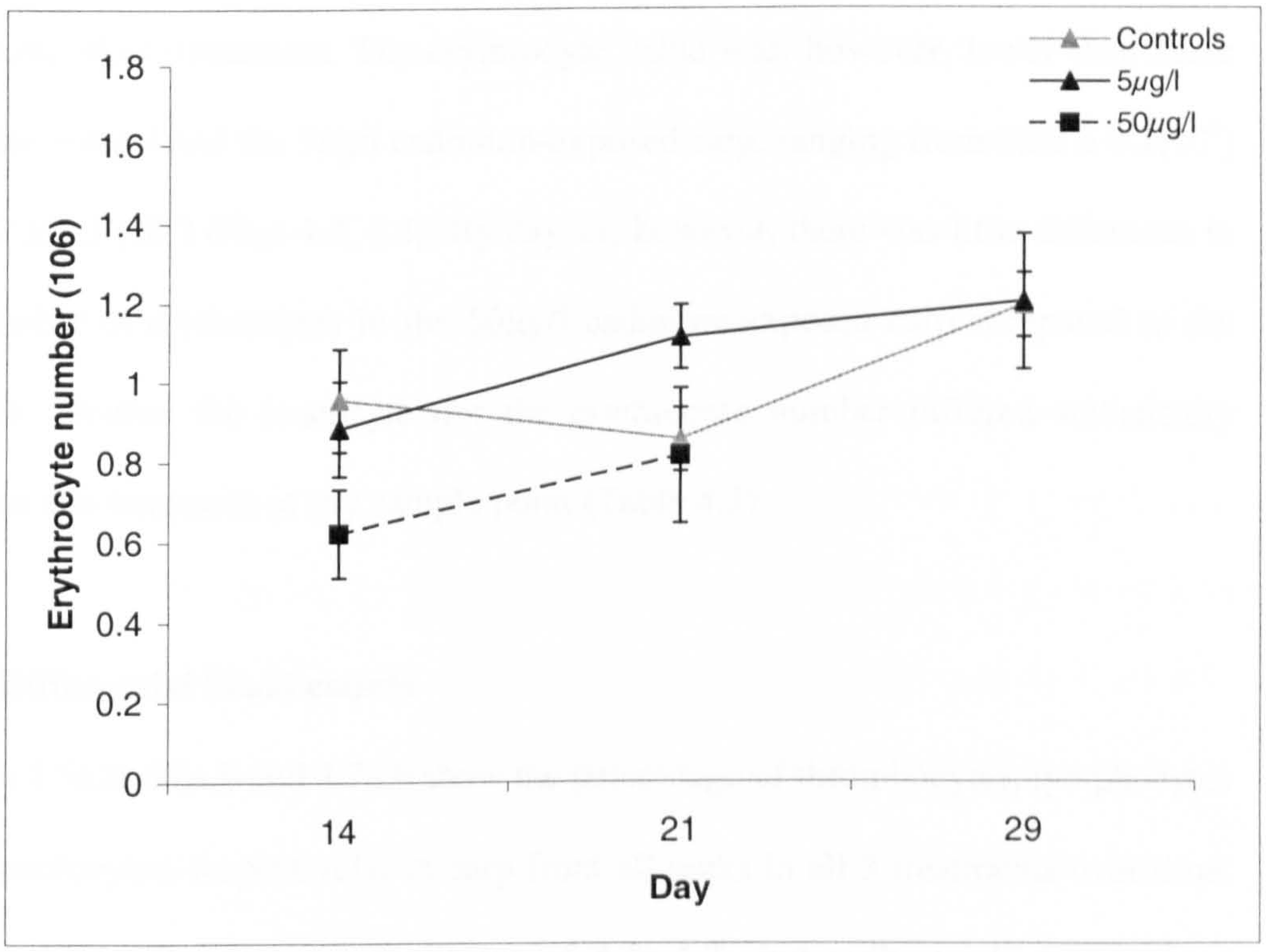


Fig. 4.4. The pooled total (mean±S.E.; n=6) number of erythrocytes (10^6)/ml of blood in control carp and in carp exposed to 5 and 50μg/l cadmium for 14, 21 and 29 days.

pooled within treatments, it can clearly be seen that the leucocyte count of control carp at day 14 [14.7 ± 5.0 (10^3)] was greater than that of the 5μg/l cadmium carp, [6.33 ± 4.2 (10^3)], and was similarly greater at day 21, 15.7 ± 2.8 (10^3) in the controls compared to 10.7 ± 1.8 (10^3) in the 5μg/l cadmium-exposed carp. It should be noted here that, despite the leucocyte and erythrocyte counts of the controls and the 5μg/l carp differing at days 14 and 21, by the end of the trial (day 29) the levels of both these cell types were almost identical in both treatments, *i.e.* 14.3 ± 1.7 and 15.0 ± 9.2 (10^3) leucocytes and 1.19 ± 0.08 and 1.20 ± 0.17 (10^6) erythrocytes in control and 5μg/l cadmium-exposed carp, respectively.

The pooled leucocyte value from the 50μg/l cadmium-exposed carp at day 14 was greater than that of the 5μg/l cadmium-exposed carp and, at day 21, was greater than both other treatments. The erythrocyte value was, however, lower than those from the control and the 5μg/l cadmium-exposed carp, ranging from 0.62 ± 0.1(10^6) to 0.82 ± 0.2 (10^6) (Figs 4.2, 4.4). By day 21, however, there was little difference in the number of erythrocytes in the 50μg/l cadmium-exposed carp compared to the controls. Neither the leucocyte nor the erythrocyte number differed statistically between any treatment at any sample point (Table 4.2).

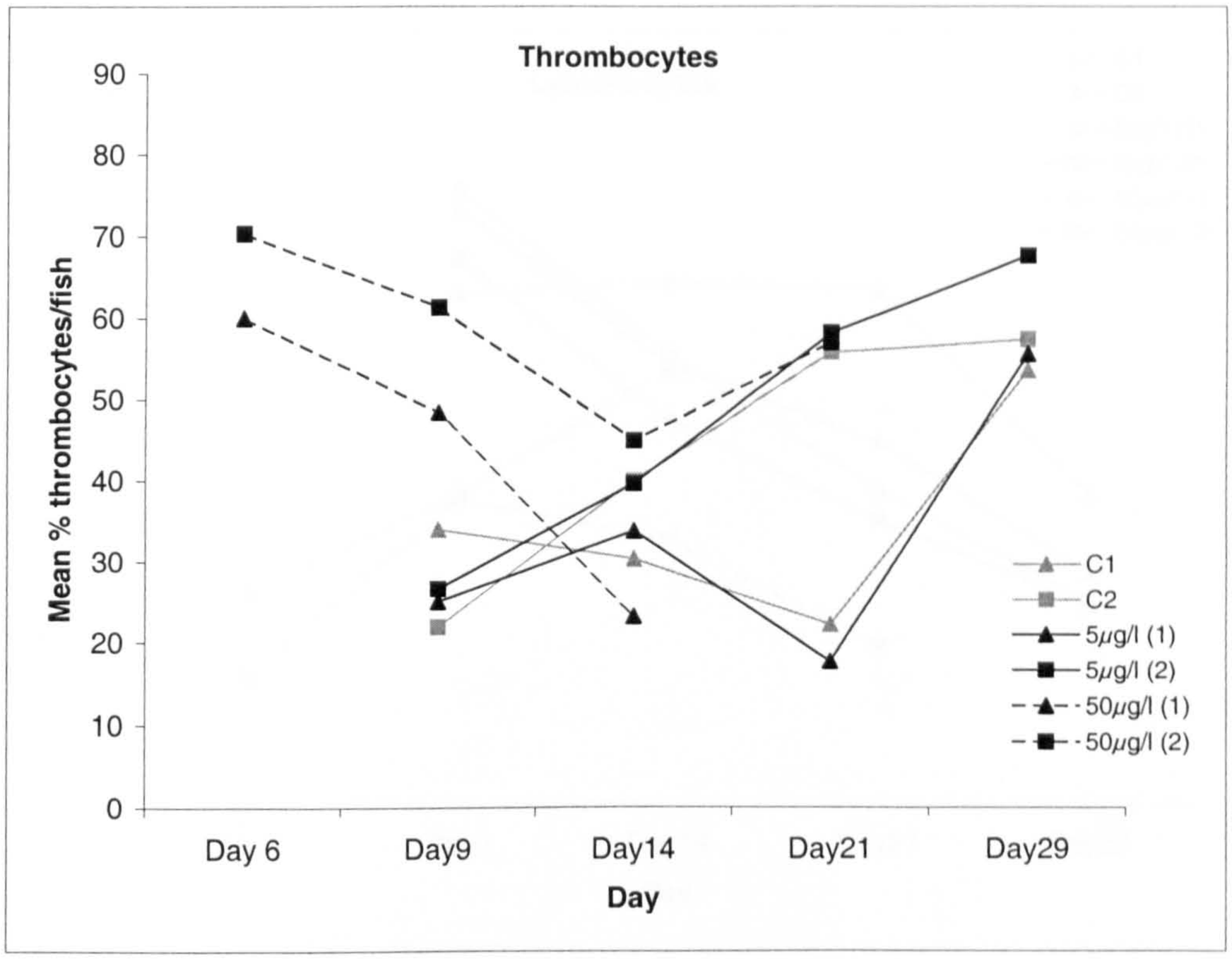
4.3.4. Differential blood counts

Figures 4.5a,b, 4.6a,b and 4.7a,b show the percentage of thrombocytes, lymphocytes and granulocytes, respectively, in carp from all tanks in all 3 treatments over time. Figures 4.5b-4.7b are exact replicates of 4.5a-4.7a but with standard error bars added.

It is hard to determine the pattern in thrombocyte number over time within each treatment, as such different responses were displayed by each replicate. However, on pooling the data, shown in Figure 4.8a, it is clear that there is little difference in the percentage of thrombocytes in control and 5μg/l cadmium-exposed carp and that in these 2 treatments the percentage of thrombocytes increases with exposure duration, with control values ranging from 26.7 ± 4.5 to 55.3 ± 7.8% and test values ranging from 26.1 ± 9.1 to 61.5 ± 6.9% at days 9 and 29, respectively. The proportion of thrombocytes in the 50μg/l cadmium-exposed carp at days 6 (65.9 ± 8.9%) and 9 (54.8 ± 9.8%) were both statistically greater than the controls at day 9 (26.7 ± 4.5%) (Fig. 4.8a, Table 4.2).

In contrast to the percentage of thrombocytes from the 5μg/l cadmium-exposed carp, which increased with exposure duration, the lymphocyte number was found to decrease in both replicate tanks (Fig. 4.6a,b). Despite the carp from the 2 replicate 50μg/l cadmium tanks showing variation in the pattern of lymphocyte number over time, they both remained consistently lower than the values obtained from the other 2 treatments. The lower percentage of lymphocytes in the carp exposed to 50μg/l cadmium can be seen clearly when the data within each treatment are pooled, as in Figure 4.8b. Indeed, at day 21, the percentage of lymphocytes in this group of carp (50μg/l) was statistically lower than the controls, 19.8 ± 3.5% and 52.3 ± 8.9%, respectively (Table 4.2). As with the thrombocytes, there was little difference in the percentage of lymphocytes between control and 5μg/l cadmium-exposed carp, and both populations showed a decline in lymphocytes with time (Fig. 4.8b).

a)



b)

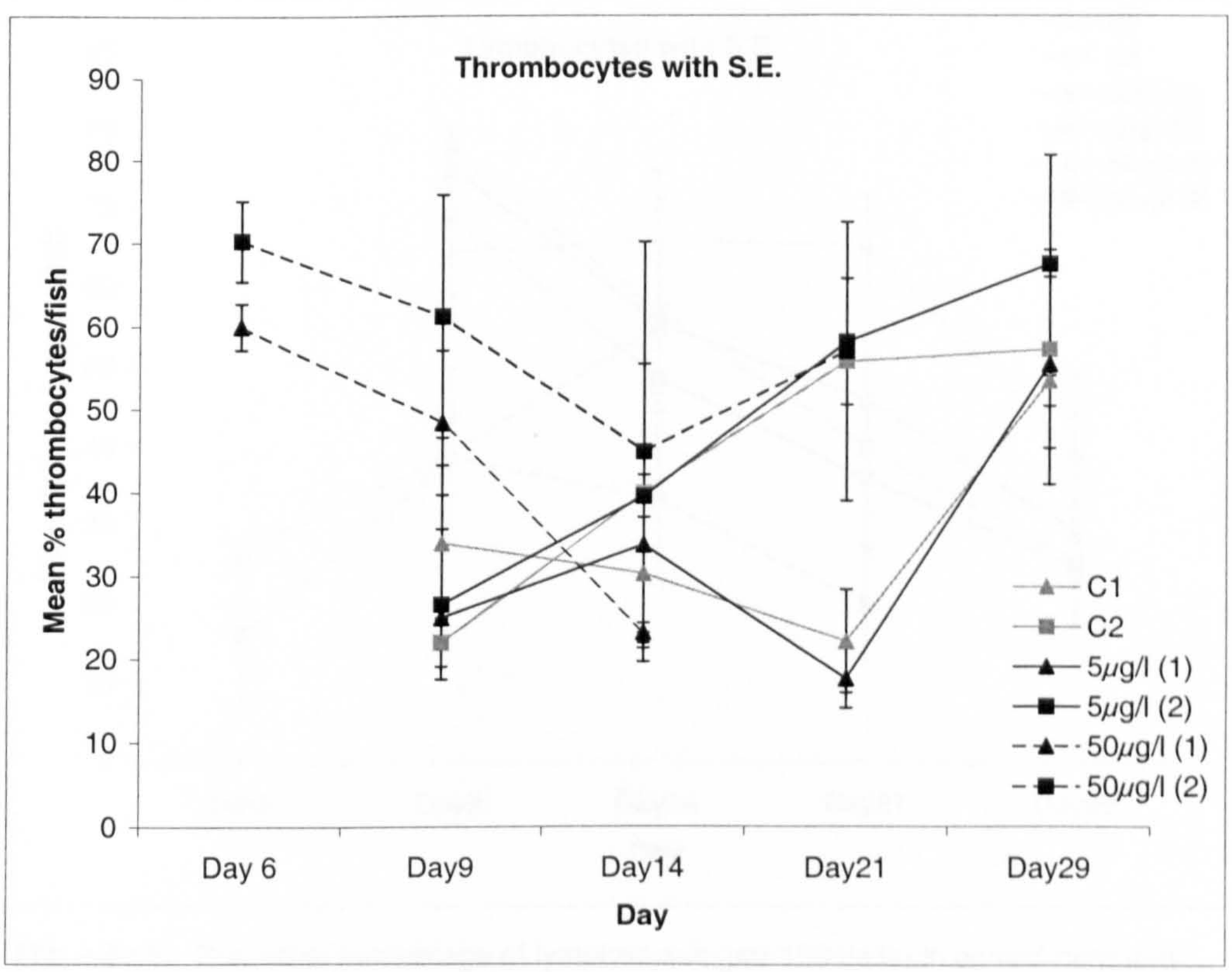
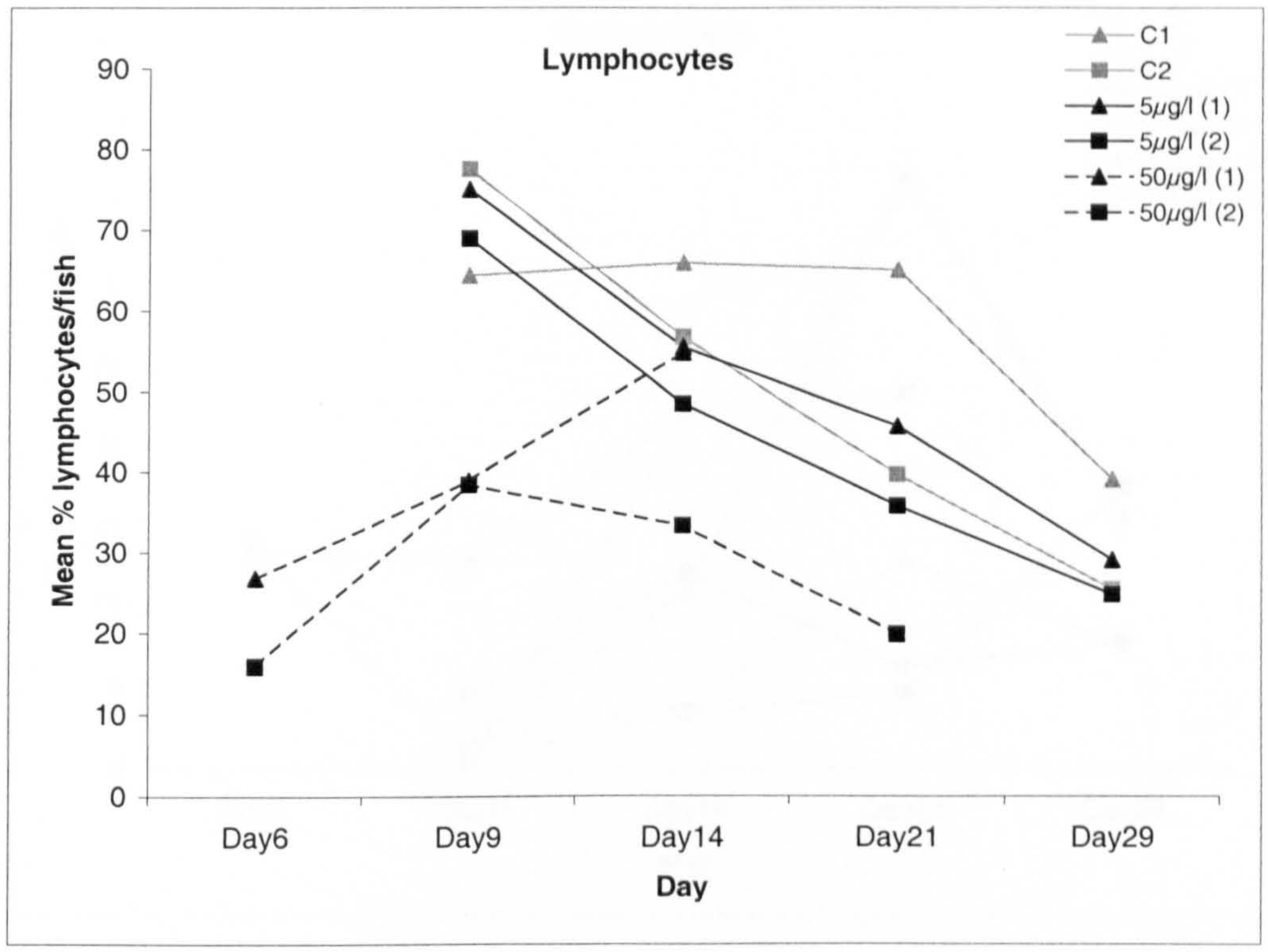


Fig. 4.5a,b. The mean percentage of thrombocytes (per 100 cells) in control carp and in carp exposed to 5 and 50μg/l cadmium at 6, 9, 14, 21 and 29 days post-start of the trial. n = 3. Fig. 4.5b as Fig. 4.5a ± S.E. of the mean.

a)



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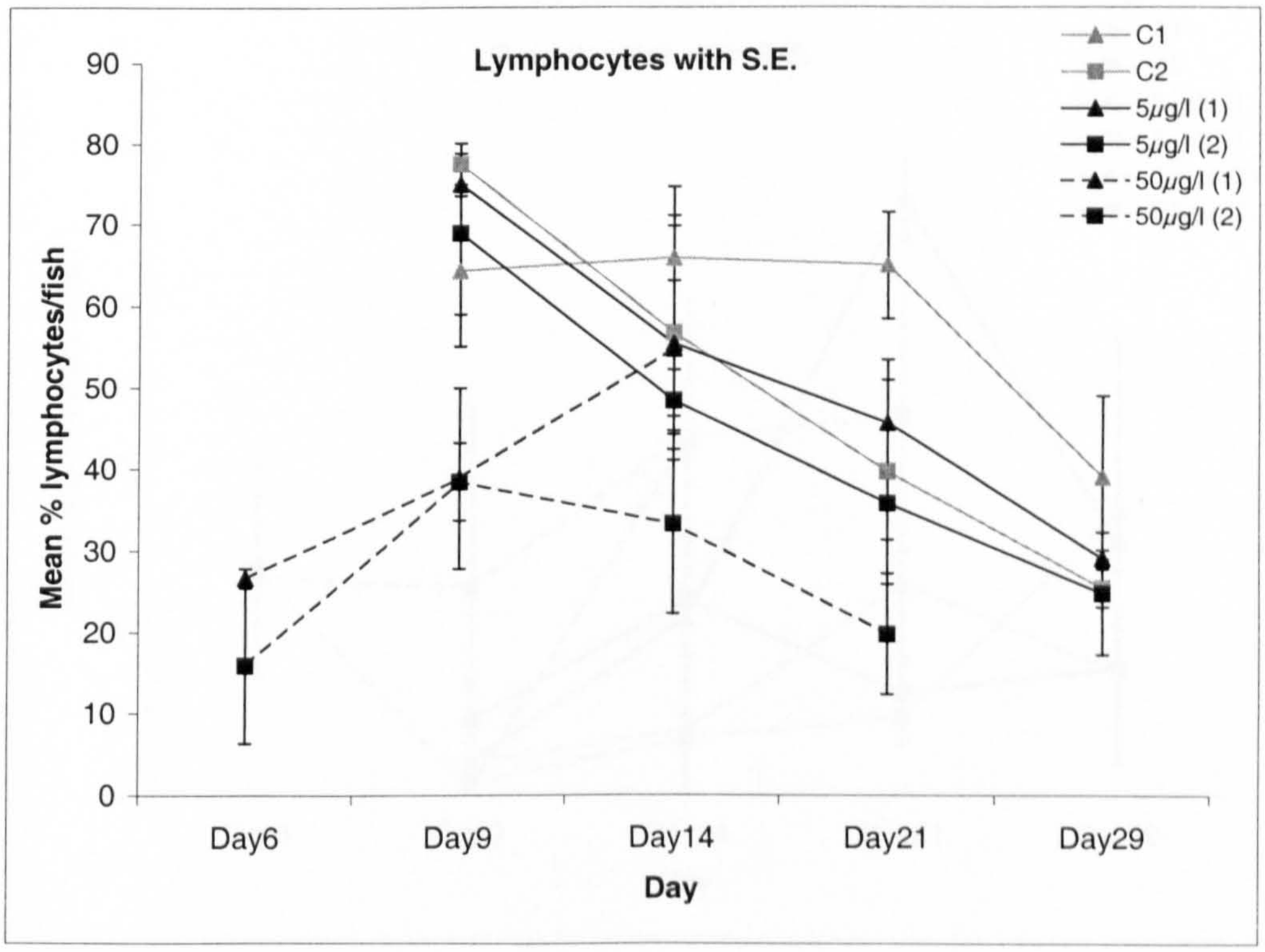
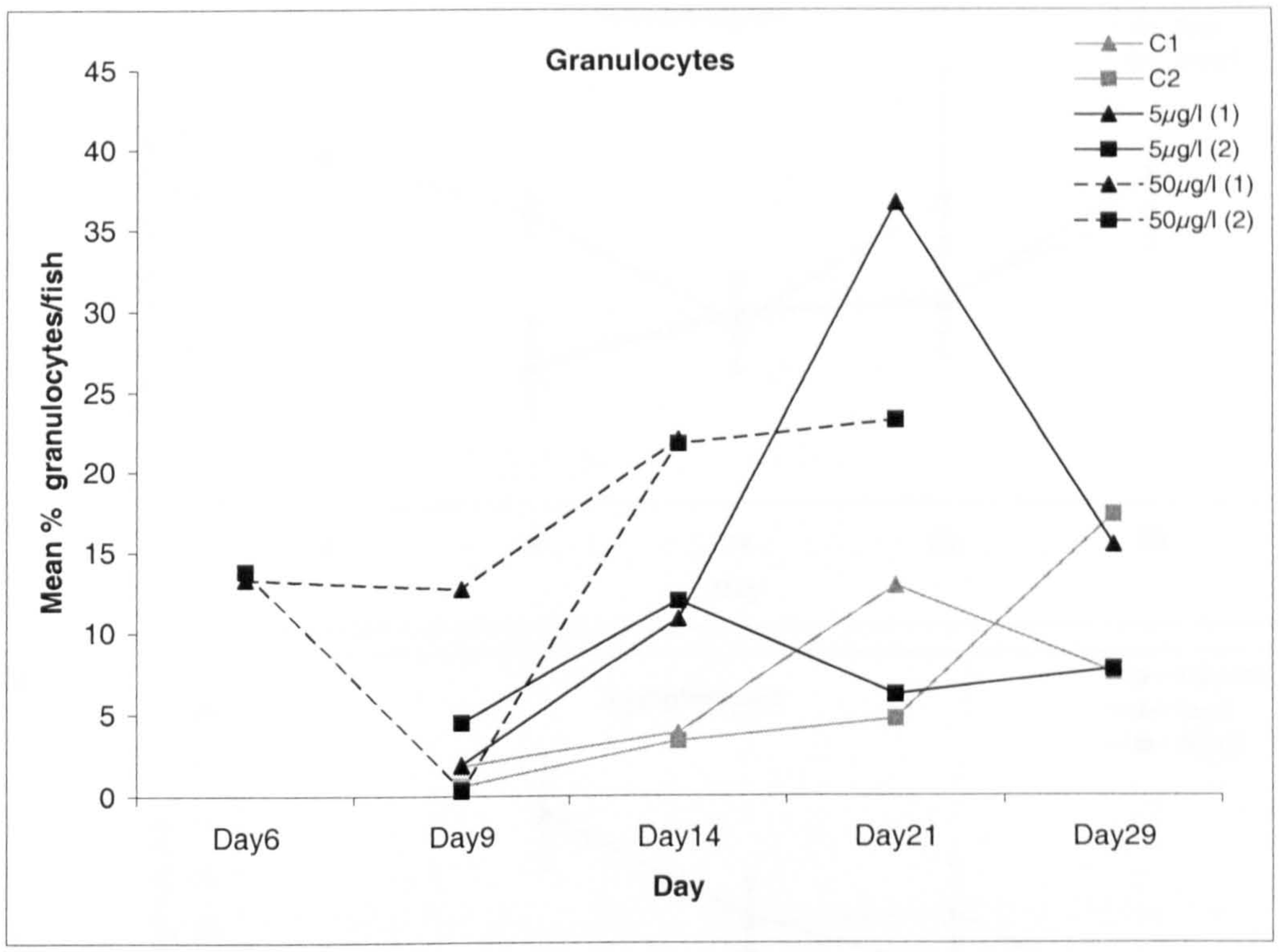


Fig. 4.6a,b. The mean percentage of lymphocytes (per 100 cells) in control carp and in carp exposed to 5 and 50μg/l cadmium at 6, 9, 14, 21 and 29 days post-start of the trial. n = 3. Fig. 4.6b as Fig. 4.6a ± S.E. of the mean.

a)



b)

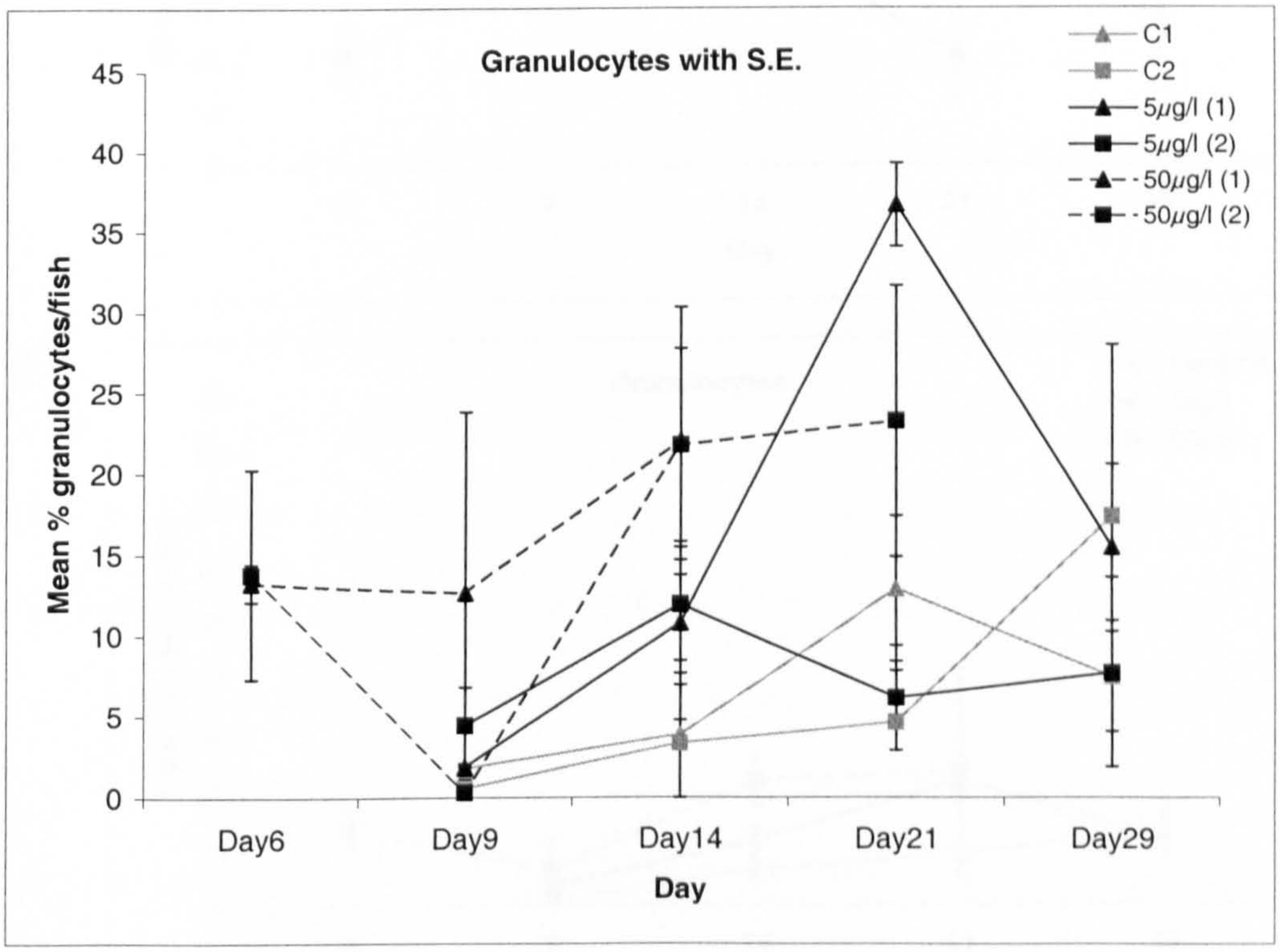
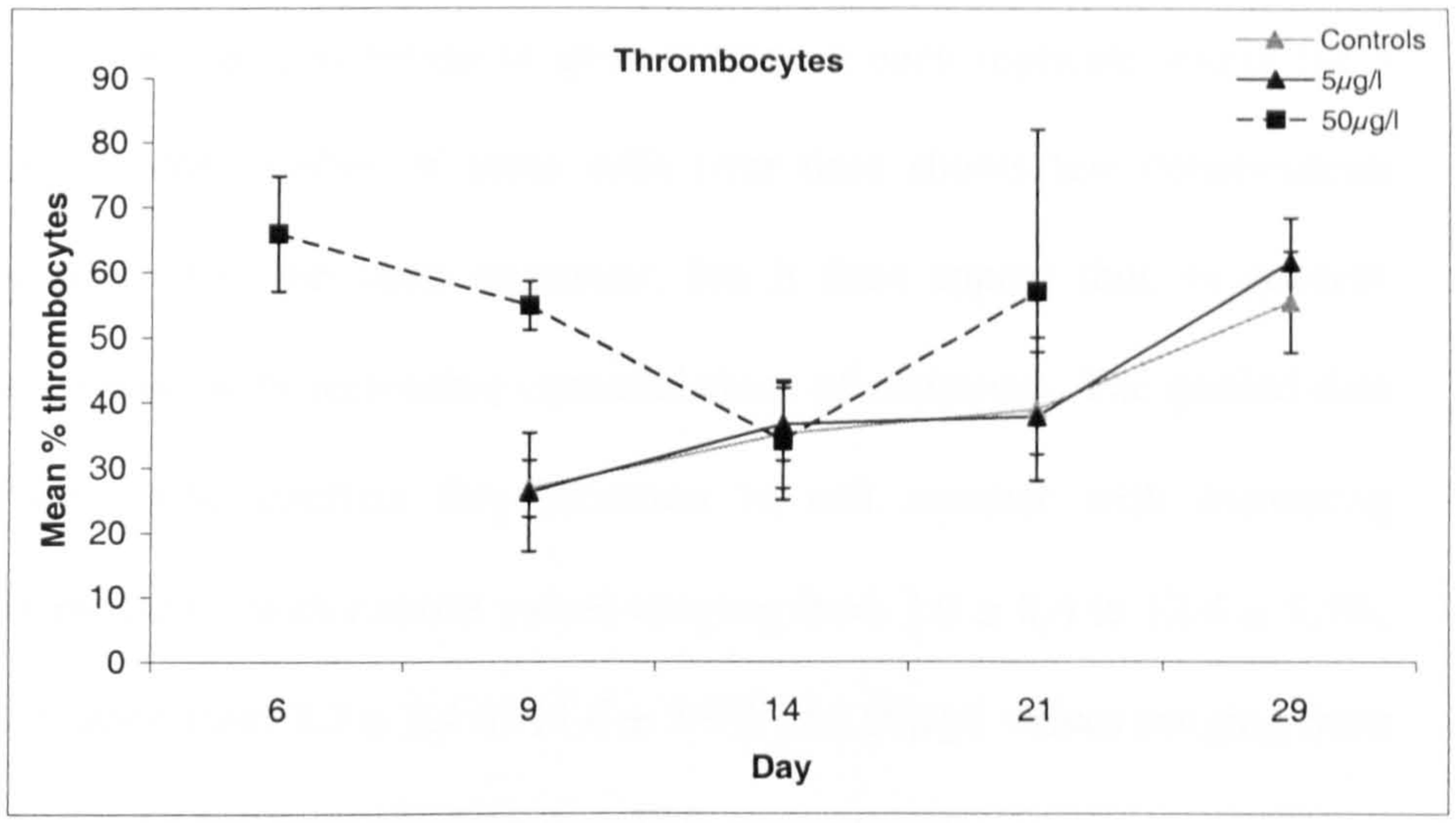
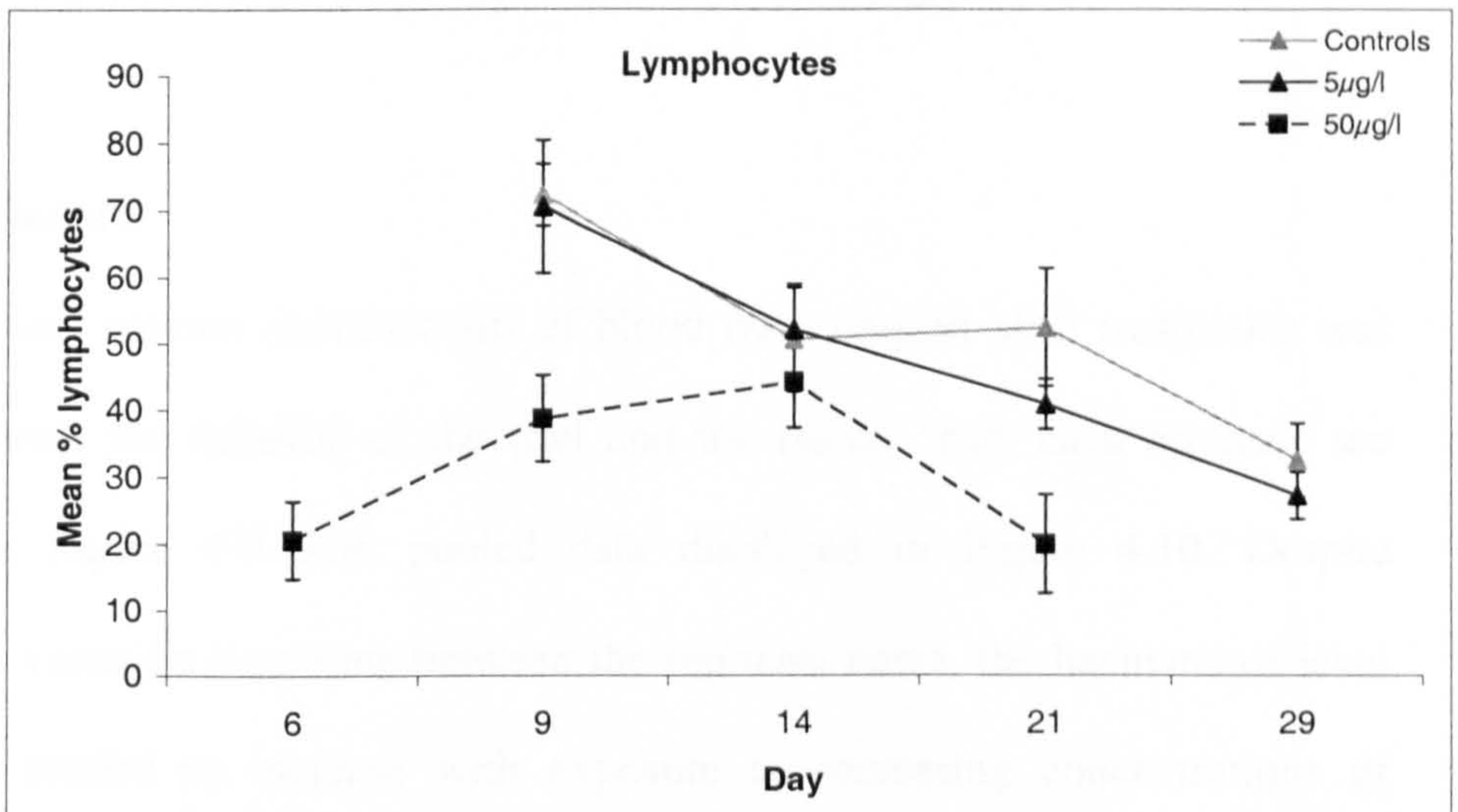


Fig. 4.7a, b. The mean percentage of granulocytes (per 100 cells) in control carp and in carp exposed to 5 and 50μg/l cadmium at 6, 9, 14, 21 and 29 days post-start of the trial. n = 3. Fig.4.7b as Fig. 4.7a ± S.E. of the mean.

a)



b)



c)

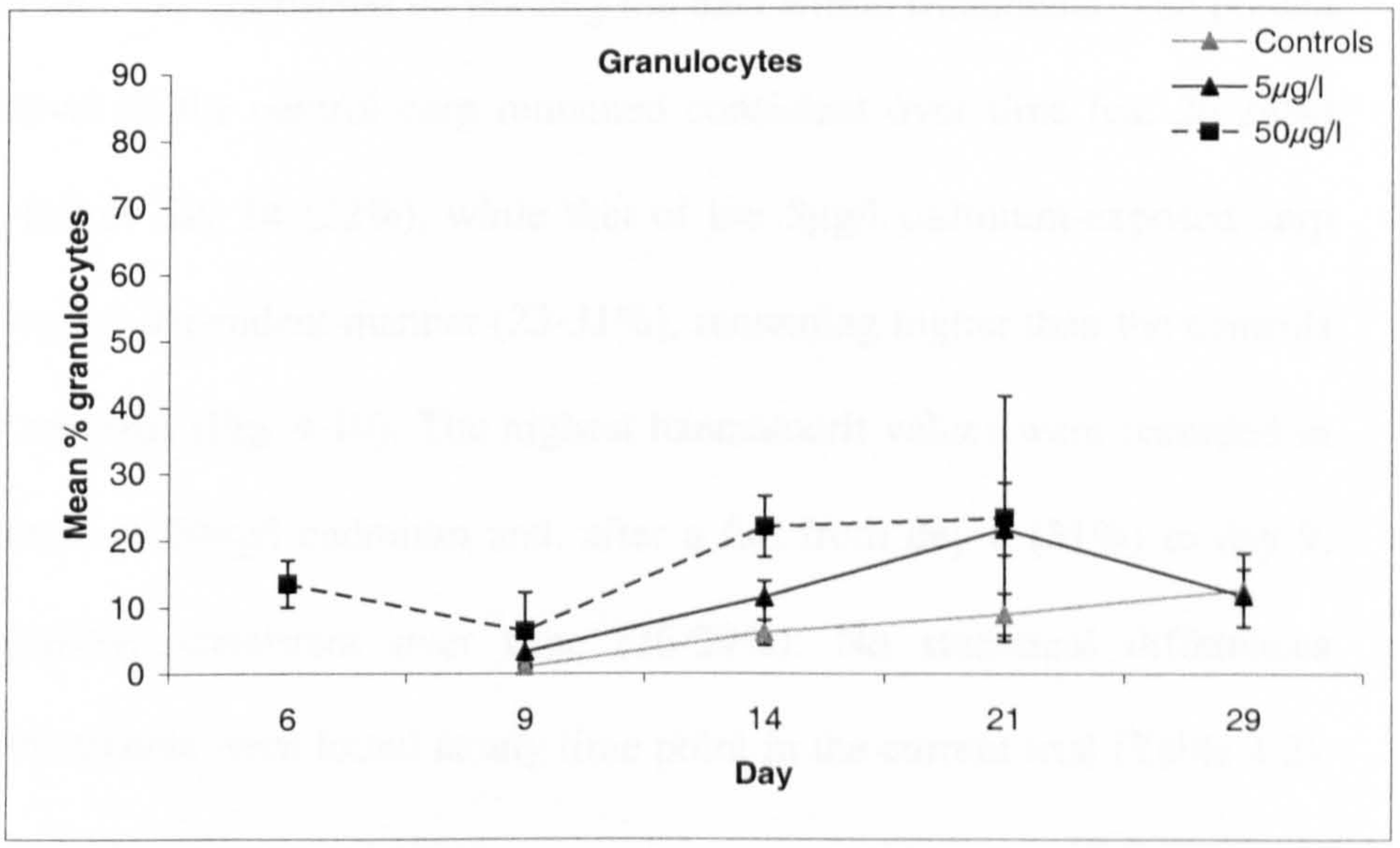


Fig. 4.8a-c The mean pooled percentage (± S.E.) of (a) thrombocytes, (b) lymphocytes and (c) granulocytes from control carp and from carp exposed to 5 and 50μg/l cadmium for 9, 14, 21 and 29 days. n = 6. Day 6 data from 50μg/l cadmium-exposed carp only.

Figure 4.7a, b shows the percentage of granulocytes in each replicate within the 3 treatments. Again, the number of these cells over time shows few consistencies between replicates from the same treatment, but it does appear that, in general, granulocytes increase with increasing concentrations of cadmium. The pooled data shown in Figure 4.8c confirm this elevation in cell number with increasing cadmium concentration, with control values ranging from 1.0 ± 0.4 to 12.4 ± 5.5%, 5μg/l values ranging from 3.3 ± 2.4 to 11.6 ± 3.9% and 50μg/l values ranging from 6.5 ± 3.5 to 23.2 ± 18.4% between days 9 and 29, respectively.

4.3.5. Haematocrit

The packed cell volume (haematocrit) of blood from carp in all 3 treatments was determined over the duration of the trial and the results from each replicate are presented in Figure 4.9 with pooled data displayed in Figure 4.10. Despite considerable variation over time between the replicate tanks, the haematocrit level of the carp tended to increase with exposure to increasing concentrations of cadmium and this was confirmed on pooling the data within treatments. The pooled haematocrit level of the control carp remained consistent over time (ca. 26-28%) except for a fall at day 14 (22%), while that of the 5μg/l cadmium-exposed carp increased in a time-dependent manner (23-31%), remaining higher than the controls from day 14 onwards (Fig. 4.10). The highest haematocrit values were recorded in the carp exposed to 50μg/l cadmium and, after a fall from day 6 (31%) to day 9, remained relatively consistent over time (26-29%). No statistical differences between the treatments were found at any time point in the current trial (Table 4.2).

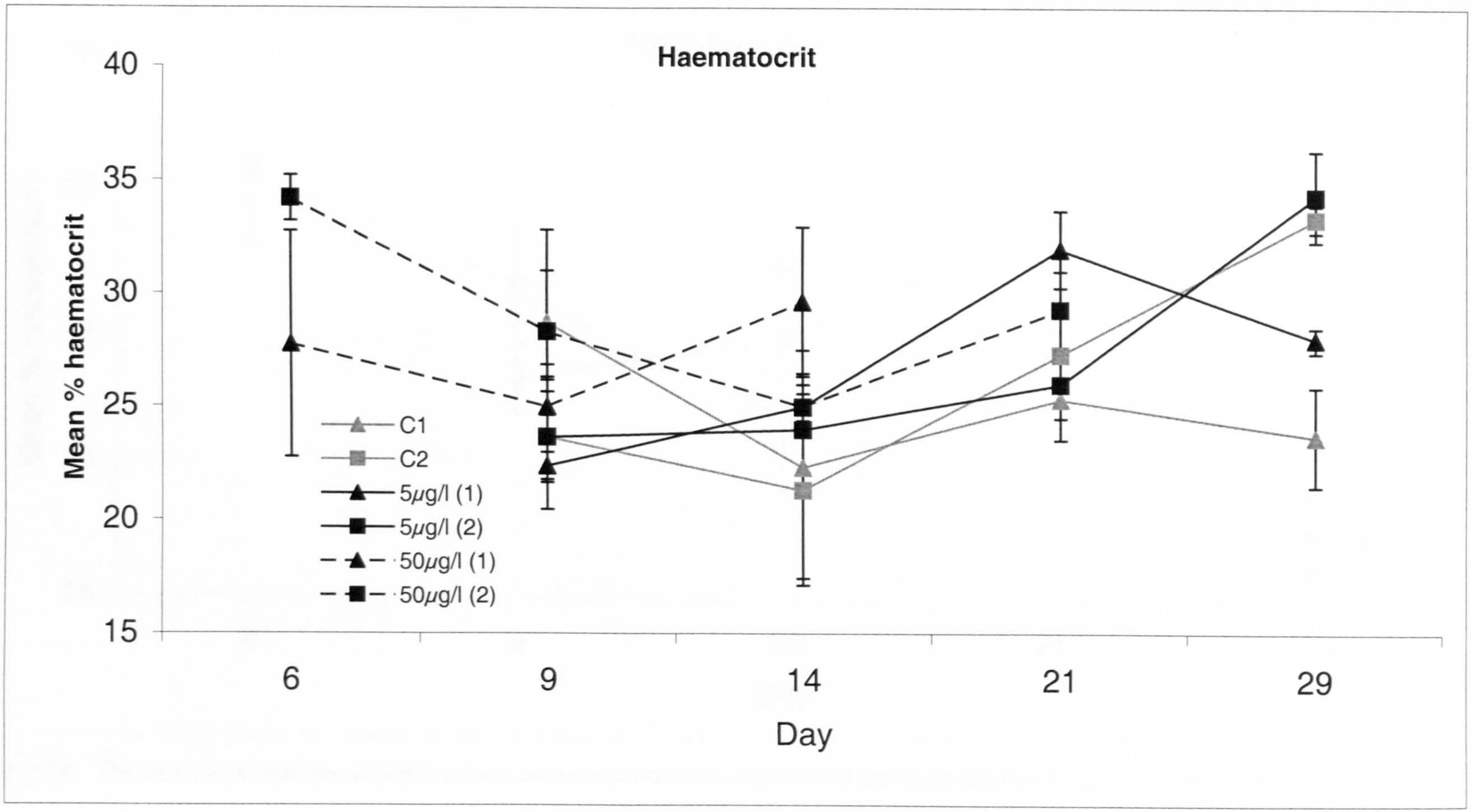


Fig. 4.9. The mean percentage haematocrit (± S.E.) in control carp and in carp exposed to 5 and 50μg/l cadmium for 6, 9, 14, 21 and 29 days. (n=3).

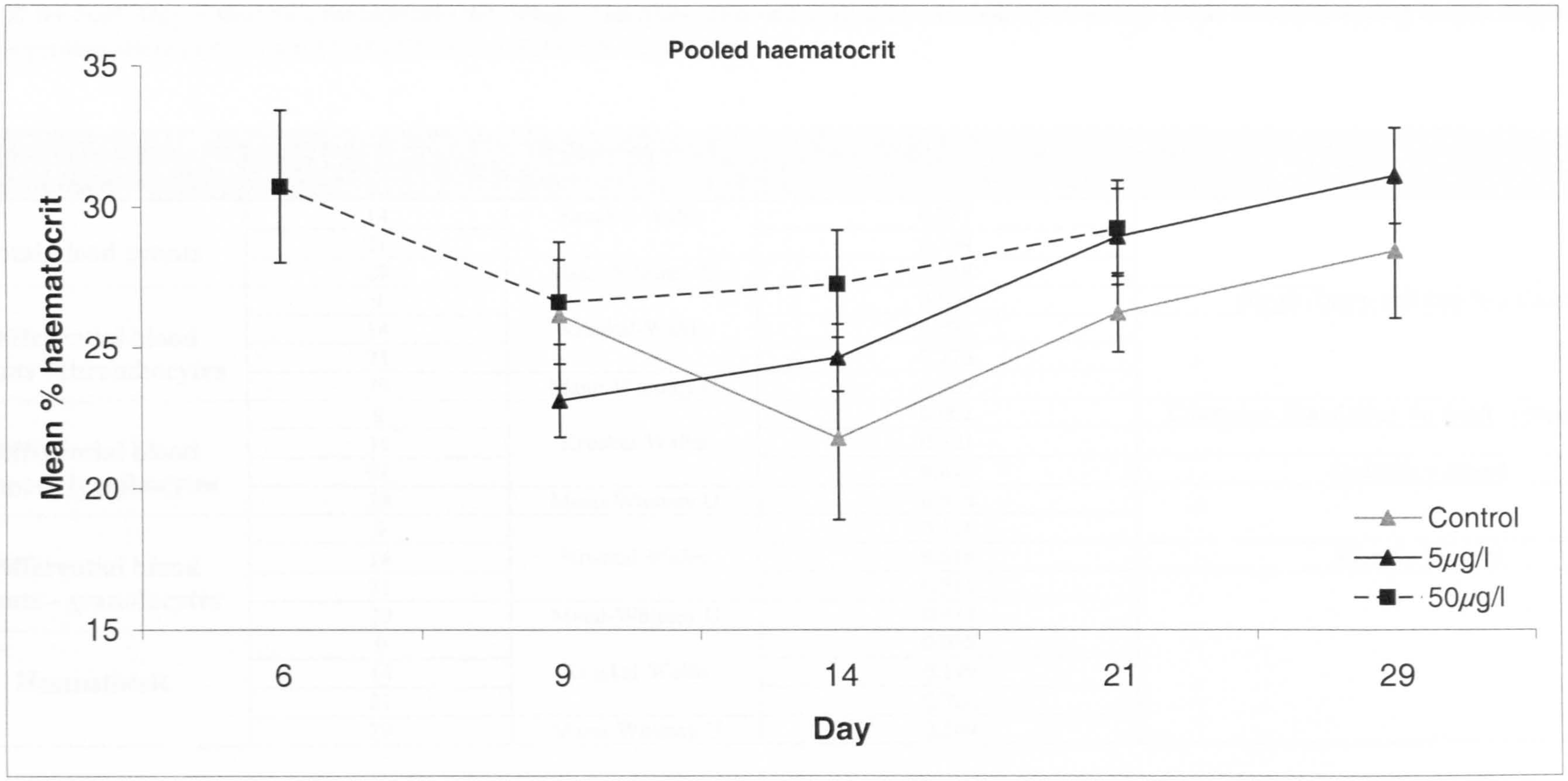


Fig. 4.10. The mean percentage haematocrit (± S.E.) in control carp and in carp exposed to 5 and 50μg/l cadmium for 6, 9, 14, 21 and 29 days. (n = 6).

Table 4.2. Statistical summary of some haematological parameters of carp maintained in 0, 5 and 50μg/l cadmium at 9, 14, 21 and 29 days post-start of the trial. Day 9 statistics incorporate the 50μg/l cadmium-exposed carp that were sampled at day 6 due to deteriorating health. Figures in bold represent statistically significant differences between treatments.

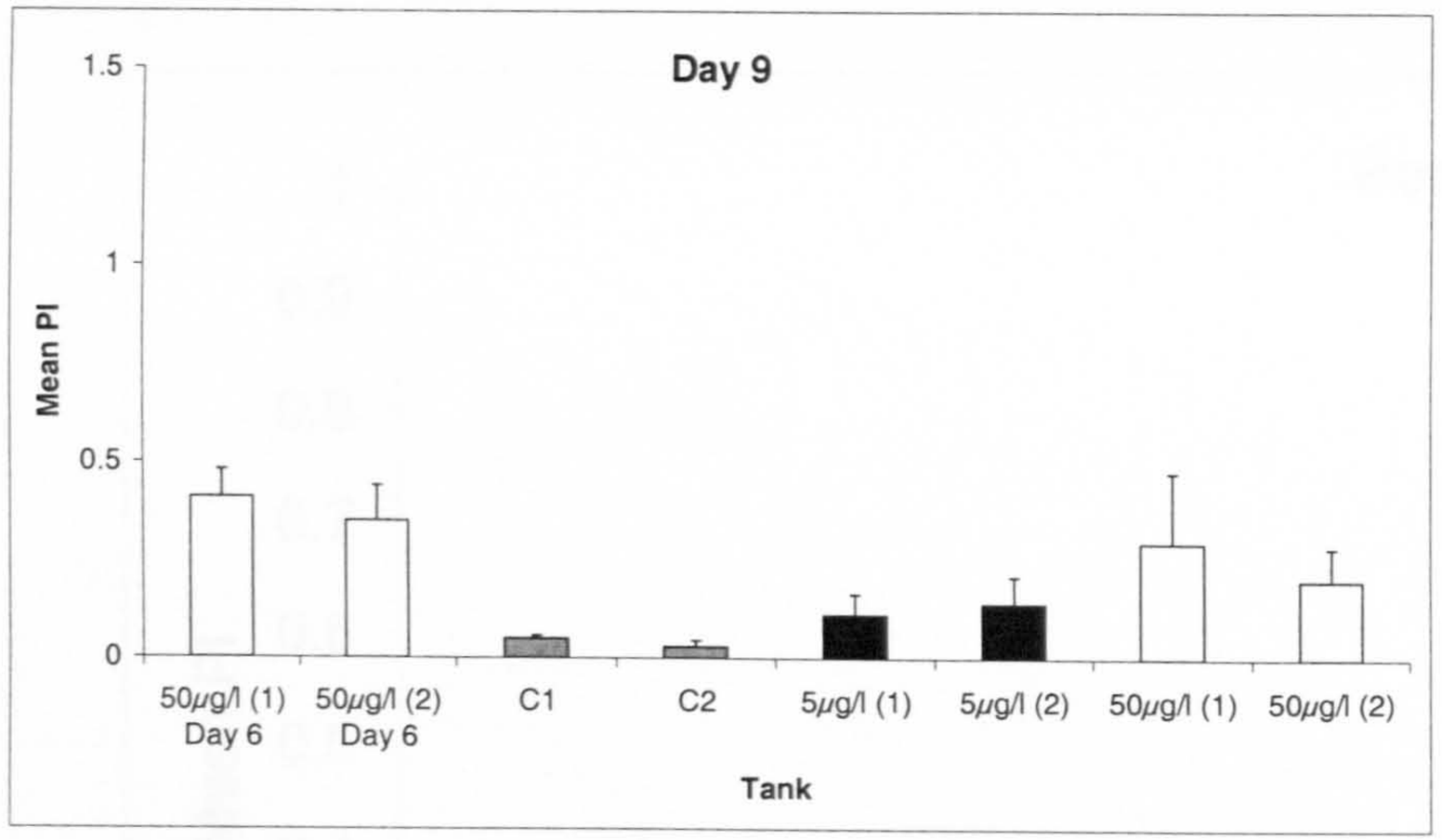
Haematological parameter	Sample day	Statistical test	Statistical significance	Treatments between which differences lie
Total blood counts	14	Kruskal-Wallis	0.287	
	21		0.394	
	29	Mann-Whitney U	0.818	
Differential blood counts - thrombocytes	9	Kruskal-Wallis	**0.004**	**50μg/l (Day 6 and Day 9) > Control**
	14		0.895	
	21		0.775	
	29	Mann-Whitney U	0.589	
Differential blood counts - lymphocytes	9	Kruskal-Wallis	**0.002**	**Controls > 50μg/l (Day 6); 5μg/l > 50μg/l (Day 6)**
	14		0.321	
	21		**0.049**	**Controls > 50μg/l**
	29	Mann-Whitney U	0.818	
Differential blood counts - granulocytes	9	Kruskal-Wallis	0.116	
	14		**0.018**	**50μg/l > Control**
	21		0.511	
	29	Mann-Whitney U	0.937	
Haematocrit	9	Kruskal-Wallis	0.085	
	14		0.199	
	21		0.326	
	29	Mann-Whitney U	0.589	

4.3.6. Phagocytosis by kidney phagocytes

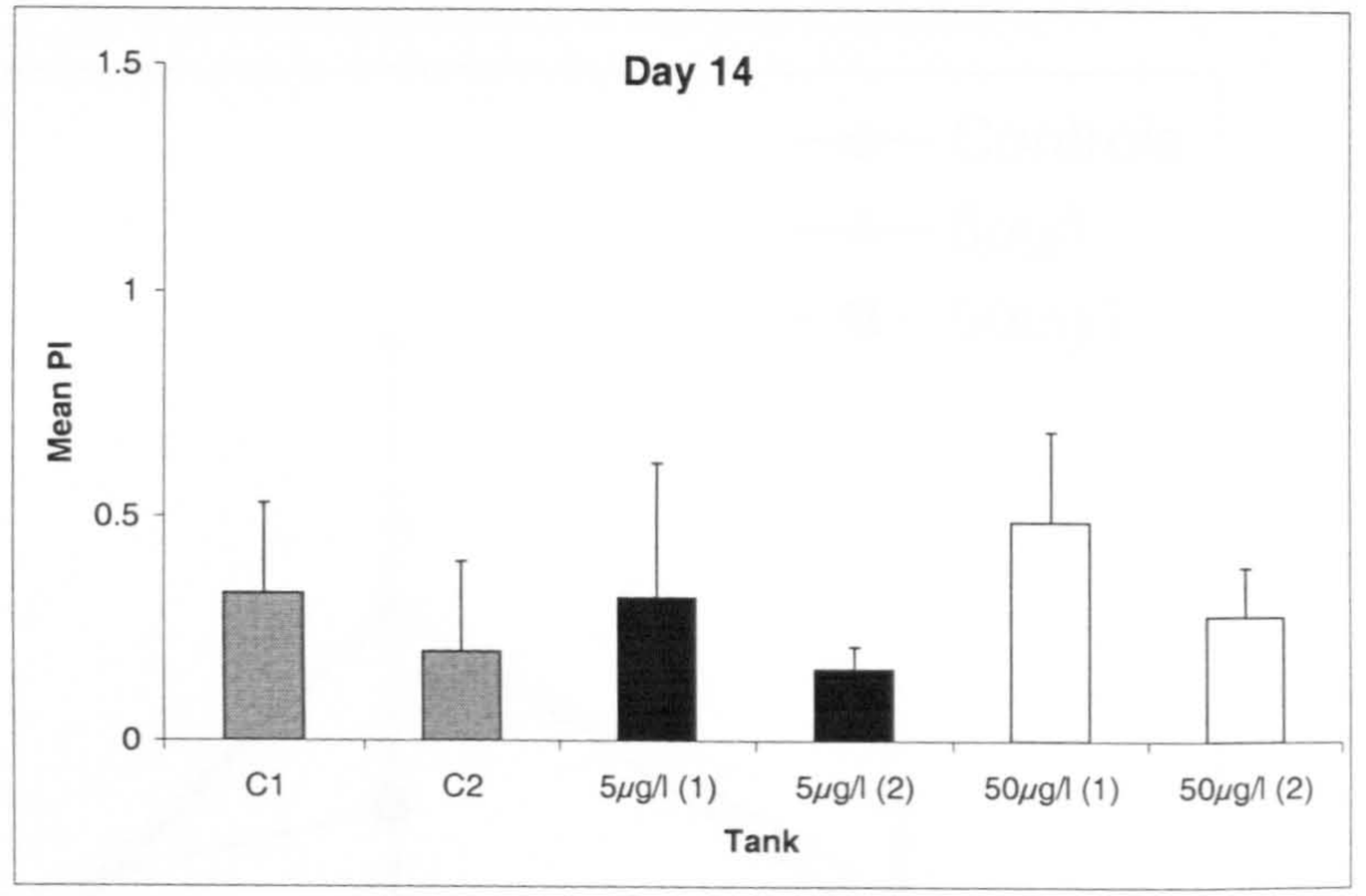
Two indices of phagocytic activity by carp phagocytes were determined during the current trial. The phagocytic index (PI) is the ability of phagocytes to engulf yeast, and the phagocytic ratio (PR) is the proportion of phagocytosing phagocytes. The PI is shown in Figures 4.11-4.12 and the PR in Figures 4.13-4.14. Within each treatment the PI and the PR, in general, followed a similar pattern over time and will thus be discussed together and referred to as the phagocytic activity. Any differences between the 2 indices will, however, be commented on.

At day 9 of trial, the phagocytic activity was seen to increase with exposure to increasing concentrations of cadmium and the activity between replicates of the same treatments was consistent (Figs 4.11a, 4.13a). Between day 9 and day 14 phagocytic activity increased in all replicates, but from this time point onwards remained constant (Figs 4.11b-d, 4.12b-d). As has been seen with the haematological parameters discussed so far, when the data for each treatment were pooled, there was little difference in phagocytic activity between control carp and those exposed to 5μg/l cadmium, with control PI and PR ranging from 0.09 ± 0.04 to 0.41 ± 0.05 and from 4.1 ± 2.0 to 20.7 ± 2.3, respectively, and the PI and PR of 5μg/l carp ranging from 0.13 ± 0.03 to 0.38 ± 0.11 and from 7.3 ± 2.0 to 22.3 ± 4.2, respectively. There were no statistical differences between these 2 treatments at any time point (Figs 4.11-4.14, Table 4.3). However, control carp at day 9 did have statistically lower PI and PR values (0.09 ± 0.04 and 4.1 ± 2.0, respectively) than 50μg/l carp at day 6 (0.38 ± 0.05 and 18.0 ± 2.0, respectively) and from that at day 9 (0.25 ± 0.09 and 10.3 ± 3.1, respectively) (Figs 4.12, 4.14, Table 4.3). At the start of the trial, days 9-14, the pooled PI of the carp exposed to 50μg/l cadmium was

a)



b)



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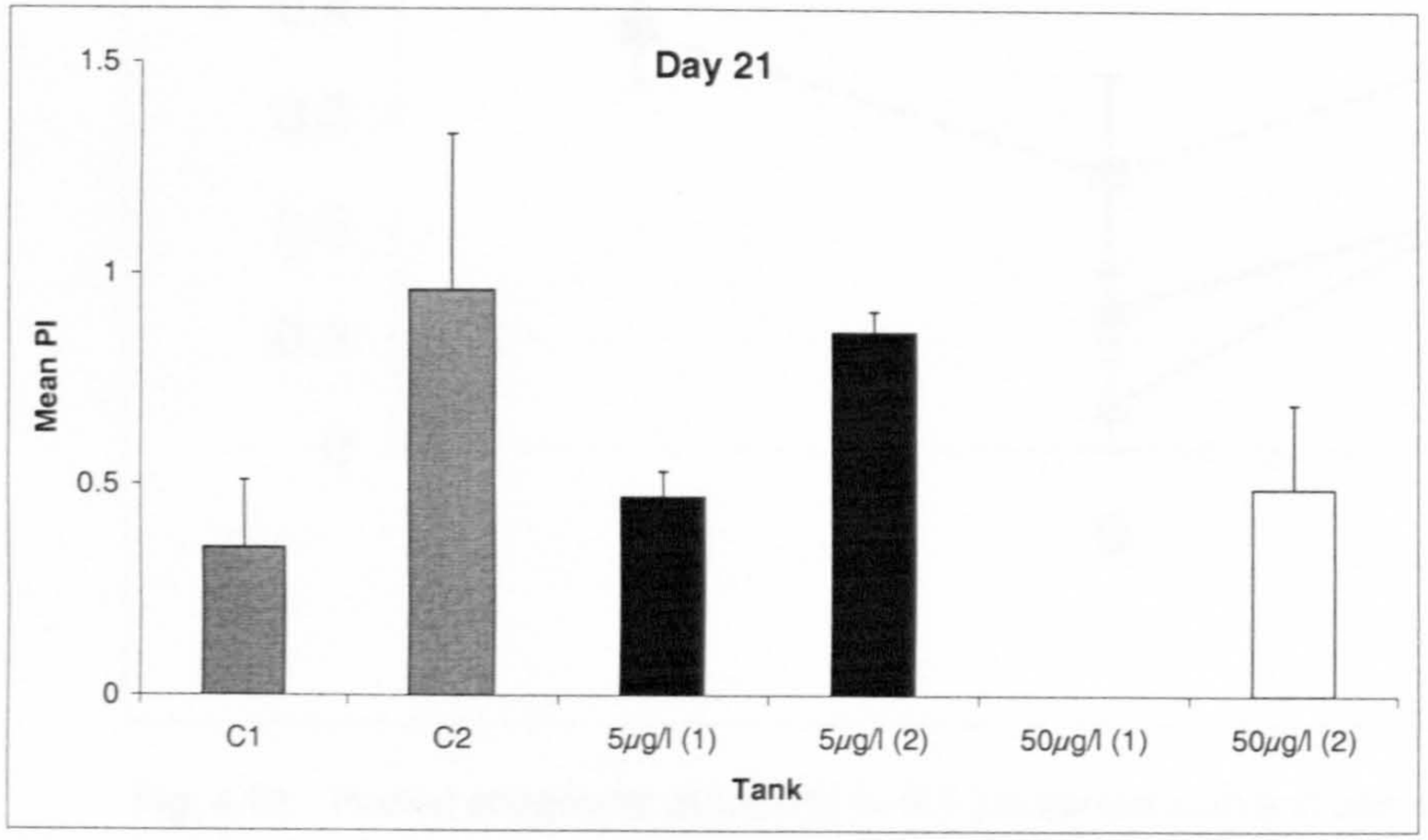

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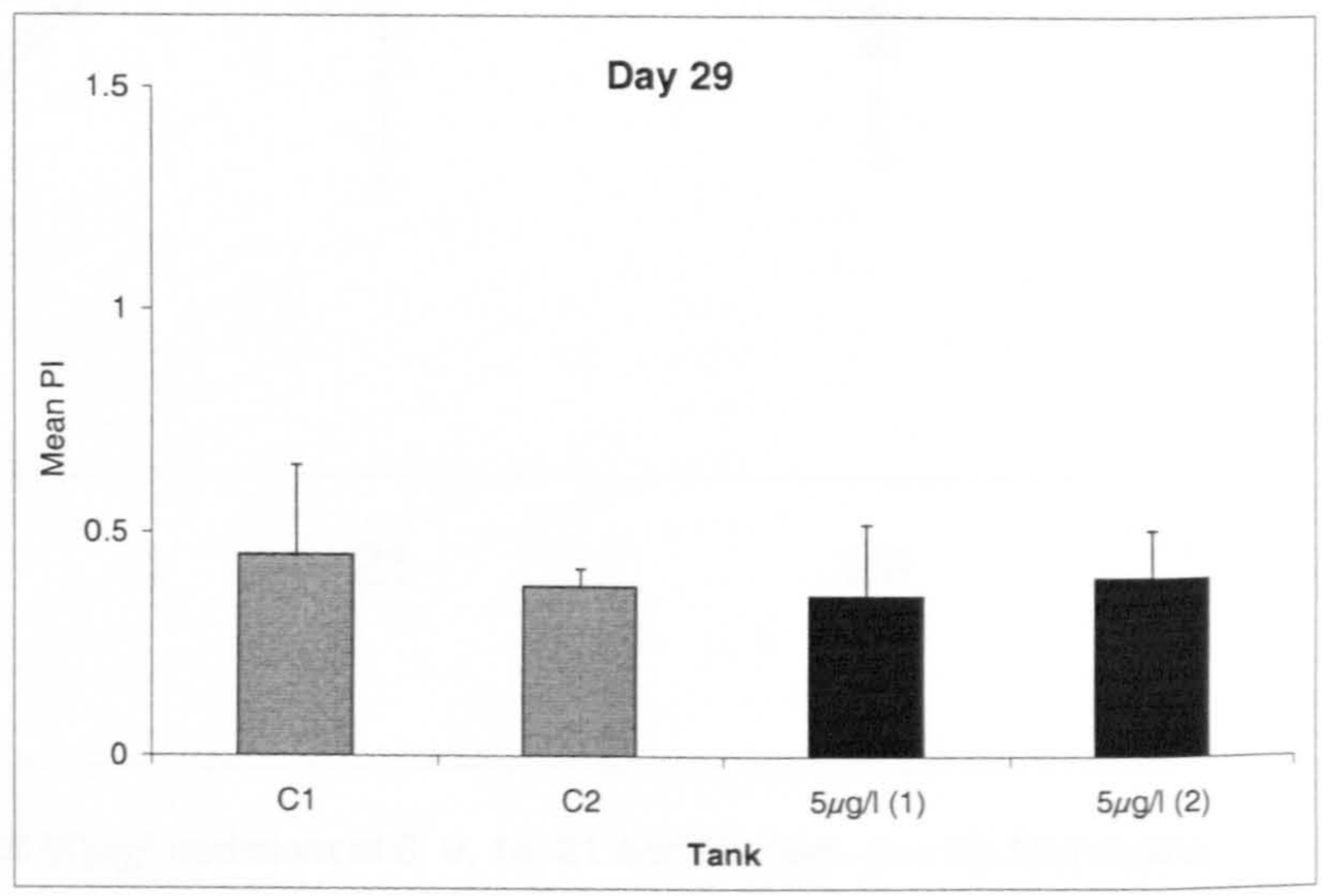


Fig. 4.11 a-d. The mean phagocytic index (PI) (± S.E.) of kidney phagocytes from control carp and from carp exposed to 5 and 50μg/l cadmium for (a) 9, (b) 14, (c) 21 and (d) 29 days. (n = 3). Day 6 data for 50μg/l cadmium-exposed carp is included alongside the data gathered on day 9.

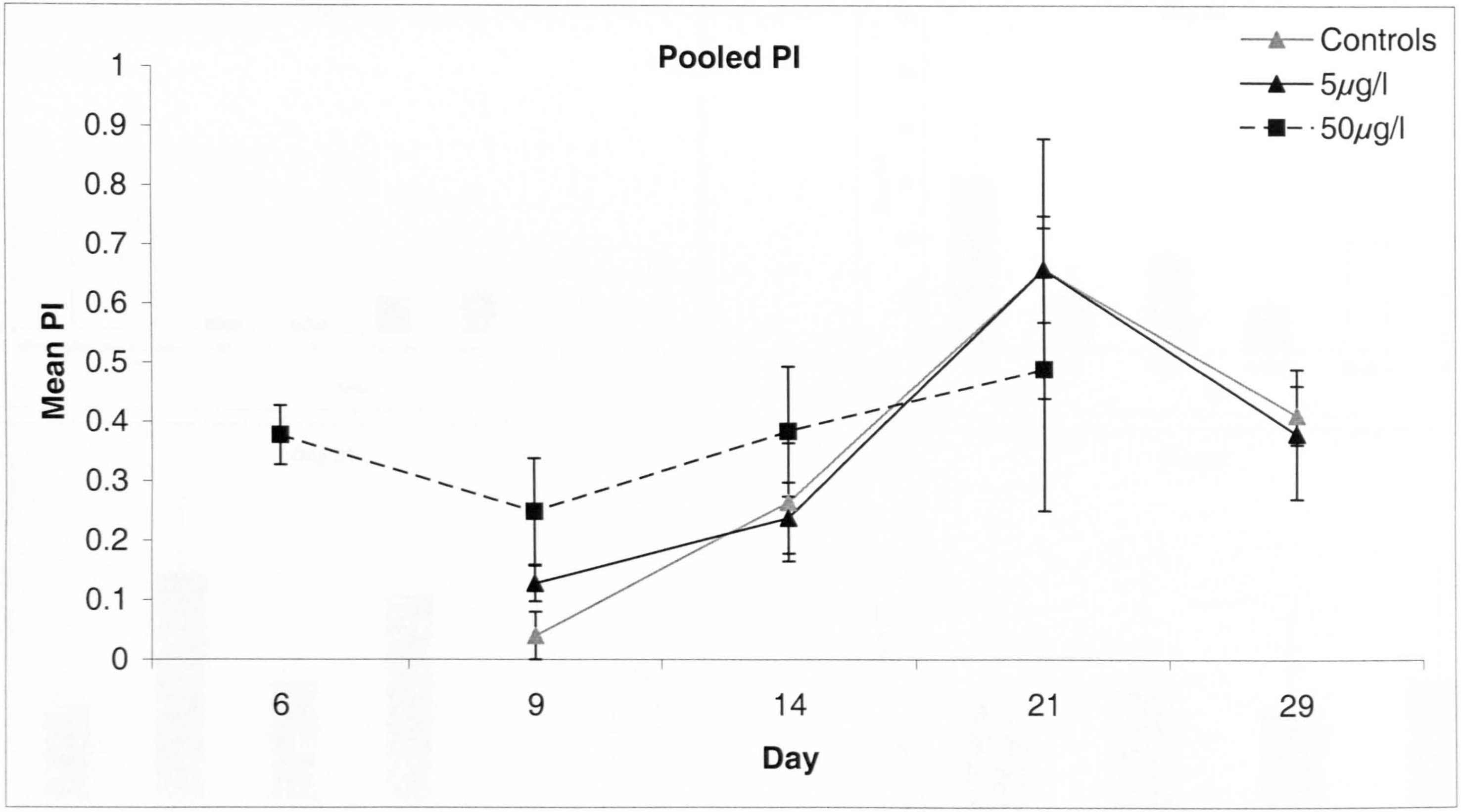
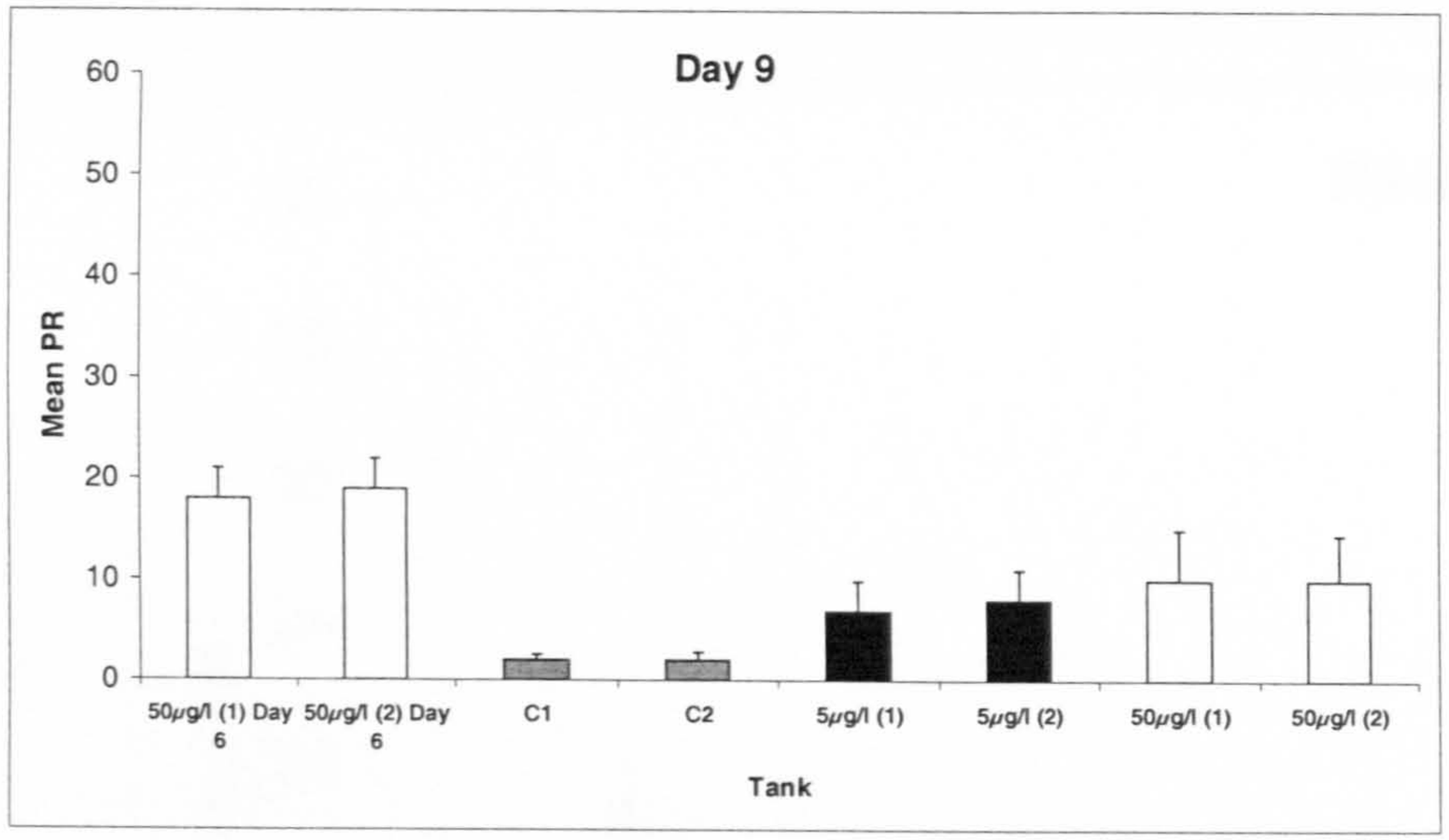
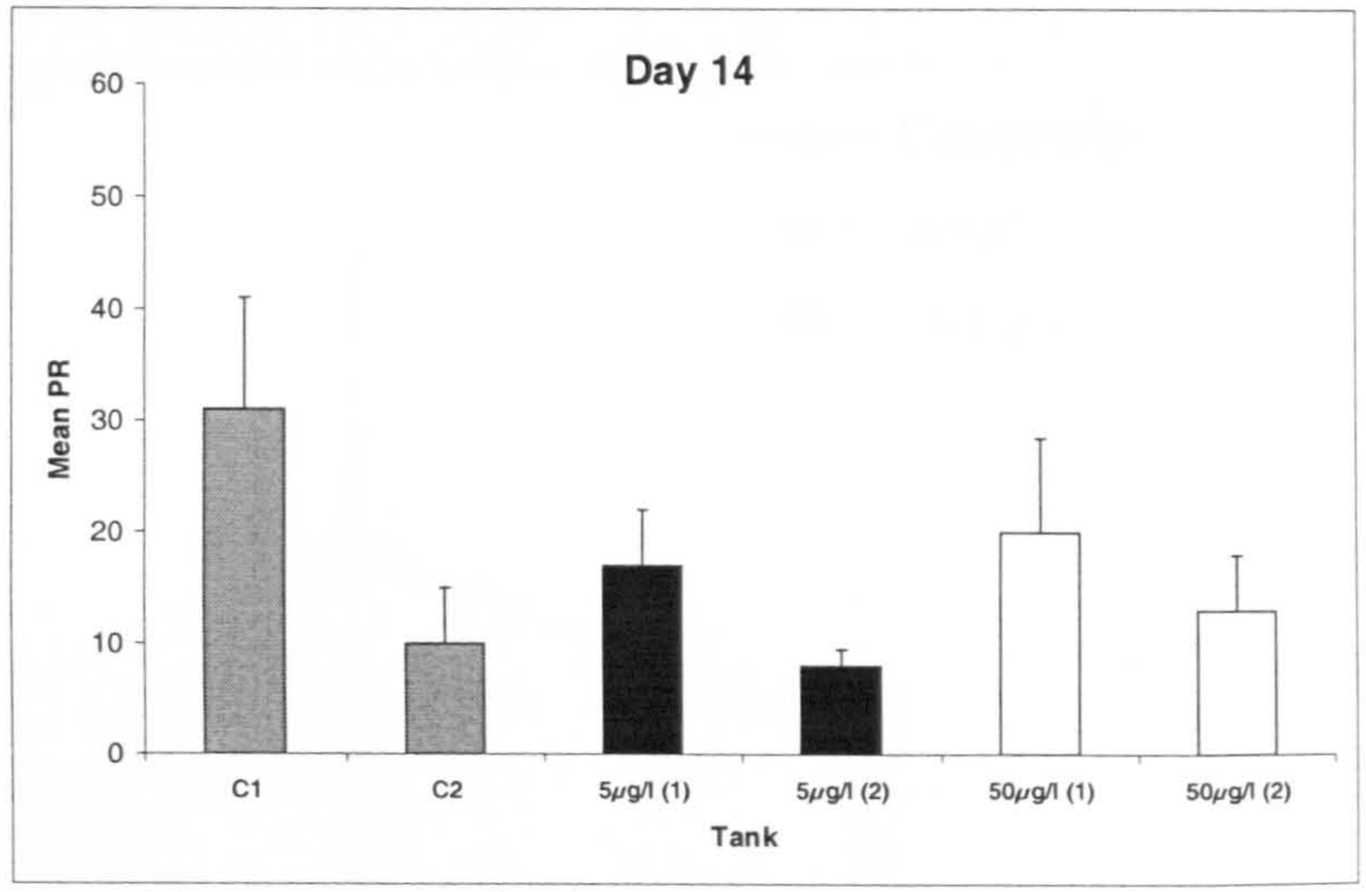


Fig. 4.12. Pooled phagocytic index (PI) (± S.E.) in control carp and carp exposed to 5 and 50μg/l cadmium at 6, 9, 14, 21 and 29 days. (n = 6). Day 6 data for 50μg/l cadmium-exposed carp only.

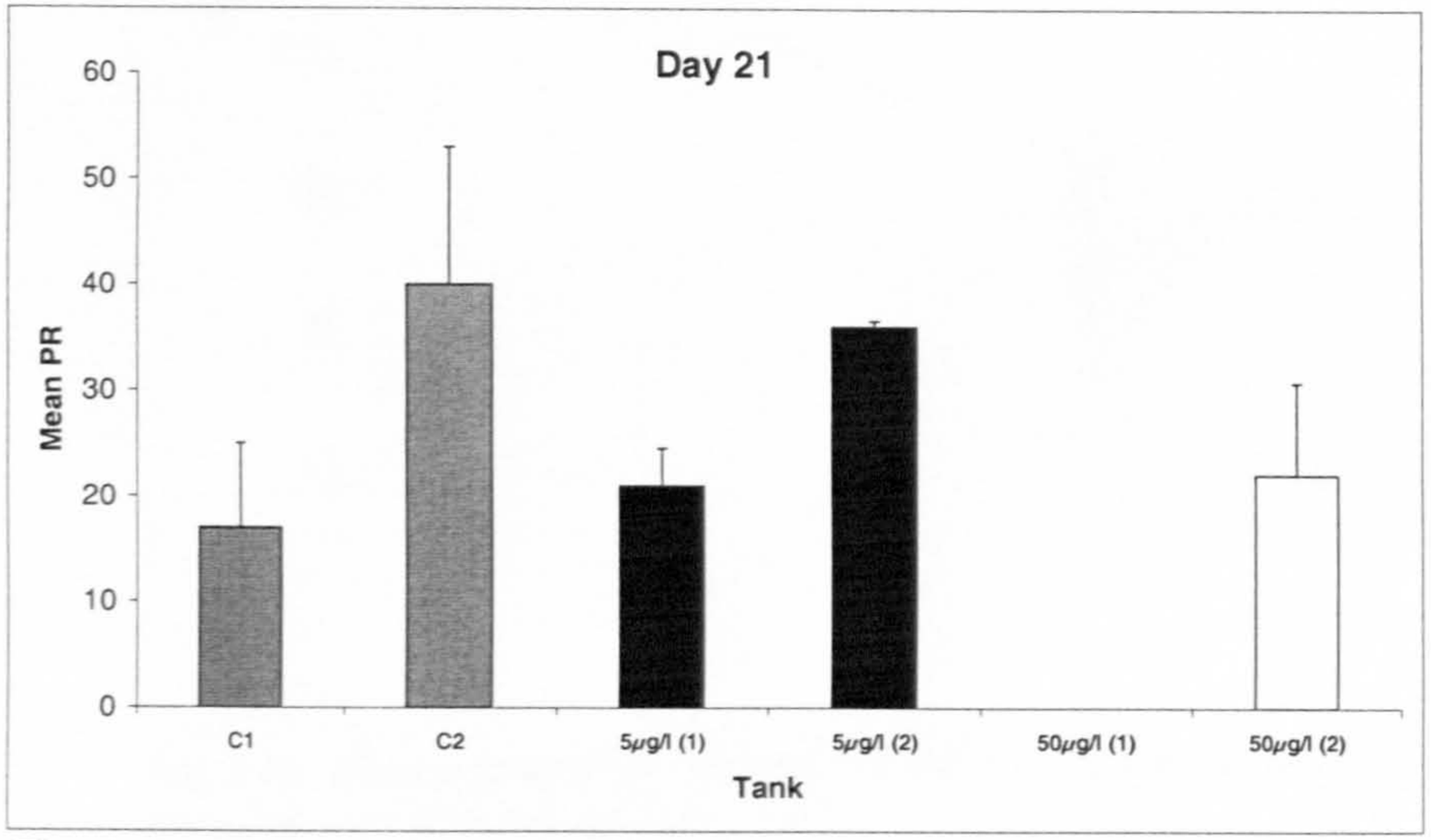
a)



b)



c)



d)

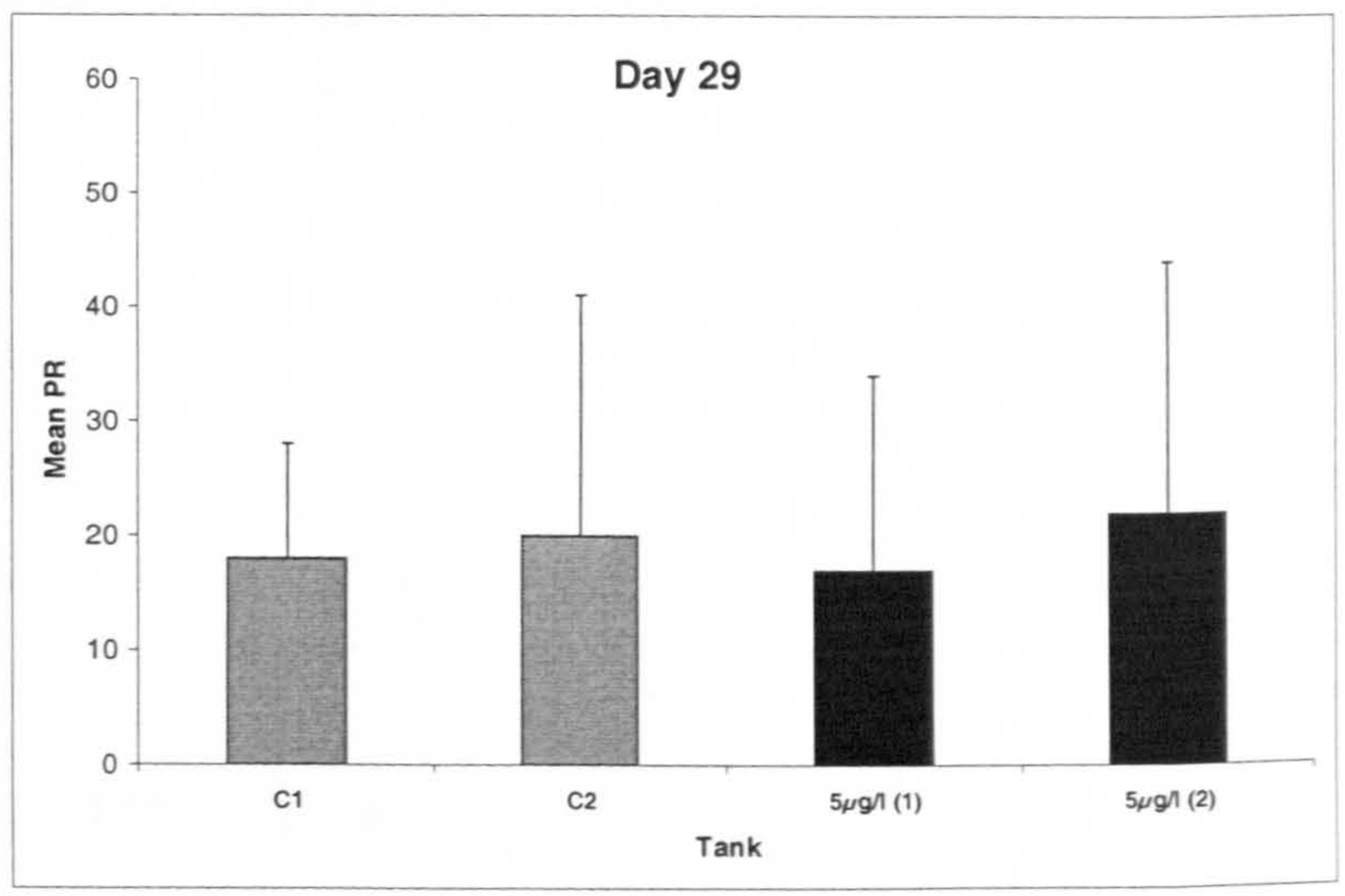


Fig. 4.13 a-d. The mean phagocytic ratio (PR) (± S.E.) of kidney phagocytes from control carp and from carp exposed to 5 and 50µg/l cadmium for (a) 9, (b) 14, (c) 21 and (d) 29 days. (n = 3). Day 6 data for 50µg/l cadmium-exposed carp only.

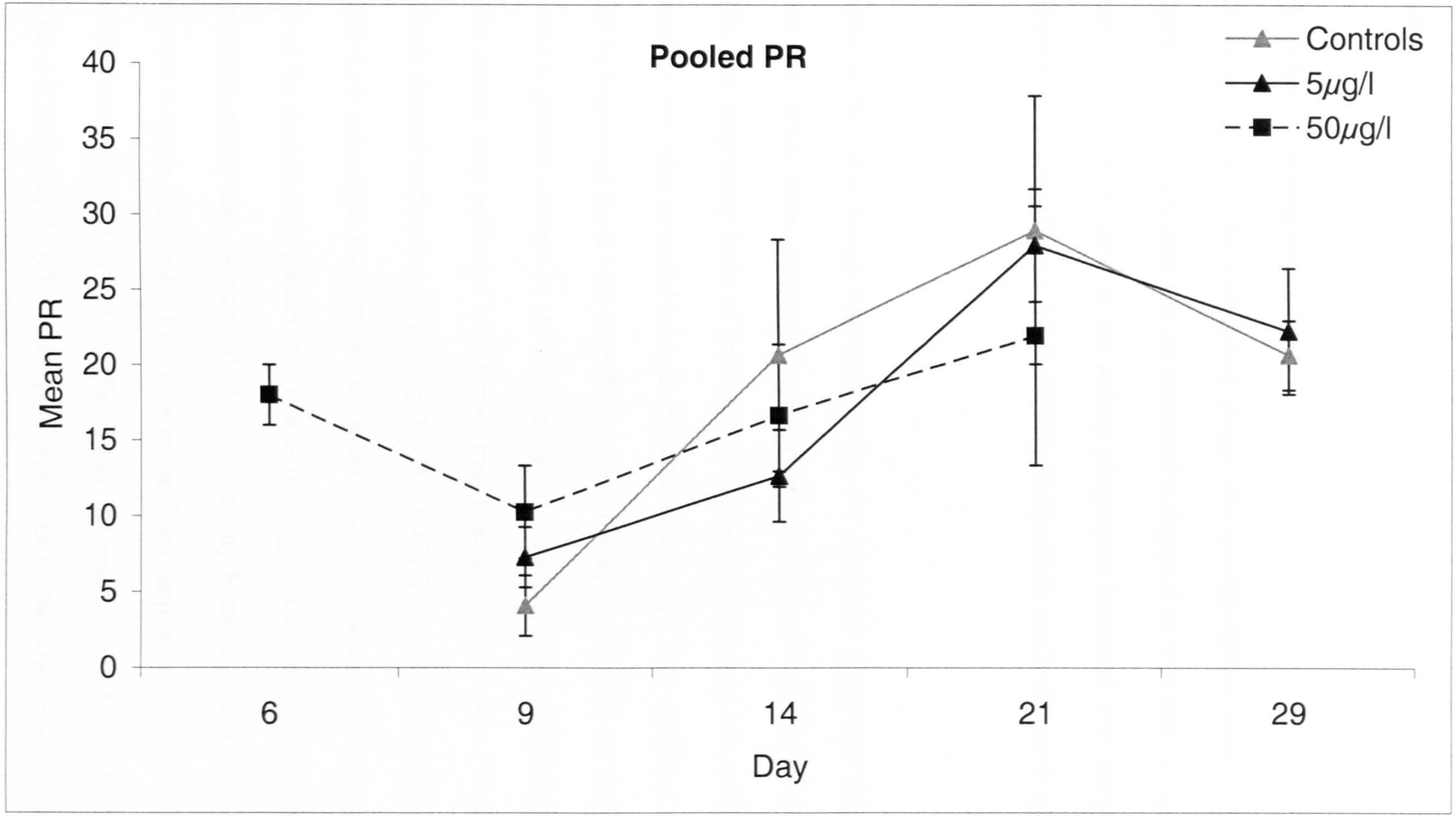


Fig. 4.14. Pooled phagocytic ratio (PR) (± S.E.) in control carp and carp exposed to 5 and 50μg/l cadmium at 6, 9, 14, 21 and 29 days. (n = 6). Day 6 data for 50μg/l cadmium-exposed carp only.

greater than the other 2 treatments, while the pooled PR was only greater at day 9 (Figs 4.12, 4.14).

4.3.7. Phagocyte respiratory burst

The mean respiratory burst of kidney phagocytes in each replicate tank is shown in Figure 4.15 and the pooled data are presented in Figure 4.16. The data are calculated from the respiratory burst of the kidney phagocytes incubated with phorbol 12-myrsitate 13-acetate (PMA). All respiratory burst values can be found in Appendix 3.

Unlike in the other assays discussed so far, the pattern in phagocyte respiratory burst was much more consistent between the replicates of each treatment (Fig. 4.15). The respiratory burst of the 5μg/l cadmium-exposed carp was particularly consistent and, after an initial increase from day 9 to day 14, both replicates showed a fall in respiratory burst with time. By contrast, the replicates from the 50μg/l carp showed a gradual increase in the respiratory burst over time. The respiratory burst of the control carp replicates varied more over time than in the other 2 treatments, but both showed a sharp increase at day 21 (Fig. 4.15). The pooled data presented in Figure 4.16 summarise the patterns described above and shows that the respiratory burst of the controls was greater than that of the 5μg/l cadmium-exposed carp at all time points except day 14, being 0.55 ± 0.19 at day 9 and 0.27 ± 0.15 at day 29 compared to 0.3 ± 0.08 and 0.15 ± 0.04 in the 5μg/l carp at the same time points. The respiratory burst activity of the 50μg/l carp increased from 0.55 ± 0.19 at day 6 to 1.62 ± 0.74 by day 21 (Fig. 4.16). However, none of the differences between the 3 treatments were found to be statistically significant (Table 4.3).

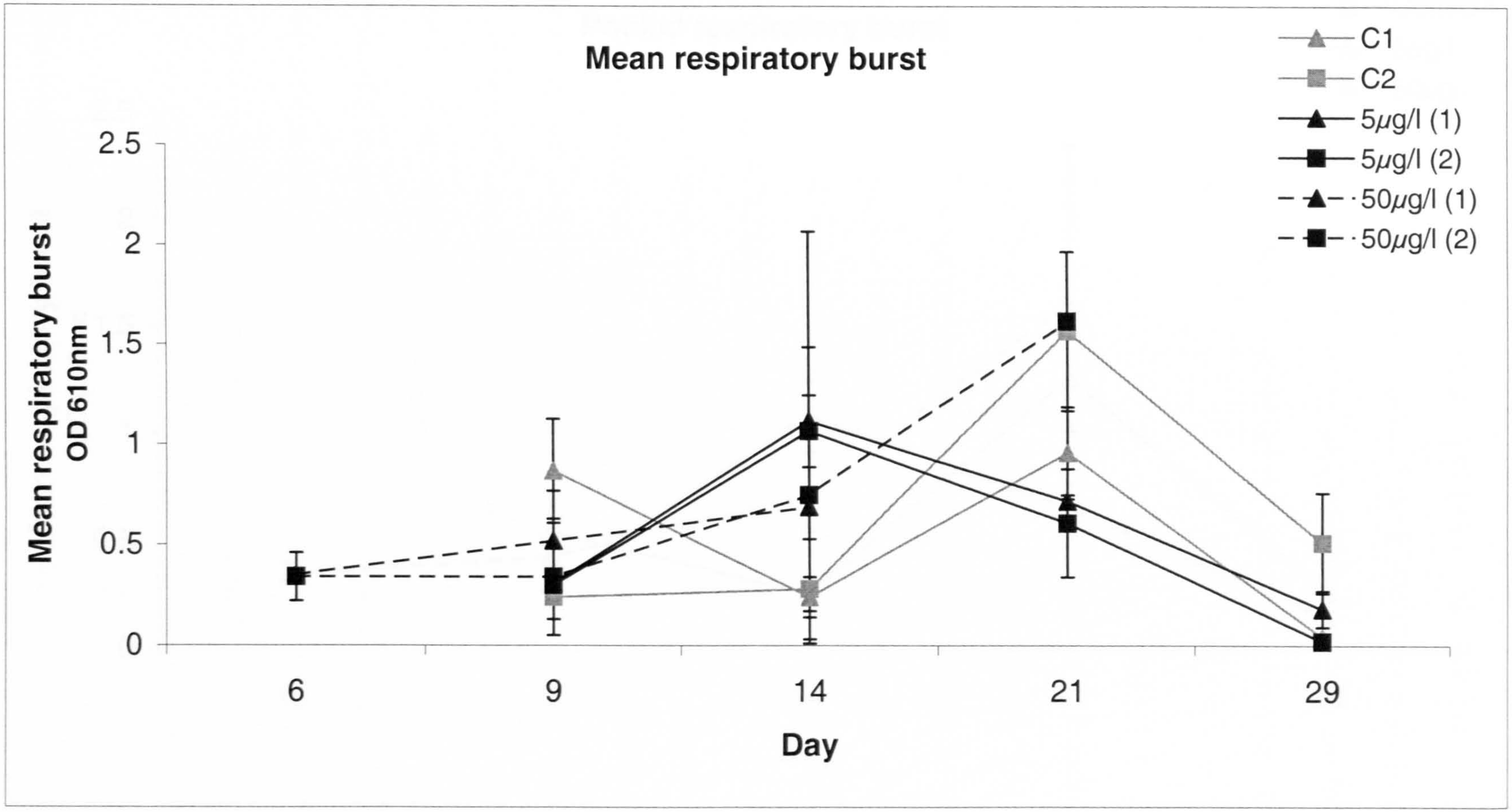


Fig. 4.15. The mean respiratory burst (± S.E.) in control carp and in carp exposed to 5 and 50µg/l cadmium for 6, 9, 14, 21 and 29 days. (n = 3).

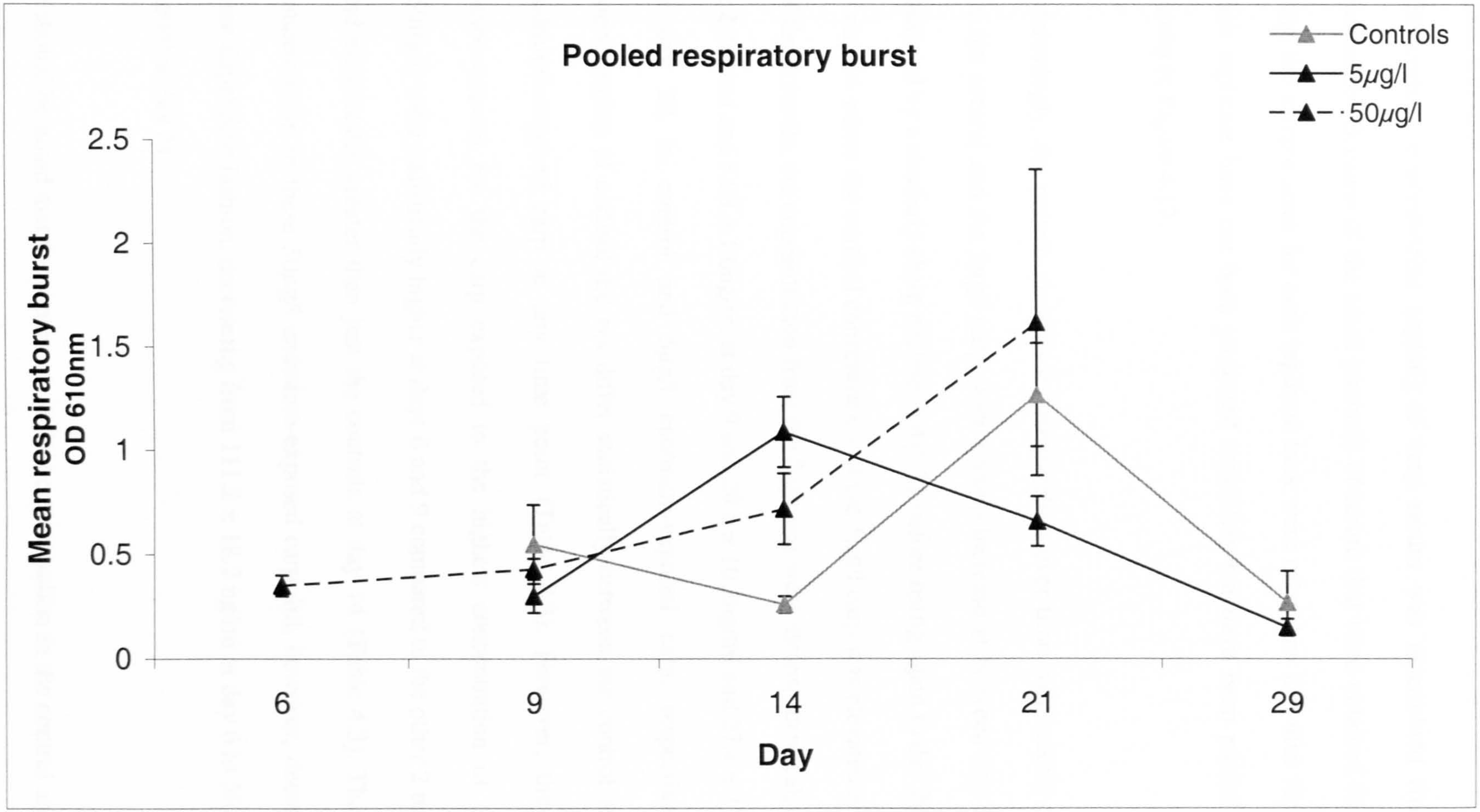


Fig. 4.16. The mean pooled respiratory burst (± S.E.) in control carp and in carp exposed to 5 and 50μg/l cadmium for 6, 9, 14, 21 and 29 days, (n = 6).

4.3.8. Cortisol

The cortisol concentration (ng/ml) of carp serum was determined from all 3 treatments. Because of the small amounts of serum that were obtained from some carp, the sample sizes for each replicate tank were often low and thus the data for each replicate have not been presented separately but have been pooled and are shown in Figure 4.17.

Interestingly, the pattern in cortisol concentration over time was exactly the same for the control and the 5μg/l carp, with a sharp increase at between day 9 and 14 followed by a similarly sharp decline to day 21 before rising again to day 29. Except at day 14 where the cortisol concentration of the 5μg/l carp was elevated above that of the controls, the concentration from the 2 groups were almost identical, *e.g.* 5.70 ± 2.2ng/ml and 5.98 ± 1.6ng/ml at day 9 and 29.8 ± 10.2ng/ml and 27.4 ± 13.0ng/ml at day 29, in control and 5μg/l cadmium-exposed carp, respectively. The concentration of cortisol did not differ statistically between the control and 5μg/l cadmium-exposed carp at any time point (Table 4.3). However, the cortisol concentrations for the carp exposed to the highest concentration of cadmium (50μg/l) were statistically higher at days 6 and 9 compared to the other 2 treatments and statistically greater than just the controls at day 14 (Table 4.3). The cortisol concentration in these 50μg/l cadmium-exposed carp did, however, decrease in a time-dependent fashion, decreasing from 111.2 ± 18.2 ng/ml at day 6 to 56.9 ± 32.4 ng/ml at day 21.

It should be noted that the pattern of cortisol concentration in the control and 50μg/l cadmium-exposed carp inversely reflected the pattern in the respiratory burst and

phagocytic activity of these 2 groups, *i.e.* a fall in the cortisol concentration resulted in an increase in phagocyte activity. The correlation of these 2 parameters was, however, not apparent in the respiratory burst of carp exposed to 5μg/l cadmium.

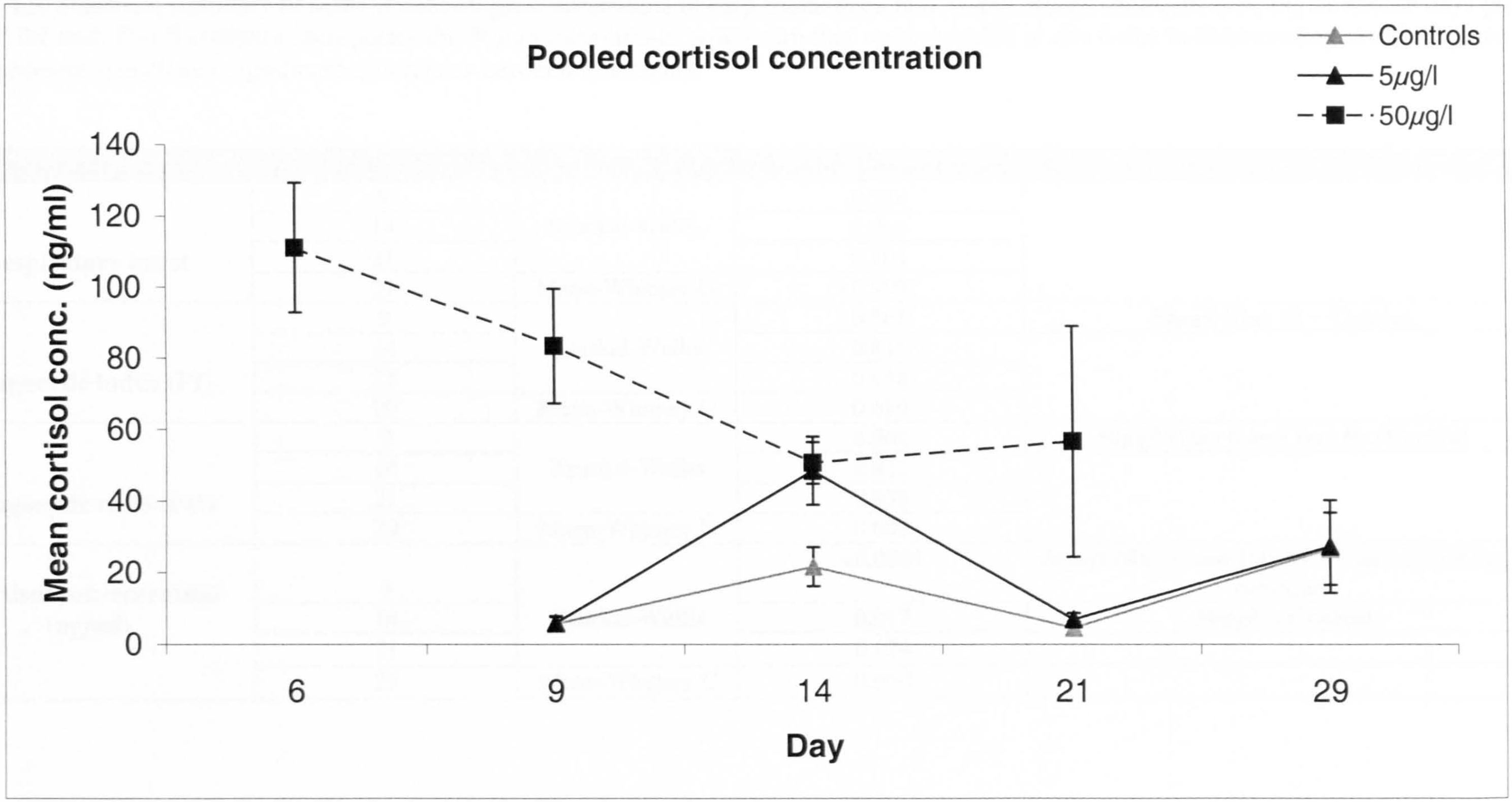


Fig. 4.17. The mean cortisol level (± S.E.) in control carp and in carp exposed to 5 and 50μg/l cadmium for 6, 9, 14, 21 and 29 days. Day 6 data for 50μg/l cadmium-exposed carp only.

Table 4.3. Statistical summary of some immunological parameters of carp maintained in 0, 5 and 50μg/l cadmium at 9, 14, 21 and 29 days post-start of the trial. Day 9 statistics incorporate the 50μg/l cadmium-exposed carp that were sampled at day 6 due to deteriorating health. Figures in bold represent statistically significant differences between treatments.

Immunological parameter	Sample day	Statistical test	Statistical significance	Treatments between which differences lie
Respiratory burst	9	Kruskal-Wallis	0.588	
	14		0.064	
	21		0.104	
	29	Mann-Whitney U	> 0.999	
Phagocytic index (PI)	9	Kruskal-Wallis	**0.002**	**50μg/l (Day 6) > Control**
	14		0.815	
	21		0.858	
	29	Mann-Whitney U	0.699	
Phagocytic ratio (PR)	9	Kruskal-Wallis	**0.001**	**50μg/l (Day 6 and Day 9) >Control**
	14		0.815	
	21		0.858	
	29	Mann-Whitney U	0.699	
Cortisol concentration (ng/ml)	9	Kruskal-Wallis	**<0.0001**	**50μg/l (Day 6 and Day 9) > Control and 5μg/l cadmium**
	14		**0.017**	**50μg/l > Control**
	21		0.124	
	29	Mann-Whitney U	0.970	

4.4. Discussion

The aim of this chapter was to determine the effect of two environmentally realistic cadmium concentrations, 5 and 50μg/l, on selected aspects of the immune response of the common carp and to begin to elucidate the effects of low concentrations of cadmium on host-parasite interactions using the data collected in Chapter 3 (The effect of cadmium on the reproductive biology and survival of *D. extensus*).

The host responses investigated in this current trial were chosen based on some of the available literature regarding heavy metals and the immune responses of fish (Sövényi & Szakolczai, 1993; Hutchinson & Manning, 1995; Zeilkoff *et al.*, 1995; Anderson, 1996; Rice *et al.*, 1996; Witeska, 1998; Sanchez-Dardon *et al.*, 1999; Bols *et al.*, 2001).

The effect of 50μg/l cadmium on the carp used in this trial resulted in many individuals displaying abnormal behaviour, incorporating extreme hyperactivity whereby the carp would swim rapidly around the tank and then jump vertically from the tank. These periods of hyperactivity were followed by periods when the carp lay motionless on the bottom of the tank. Common carp exposed to cadmium a thousand times more concentrated than that used in this current trial (5-35 mg/l) have also been recorded as displaying this behaviour (Sövényi & Szakolczai, 1993). It is interesting, therefore, that carp in this trial showed deviations from normal health at such a low concentration of cadmium. It may be that the increased toxicity is a function of the hardness of the incoming tank water (ca. 24-26 mg/l $CaCO_3$). "Soft" water has been standardised at ca. 50 mg/l $CaCO_3$ (ppm) (Sprague, 1973) and it has been recorded that cadmium is more toxic in soft than in hard water

(Mason, 1991). In hard water many metals precipitate out as non-toxic salts or form soluble, less toxic compounds (Robinson, 1998). Other trials investigating the effect of cadmium on common carp have used much higher concentrations of cadmium (5-35 and 10 mg/l) but have been carried out in water of hardness 138 and 162 mg/l $CaCO_3$, respectively (Sövényi & Szakolczai, 1993; Witeska, 1998). At these levels of water hardness, cadmium was likely to be markedly less toxic than that in this current trial, enabling the fish to tolerate the higher concentrations of cadmium which they were exposed to. No other obvious compounding factors could be identified to account for the morbidity of the 50μg/l cadmium-exposed carp in this trial. The absence of this "abnormal" behaviour in the carp exposed to 5μg/l cadmium suggests that this lower concentration of cadmium is below the toxicity threshold of this fish species. The difference in behaviour between the carp in the two different concentrations of cadmium may also explain why the effects of this metal on the immune responses of the 50μg/l carp were more pronounced than those of the 5μg/l cadmium-exposed carp, which tended to be subtle alterations to the immunological and haematological parameters. However, it should be noted that, wherever the immune responses of the control and 5μg/l cadmium-exposed carp did differ, the direction of change in the 5μg/l carp was generally the same as that of the carp exposed to 50μg/l. This was true of all the immune parameters investigated in this trial, except the respiratory burst of the kidney phagocytes, suggesting that cadmium can indeed alter some aspects of the carp innate immune functions even at low concentrations.

Another particularly interesting observation made in the current trial is the apparent ability of the 5μg/l cadmium-exposed carp to adapt with time to their new

conditions. Subtle differences from the controls in total leucocyte number, the percentage of granulocytes, the phagocytic index and ratio and the respiratory burst of the carp exposed to 5μg/l of cadmium tended to be between day 9 and day 14, or in some cases between day 9 and day 21. However, by day 29, the values for these immune responses were similar in both the controls and the 5μg/l cadmium-exposed carp. It could thus be suggested that the exposure of carp to 5μg/l cadmium results in a two stage response, where from the beginning of the exposure period to day 14-21 the immune system of the carp is altered to some extent, while continued exposure results in the carp adapting to metal exposure. This adaptation may arise from the production of metallothioneins (MT), cysteine rich proteins that have a high affinity for heavy metals. Once bound to the MT, cadmium is prevented from disrupting cellular functions. Metallothioneins have been shown to be produced in the kidney, gills and liver of carp in response to cadmium exposure (Kito, Ose & Sato, 1986; De Smet & Blust, 2001). Thus, over time, the production of metallothioneins by the carp exposed to 5μg/l cadmium may have increased, resulting in less cadmium being available to interfere with the functioning of the immune system, leading to these carp "adapting" to their conditions and functioning in a similar manner to the control carp. The general lack of immunological adaptation in the carp exposed to 50μg/l cadmium could be explained by their inability to produce sufficient concentrations of metallothionein to cope with the level of cadmium they were exposed to. Such an observation was made by Kito *et al.* (1986) on exposing carp to 15 mg/l cadmium for 18 hours. The cadmium levels were found to be in excess of the metallothionein-binding capacity and cadmium then became bound to the high molecular weight proteins and this binding resulted in cadmium toxicity (Kito *et al.*, 1986).

With regards to the total blood counts of the carp in the current trial, it should be noted that the total leucocyte counts in control carp were lower than those from control carp in other trials. For example, Mohan (1990) recorded control values ranging from 28-35 $(10^3)/mm^3$ and Witeska (1998) values from 40-70 $(10^3)/mm^3$, while the values for control carp in this current trial were approximately 14-15 $(10^3)/mm^3$. Klontz (1994) stated that no "normal" values for leucocytes can be applied to fish due to the responsiveness of the blood system to external stimuli. Due to limited numbers of carp of the same age and health status that were available for this trial, it was not possible to gain baseline leucocyte data for this batch of fish. However, in future experiments, baseline leucocyte data should be collected and used to compare with the findings from the experimental carp. There are, however, two hypotheses to explain the differences in leucocyte number between the carp in the current trial and those from other trials. Differences in the temperature at which the trials were run has been discounted as a possible hypothesis, as, although Mohan (1990) carried out his trial at 26°C, Witeska (1998), who recorded the highest leucocyte counts carried out his trial at 17°C, only one degree higher than in the current trial. Thus, the first hypothesis is that the carp used in each trial were of different ages and thus their immune systems may have been at different stages of development. The ontogeny of the fish immune response is a vast subject but has been comprehensively reviewed by Tatner (1996). A second hypothesis to explain the lower leucocyte number recorded in the current trial is that the trials were run at different times of the year. Despite maintaining *O. mykiss* at a constant temperature (18°C) all year round, Yamaguchi, Teshima, Kurashige, Saito & Mitsuhashi (1980, cited by Tatner, 1996), found a lower antibody production in fish immunised against the bacterium *Aeromonas salmonicida* in autumn, compared to those immunised in

the spring. It is possible that circadian rhythms could also have contributed to the observed differences in leucocyte number between the carp in the current trial and those in previous trials.

Significant leucopenia is a common response to cadmium exposure and has been recorded in several studies (Mohan, 1990; Ghazaly, 1992; Witeska, 1998). Although not significant, the total number of leucocytes in the 50μg/l carp in the current trial was lower (7.3 ± 3.0 (10^3)/ml) than that of the controls (14.7 ± 5.0 (10^3)/ml at ca. day 14, suggesting a possible mild immunosuppression. This is supported by the differential blood counts in the 50μg/l group, which revealed a significantly lower number of lymphocytes compared to both the control and the 5μg/l group at both days 9 and 21 of the trial. Exposure of *Tilapia zillii* (Gervais) to 17.7 mg/l and 24.78 mg/l cadmium for 48 hours also resulted in a significant decrease in lymphocyte number (Ghazaly, 1992). Witeska (1998) and Kotsanis *et al.* (2000) have also recorded similar decreases in lymphocyte number as a result of cadmium exposure. However, by contrast, *Perca fluviatilis* L. taken from cadmium polluted areas of the River Emån in Sweden, were found to have both a significantly greater total white blood count and a significantly greater number of lymphocytes than fish sampled from control reference sites (Sjöbeck, Haux, Larsson, & Lithner, 1984).

Elevated numbers of thrombocytes in fish have been associated with environmental stressors (Wedemeyer & McLeay, 1981), as has a significant increase in the number of granulocytes (neutrophilia) (Ellis, 1981; Sövényi & Szakolczai, 1993). Both of these features were observed in the carp exposed to 50μg/l cadmium in the early stages of the trial (days 6-14). A reduction in the number of lymphocytes coupled

with an elevated number of neutrophilic granulocytes has been attributed to increases in the secretion of corticosteroids, such as cortisol (Schreck, 1996). The concentration of cortisol in the 50μg/l carp was higher than both the controls and the 5μg/l cadmium-exposed carp throughout the trial and this may explain the obvious difference in the number of each type of leucocyte recorded in these fish.

There were no statistically significant differences in any leucocyte type between the 5μg/l group and the controls, although the percentage of granulocytes was elevated in the cadmium-exposed carp at all time points until day 29 (Fig. 4.8c) and the percentage of lymphocytes was, in general, lower than in the controls (Fig. 4.8b). Considering that these subtle differences were in the same direction as those seen in the 50μg/l carp, the lack of significant differences between these two groups is possibly the result of the cadmium concentration being too low to elicit a strong response from the carp.

Ghazaly (1992) observed metal exposure to induce alterations in haematological parameters generally through changes in blood water content. Wedemeyer & McLeay (1981) and Morgan & Iwama (1997) stated that changes in erythrocyte number (approximated by the haematocrit level) are a good indicator of either haemodilution or haemoconcentration. An increase in haematocrit was associated with an increase in cadmium concentration in the current trial. It is likely that these increases were associated with haemoconcentration as opposed to an increase in erythrocyte number as the erythrocyte number of the 50μg/l cadmium-exposed carp, those with the greatest percentage haematocrit, was lower than those in the other treatments. It should also be noted that the only other study to find an increase in the

haematocrit on exposure to cadmium was carried out by Witeska (1998). However, Witeska (1998) attributed this increase to manipulation stress rather than to the impact of cadmium. Sakanari, Moser & Reilly (1984), Sjöbeck *et al.* (1984), Tort & Torres (1988) and Ghazaly (1992) have all recorded significantly lower haematocrit levels in cadmium-exposed groups when compared to the controls. In the current trial, it is possible that some aspect of the experimental system may have caused the carp to become stressed over time, particularly given that the haematocrit level of the control carp also increased over time. However, cadmium must also have been impacting on the haematocrit level to some extent, as seen in its increase with increasing concentrations of cadmium. Despite the elevation in haematocrit caused by the cadmium exposure, all values fell within the range suggested for clinically healthy carp (26-39%) by Klontz (1994).

The lack of significant differences in the respiratory burst between any treatment on any individual sample day could possibly be attributed to the large amounts of variation that resulted within each treatment at each time point (Fig. 4.15). Such within-treatment variation in respiratory burst was also recorded in control groups of *Limanda limanda* with marked variation between individual fish sampled simultaneously from the same treatments (Hutchinson & Manning, 1995). The respiratory burst of the control group in the current trial was the least consistent in its pattern of response over time. These fluctuations are possibly due to the natural heterogeneity of fish from the same population and the respiratory burst may vary due to variations in the immune status of the individual fish sampled at each time point. The more consistent responses of the treated groups may be due to the effect of cadmium on the fish immune system masking this natural variation in response.

Interestingly, the respiratory burst of the 50μg/l cadmium-exposed carp was seen to increase in a time-dependent manner and, except for day 9, was consistently higher than the controls (Fig. 4.16). This finding is consistent with those of Enane *et al.* (1991), who exposed to *O. mykiss* to 2μg/l cadmium for 17 days, and to Zelikoff *et al.* (1996, cited by Zelikoff, 1997) who exposed medaka to 6 and 60μg/l cadmium and found a significant enhancement of the respiratory burst after five days. Of note is that Youn, Borghesi, Olson & Lynes (1995) found that the production of oxygen radicals via the respiratory burst in murine macrophages was enhanced by their incubation with metallothioneins (Zn, Cd-metallothionein) at concentrations of 20μM and above. Lower concentrations of metallothioneins did not alter the oxygen radical production (Youn *et al.*, 1995). In order to determine whether the cadmium and zinc components of the metallothionein contributed to the increased respiratory burst activity, concentrations of these two metal cations, equivalent to that found in 20μM metallothionein, were added by Youn *et al.* (1995) to the macrophage cultures. However, there were no resulting increases in the production of oxygen radicals, suggesting that the metallothioneins alone were responsible for the stimulated respiratory burst activity (Youn *et al.*, 1995). The presence of metallothioneins could explain the increasing respiratory burst activity of the 50μg/l cadmium-exposed carp in the current trial. It is possible that the metallothionein production increased over time in response to the cadmium exposure, resulting in continued stimulation of the phagocyte respiratory burst. In the 5μg/l cadmium-exposed carp the initial increase in respiratory burst activity between days 9 and 14 may similarly have been the result of increasing levels of metallothioneins. The subsequent decline in the respiratory burst of this group of carp is harder to explain. The majority of studies investigating the effect of cadmium on the production of

reactive oxygen intermediates (ROS) have, however, found significant inhibition of the respiratory burst by cadmium levels as low as 1-5μg/l and as high as 1.3-5.5 mg/l (Hutchinson & Manning, 1995; Zelikoff *et al.*, 1995; Sanchez-Dardon *et al.*, 1999), findings similar to those recorded on exposing carp to 5μg/l cadmium. In the trial by Hutchinson & Manning (1995), the impaired respiratory burst in *Limanda limanda*, on exposure to 1.3, 2.7 and 5.5 mg/l cadmium, was attributed to the high levels of cadmium that accumulated in the kidneys of the fish and subsequently impaired the adjacent lymphoid tissues. No specific explanations were given by either Zelikoff *et al.* (1995) or Sanchez-Dardon *et al.* (1999) for how cadmium at these low concentrations may have caused the reduction in the respiratory burst. However, in the current trial, it may be that after a rapid increase in the concentration of metallothioneins in response to the initial 5μg/l cadmium-exposure, the production of metallothioneins may have decreased with the adaptation of the carp to their conditions, and may have fallen below the level that can stimulate superoxide production, resulting in a decrease in the respiratory burst to near control levels. In order to test this hypothesis, future experiments would need to measure the concentration of metallothioneins produced by each cadmium-exposed group of carp. The phagocytes could then be exposed *in vitro* to these concentrations of metallothionein and the respiratory burst response from both groups determined to see if metallothionein levels could account for the observed differences in superoxide production.

Of particular interest in the current trial, is that it would appear, at least in the case of the control carp and the carp exposed to 50μg/l cadmium, that the respiratory burst activity was correlated to changes in the cortisol concentration (Figs 4.16,

4.17). As the cortisol concentration of the 50μg/l cadmium-exposed carp fell over time, there was an increase in the respiratory burst activity of the kidney phagocytes. Similarly, positive increases in the cortisol concentration of the control carp were reflected by decreases in their respiratory burst. Given the findings in this trial, it is entirely possible that the respiratory burst by carp phagocytes can be influenced to some degree by cortisol. However, until further trials can be carried out to prove this hypothesis, the results should be viewed with some caution given that the cortisol and respiratory burst responses of the 5μg/l cadmium-exposed carp were not correlated. Also, given the apparent immunosuppressive effect of cortisol on the respiratory burst, it would be expected that the very high levels of this hormone in the 50μg/l carp would have caused inhibition of this activity to a level below that of the controls. Nonetheless, these preliminary findings are of great interest and are worth pursuing further by incubating phagocytes with cortisol of varying concentrations and determining how the respiratory activity may be altered.

Phagocytosis by carp phagocytes was seen to increase in a dose-dependent manner after the fish had been exposed to cadmium for 9 days. Similarly, *O. mykiss* exposed to 2μg/l cadmium for 8 days showed an increase in phagocytosis above the controls (79% and 40%, respectively) (Zelikoff *et al.*, 1995), as did exposing *O. mykiss* to the same cadmium concentration for 17 days (Enane *et al.*, 1991). Zelikoff *et al.* (1995) suggested that the increase in phagocytosis after 8 days exposure could be attributed to augmentation resulting from low cadmium exposure, while higher cadmium concentrations act as immunosuppresants. The same authors saw a fall in phagocytic activity at later time points in the trial (days 17 and 30), although the activity of the cadmium-exposed phagocytes never fell below that of the controls,

and they attributed this fall to an increase in the intracellular concentration of cadmium. This hypothesis, suggested by Zelikoff *et al.* (1995), is a possible explanation for the activity of the carp phagocytes in the current trial, as, from day 14 onwards, the phagocytic activity (PR) of both cadmium-treated groups was lower than the controls. The enhanced phagocytosis at day 9 in both cadmium-treated populations of carp could possibly be attributed to increasing levels of C-reactive protein (CRP). CRP is an opsonin and, as such, mediates the ligand-receptor interactions between the phagocyte and the adhering cell, enhancing phagocytosis (Secombes & Fletcher, 1992). Paul *et al.* (1998) exposed the fish *Catla catla* (Hamilton) to 60 mg/l cadmium for 3, 6,12, 24, 36 and 48 hours and found that levels of CRP increased above the controls from 3 to 24 hours before showing a gradual decrease after 48 hours. The cadmium concentrations used in the current trial (5 and 50μg/l) were much lower than those used by Paul *et al.* (1998), and it is thus possible that the increase in CRP above the controls would have taken longer than the 24 hours recorded by these authors and may have occurred at around day 6 or 9, subsequently enhancing phagocytosis at this time. CRP has been recorded as increasing in response to stress and tissue necrosis (Paul *et al.*, 1998). Mohan (1990) found that exposing carp to 50μg/l cadmium caused necrosis in the secondary lamellae of the gills after only 6 days. Coupled with the increased "stress" response shown by the carp exposed to 50μg/l cadmium in this current trial, the production of CRP by these carp could have been greater than that of fish from the other two treatments, thus explaining the greater phagocytosis in these fish compared to those exposed to 5μg/l cadmium. Future work should investigate the levels of CRP in carp exposed to both concentrations of cadmium over time to determine how this may be contributing to the observed effects on phagocytosis. It is also worth noting that the

phagocytic activity of the carp in all trials appeared to be negatively correlated to the cortisol concentration. Schreck (1996) suggested that mediation of phagocytes by cortisol is possible but *in vitro* incubation of *O. mykiss* phagocytes with cortisol failed to show any effects. However, it is possible that in this current trial cortisol was able to directly influence the activity of carp phagocytes.

Of further note is that, many of the immune parameters of the control carp were observed to increase over time. It is possible that moving the carp to the experimental system had altered the immunological parameters of all fish and that a two week acclimation period had not been long enough for the immune parameters to return to "normal values" which was still occurring during the trial. However, it is most likely, given the increase in cortisol concentration in the control carp over time (except day 21), that some aspect of the experimental system resulted in their becoming more "stressed", leading to subsequent increases in some of the immunological parameters. All efforts were made to minimise stress to the carp during the trial, but it may be that daily monitoring of flow-rates and the noises and vibrations of the aquarium facility were enough to result in a minor stress response in the fish.

Elevated cortisol levels as a result of cadmium exposure has been recorded by Mohan (1990), Pratap & Wendelaar Bonga (1990), Pelgrom *et al.* (1995), Tort, Kargacin, Torres, Giralt, & Hidalgo (1996) and Ricard *et al.* (1998). Interestingly, the highest cortisol concentration recorded in 50μg/l cadmium-exposed carp by Mohan was 28ng/ml while in the current trial the highest value obtained in carp exposed to the same cadmium concentration was 110ng/ml. The differences

between these two concentrations may reflect the difference in the health of the two groups of carp, with severe deviation from normal behaviour and health seen in the carp in the current trial, with no such abnormal behaviour being recorded by Mohan (1990). Although, both trials were carried out in the Institute of Aquaculture, the water hardness in Mohan's trial was twice that of the current trial and the lower water hardness in the current trial may have resulted in the increased cadmium toxicity. However, in both studies, the cortisol concentration decreased over time, suggesting that those carp remaining viable over the course of the trial were able to acclimate to some extent to cadmium exposure.

From this trial it is apparent that the maximum permissible level of cadmium (5μg/l) causes subtle alterations to several aspects of the carp immune response in the early stages of exposure, followed by some degree of adaptation that results in similar immunological values in these carp and the controls by the end of the trial. These alterations are exaggerated by exposure of carp to 50μg/l cadmium. From the analysis of the cortisol concentration, it appears that this hormone may be able to directly influence some of these immune parameters and that the concentration of this hormone is affected by cadmium exposure.

Except for detailed work by Buchmann and co-workers regarding the interactions between gyrodactylids and their hosts (Buchmann, 1997, 1999; Buchmann & Bresciani, 1998, 1999; Stolze & Buchmann, 2001; Buchmann & Lindenstrom, 2002) and one early study by Vladimirov (1971), investigating the immunity of carp to dactylogyrids, there is a paucity of information regarding the interactions between monogeneans and their hosts. Even less literature is available on how these

interactions are affected by heavy metals. The following discussion will try to elucidate the interactions between *D. extensus* and *C. carpio* when influenced by cadmium exposure. It should be noted that the studies were carried out independently from one another and a trial assessing parasite biology and carp immunology simultaneously may have resulted in slightly different observations of host immunity being made, due to the complex interactions that exist between host and parasite. Elucidating the interactions of this host-parasite system in the presence of 50μg/l cadmium posed difficulties due to the evident stress and deterioration of fish health observed in the carp used in the immunology trial. As a result, the *D. extensus* reproduction trial was carried out the reduced level of 30μg/l cadmium to ensure survival of the carp. Thus, the assumption that the immunological results obtained at 50μg/l cadmium would be similar in the carp exposed to 30μg/l has to be made. Tables 4.4 and 4.5, at the end of this discussion, summarise the changes in egg production of *D. extensus* and the changes in carp immune responses at each time point in the separate trials.

The most important feature of both these studies is that the first 9-14 days of cadmium exposure appears to have the greatest impact on both the reproductive potential of *D. extensus* and on the innate immune responses of *C. carpio*. At day 9/10, *D. extensus* exposed to both 5 and 30μg/l cadmium produced a greater number of eggs than their control counterparts in all trials, and the difference in several of the immune parameters in 5 and 50μg/l cadmium-exposed carp compared to the controls was the greatest during this period. Vladimirov (1971) found that the increase in immunological resistance of carp infected with *D. vastator* resulted from the activation of their protective mechanisms, including phagocytic reaction,

complement activity and specific antibody formation. It was found that all three of these parameters increased in carp with increasing infection intensities of *D. vastator* (see Vladimirov, 1971). He found that on incubating *D. vastator in vitro*, in either the blood serum or mucus taken from carp with increasing intensities of infection, that egg production of the parasites also increased. While these results were obtained *in vitro*, it is possible that the cells and substances within these parameters can exert some influence on the reproductive biology of dactylogyrids *in vivo*. Of note, in the current trial is that there appeared to be some correlation between the activity of the phagocytes (principally the PR) and egg production by *D. extensus*, with oviposition being, in general, greater in the parasites from the treatment with the highest PR. There were no apparent links between parasite egg production and the other immune parameters investigated in the current trial, although a simultaneously run trial, investigating parasite biology with carp responses, may have shown different results.

At the start of the trial (ca. day 9), the slightly greater production of eggs by *D. extensus* exposed to 5μg/l cadmium could have been related to the subtlety enhanced phagocytic activity in the cadmium-exposed carp compared to the controls. Given that *D. extensus* exposed to 30μg/l cadmium produced a greater number of eggs than both the controls and the 5μg/l parasites at day 9 and that the 50μg/l carp had a statistically greater phagocytic activity than both other treatments at this time, this theory seems to be entirely possible. Interestingly, from day 14-21 oviposition by control *D. extensus* was, in general, greater than that of the cadmium-exposed parasites. However, the greater production of eggs by control *D.extensus* was not the result of suppressed oviposition in the cadmium-treated parasites but

can instead be attributed to increased oviposition over time by the control parasites. The increasing oviposition in the control parasites over time (Experiments 2 and 4) also appears to be linked to a rise in the phagocytic activity of the control carp. While it is not known whether macrophages can affect ectoparasites *in vivo*, Buchmann & Bresciani (1999) have found that peritoneal macrophages can bind *in vitro* to *Gyrodactylus derjavini*, binding to the cephalic ducts and the body. It is possible that these and other phagocytic cells can bind to the opisthaptor and cephalic ducts and can be ingested by *D. extensus* as its grazes on the surface of the gills. The attachment of these cells to the parasites may impair their normal functioning and result in a form of "stress" response that leads to their producing more eggs. Macrophages and neutrophils produce the cytokine interleukin-1 (IL-1), which Cohan, Scott, Dinarello & Prendergast (1991) recorded as causing mucus cells to secrete their contents, leading to greater mucus production. It is possible that an increase in mucus, caused by the greater number of activated macrophages in the gills of the carp from the treatment with the greatest phagocytic ratio, results in the dactylogyrids becoming exposed to greater quantities of substances, such as lysozyme, esterases, arylamidases, complement and specific immunoglobulins that Shephard (1994) and Buchmann & Bresciani (1999) reported as being present in the mucus of fish and acting against parasites. These again may elicit a "stress" response from the parasites and more eggs may be produced. Of note, are the findings by Jacobs (1995), that stressing carp by netting them from their tanks for 30 seconds, three times daily, results in an increase in the population size of dactylogyrids compared to the controls. No aspects of the host immune response were studied by Jacobs (1995), but it can be assumed that the stress of netting induced alterations to the immune response, which led to a greater number of eggs

being produced by the parasites, thus leading to an increase in population size. It is possible that, as detailed above, the presence of phagocytic cells may have been in part responsible for the greater egg production. However, without further work to assess the impact of phagocytes and their properties on *D. extensus* reproduction, the extent to which these cells may impact on dactylogyrids remains unknown. Nonetheless, this preliminary finding remains of great interest.

It is not known from this current trial if antibody production could also have had an impact on the egg production by *D. extensus*. Vladimirov (1971) found that a doubling of the antibody titre only resulted in a small increase in *in vitro* egg production by *D. vastator* from 1.5 ± 0.07 to 1.6 ± 0.06 eggs/25 parasites, while Grayson, John, Wadsworth, Greaves, Cox, Roper, Wrathmell, Gilpin & Harris (1995) found that adult female copepod *Lepeophtheirus salmonis* on *Salmo salar* L., immunised with extracts from adult caligid copepods, produced significantly fewer eggs than female caligids on un-immunised fish. The effect of cadmium on the antibody responses of fish gives conflicting results with Thuvander (1989), who found an enhancement of the response after exposing *O. mykiss* to 3.6μg/l cadmium for five weeks, and Albergoni & Viola (1995), who recorded an initial reduction in the response after exposing *Ictalurus melas* to 10-30μg/l cadmium for one week, followed by a return to near control levels after two weeks. Thus, future experiments could determine the antibody levels in control carp and in those exposed to cadmium and attempt to correlate this to egg production in *D. extensus* at specific time points.

Despite the influx of eosinophilic granular cells (EGCs) to the gills observed by Mohan (1990), it is unlikely that they elicit an effect on the reproductive biology of *D. extensus*. EGCs along with neutrophils, and to a lesser extent basophils, compose the group of leucocytes referred to as granulocytes. The number of granulocytes in both cadmium-exposed populations of carp remained consistently higher than the controls throughout the trial, showing no apparent correlation with egg production by *D. extensus*. Eosinophilia has been recorded in response to hookworm, ascaridids, strongyles, schistosomes and trichinellids (Cohen & Ottesen, 1981), although the exact role of EGCs in helminth infections is unknown. However, Cohen & Ottensen (1981) have suggested that they may act as inflammatory-modulating cells in response to tissue damage caused by attachment or invasion of the host by the parasite.

The apparent lack of correlation between the respiratory burst activity of the carp phagocytes and the rate of oviposition in *D. extensus* was not unexpected, as, while reactive oxygen species (ROS) have been shown to have larvacidal properties against the metacercaria of the digenean *Diplostomum spathaceum* Rudolph, 1819 (Whyte, Chappell & Secombes, 1989) and against the metacestode stage of *Diphyllobothrium dendriticum* (Nitzsch, 1824) (Sharp, Pike & Secombes, 1991), incubation of *Gyrodactylus derjavini* with peritoneal macrophages of *O. mykiss* only elicited a weak respiratory burst from these cells (Buchmann & Bresciani, 1999). Thus it is likely that ectoparasites are less affected by phagocyte respiratory burst than endoparasites.

Despite only subtle differences being seen in the rate of oviposition of control and cadmium-exposed *D. extensus*, they do appear to be linked with subtle changes in some aspects of the innate immune system of carp exposed to the same conditions. However, with regard to the higher concentration of cadmium (30μg/l), used in the current trial, it is possible that cadmium impacts directly on the parasite. After the initial increase in the proportion and activity of phagocytes (day 9), the number and activity of these cells fell below the controls and with this so too did the egg production by the parasites. However, it is also possible that over time cadmium accumulates in the parasites (see Chapter 5) and reduces their reproductive potential in comparison to the controls. This could also explain the lower number of eggs produced by parasites exposed to 30μg/l cadmium compared to the controls from day 14 of the trial (Experiment 4) onwards. Parasites exposed to 5μg/l cadmium may be able to detoxify the cadmium more effectively than those exposed to the higher level of the metal, resulting in their reproductive output being less affected.

The current findings need to be elucidated further, with future work looking at how certain aspects of the innate immune response interact *in vitro* with dactylogyrids. This needs to be coupled with electron microscope studies of dactylogyrids attached to the gills in order to determine which cell types are able to attach directly to the parasite and potentially stimulate egg production.

While cadmium may exert direct effects on *D. extensus* with regards to its reproductive biology, it is most likely that host responses are responsible for the differences that occur due to the egg production of the control parasites, *i.e.* in the absence of cadmium, showing some increase over time (Experiments 2 and 4). The

immunological parameters between the control and cadmium-exposed carp, particularly those exposed to 5μg/l, were very subtle and it may be this subtlety that explains why the egg production and survival of dactylogyrids were not significantly affected by exposure to cadmium.

These trials have been the first to try and elucidate dactylogyrid-host interactions in the presence of cadmium through independent investigations of parasite biology and host responses. It can be seen that the phagocytic activity of carp kidney phagocytes appears to show some association with the egg production of *D. extensus*. This may be through the direct effects of the macrophages on the parasites or through their production of cytokines and enzymes, which may act as stressors to the parasites and result in an increased rate of oviposition in response. Future work should attempt to simultaneously assess the effects of cadmium on host responses and dactylogyrid egg production in order to elucidate these findings further.

Table 4.4. Summarised data of egg production by *Dactylogyrus extensus* and of carp haematological and immunological responses when exposed to 5µg/l cadmium for 9, 14, 21, 29 days, compared to control values. No statistical differences between treatments for either parasite egg production or host immune responses were observed.

Day and experiment number	Egg production by *D.extensus* exposed to 5µg/l cadmium (higher or lower than controls)	Response from carp exposed to 5µg/l cadmium (higher or lower than the controls)					
		Phagocytosis	Respiratory burst	Haematocrit	Total white blood count	Differential blood counts	Cortisol concentration
9 - Exp 1	Higher	Higher PI and PR	Lower	Lower	N/A	Higher % granulocytes	Approx. equal
10 - Exp 3							
9 - Exp 4							
14 - Exp 2	Higher	Lower PI and PR	Higher	Higher	Lower		Higher
14 - Exp 4	Lower						
21 - Exp 2	Lower	Approx. equal PI; lower PR	Lower	Higher	Lower		Approx. equal
21 - Exp 4							
29 - Exp 1	Higher	Lower PI; higher PR	Lower	Higher	Approx. equal	Approx. equal for all cell types	
31 - Exp 1	Lower						
30 - Exp 2	Higher						
30 - Exp 4	Higher						

Table 4.5. Summarised data of egg production by *Dactylogyrus extensus* exposed to 30μg/l cadmium and carp haematological and immunological responses when exposed to 50μg/l cadmium over time in comparison to control values.

Day	Egg production by *D. extensus* exposed to 30μg/l cadmium (higher or lower than the controls)	Response from carp exposed to 50μg/l cadmium (higher or lower than the controls)					
		Phagocytosis	Respiratory burst	Haematocrit	Total white blood count	Differential blood counts	Cortisol concentration
9	Higher	Statistically higher PI and PR	Lower	Higher	N/A	Granulocytes higher Lymphocytes statistically lower Thrombocytes statistically higher	Higher
14	Lower	Higher PI Lower PR	Higher	Higher	Lower	Granulocytes statistically higher	
21		Lower PI and PR	Higher	Higher	Higher	Lymphocytes statistically lower	
30		No immunological data from carp					

Chapter 5

Accumulation of cadmium by *Dactylogyrus extensus* and common carp

5.1. Introduction

5.1.1. Accumulation of heavy metals in fish parasites

Over a ten year period, Pascoe and co-workers carried out some of the first investigations on the accumulation of heavy metals by fish parasites and their hosts (Pascoe & Cram, 1977; Pascoe & Mattey, 1977; Brown & Pascoe, 1989). The earliest work demonstrated that the plerocercoids of the cestode *Schistocephalus solidus* (Müller, 1776), located within the peritoneal cavity of sticklebacks (*Gasterosteus aculeatus* L.), accumulated less cadmium than their host (Pascoe & Mattey, 1977) and that infected hosts had a reduced survival time in the presence of cadmium compared to their uninfected conspecifics (Pascoe & Cram, 1977). In this same experiment, it was noted that the parasites had a greater survival time than their fish hosts and remained alive even when the hosts had died as a result of cadmium toxicity (Pascoe & Cram, 1977). A similar pattern, of less cadmium being accumulated by parasites than their hosts, was noted by Brown & Pascoe (1989) investigating the cystacanths of the acanthocephalan *Pomphorhynchus laevis* (Müller, 1776) and its intermediate host *Gammarus pulex* L.

At around the same time as these investigations were being conducted on aquatic parasites, the elemental composition of mammalian parasites (*e.g.* the digenean *Fasciola hepatica* L. and the nematodes *Ascaris lumbricoides suum* (now *Ascaris suum* Goeze, 1782) and *Ascaridia galli* (Schrank, 1788) compared to their hosts was becoming of interest (Jacutowicz, 1977; Jacutowicz & Korpaczewska, 1977;

Greichus & Greichus, 1980; Chowdhury & Singh, 1989, 1995, 1996; Pandey & Choudhry, 1989; Tandon & Roy, 1994).

By 1994, the ability of fish parasites, in particular the acanthocephalans, to accumulate heavy metals had become a great focus of attention for Sures and co-workers (Sures, Taraschewski & Jackwerth, 1994a,b,c). Early work by this group concentrated on the uptake of lead (Pb) by three acanthocephalans (*Acanthocephalus lucii* (Schrank, 1788), *Paratenuisentis ambiguus* (Müller, 1776) and *Pomphorhynchus laevis*) and their respective hosts (*Perca fluviatilis* L., *Anguilla anguilla* L. and *Leuciscus cephalus* (L.)) and the nematode *Anguillicola crassus* Kuwahara, Niimi & Itagaki, 1974 which also parasitises the eel (*Anguilla anguilla*) (Sures *et al.*, 1994a,b,c). Concentrations of lead in all three acanthocephalans were found to be significantly greater than that in any host tissue (muscle, liver, intestine), a phenomenon also recorded in a similar but less comprehensive study by Galli, Crosa & Ambrogi (1998) comparing *Acanthocephalus anguillae* (Müller, 1780) with its host, the chub *L. cephalus*. The concentration of lead in the nematode *Anguillicola crassus* was, however, lower than that found in the host tissues (Sures *et al.*, 1994a,b,c). The low concentration of lead observed in *A. crassus* was attributed to its ability to excrete heavy metals (Sures *et al.*, 1994b) but could equally be attributed to the difference in microhabitat occupied by the nematode (swimbladder) compared to that of the acanthocephalans (intestine) (Sures *et al.*, 1994b).

A relationship between heavy metal accumulation and the microhabitat of parasites was demonstrated in further experimental studies by Sures & Taraschewski (1995),

Siddall & Sures (1998) and Sures & Siddall (2001). In contrast to the adult worm in the fish host, larvae of *A. lucii* were found to accumulate less cadmium and lead than their host (Sures & Taraschewski, 1995). The larvae, found in the haemocoel of the intermediate host *Asellus aquaticus* L., are surrounded by liquid of host origin and are thus indirectly exposed to the heavy metals. Adults, however, are located in the fish intestine where they are exposed directly to the heavy metals that have been unaltered by the host (Sures & Taraschewski, 1995). Similarly, cystacanths of *Pomphorynchus laevis*, in the amphipod *Gammarus pulex* L., also accumulated less lead than their host, while the immature worm in the intestine of *Leuciscus cephalus* was capable of accumulating lead to significantly greater concentrations than the fish host, proving that lead uptake can occur immediately after infection of the host (Siddall & Sures, 1998).

Microhabitat also appears to be an important factor when considering lead accumulation in adult acanthocephalans. Adult *P. laevis* persist in the intestine of *L. cephalus,* but in goldfish (*Carassius auratus auratus* (L.)) they penetrate the intestinal wall and are found in the body-cavity (Sures & Siddall, 2001). Exposure to lead for three weeks resulted in adult parasites in *L. cephalus* accumulating lead to a concentration of 7.3μg/g, while *P. laevis* from experimentally exposed *C. auratus* accumulated no lead (Sures & Siddall, 2001). The conclusion from this experiment was that lead accumulation is associated with the intestinal location of the parasite (Sures & Siddall, 2001). Further work demonstrated that *P. laevis* attached to the posterior intestine accumulated the most lead and that parasitisied *L. cephalus* accumulate less lead in their tissues than their uninfected conspecifics (Sures & Siddall, 2003).

The mode of application of heavy metals, dietary or waterborne, has also been found to have a significant influence on the distribution and concentration of lead in fish tissues and thus an effect on some endoparasites of fish (Zimmerman, Sures & Taraschewski, 1999). Exposure to lead via water results in the greatest concentrations being found in fish blood, and the nematode *A. crassus*, feeding on the blood, took up more lead from this source than from lead passing into the gut in food (Zimmerman *et al.*, 1999). Unlike for *A. crassus*, the mode of application had no significant affect on the uptake of lead by the acanthocephalan *Paratenuisentis ambiguus* (see Zimmerman *et al.*, 1999).

As acanthocephalans are able to accumulate high levels of lead, Sures & Siddall (1999) postulated that the parasites might act as a lead sink for their hosts. A five-week experiment, exposing infected and uninfected *L. cephalus* to lead (0.01 and 0.015 mg/l) resulted in a 50% reduction in the lead concentration of infected fish intestines compared to uninfected counterparts, implying some form of "benefit" to the fish from parasitic infection (Sures & Siddall, 1999; Sures, 2001). Turčeková & Hanzelová (2002) also recorded that *Perca fluviatilis* infected with both *Proteocephalus percae* (Müller, 1780), a cestode, and *Acanthocephalus lucii* had lower concentrations of cadmium and zinc in their livers than uninfected fish, again suggesting that some parasites may "benefit" the host.

Fish bile has been shown, by *in vitro* studies, to have an essential role in the uptake of lead by acanthocephalans and the liver fluke *Fasciola hepatica* by forming organo-metallic complexes with lead ions, which can then be taken up from the bile by the parasites (Sures & Siddall, 1999; Sures, 2001). Using inductively coupled

plasma mass spectrometry (ICP-MS) it has also been reported that *Acanthocephalus lucii* and its host, *Perca fluviatilis*, "compete" for a variety of essential elements (Sures, 2002), since a negative correlation was recorded between the concentrations of some elements (*e.g.* calcium and zinc) in the host liver and the size of the parasite infrapopulation (Sures, 2002).

The overall result from all experiments with acanthocephalans was that they exhibited a strong potential for use as bioindicators of pollution due to their ability to concentrate a range of heavy metals to levels above that of their hosts. The rare metals, platinum (Pt) and rhenium (Rh), emitted from catalytic converters are the latest elements known to be bioaccumulated by an acanthocephalan (*Paratenuisentis ambiguus*) (see Sures, Zimmerman, Sonntag, Stüben & Taraschewski, 2002b). Further proof of their bioindicator potential has come from a comparison of the concentrations of 18 different elements in *A. lucii* with the concentrations of the same elements in the zebra mussel *Dreissena polymorpha* (Pallas), a sensitive, established, free-living indicator (Sures, Taraschewski & Rydlo, 1997a). Most of the 18 elements were found in significantly greater concentrations in the acanthocephalan as compared to the mussel. It was concluded that, while *D. polymorpha* is suitable for detecting small-scale differences in heavy metal pollution, acanthocephalans, having a mobile host, can provide information on the average exposure to metals of their hosts within their natural range (Sures *et al.*, 1997a). A comprehensive review on the use of fish parasites as bioindicators of heavy metals in aquatic ecosystems has been prepared by Sures (2001), while cautionary notes about the use of parasites for this purpose have been published by Kennedy (1997) and Overstreet (1997).

Mammalian acanthocephalans, *Moniliformis moniliformis* (Bremser, 1811) and *Macracanthorhynchus hirudinaceus* (Pallas, 1781), and both *Ascaris suum* and *Fasciola hepatica* have also been subject to comprehensive studies with regard to metal accumulation (see Sures, Jürges & Taraschewski, 1998, 2000a; Scheef, Sures & Taraschewski, 2000; Sures, Franken & Taraschewski, 2000b; Sures, Scheef, Klar, Kloas & Taraschewski, 2002a).

While acanthocephalans of fish have been relatively extensively investigated with regard to heavy metal accumulation, less literature is available for cestodes and nematodes. Sures, Taraschewski & Rokicki (1997b) investigated the lead and cadmium content of two cestodes, *Monobothrium wageneri* Nybelin, 1922 and *Bothriocephalus scorpii* (Müller, 1776) parasitising *Tinca tinca* L. and *Scopthalamus maximus* (L.), respectively. Both cestodes contained a significantly higher cadmium concentration than the muscle, liver and intestine of their fish hosts, while only *M. wageneri* contained lead at a higher concentration than in the host tissues (Sures *et* al., 1997b). Similar patterns of element accumulation, above host levels, have been recorded by Riggs *et al.* (1987) in the fish cestode *Bothriocephalus acheilognathi* Yamaguti, 1934, in plerocercoids of *Ligula intestinalis* (L.) and in adult female nematodes of the species *Philometra ovata* (Zeder, 1803) (see Tenora, Baruš, Kráčmar & Dvořáček, 1999). Interestingly, Sures *et al.* (1997b) reported a significantly higher concentration of accumulated lead and cadmium in the gravid, posterior parts of *B. scorpii* and found that the weight of the parasites was positively correlated with their lead burden. Metal burdens have also been recorded as differing between plerocercoids of *L. intestinalis* of different ages (0+; 1+; 2+) with lead, cadmium and chromium (Cr) being in significantly higher

levels in older individuals, suggesting that their accumulation is a gradual and long term process (Baruš, Tenora, Kráčmar & Prokeš, 2001). Conversely, nickel (Ni) was found in significantly greater concentrations in younger specimens (Baruš *et al.*, 2001).

The elemental profile of infected and uninfected eel (*Anguilla anguilla*) muscle revealed that the individuals parasitised with the nematode *Anguillicola crassus* had lower concentrations of iron (Fe), manganese (Mn), copper (Cu) and cobalt (Co) than their uninfected counterparts (Baruš, Tenora, Kráčmar & Dvořáček, 1999). More recently, Bergey, Weis & Weis (2002) reported a similar phenomenon in *Fundulus heteroclitus* (L.) parasitised by the larval nematode *Eustrongylides* sp. Mercury (Hg) was found in significantly lower concentrations in the parasitised fish and the parasites accumulated less mercury than their host (Bergey *et al.* 2002).

Elemental profiles of the nematodes *Pseudalius inflexus* (Rudolphi, 1808) and *Anisakis simplex* (Rudolphi, 1809) (*sensa lato*), parasitising cetaceans have been investigated recently (Szefer, Rokicki, Frelek, Skora & Malinga, 1998; Abollo & Pascual, 2001). Inorganic element concentrations were, in general, higher in adult anisakids compared to their larval forms (Abollo & Pascual, 2001) and chromium, copper, iron and zinc were more concentrated in *P. inflexus* compared to the levels in its host (Szefer *et al.*, 1998).

From the literature it is apparent that most research regarding heavy metals and fish parasites has been concerned with endoparasites. Accumulation of metals by ectoparasites has largely been ignored. However, one study has investigated the lead

content of the monogenean *Ancyrocephalus mogurndae* (Yamaguti, 1940) and compared it to the concentration in the tissues of its host, the mandarin fish *Siniperca chuatsi* (Basilewsky) (Gao & Nie, 2000). The concentration of lead in this monogenean was found to be significantly higher than that in the host tissues (Gao & Nie, 2000). Unfortunately, there is at present no English translation of Gao & Nie's (2000) work and so no further information regarding their methodology and sampling techniques is known.

5.1.2. Accumulation of cadmium in fish tissues

The accumulation of cadmium by fish tissues has been investigated thoroughly and thus the literature relating to this metal only will be reviewed below. The distribution of metals in fish differs between various tissues (Kraal, Kraak, de Groot & Davids, 1995) and thus each tissue type will be reviewed separately.

5.1.2.1. Accumulation of cadmium in fish gills

The gills of fish are considered to be the major site for cadmium uptake (De Smet & Blust, 2001) and thus high levels of cadmium have been recorded in this organ (see Giles, 1988; Suresh, Sivaramakrishna & Radhakrishnaiah, 1993; Kraal *et al.*, 1995; De Conto Cinier, Petit-Ramel, Faure & Garin, 1997; Tayal, Kaur & Mathur, 2000; De Smet & Blust, 2001). As cadmium has a similar ionic radius to calcium (Rainbow, 1995), it is possible that cadmium ions enter the gills not only by facilitated diffusion but also by active transfer via the calcium pump. Indeed, Verbost, Flick, Lock & Wendelaar Bonga (1987) demonstrated that cadmium exposure inhibits calcium uptake in the gills, possibly by competing for the Ca-ATPase of branchial chloride cells. Further evidence for the role of gills in the

uptake of cadmium from water comes from a study by Kraal *et al.* (1995) where common carp (*Cyprinus carpio*) were exposed to waterborne cadmium (80μg/l) or to cadmium-contaminated food (chironomid larvae containing 99μg/l cadmium) for 33 days. Accumulation of cadmium in the gills was highest in carp exposed to the cadmium-contaminated water compared to those exposed to cadmium-contaminated food (75 mg/kg and 2.2 mg/kg respectively) (Kraal *et al.*, 1995).

Giles (1988) found that cadmium levels in the gills of rainbow trout (*Oncorhynchus mykiss*), exposed to 3.6 and 6.4μg/l cadmium, increased 30-fold in the first 10 days of the trial before reaching a steady state. This finding suggests a possible equilibrium between the absorption of cadmium by the gills and the translocation of the metal to other sites in the body (Giles, 1988).

5.1.2.2. Accumulation of cadmium in the liver and kidney of fish

Reports on the uptake of cadmium by fish liver and kidney can be split into two groups depending on the presence or absence of a plateau in the cadmium accumulation kinetic curves (De Conto Cinier, Petit-Ramel, Faure, Garin & Bouvet, 1999). Plateaus in cadmium accumulation have been reported in the liver and kidney of common carp exposed to 450μg/l cadmium for 140 days (De Conto Cinier *et al.*, 1997) and in the liver of rainbow trout exposed to both 3.6 and 6.4μg/l cadmium for a period of between 52 and 129 days (Giles, 1988). By day 178, however, Giles noted that there was a rapid increase in cadmium accumulation in the liver. Continuing accumulation of cadmium in liver and kidney, (*i.e.* not reaching saturation point) has been observed in *C. carpio* exposed to 53 and 443μg/l cadmium (De Conto Cinier *et al.*, 1999) and in the kidney of *O. mykiss* (see Giles,

1988). The kidney's ability to accumulate more cadmium than the liver has been recorded by several authors, including Giles (1988), Suresh *et al.* (1993), Kraal *et al.* (1995), De Conto Cinier *et al.* (1997, 1999) and De Smet & Blust (2001). The liver is thought to be the initial site for cadmium storage, where it is bound to metallothioneins before being moved to the kidneys, which act as the main storage site for the metal (Gill, Leitner, Porta & Epple, 1993). Carp, which are capable of developing a tolerance to cadmium levels of 1 and 5 mg/l, have been shown to produce increasing levels of metallothioniens in the hepato-pancreas and kidney during exposure to this metal (Kito *et al.*, 1986).

5.1.2.3. Accumulation of cadmium in the muscle of fish

Accumulation of cadmium in fish muscle is important because of the potential impact on human health (De Conto Cinier *et al.*, 1999). The permitted level of cadmium in food fish muscle is set at 0.05 mg/kg (EC Regulation 466/2001).

Cadmium concentrations in fish muscle are consistently lower than those found in the gills, liver and kidney (Giles, 1988; Suresh *et al.*, 1993; Kraal *et al.* 1995; De Conto Cinier *et al.*, 1997, 1999; Tayal *et al.*, 2000; De Smet & Blust, 2001). Interestingly, levels of cadmium in muscle appear to increase most when the liver and kidney have reached saturation point (De Conto Cinier *et al.*, 1997, 1999). De Conto Cinier *et al.* (1999) suggested that at the point of saturation, the concentration of cadmium exceeds the fish's ability to produce metallothioneins responsible for detoxifying the metal, and thus the metal is translocated to other organs such as the muscle. Suresh *et al.* (1993) also supported this theory of metal redistribution to the muscle. On the basis of this, De Conto Cinier *et al.* (1997) considered muscle to be

the site for long-term cadmium accumulation. However, contradictory to this hypothesis, a further trial involving a 43-day period of depuration resulted in rapid and immediate loss of cadmium from the muscle, while there was no loss of cadmium from either the kidney or the liver (De Conto Cinier *et al.*, 1999). The amount of cadmium accumulated in fish muscle appears to be dependent on the concentration they are exposed to, the exposure duration and the age and species of the fish under investigation. As an example, carp fingerlings exposed to 3.4 mg/l of cadmium accumulated 0.821μg/g after 30 days exposure (Suresh *et al.*, 1993), while *Colisa fasciatus* Bloch & Schneider (age of fish not stated) exposed to 1 mg/l cadmium accumulated 7.7μg/g in 25 days (Tayal *et al.*, 2000).

5.1.2.4. Accumulation of cadmium in the fish intestine

Contrary to the findings of the studies discussed in section 5.1.2.2, where the kidney has been identified as the major site for cadmium accumulation, Kraal *et al.* (1995) found that the intestine of carp accumulated more cadmium than the kidney, liver and gills. After 35 days, the concentration of cadmium in the carp gut was 174 mg/kg, while in the kidney the concentration was 2.2-2.5 mg/kg (Kraal *et al.*, 1995). These authors suggested that the high levels of cadmium in the intestine was due to the excretion of this metal into the intestine by the bile (Kraal *et al.*, 1995).

5.1.3. Aims of the present study

In light of the paucity of information regarding accumulation of heavy metals by ectoparasites, the aim of this chapter was to determine the accumulation ability of *D. extensus* at both 5 and 30μg/l cadmium. Cadmium accumulation in various organs of the common carp, exposed to 5, 30 and 50μg/l cadmium was also

determined. It should be noted here that several problems were encountered in attempting to determine the accumulation ability of *Dactylogyrus extensus*. A limiting factor within this study was calculated to be the number of individual *D. extensus* that would be required to register a measurable concentration of cadmium that would be above the detection limits of the atomic adsorption spectrometer. The small size of these monogeneans, in comparison to the acanthocephalans and cestodes investigated by Sures, meant that to determine the concentration of cadmium in *D. extensus* a minimum of 50 individuals was required for one sample (data not presented). Given the high number of *D. extensus* individuals required for one sample, the overall number of replicate samples was lower than desired.

5.2. Materials and methods

5.2.1. Determining cadmium concentrations in *Dactylogyrus extensus* and common carp tissues.

5.2.1.1. Source of *Dactylogyrus extensus* and carp tissues.

The same general methodology was used for all the following experiments but the experiments differed from each other, either in terms of the timing and number of sample days, the cadmium concentration used or the type of carp tissues sampled. Each experiment (A-D) and the sampling details specific to them are listed below.

A. *D. extensus* were removed from control carp (5-7.5 cm) and from carp exposed to 5μg/l cadmium at 9 and 29/31 days in Experiment 1, Chapter 3 (section 3.2.2.1). Blood, gill and kidney samples were taken from carp in the same experiment on the same sample dates. Parasites and carp tissue were fixed in 80% alcohol until analysed by atomic adsorption spectrometry (AAS) (see sections 5.2.1.2 and 5.2.1.3)

B. *D. extensus* were removed from control carp (5-7.5 cm) and from carp exposed to 5μg/l cadmium at 14 and 30 days in Experiment 2, Chapter 3 (section 3.2.2.2). Some parasites were removed from the carp directly after their arrival from the fish farm and processed immediately for their cadmium content. Gill, muscle and kidney samples were taken from the carp at the same sampling times. Parasites and fish tissue were processed immediately, *i.e.* not fixed in alcohol, as detailed in sections 5.2.1.2 and 5.2.1.3.

C. *D. extensus* were removed from control carp (5-7.5 cm) and from carp exposed to 5 and 30μg/l cadmium for 9 days (there were too few parasites at day 30 to process). This trial was run solely for the purpose of determining the cadmium accumulation in *D. extensus* and fish gills (no other carp tissues were analysed). Gill samples were taken from the carp at day 9 post-start of the trial and the parasites and gills were processed immediately, *i.e.* not fixed in alcohol, as detailed in sections 5.2.1.2 and 5.2.1.3.

D. Gill, liver, kidney, spleen and muscle samples were taken from the carp (8-12 cm) used in the immunology trial (Chapter 4). These carp were not infected with any parasites. Samples were taken from control carp and from carp exposed to both 5 and 50μg/l cadmium at 9, 21 and 29 days post-start of the trial. As the kidneys of most carp were used for a range of immunology assays, only a few kidney samples were available for processing. The tissue samples were processed immediately, *i.e.* not fixed in alcohol, as detailed in section 5.2.1.3.

5.2.1.2. Carp tissue samples

The skin was removed from all of the fish muscle samples before processing to avoid any cadmium bound to the skin contaminating the muscle samples. All muscle samples were removed from the flanks of the carp and the dry weight of the samples ranged from 5-21 mg. Gill samples consisted of one complete gill arch (randomly selected) from each carp, irrespective of the fish size. Where available the entire kidney was removed from each fish (2-8 mg dry wt.) along with the entire liver (5-20 mg dry wt.) and spleen (3-11 mg dry wt.).

5.2.1.3. Determining the cadmium concentration of *D. extensus* by graphite furnace atomic adsorption spectrometry (AAS).

Only adult *D. extensus* were selected from the carp for analysis of their cadmium concentration as this standardised the methodology and prevented any problems with variation in cadmium accumulation abilities that may occur with the age of the parasite. An adult parasite was classified as having dark vitelline follicles present.

D. extensus were removed from control and 5μg/l cadmium-exposed carp gills and were placed in two separate Petri dishes to avoid contaminating the controls with cadmium. Each Petri dish contained tank water from which the parasites had been removed. To prevent biasing the samples, all traces of gill tissue were carefully removed from the parasites using a seeker and a scalpel blade and were disposed of. Specimens of *D. extensus* were either placed in bijou's containing 5 ml 80% alcohol or were processed immediately. All specimens were processed in the same way irrespective of whether they were fixed in alcohol or were processed immediately.

Due to the small size of the *D. extensus* (ca. 1-1.5 mm), it was not known how many dried specimens would be needed for their cadmium concentration to be above the detection limits of the atomic adsorption spectrometer (0.5μg/l). Thus, initial samples of 250 control parasites and 250 parasites exposed to 5μg/l cadmium were processed and their cadmium concentration determined. To further ensure that a cadmium reading was obtained these parasites were processed without any prior rinsing (see below). Future samples consisted of 50 individual *D. extensus*.

The uptake of cadmium by any organism involves two-stages whereby cadmium rapidly adsorbs or binds to the tissue or cell surface, followed by its slow penetration in to the interior (Siriwardena, Rana & Baird, 1995). To ensure that only the concentration of internally bound cadmium was determined in each *D. extensus* sample, it was necessary to remove all traces of externally bound cadmium from the specimens. Thus, all parasites, including the controls, were rinsed for 5 min in a solution of ethylenediaminetetracetate (EDTA) and nanopure water following the method of Siriwardena *et al.* (1995). In each case the concentration of EDTA in the rinse was 10 times more concentrated than the cadmium concentration in the exposure medium. For example, for the 5μg/l cadmium-exposed *D. extensus,* the EDTA rinse was 50μg/l. The same concentration of EDTA rinse was also used for the control parasites so that both populations were treated identically.

Prior to the start of each experiment, 100 Eppendorfs were acid washed using the methodology provided by V. Din at The Natural History Museum, London. The acid washing removed any traces of cadmium and other metals that may have been present and may have contaminated the samples. The Eppendorfs were soaked in a beaker of 10% nitric acid (10 ml concentrated nitric acid: 90 ml distilled water) for 48 h in a fume cupboard. The acid was then carefully poured from the beaker and the tubes rinsed 3 times in distilled water. Enough distilled water was then added to the beaker to cover the Eppendorfs and the water was boiled briefly using a Bunsen burner. On cooling, the tubes were removed from the beaker and dried in a Gallenkamp drying cabinet at 45°C. Once completely dried, the tubes were only handled by gloved hands to avoid transfer of grease to the tubes that may have affected the overall weight of the tube and thus the samples. The empty tubes were

labelled and were then weighed on an OHAUS GA200D balance and the weights recorded.

Groups of *D. extensus*, prepared as above, were then carefully placed in the labelled, acid-washed Eppendorfs using a Pasteur pipette. Transferring the parasites in this manner meant that some EDTA solution was also transferred to the tubes. The EDTA solution was carefully removed from the tubes using a pipette before they were placed in Eppendorf racks and dried in the drying cabinet set at 45°C.

The samples of *D. extensus* were dried for 48 h and were then removed from the drying cabinet and placed in a desiccator for 2 h and brought down to room temperature. Each tube (+ sample) was then weighed to 5 decimal places on an OHAUS GA200D balance. To ensure that the samples were completely dried they were returned to the desiccator for a further 2 h, after which time they were weighed again. If there was no difference between the first and second weight the samples were regarded as being totally dry and digestion, the next stage of the cadmium determining process, could be carried out.

Into each Eppendorf, 1 ml of Aristar concentrated nitric acid (BDH Ltd) was carefully pipetted. The Eppendorfs were then placed in a tube rack resting on a wire mesh cradle in a 40°C water bath, with the heat aiding the digestion of the tissue sample. The entire digestion process was carried out in a fume cupboard. At regular intervals it was necessary to open the lids of the tubes to release the gases that built up during the digestion process, thus preventing the tubes from exploding. Tubes

were kept in the water bath until all tissue was digested and the solution in the Eppendorfs was transparent and yellow.

Once digested, the samples were stored in a refrigerator at 4°C until a time when they could be analysed. Cadmium levels in the parasites were analysed at 222.8nm using a Unicam 939 QZAA Spectrometer fitted with an FS90 Furnace Autosampler, with a cadmium detection limit of 0.5μg/l. Acid digests of test parasites were diluted with distilled water before analysis (0.5 ml digest: 0.5 ml water) and the mixture (1 ml) placed in the small, plastic sample cups of the auto sampler. Acid digestions of the control parasite were left undiluted (1 ml acid digest only, no water) to ensure that any cadmium present would be detectable by the spectrometer.

The cadmium concentrations recorded in each sample by the atomic adsorption spectrometer were adjusted for the weight of the samples and the values expressed as μg cadmium/g dry weight of *D. extensus*:

$$\mu g\ Cd/sample\ (reading\ from\ AAS) \times \frac{total\ volume\ of\ digested\ sample}{1000} \div dry\ wt$$

As the mean weight of 50 control (n=11) and 50 cadmium-exposed (n=9) *D. extensus* did not differ significantly (one-way ANOVA P=0.28), the dry weight of the samples was standardised by using the mean weight from all samples over all experiments (0.18 ± 0.02 mg).

5.2.1.4. Determining the cadmium concentration of carp tissues by graphite furnace atomic adsorption spectrometry.

Small samples of each tissue, as detailed in section 5.2.1.1, were removed from the carp and placed in labelled, acid-washed Eppendorfs and dried as detailed in section 5.2.1.3. All tissue samples, except those collected during the immunology trial (section 5.2.1.1 D), were then digested in the same way as for the parasites (section 5.2.1.3). The fish used during the immunology trial were larger and thus the tissues tougher and less digestible than those from the smaller fish used in the other trials. Thus, once the samples were dried they were carefully removed from the Eppendorfs using EDTA-rinsed forceps and placed in 5 ml glass tubes (Hach) with plastic screw-top lids. Concentrated nitric acid (3 ml) was added to each tube and the tubes placed in a Techne Dri-Block DB-3H. The tubes were heated at 100°C in the Dri-Block until the entire tissue sample had been digested and the acid solution was transparent and yellow. As before, the digestion process was carried out in a fume cupboard. Before analysing the cadmium concentration, all samples of fish tissue (control and cadmium-exposed) were diluted by half with distilled water. Tissue samples from carp exposed to 30 or 50μg/l cadmium were diluted 1:3 (250μl carp tissue digest: 750μl H_20) to enable the spectrometer to read the cadmium concentrations. The cadmium concentration in each tissue type was adjusted for the dilution factor and then expressed as μg/g dry weight using the calculation expressed in section 5.2.1.3.

5.2.1.5. Inductively coupled plasma mass spectrometry (ICP-MS)

Two samples [one control (50 parasites) and one 5µg/l cadmium-exposed (50 parasites)] had previously been sent to Elemental Research Inc., Vancouver, Canada, where the samples were screened on a Perkin Elmer/Sciex ELAN 6000 inductively coupled plasma mass spectrometer to determine the concentrations of the most common 66 elements. This initial analysis was to determine whether the small amount of digested parasite material was substantial enough for the range of elements to be detected. Once it had been determined that ICP-MS was possible on the *D. extensus* samples, further samples were processed and, in the absence of an ICP-MS at the Institute of Aquaculture, these acid-digested samples were sent to The Natural History Museum (NHM), London for analysis. Rather than screen for all 66 elements, The Natural History Museum typically analyses 35-40 elements and thus fewer elements were determined than by the Canadian ICP-MS. At The Natural History Museum, a Thermo Elemental PlasmaQuad 3 ICP-MS was used for elemental analysis. Each acid digested sample of *D. extensus* was diluted a further 10 times in order to get the acid matrix to a manageable level. Indium (1ppb) and Rhenium (2ppb) were added to the samples to act as internal standards to correct for instrument drift and volume differences in the sample uptake. Samples were each made up to 3-5 ml, 2 ml of which was used by the mass spectrometer during analysis (*pers. comm.* Dr T Jeffries, The Natural History Museum, London).

Water samples were also sent to The Natural History Museum for ICP-AES analysis to determine the concentration of Ca^{2+} ions in the water as the Ca^{2+} concentration in water can affect the toxicity of cadmium (see section 5.4). The cadmium

concentration of the water samples was also analysed by ICP-AES to confirm the accuracy of the values obtained from the AAS in the Institute of Aquaculture.

5.2.2. Statistical analysis

Due to the large number of individual *D. extensus* needed to create one sample for analysis of cadmium concentration only a small number of samples were available for analysis. When there were 3 or more replicate samples, invariably non-parametric statistics (Kruskal-Wallis or Mann-Whitney U tests) were used to analyse the data. The results obtained from the ICP-MS of the samples were also analysed using non-parametric statistics.

In the light of the small sample sizes and the non-normality of the data, the cadmium concentrations determined from the carp tissues were also subjected to non-parametric statistical analysis. The standard error of the mean is shown on each graph.

5.3. Results

5.3.1. Water quality

A summary of the water quality parameters from the cadmium accumulation experiments is presented in Table 5.1.

Table 5.1. Summary of the water quality data from all cadmium accumulation experiments. Mean ± S.E. values are presented.

Experiment (letters refer to experiments listed in section 5.2.1.1)	Treatment (nominal cadmium values)	Actual cadmium concentration of water.	Total hardness of water (ppm $CaCO_3$)	Concentration of Ca^{2+} ions in water (ppm in solution)
A	Control	0.11 ± 0.01 (n = 36)	26.25 ± 0.16 (n = 24)	Not analysed
	5μg/l	8.07 ± 0.32 (n = 36)	24.64 ± 0.18 (n = 24)	Not analysed
B	Control	0.06 ± 0.01 (n = 39)	24.50 ± 0.71 (n = 21)	8.00 ± 0.15 (n = 4)
	5μg/l	7.45 ± 0.48 (n = 39)	24.77 ± 0.74 (n = 21)	7.60 ± 0.16 (n = 4)
C	Control	0.64 ± 0.11 (n = 17)	26.30 ± 0.62 (n = 10)	9.53 ± 0.15 (n =12)
	5μg/l	8.43 ± 0.27 (n = 22)	25.40 ± 0.78 (n = 10)	9.35 ± 0.09 (n =12)
	30μg/l	29.88 ± 0.85 (n = 20)	25.10 ± 0.8 (n = 10)	9.15 ± 0.07 (n = 12)
D	Control	0.39 ± 0.08 (n = 30)	24.86 ± 0.42 (n = 22)	Not analysed
	5μg/l	6.86 ± 0.27 (n = 30)	25.18 ± 1.10 (n = 22)	Not analysed
	50μg/l	50.88 ± 1.72 (n =30)	26.07 ± 0.7 (n = 22)	Not analysed

The total water hardness (ppm $CaCO_3$) did not differ significantly between treatments in any trial. The concentration of Ca^{2+} ions in control and test water, as analysed by ICP-AES, also showed no significant difference between any treatment in Experiments B and C (P = 0.10 and 0.2, respectively).

5.3.2. Accumulation of cadmium by *D. extensus*

Figure 5.1 shows the concentration of cadmium (μg/g dry wt) in those *D. extensus* that were not rinsed in EDTA and thus the concentration of cadmium determined by atomic adsorption spectrometry should have been a reflection of both internally and externally bound cadmium. Although batches of 250 parasites were processed and analysed for cadmium concentration, the results shown in Figure 5.1 have been adjusted to represent the cadmium concentration in 50 parasites so that the results are comparable with all other data. The *D. extensus* exposed to 5μg/l cadmium had a greater concentration of cadmium than their control counterparts (7.2 and 1.5μg/g dry wt, respectively).

As above, the concentration of cadmium in the EDTA-rinsed *D. extensus* that had been exposed to 5μg/l cadmium was greater than the control values (also EDTA-rinsed) at both 9 (Fig. 5.2) and 29/31 (Fig. 5.3) days post-start of the trial (day 9, 20.7 and 12.6μg/g, day 29/31, 13.0 and 3.5μg/g, respectively). Due to the small sample sizes for control and 5μg/l cadmium-exposed *D. extensus* (n=3 and n=2, respectively), no statistical analysis could be carried out and, therefore, it is not known if the difference in accumulated cadmium between the 2 populations of parasites was statistically significant. Surprisingly, and in contrast to what was expected, the concentration of cadmium in non-EDTA-rinsed parasites (Fig. 5.1) was lower than that in the EDTA-rinsed parasites at both day 9 and day 29/31 (Figs 5.1-5.3) and was approximately the same concentration as the EDTA-rinsed parasites from Experiments B and C. All the *D. extensus* in Experiment A had been stored in alcohol for 1 month prior to analysis.

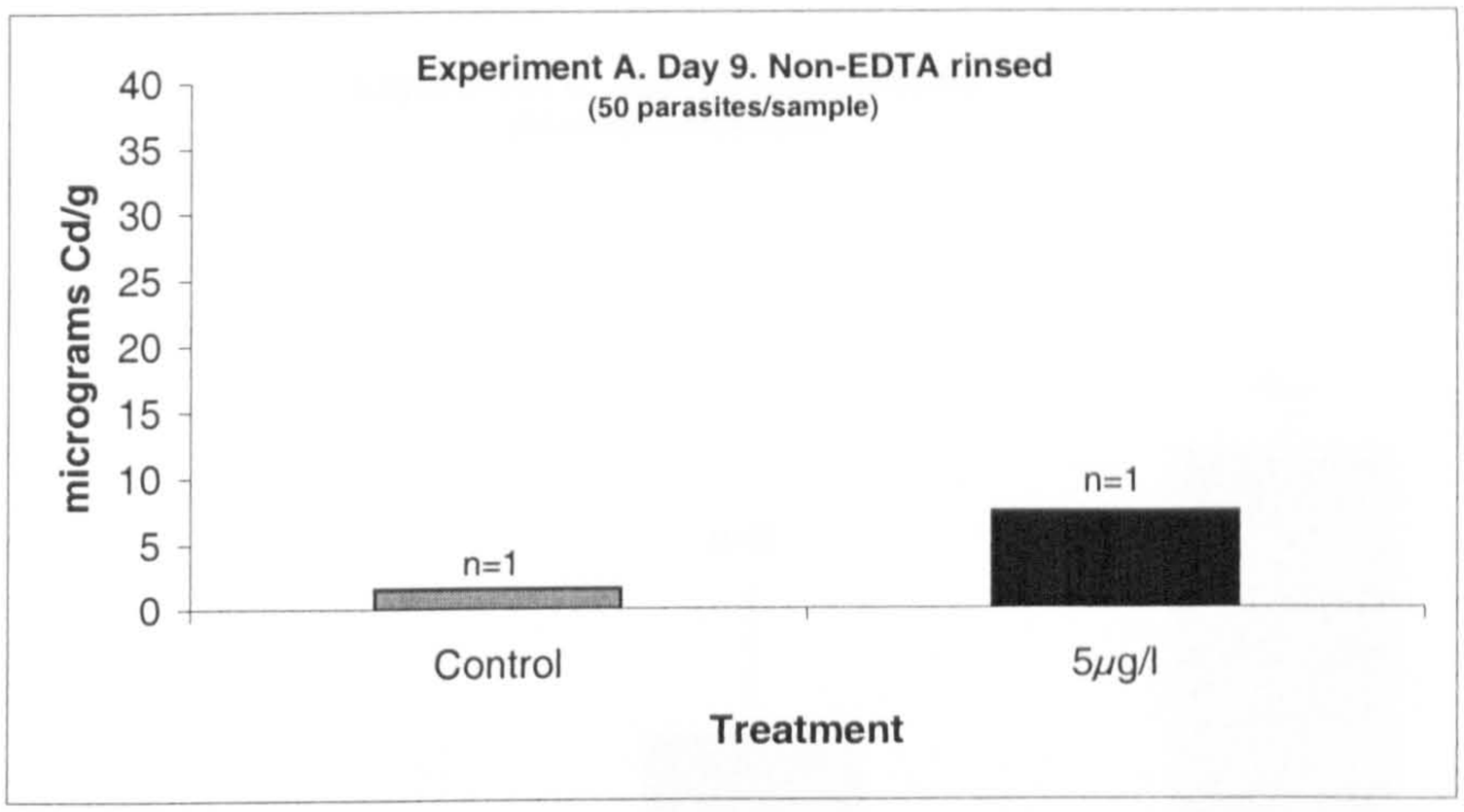


Fig. 5.1 The concentration of cadmium (μg/g dry wt.) (± S. E.) in non-EDTA rinsed *D. extensus* (50/sample) after 9 days exposure to 5μg/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 A.

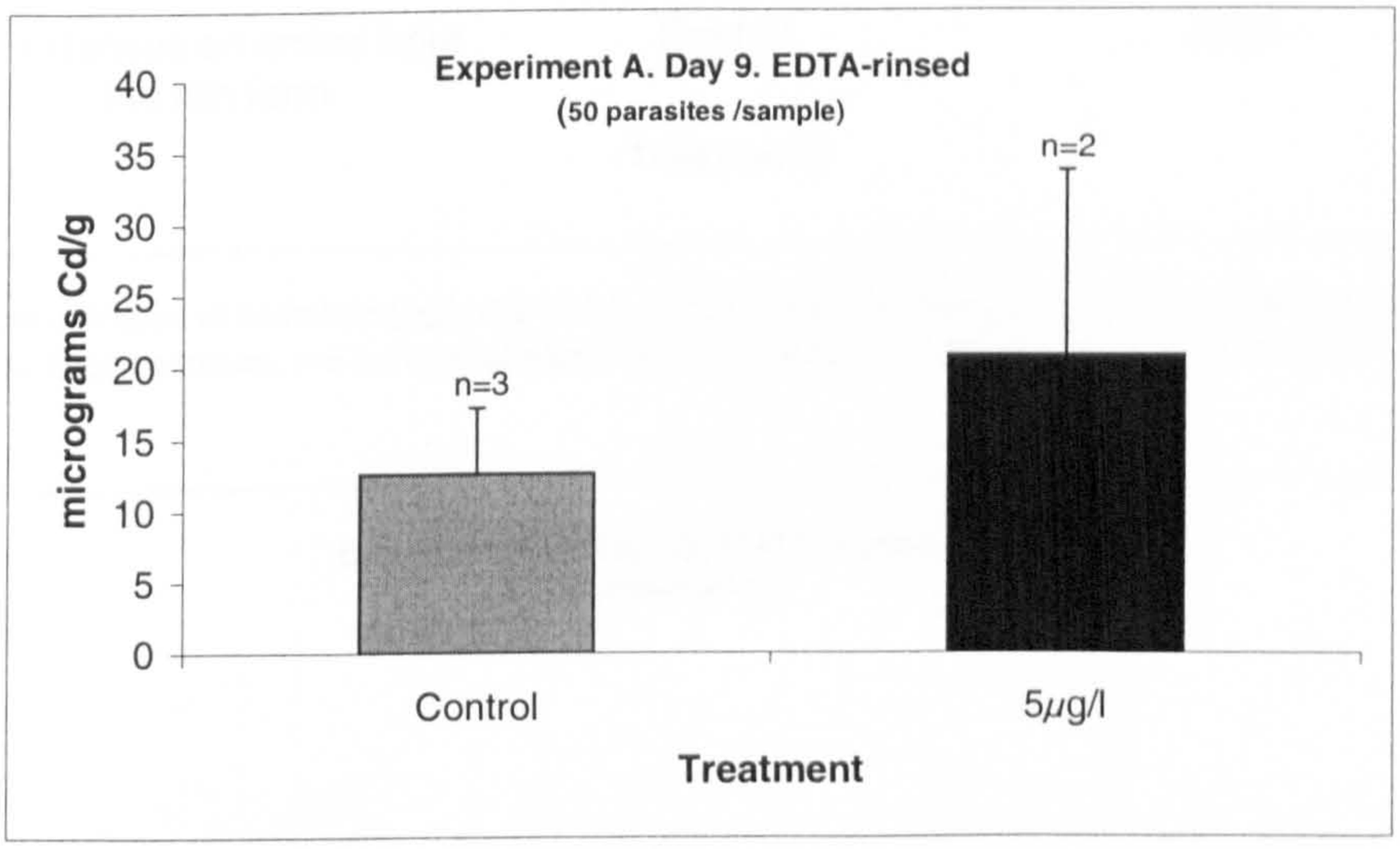


Fig. 5.2 The concentration of cadmium (μg/g dry wt.) (± S.E.) in EDTA rinsed *D. extensus* (50/sample) after 9 days exposure to 5μg/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 A.

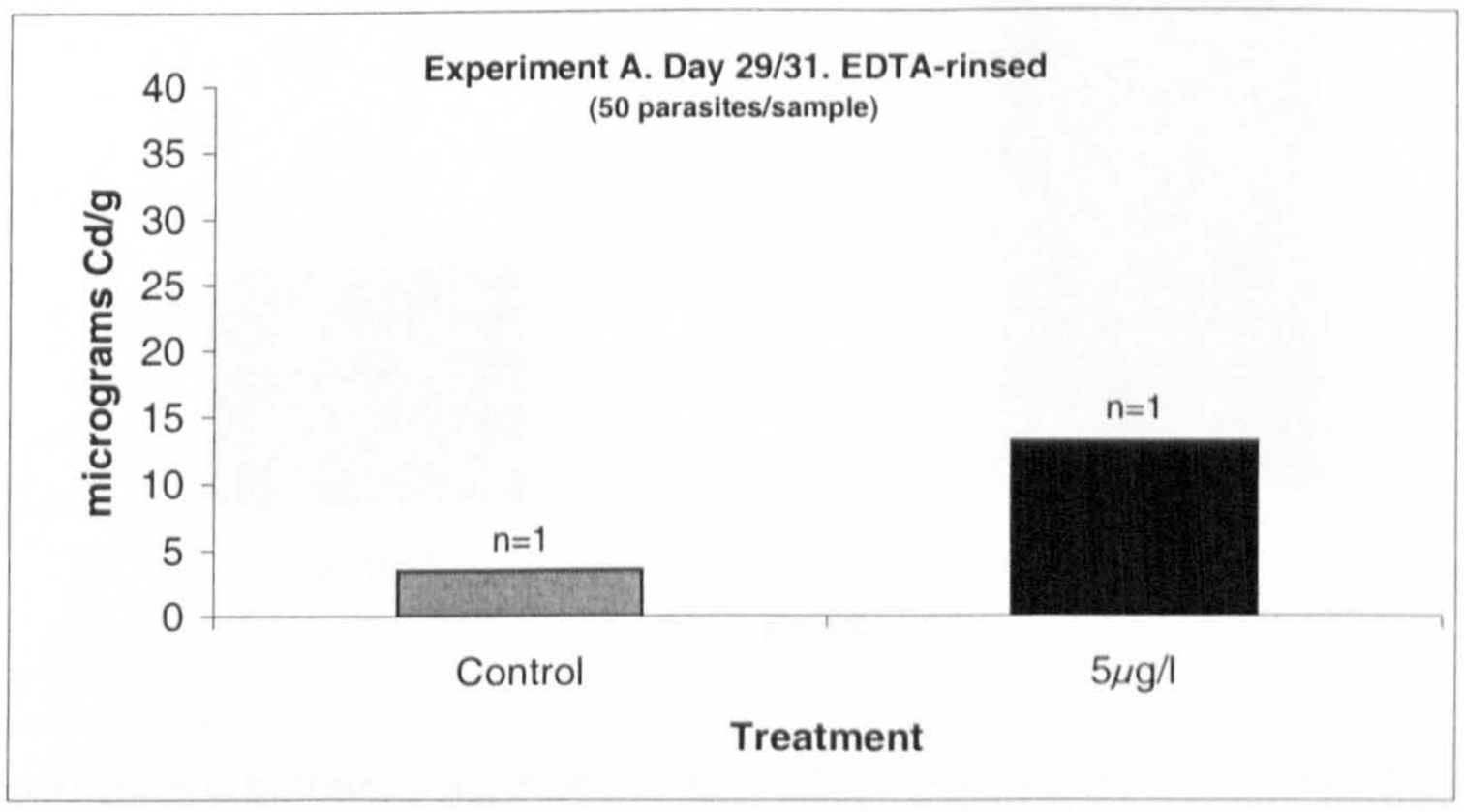


Fig. 5.3 The concentration of cadmium (μg/g dry wt.) (± S.E.) in EDTA-rinsed *D. extensus* (50/sample) after 29/31 days exposure to 5μg/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 A.

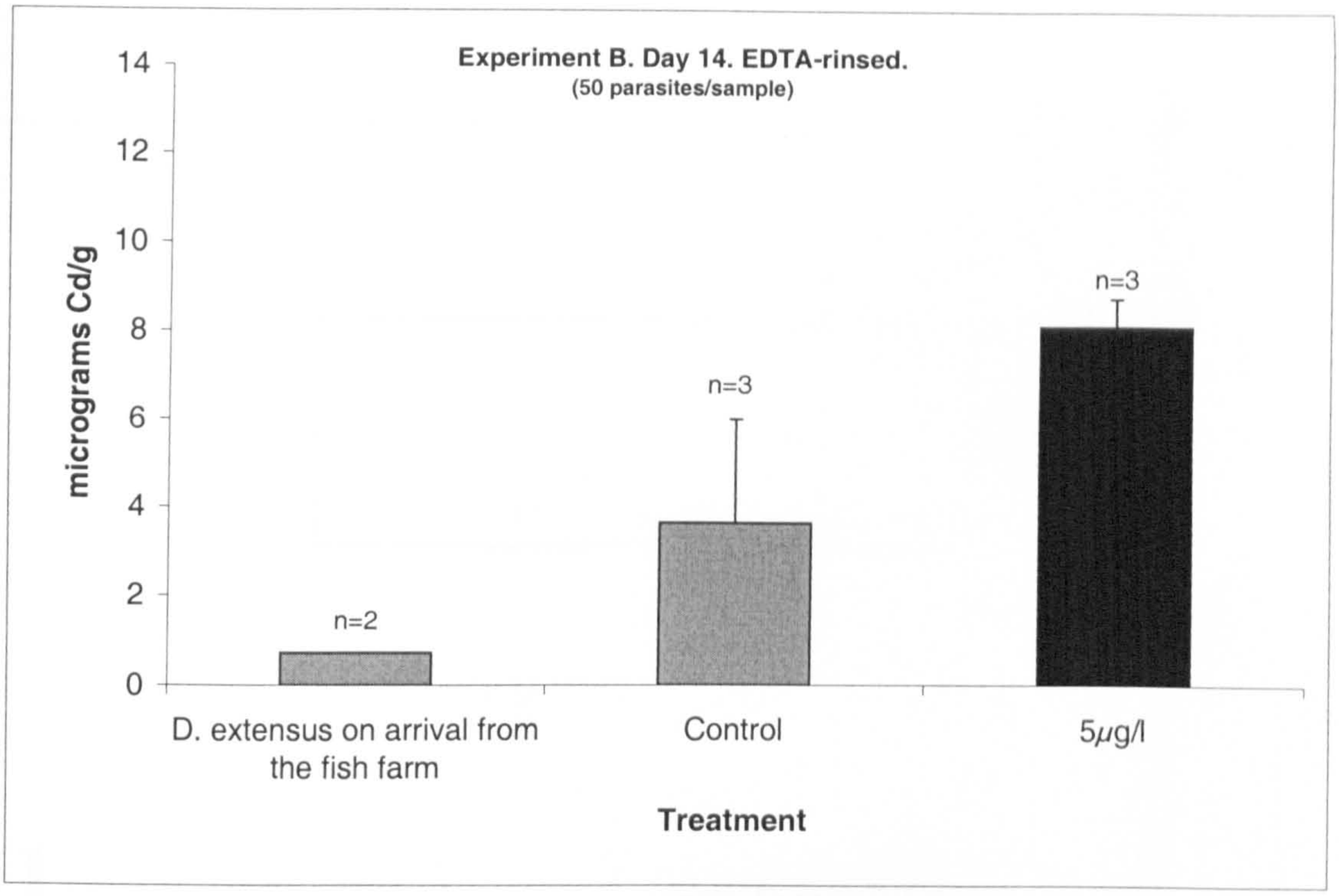


Fig. 5.4 The concentration of cadmium (µg/g dry wt.) (± S. E.) in EDTA-rinsed *D. extensus* (50/sample) after 14 days exposure to 5µg/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 B.

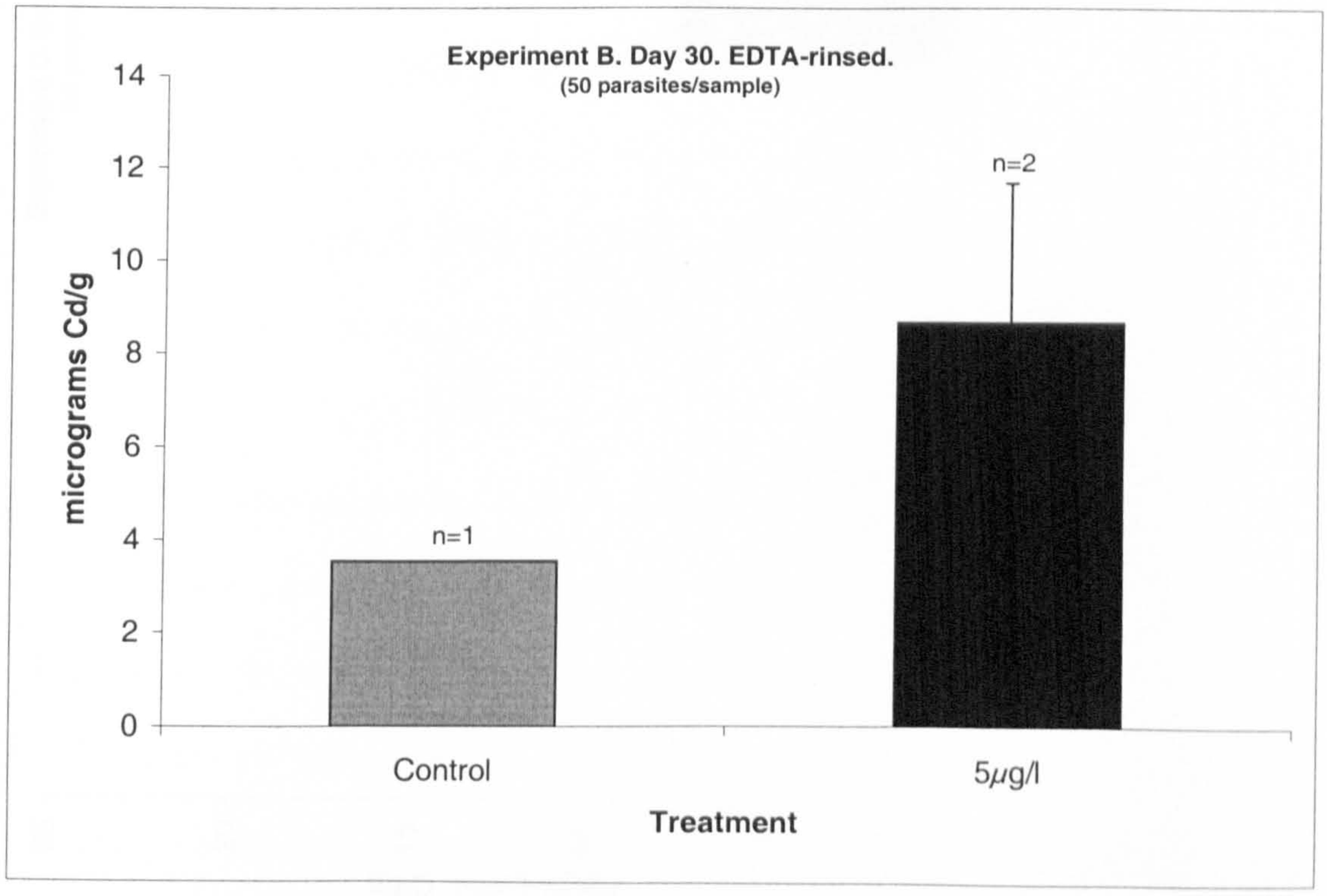


Fig. 5.5 The concentration of cadmium (µg/g dry wt.) (± S.E.) in EDTA-rinsed *D. extensus* (50/sample) after 30 days exposure to 5µg/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 B.

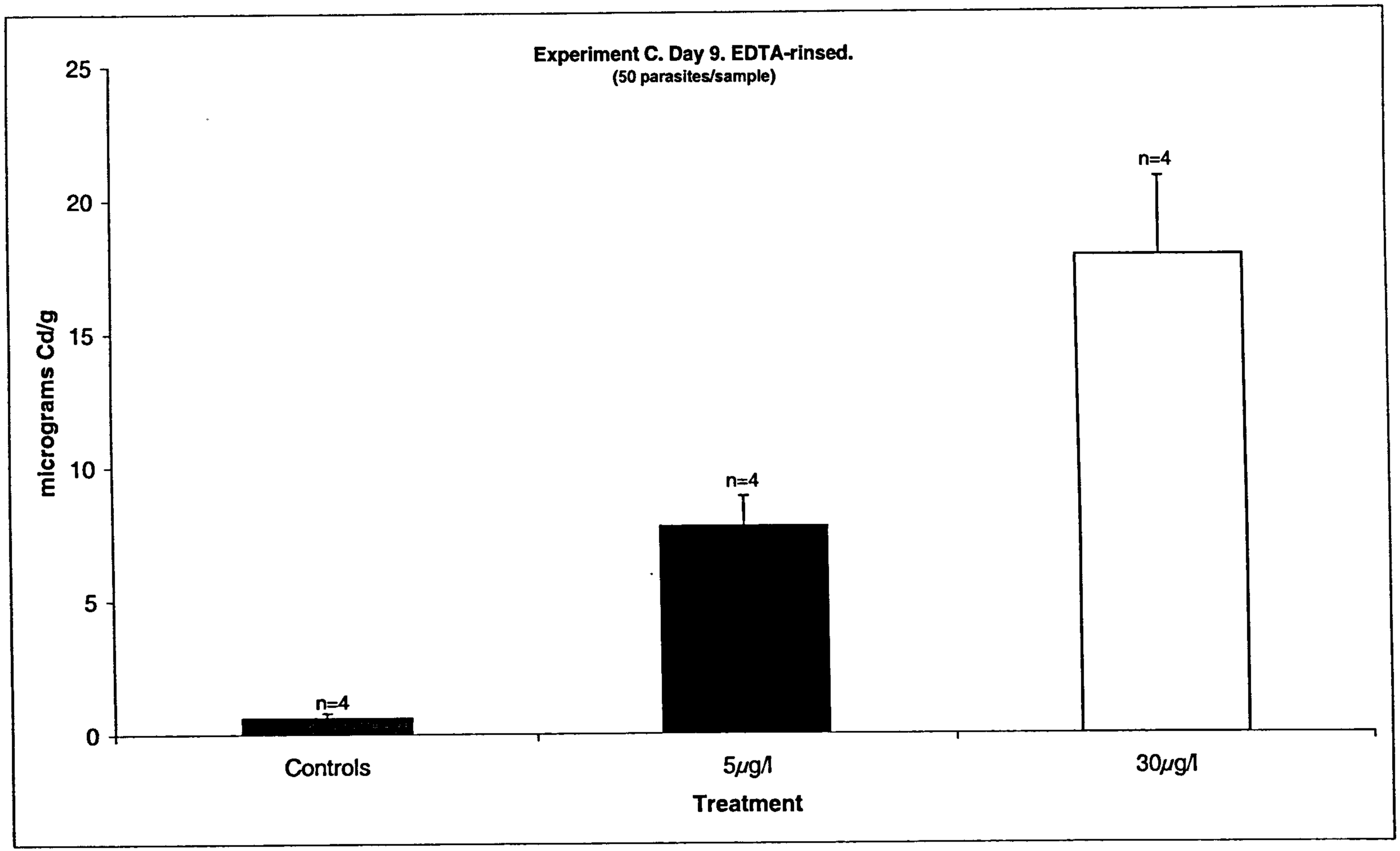


Fig. 5.6 The concentration of cadmium (*µg*/g dry wt.) (± S.E.) in EDTA-rinsed *D. extensus* (50/sample) after 9 days exposure to 5*µg*/l and 30*µg*/l cadmium. n = number of samples. For source of *D. extensus* see section 5.2.1.1 C.

Some *D. extensus*, from carp destined for use in Experiment B, were analysed directly on their arrival from the fish farm and were found to have a lower concentration of cadmium than the control parasites analysed after 14 days exposure to experimental conditions (0.72 and 3.66μg/g, respectively) (Fig. 5.4). Over time, the concentration of cadmium in both the control and 5μg/l cadmium *D. extensus* remained relatively constant, with control parasites having 3.66 and 3.55μg/g cadmium and parasites exposed to 5μg/l cadmium having 8.10 and 8.67μg/g at days 14 and 30, respectively (Figs 5.4-5.5). The differences in cadmium concentration in *D. extensus* from Experiment B, were not statistically different over time in either population ($P = 0.088$).

In Experiment C, *D. extensus* were exposed to both 5μg/l and 30μg/l cadmium and the cadmium burdens of the parasites from the 2 different concentrations are shown in Figure 5.6. The concentration of cadmium accumulated by *D. extensus* increased with exposure to increasing concentrations of cadmium, resulting in a cadmium burden in the 30μg/l cadmium-exposed parasites (17.91μg/g) that was statistically higher than that of the controls (0.6μg/g) ($P = 0.0002$).

5.3.3. The elemental profile of *D. extensus* as determined by ICP-MS

Samples of control, 5 and 30μg/l cadmium-exposed *D. extensus* were analysed by ICP-MS and the concentrations (μg/g) of the specific elements investigated during the analysis are presented in Table 5.2. In addition to the elements found in *D. extensus* by the analysis at The Natural History Museum, a more comprehensive elemental profile carried out by Elemental Research Inc., Vancouver revealed the

presence of a range of other elements, which are listed in Table 5.3. The concentrations of these elements are not provided, as only one sample of control and 5μg/l cadmium-exposed parasites was sent to this Vancouver laboratory for analysis.

From Table 5.2 it can be seen that in 11 of the 17 elements detected (barium; cadmium; cobalt; gallium; manganese; nickel; selenium; tin; titanium; uranium and zinc) in *D. extensus*, the concentration of each element was greatest in the parasites that had been exposed to 30μg/l cadmium. Of these elements, the concentration of cadmium (Cd) in the 30μg/l cadmium-exposed *D. extensus* (16.46 ± 2.3μg/g) was statistically greater than that in the *D. extensus* exposed to 5μg/l (5.46 ± 0.7μg/g) (P = 0.03). The cadmium concentration of the control *D. extensus* was below the detection limits of the ICP-MS machine, *i.e.* below 0.5μg/g, and thus they could not be incorporated in to the statistical analysis. However, given that the control values were undetectable and the cadmium concentration of the 5μg/l cadmium-exposed parasites was 5.5μg/g, one could assume that the values are statistically significant. An overall statistically significant difference in the concentration of titanium (Ti) between treatments (P = 0.04) was also observed. Concentrations of lead (Pb), rubidium (Rb), molybdenum (Mo) and vanadium (V) were greatest in *D. extensus* that had been exposed to 5μg/l cadmium, however; the differences were not statistically significant.

Table 5.2. The concentrations of elements in *D. extensus* after exposure to 0, 5 and 30 μg/l cadmium as determined by ICP-MS at The Natural History Museum, London. Four samples (50 parasites each) were analysed for each element. The number in parentheses represents the number of samples for each element that were above the detection limit of the ICP-MS; unless otherwise stated n=4.

Element	Element concentration in *D. extensus* (μg/g)		
	Control	5μg/l cadmium	30μg/l cadmium
Barium	4.83 ± 2.5	7.80 ± 2.20	10.70 ± 6.80
Cadmium*	Nd	5.46 ± 0.70	16.46 ± 2.30
Cobalt	8.53 ± 0.00 (n=1)	6.68 ± 6.30 (n=3)	17.68 ± 15.7 (n=3)
Gallium	0.18 ± 0.09	0.29 ± 0.07	0.88 ± 0.40
Lead	0.58 ± 0.18 (n=3)	6.28 ± 2.96 (n=3)	1.72 ± 0.89
Manganese	8.87 ± 0.08 (n=2)	3.73 ± 2.00	40.20 ± 28.7
Molybdenum	0.12 ± 0.00 (n=1)	10.2 ± 0.00 (n=1)	Nd
Nickel	40.60 ± 5.80 (n=2)	28.81 ± 25.70 (n=2)	41.10 ± 22.0 (n=3)
Rubidium	6.38 ± 0.50	8.70 ± 0.70	7.63 ± 0.70
Selenium	1.72 ± 0.90 (n=3)	1.96 ± 0.30 (n=3)	2.47 ± 1.00 (n=3)
Strontium	2.68 ± 1.60	2.39 ± 0.30	2.35 ± 0.70
Tin	0.85 ± 0.50 (n=3)	0.88 ± 0.30 (n=3)	0.99 ± 0.00 (n=1)
Titanium*	16.69 ± 5.00	14.58 ± 7.40	47.60 ± 8.8
Uranium	0.49 ± 0.50 (n=2)	0.08 ± 0.06 (n=3)	0.48 ± 0.46 (n=3)
Vanadium	0.08 ± 0.02 (n=2)	7.63 ± 7.60 (n=3)	0.54 ± 0.40 (n=3)
Zinc	1598.11 ± 215.0	2536.62 ± 778.0	2872.12 ± 505.0

* Denotes elements where statistical differences between treatments occurred. Nd = below the detection limits of the ICP-MS.

Table 5.3. Elements found in control and 5μg/l cadmium-exposed *D. extensus* as determined by ICP-MS (in addition to those found by The NHM) at Elemental Research Inc., Vancouver, Canada. No values are expressed as only one sample (50 parasites) of each treatment was analysed.

Aluminium	Chromium	Mercury	Sodium
Antimony	Cobalt	Palladium	Thorium
Arsenic	Copper	Phosphorous	Tungsten
Beryllium	Germanium	Platinum	
Bismuth	Gold	Potassium	
Boron	Iron	Rhodium	
Caesium	Lanthanum	Scandium	
Calcium	Lithium	Silicon	
Cerium	Magnesium	Silver	

5.3.4. Accumulation of cadmium by common carp

As a summary, in Experiment A the carp were exposed to 5μg/l cadmium only and the cadmium concentration of carp gills and blood was determined at days 9 and 29/31 and the cadmium concentration of kidney determined at day 29/31 only. In Experiment B, the carp were again exposed to 5μg/l cadmium but gill, kidney and muscle samples were taken at 9, 14 and 29 days. Only gill samples were taken from carp in Experiment C but they had been exposed to both 5 and 30μg/l cadmium. The carp in Experiment D had been exposed to 5 and 50μg/l cadmium and samples of gill, liver, muscle and spleen were taken at 9, 21 and 29 days of the trial. Kidney samples were only obtained at day 9. Because of these differences, all the trials are dealt with separately below.

Also, due to the different tissues of carp accumulating cadmium to very different concentrations, when comparing the individual graphs for each experiment, it should be noted that the graphs are plotted on different scales. This was necessary to enable the very low concentrations of cadmium that were accumulated in some tissues to be visualised clearly.

Figure 5.7a-c shows the cadmium concentration in control and 5μg/l cadmium-exposed carp blood, gills and kidney at 9 and 29/31 days post-start of the trial (section 5.2.1.1 A). From Figure 5.7a it can be seen that at both days 9 and 29/31, blood taken from the 5μg/l cadmium-exposed carp had a greater concentration of cadmium than that of the control carp (day 9 = 1.59 and 1.05μg/g; day 29/31 = 0.06 and 0.60μg/g, respectively). Indeed, at the end of the trial (day 29/31) the cadmium

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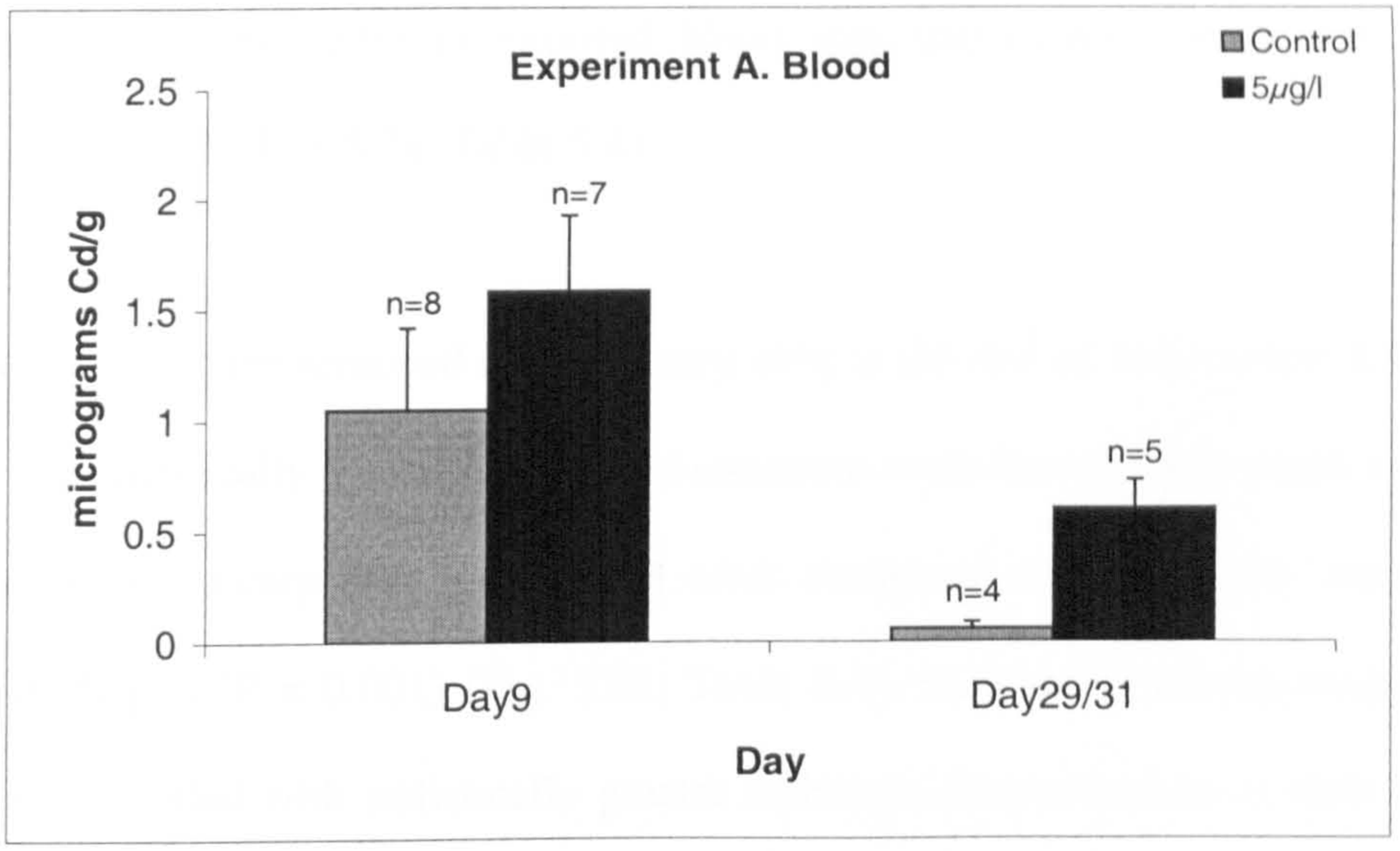

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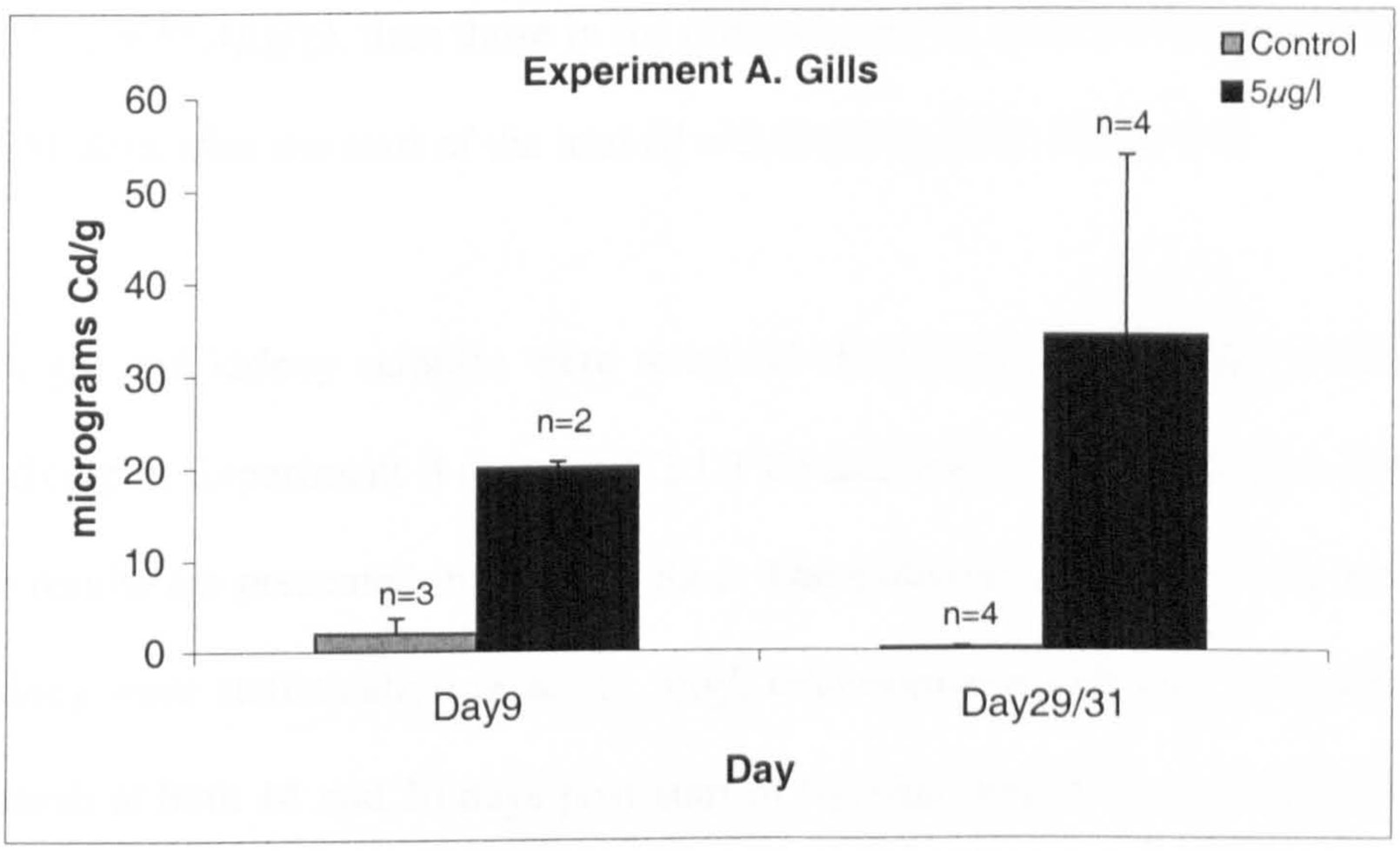

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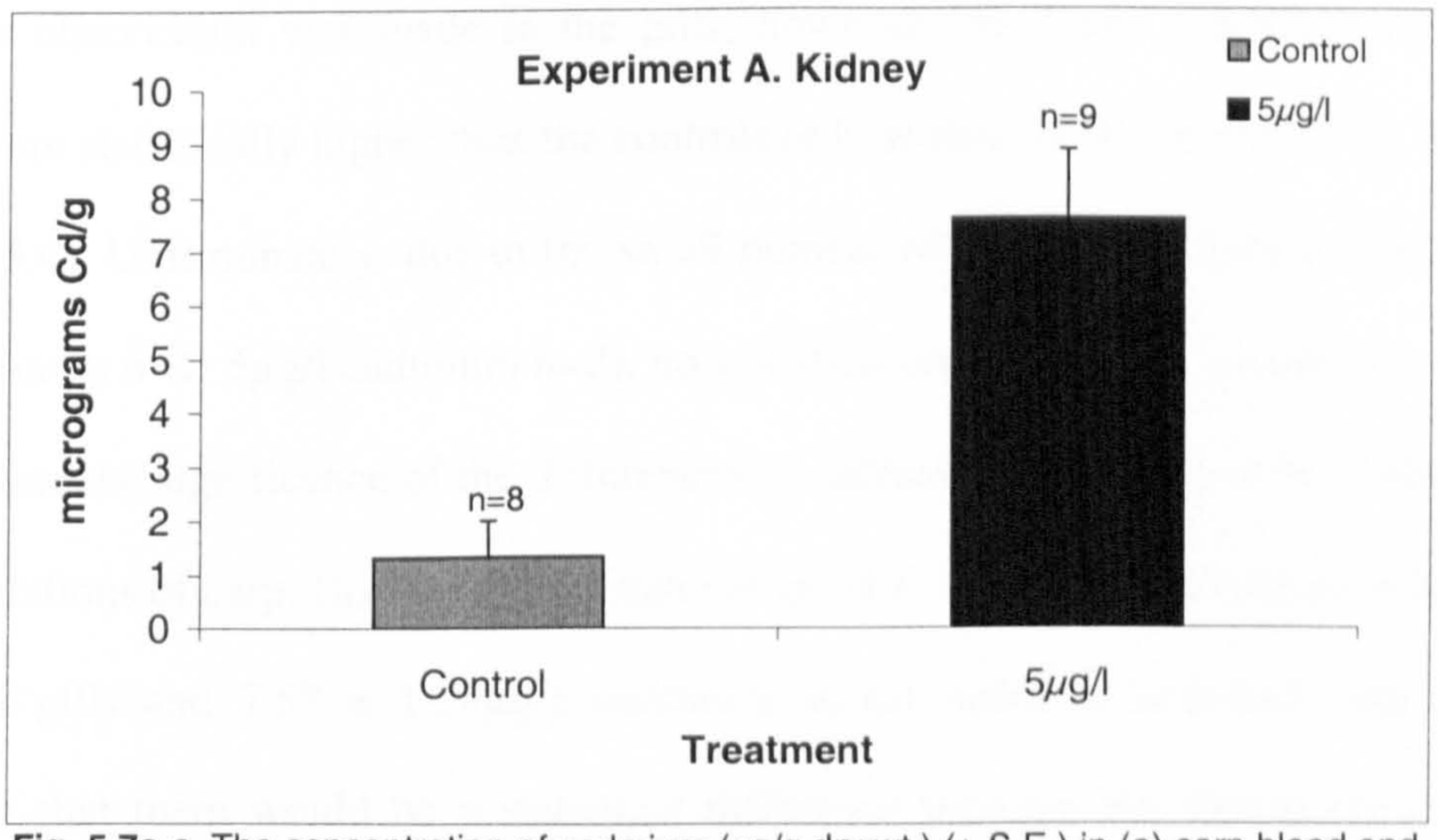


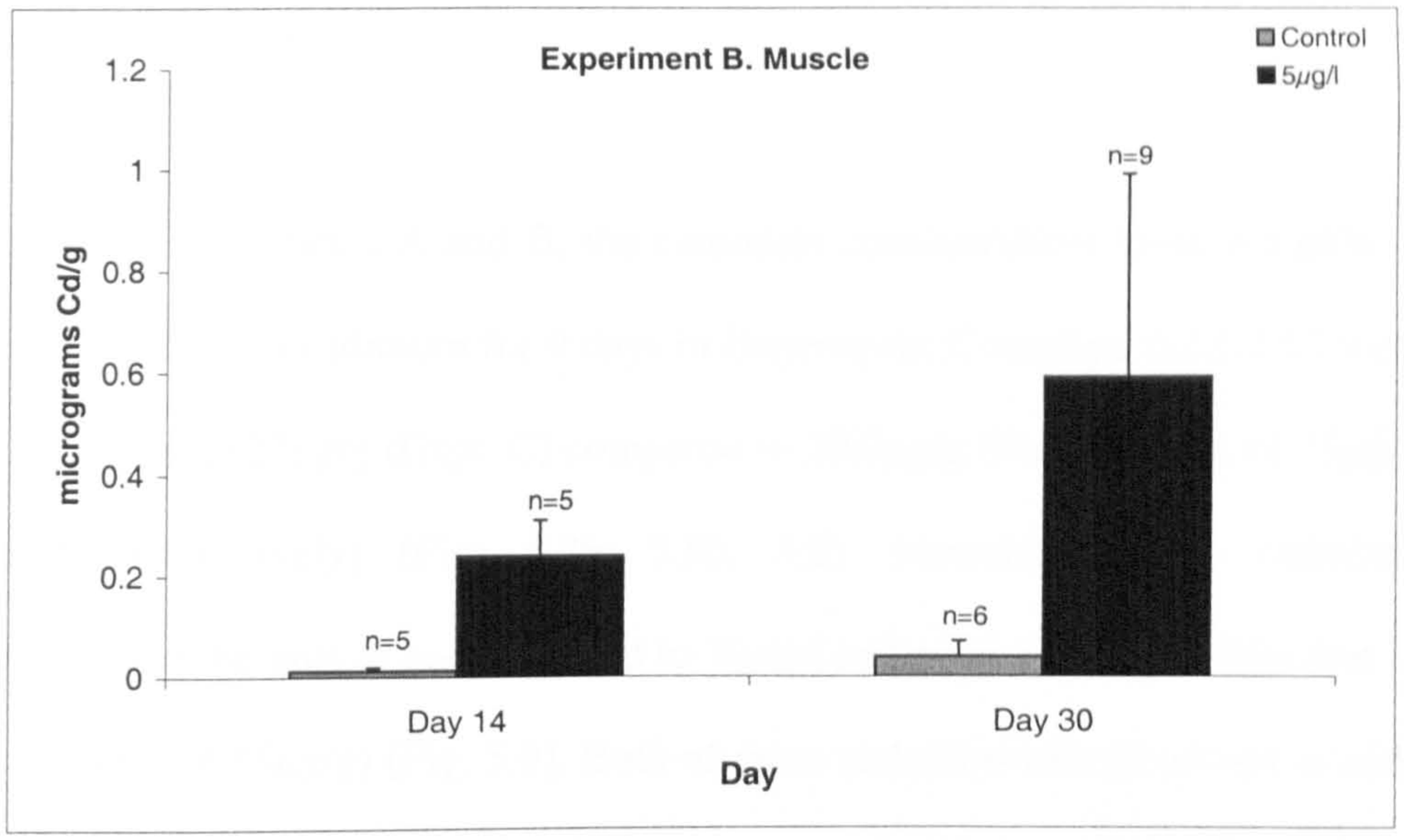
Fig. 5.7a-c. The concentration of cadmium (μg/g dry wt.) (± S.E.) in (a) carp blood and (b) gills at 9 and 29/31 days post-start of the trial and in (c) kidney after 29/31 days only. (n = number of samples). For source of carp see section 5.2.1.1 A.

concentration of the cadmium-exposed blood was statistically greater than the controls (P = 0.02) (Fig. 5.7a; Table 5.4).

Kidney samples were removed from the carp only at the end of Experiment A (day 29/31) and statistically greater amounts of cadmium were found in this organ in the cadmium-exposed carp (7.6 ± 0.66μg/g) when compared to those in the controls (1.3 ± 0.76μg/g) (P = 0.001) (Fig. 5.7c; Table 5.4). Similarly, cadmium-exposed carp were recorded with statistically greater cadmium concentrations in their gills (day 9, 34.1 ± 19.4μg/g), than those in the controls (day 9, 0.33 ± 0.1μg/g), at both 9 and 29/31 days after the start of the trial (P = 0.03) (Fig. 5.7c; Table 5.4).

Muscle, gill and kidney samples were removed from control and 5μg/l cadmium-exposed carp in Experiment B (section 5.2.1.1 B) and analysed for cadmium content and the results are presented in Figure 5.8a-c. The cadmium content of carp muscle and kidney were statistically greater in 5μg/l cadmium-exposed carp compared to the controls at both 14 and 30 days post-start of the trial (Fig. 5.8a, c; Table 5.4). A similar observation was made in the gills, however, the 5μg/l cadmium-exposed gills were statistically higher than the controls only at Day 14 (P = 0.009) (Fig. 5.8b; Table 5.4). Unfortunately, due to the small number of gill samples analysed at day 30 (controls n=1; 5μg/l cadmium n=2), no statistical analysis could be carried out to determine the significance of the differences in cadmium concentration between the 2 populations of carp. However, the mean values of 1.32 ± 0.66μg/g cadmium in the control gills and 7.57 ± 1.28μg/g cadmium in the cadmium-exposed carp gills suggest that there would be a statistical difference between the treatments if the

a)



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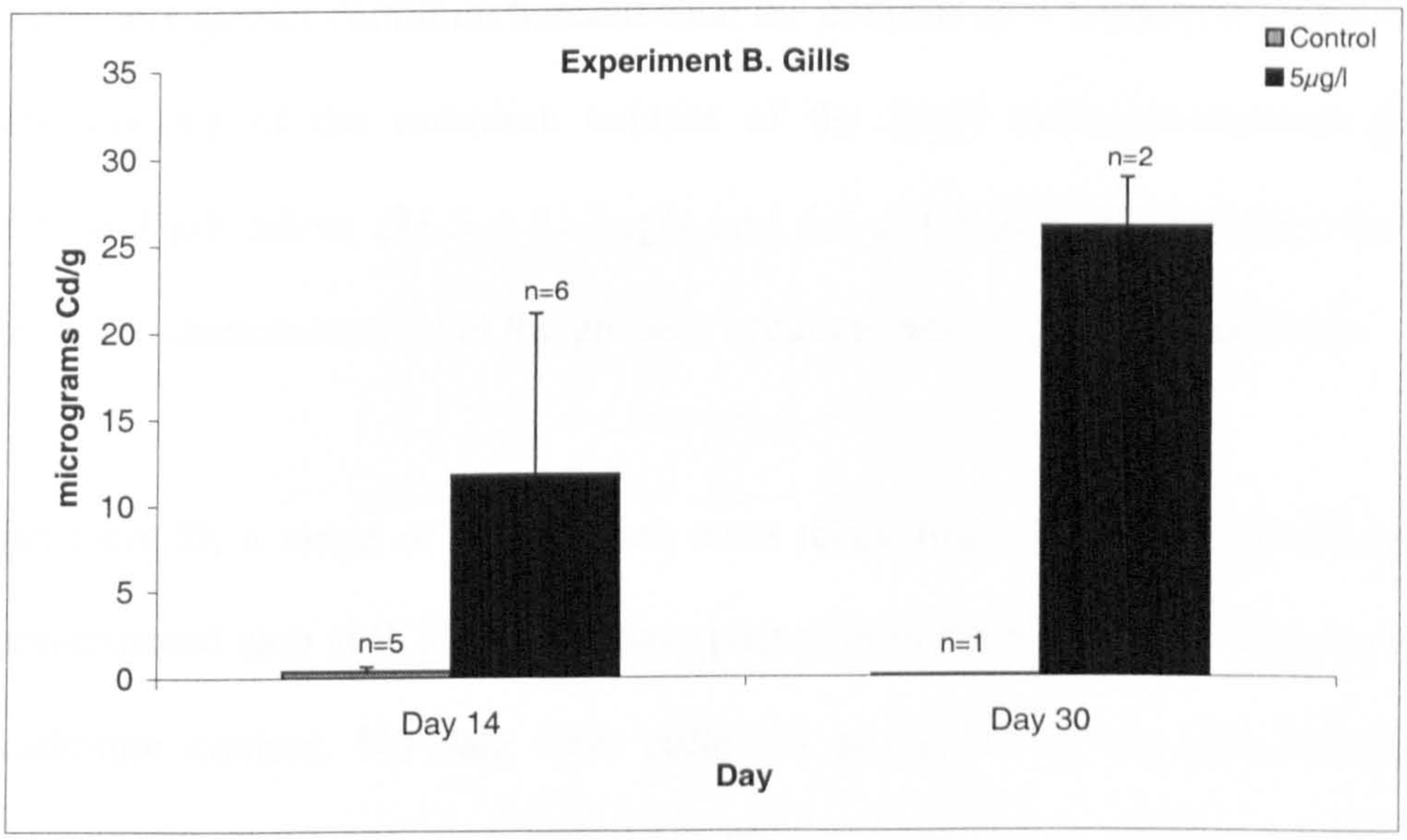

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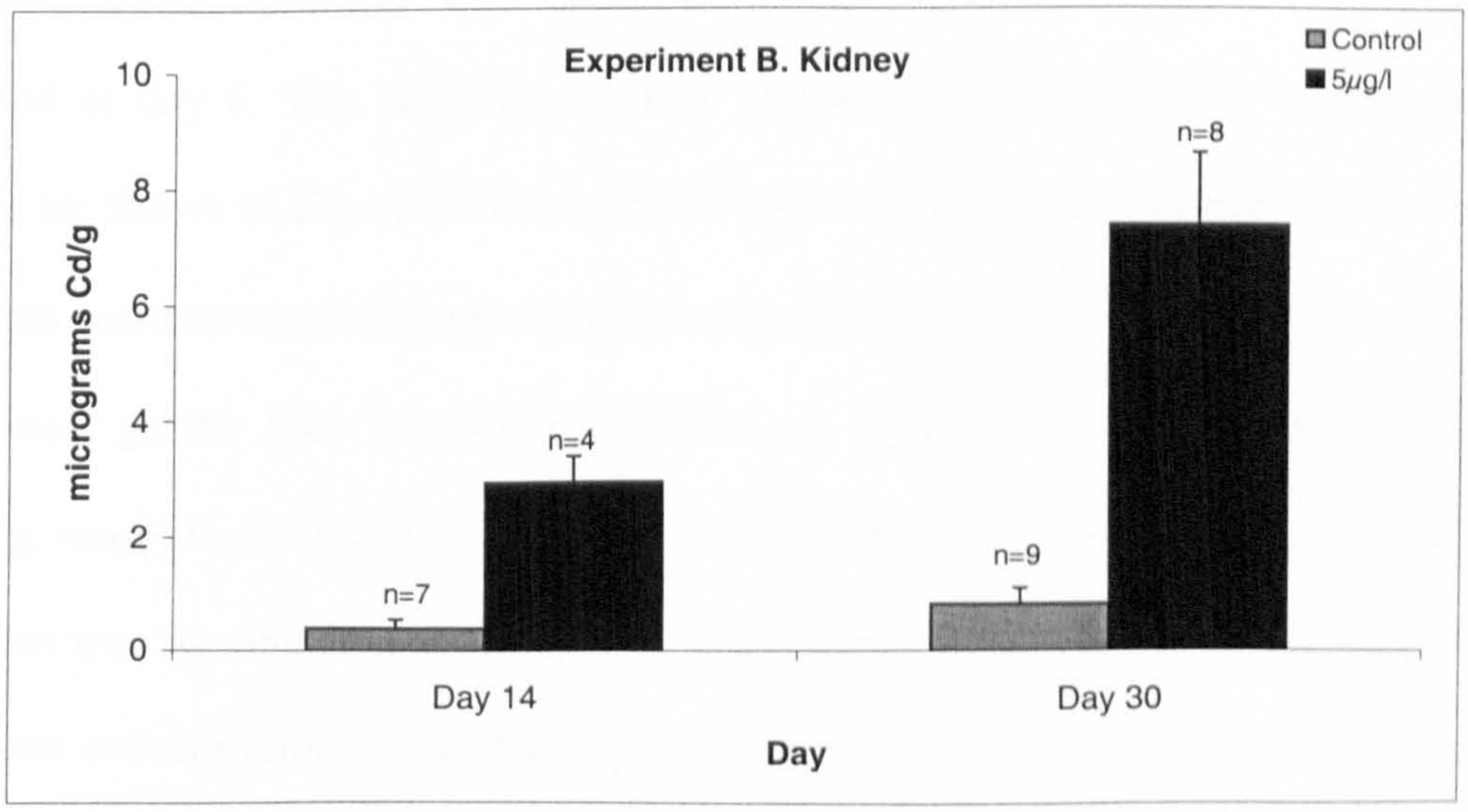


Fig. 5.8a-c. The concentration of cadmium (μg/g dry wt.) (± S.E.) in (a) carp muscle, (b) gills and (c) kidney after 14 and 30 days exposure to 5μg/l cadmium. (n = number of samples). For the source of carp see section 5.2.1.1 B.

sample size was greater.

Compared to Experiments A and B, the cadmium concentrations from the gills of carp exposed to 5μg/l cadmium for 9 days in Experiment C (section 5.2.1.1 C) were particularly high [122μg/g (Expt. C) compared to 20.0μg/g (Expt. A) and 11.75μg/g (Expt. B), respectively] (Figs 5.7b, 5.8b, 5.9). Interestingly, the cadmium concentration in the gills of carp exposed to 30μg/l cadmium was lower than that in the 5μg/l gills (34.95μg/g) (Fig. 5.9). Both of these cadmium-treated groups of carp had statistically greater cadmium burdens than the controls ($P = 0.0001$) (Table 5.4). Separate analysis of the cadmium content of the 5μg/l cadmium-exposed gill filaments and gill rakers (81.5 ± 21.7μg/g and 5.2 ± 1.1μg/g, respectively) from Experiment C, demonstrated that the greatest accumulation was in the filaments.

In Experiment D, a range of carp tissues were taken from control, 5 and 50μg/l cadmium-exposed carp at 9, 21 and 29 days post-start of the trial for the analysis of their cadmium content. No data were collected at day 14 of the trial for any treatment group and only data from the 50μg/l cadmium-exposed carp were collected at day 6. The cadmium content of the gills, liver, kidney, spleen and muscle are shown in Figure 5.10a-e. As has been recorded in Experiments A-C, the cadmium concentration of 5μg/l cadmium-exposed carp gills, at all time points, was statistically greater than that of the controls, *e.g.* at day 9, 31.8 ± 5.6 and 0.24 ± 0.1μg/g, respectively (Fig. 5.10a; Table 5.4). Interestingly, as seen in Experiment C, cadmium was accumulated to a greater concentration in the gills of carp exposed to the lower concentration of cadmium (5μg/l) compared to those exposed to 50μg/l

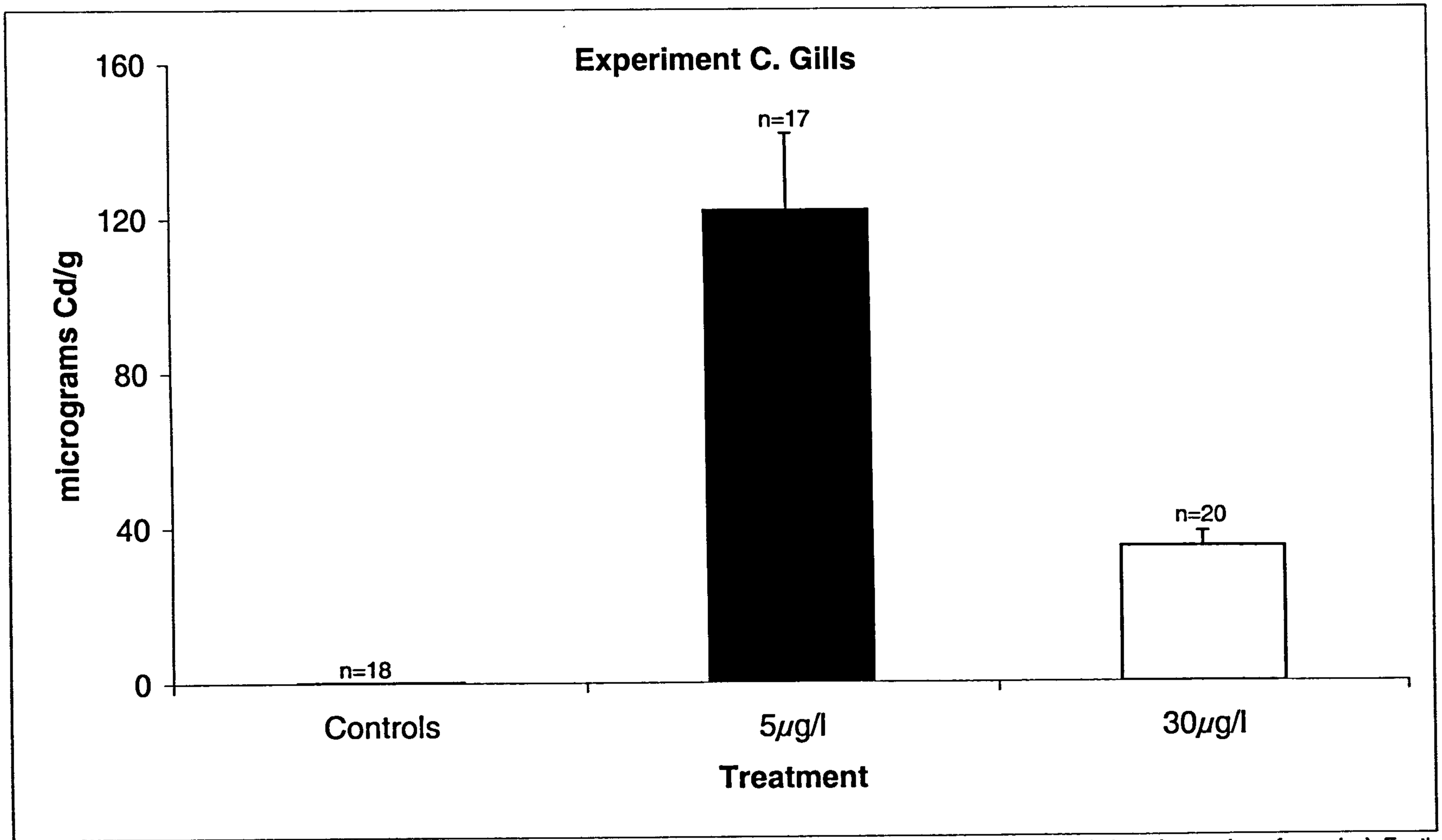


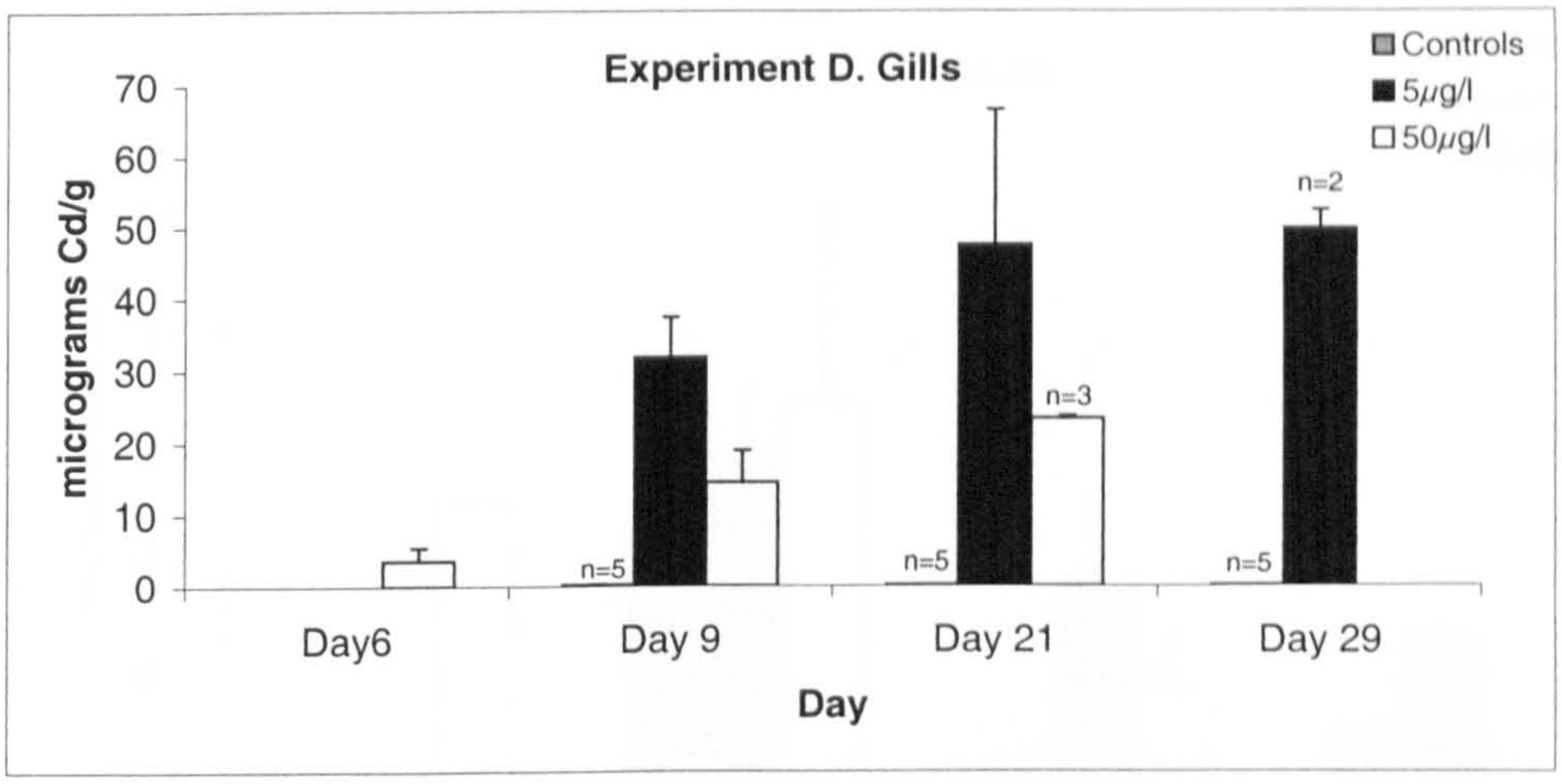
Fig. 5.9. The concentration of cadmium (μg/g dry wt.) (± S.E.) in carp gills after 9 days exposure to 5 and 30μg/l cadmium. (n=number of samples). For the source of carp see section 5.2.1.1 C.

cadmium, *e.g.* at day 21, 47.3 ± 19.1 and 23.2 ± 0.4μg/g, respectively (Fig. 5.10a). With increasing exposure duration, the concentration of cadmium in the gills of both cadmium-treated carp groups increased (Fig. 5.10a).

Figure 5.10b shows the cadmium concentration in carp kidney over time in all 3 treatments. At day 9, the concentration of cadmium in the kidneys of the carp was found to increase with exposure to increasing concentrations of cadmium. Comparing all samples from each treatment resulted in an overall significance value of P = 0.02, although a Dunn's multiple comparisons test did not highlight between which treatments the statistical difference lay (Table 5.4). Cadmium concentrations in control fish kidneys remained relatively constant over time (0.33-0.46μg/g), while in the 5μg/l group they increased from day 9 (2.0 ± 0.4μg/g) to day 21 (15.6 ± 9.8μg/g) before falling at day 29 post-start of the trial (8.2 ± 6.4μg/g) (Fig. 5.10c). There are no data for the cadmium concentrations in the kidneys of the 50μg/l cadmium-exposed carp at either day 21 or day 29 due to low numbers of carp remaining at day 21 and no carp remaining by day 29.

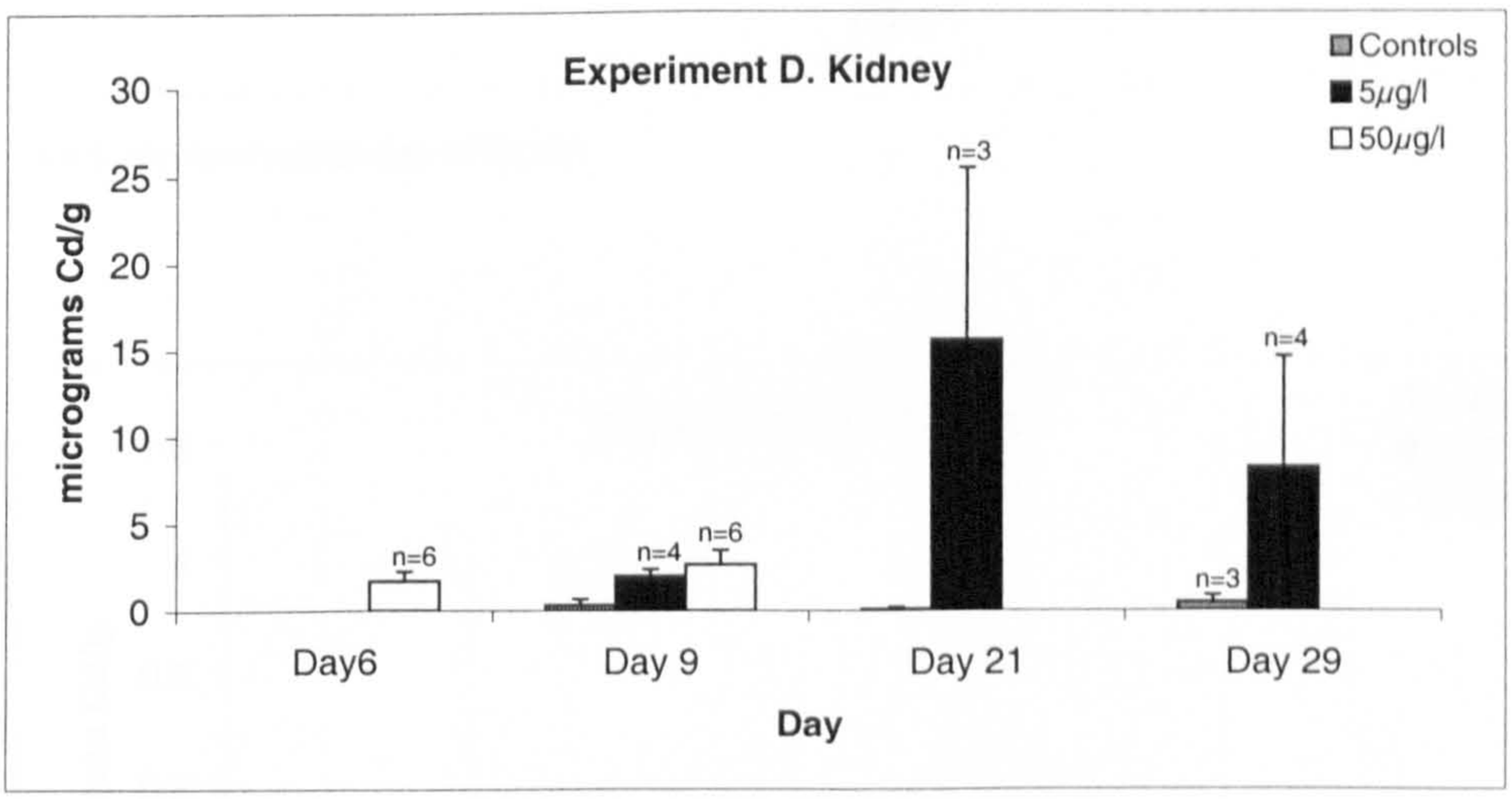
In both the control and 50μg/l cadmium-exposed fish, the concentration of cadmium in carp livers was seen to decrease with increasing exposure duration, 0.18 ± 0.07 to 0.05 ± 0.03μg/g between day 9 and 29 in the controls, and 2.4 ± 0.5 to 1.1 ± 0.1μg/g, between days 6 and 21 in the 50μg/l cadmium-exposed carp (Fig.5.10c) Conversely, the livers of 5μg/l cadmium-exposed fish accumulated cadmium over time (0.6 ± 0.3 at day 9 to 2.3 ± 0.03μg/g at day 29) (Fig. 5.10c). The greatest concentration of cadmium was recorded in the livers of the 50μg/l cadmium-

a)



n = 6 unless stated otherwise. No 50μg/l gill data for day 29.

b)



n = 5 unless stated otherwise. No 50μg/l kidney data from days 21 and 29.

c)

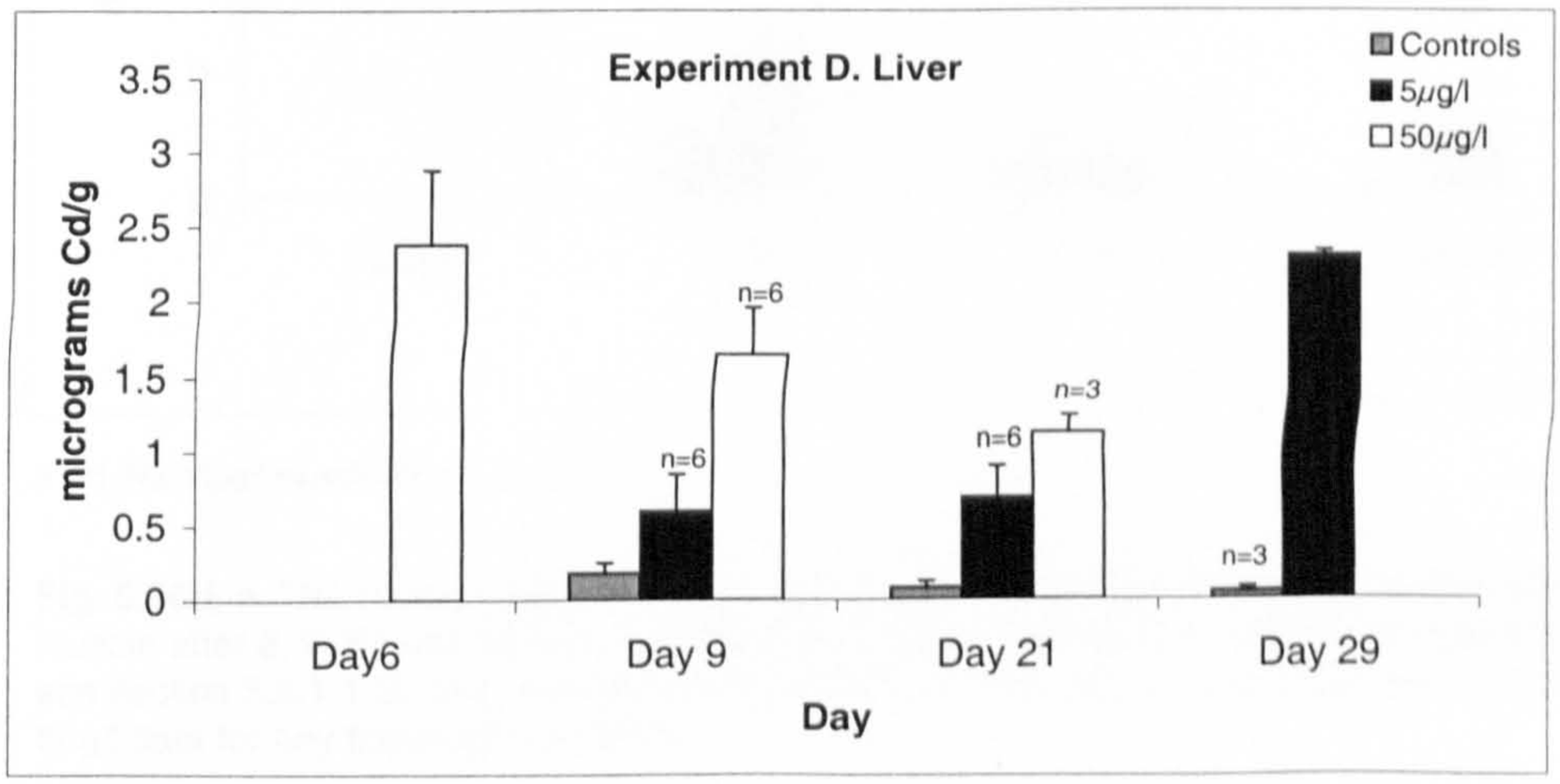
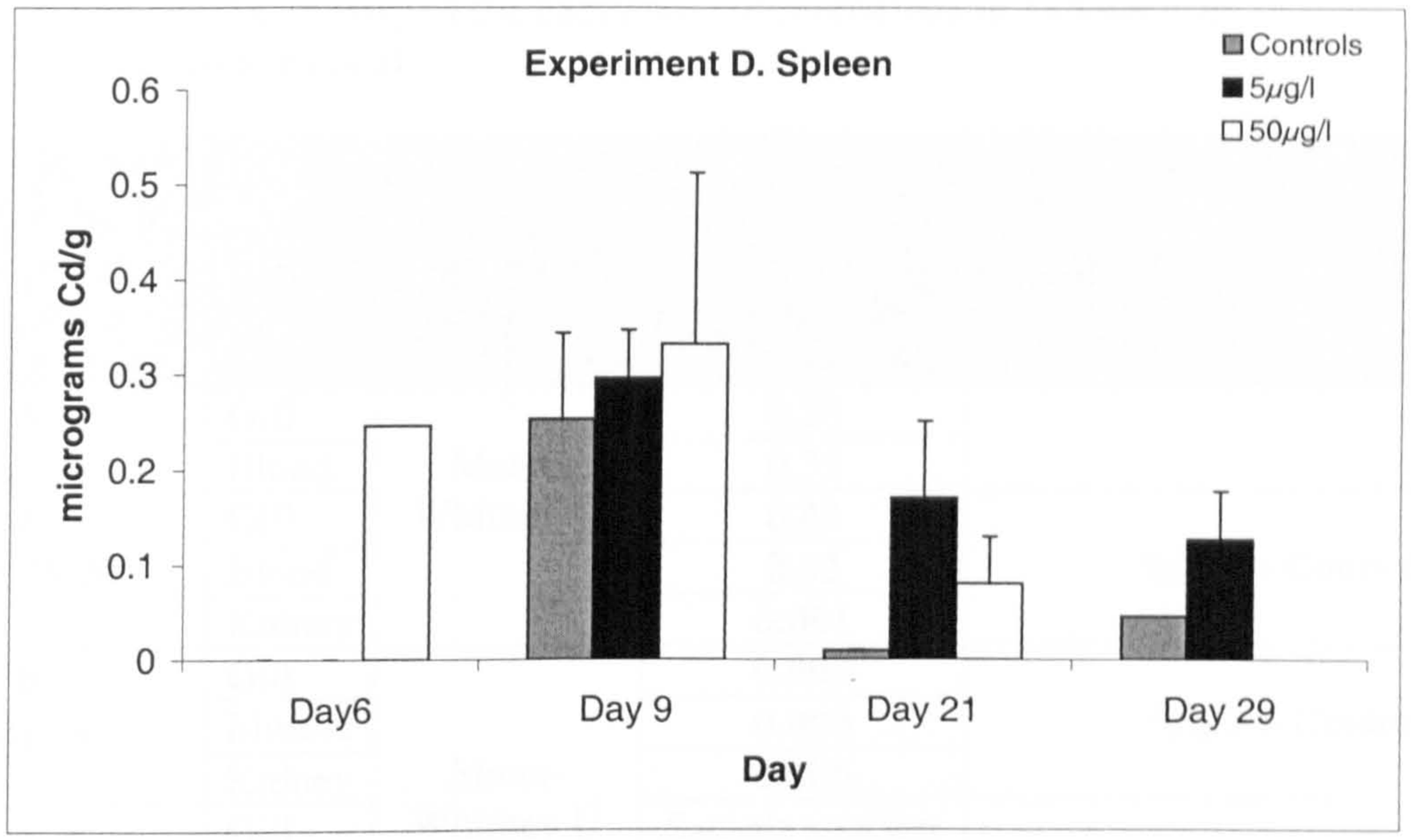


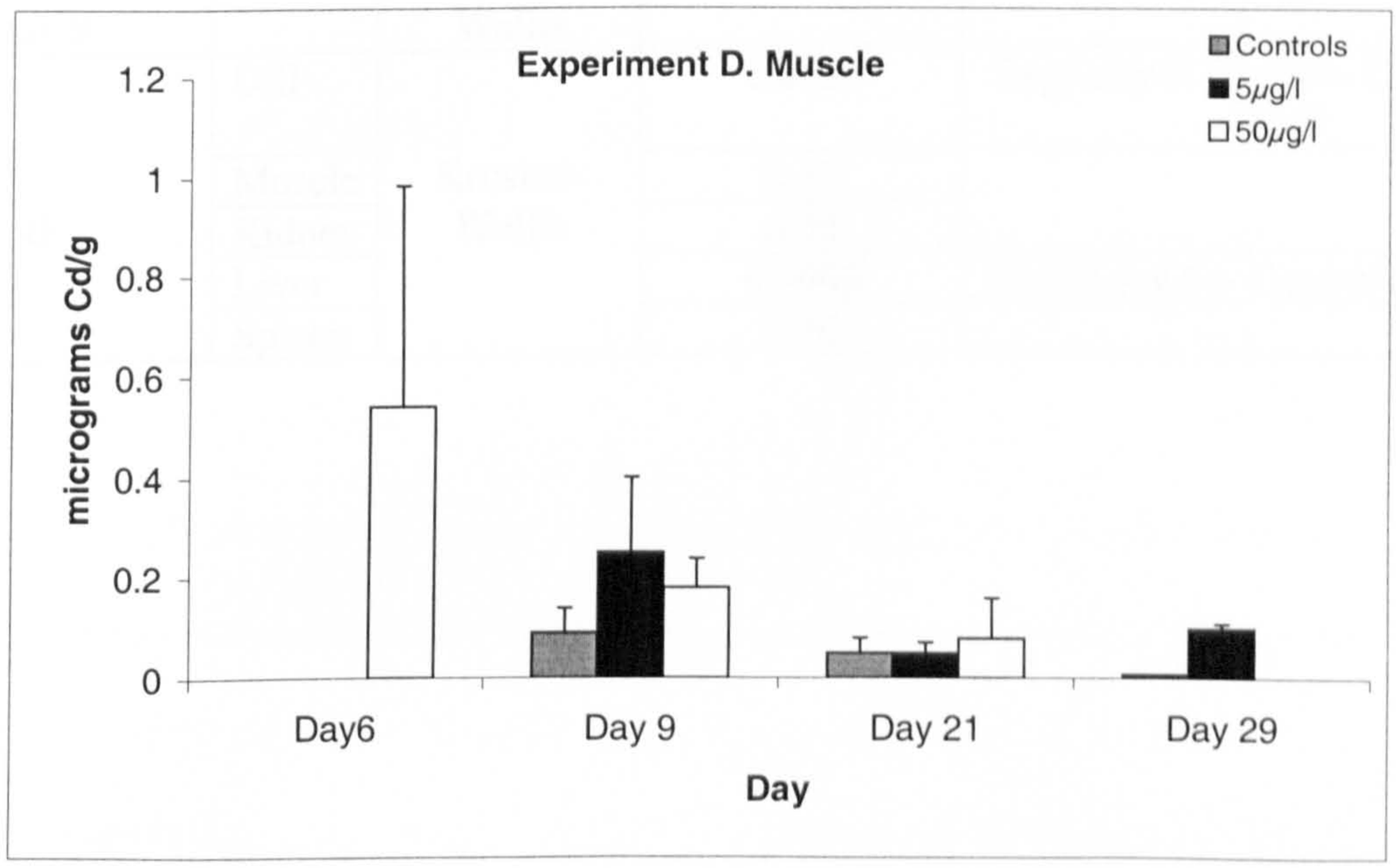
Fig. 5.10a-c. The concentration of cadmium (μg/g dry wt.) (± S.E) in carp (a) gills, (b) kidney and (c) liver after 6, 9, 21 and 29 days exposure to 5 and 50μg/l cadmium. Axes are on different scales for each tissue type. For the source of carp see section 5.2.1.1 D. There are no control or 5μg/l data for any tissue type at day 6.

d)



n = 5. No 50μg/l spleen data for day 29.

e)



n = 5. No 50μg/l muscle data for day 29.

Fig. 5.10d, e. The concentration of cadmium (μg/g dry wt.) (± S.E.) in carp (d) spleen and (e) muscle after 6, 9, 21 and 29 days exposure to 5 and 50μg/l cadmium. For the source of carp see section 5.2.1.1 D. Axes are on different scales for each tissue type. There are no control or 5μg/l data for any tissue type at day 6.

Table 5.4. Statistical summary of the cadmium concentrations in various carp tissues from Experiments A-D.

Experiment (letters refer to trials listed in section 5.2.1.1)	Tissue	Statistical test used	Statistical significance	Significant differences between
A Day 9	Gill	Mann-Whitney U	0.20	
	Blood		0.35	
A Day 29/31	Gill		**0.03**	5µg/l > Controls
	Blood		**0.02**	
	Kidney		**0.001**	
B Day 14	Gill	Mann-Whitney U	**0.009**	5µg/l > Control
	Muscle		**0.003**	
	Kidney		**0.006**	
B Day 29	Gill		Sample size too small	
	Muscle		**0.01**	5µg/l > Control
	Kidney		**<0.001**	
C Day 9	Gill	Kruskal-Wallis	**0.0001**	5µg/l and 30µg/l > Control
D	Gill	Kruskal-Wallis	**0.0001**	5µg/l day 9, 21, 29 > Control day 9, 29
	Muscle		0.37	
	Kidney		**0.02**	
	Liver		**0.0003**	50µg/l day 6 > Control day 21, 29
	Spleen		0.16	

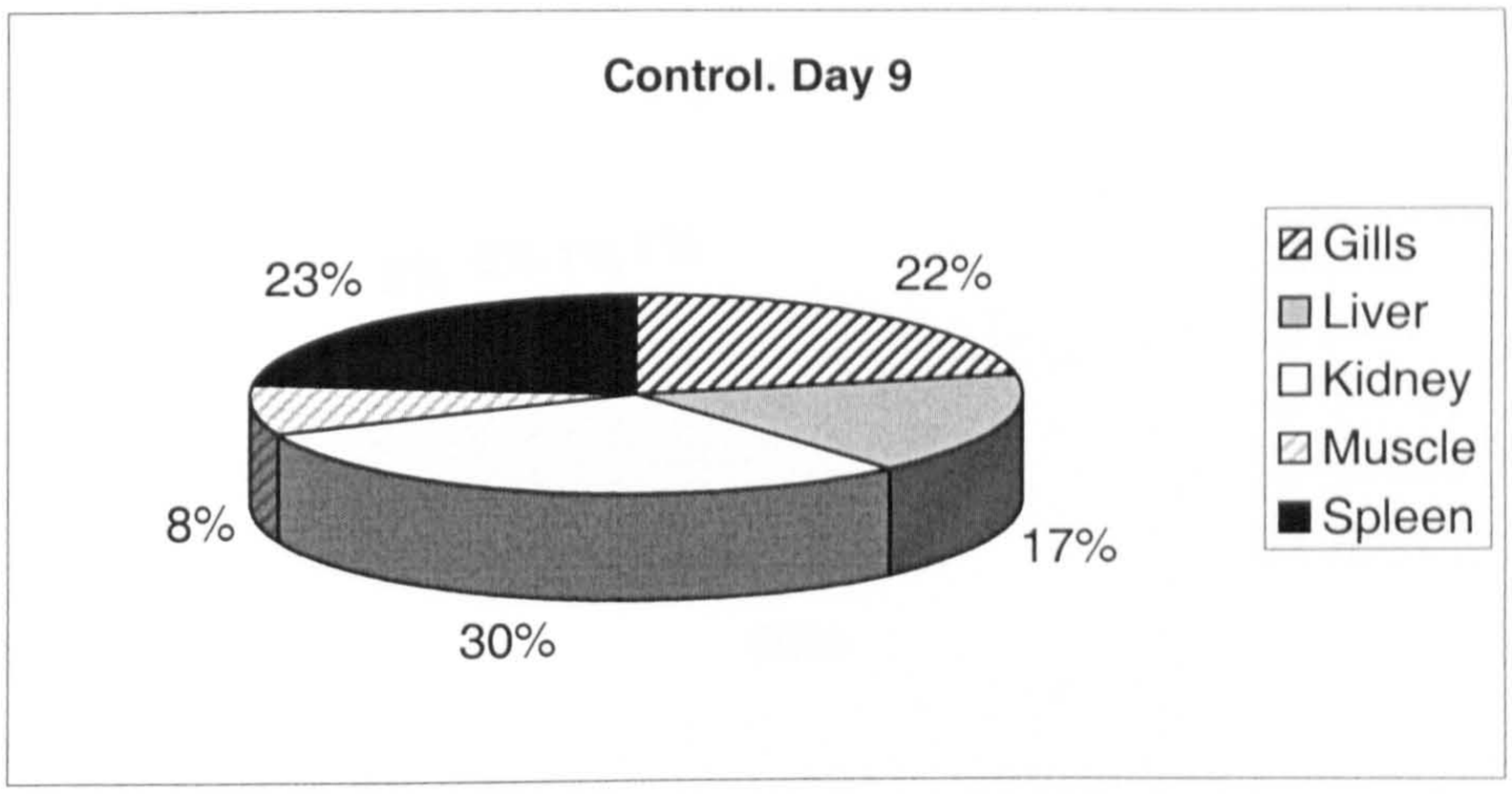
exposed carp at 6 days post-start of the trial.

The muscle and spleen of common carp in the Experiment D accumulated the least cadmium of all the tissues (Fig. 5.10d, e) and the concentration of cadmium in both these tissues did not differ significantly between treatments at any time point (Table 5.4). All 3 treatments saw a reduction in the amount of cadmium in the muscle and spleen as the experiment progressed (Fig. 5.10.d, e).

The relative shifts in cadmium concentration in the different organs can be summarised by looking at the proportion of cadmium in each tissue over time. Figures 5.11-5.13a-c show the proportion of cadmium in each tissue in the control, 5 and 50μg/l cadmium-exposed carp over time, respectively. It should be noted that, while the time series for the control and 5μg/l cadmium-exposed carp was day 9, 21 and 29, due to deteriorating health the carp exposed to 50μg/l were sampled at days 6, 9 and 21 and the piecharts for each treatment reflect these specific time series.

The sequence of cadmium accumulation in the control carp (from background levels and feed only) involved increases in the proportion of cadmium in the kidney over time (30-70% from day 9-29), coupled, in general, with a reduction in cadmium in all other tissues (Fig. 5.11). Interestingly, in the carp exposed to 5μg/l cadmium the greatest proportion of cadmium was located in the gills at all time points (>70%). However, liver and kidney cadmium burdens were found to increase over time while, as previously stated, the proportions in both the muscle and spleen decreased (Fig. 5.12). The 50μg/l cadmium-exposed carp were particularly stressed in the

a)



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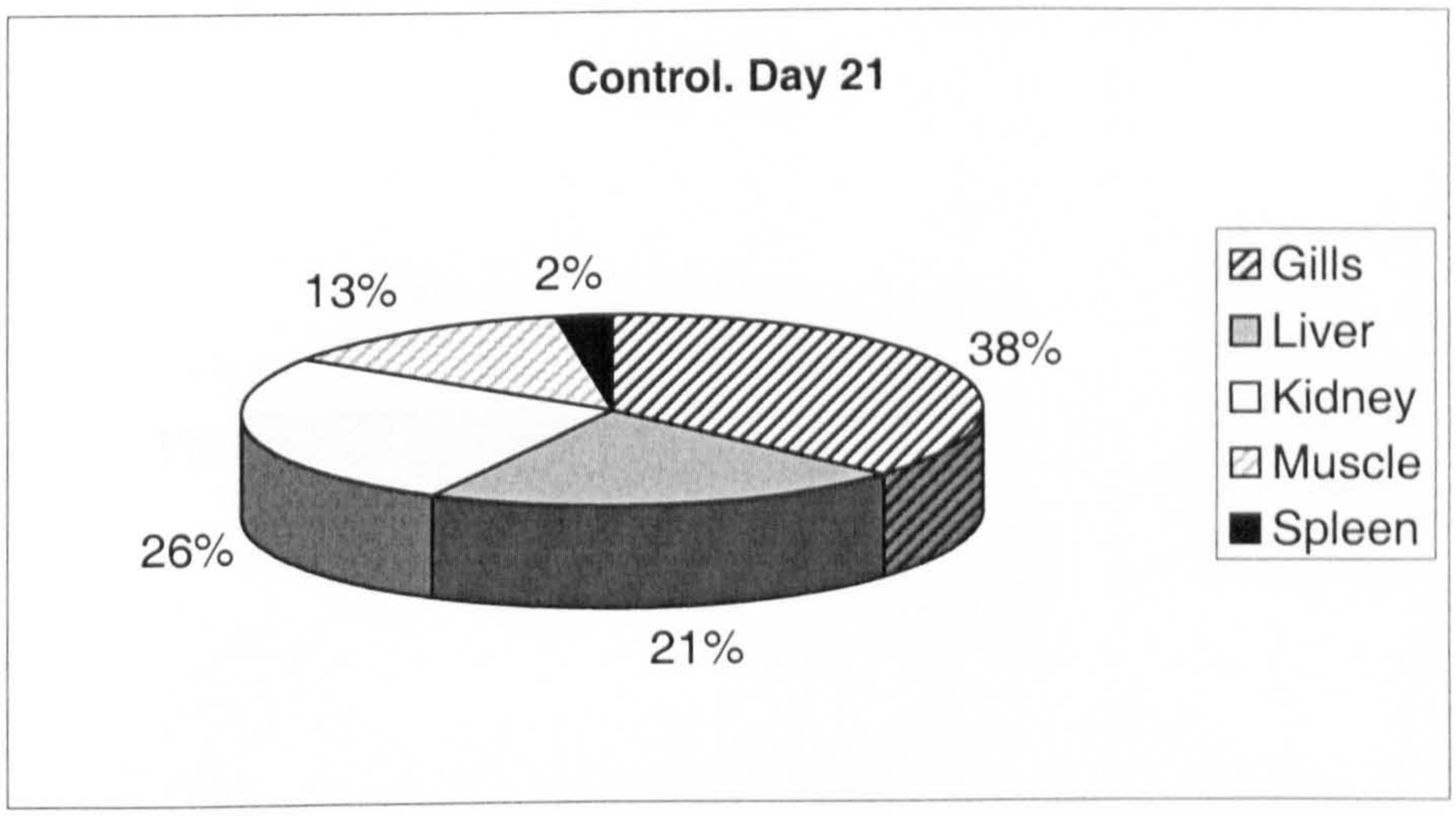

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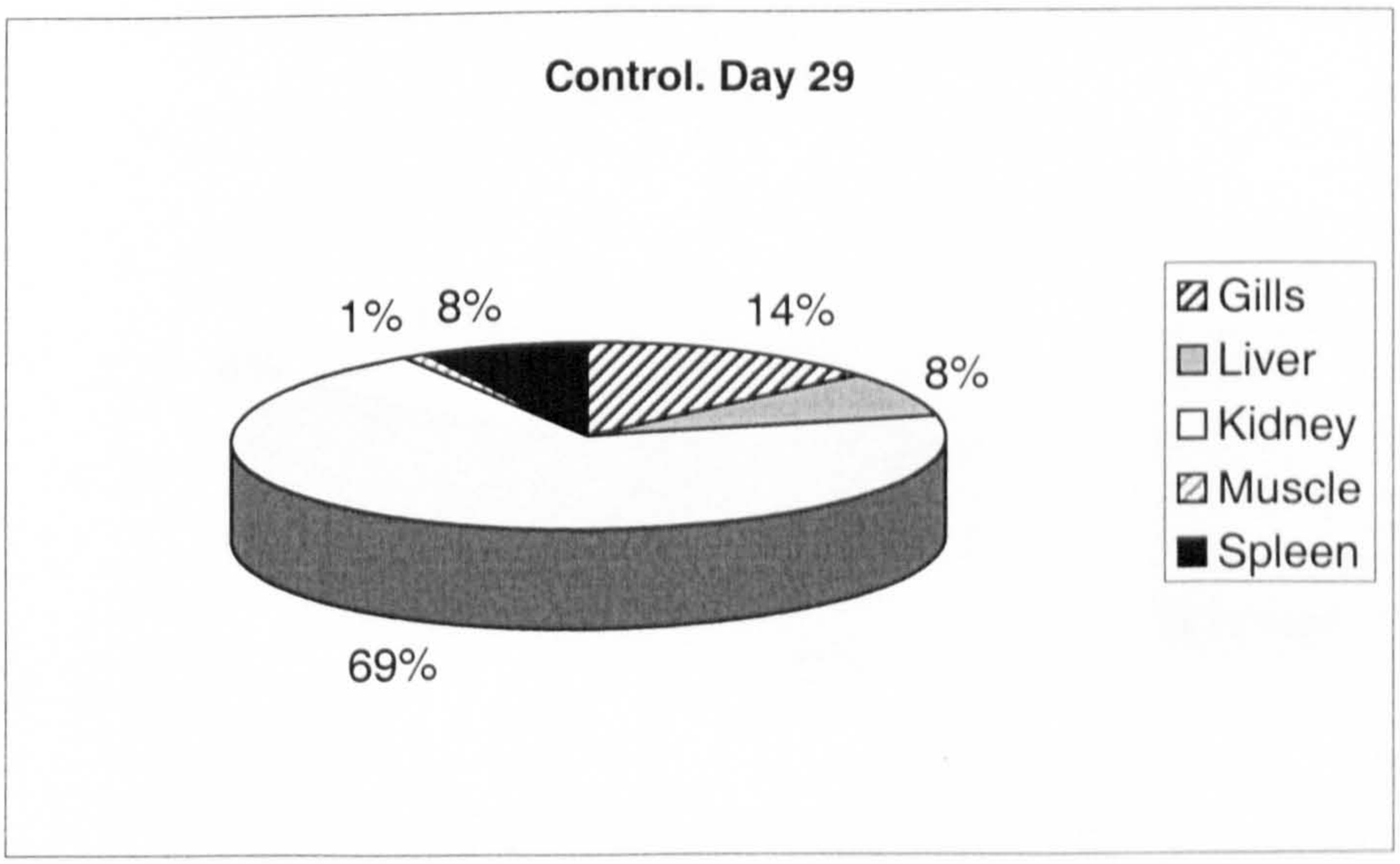
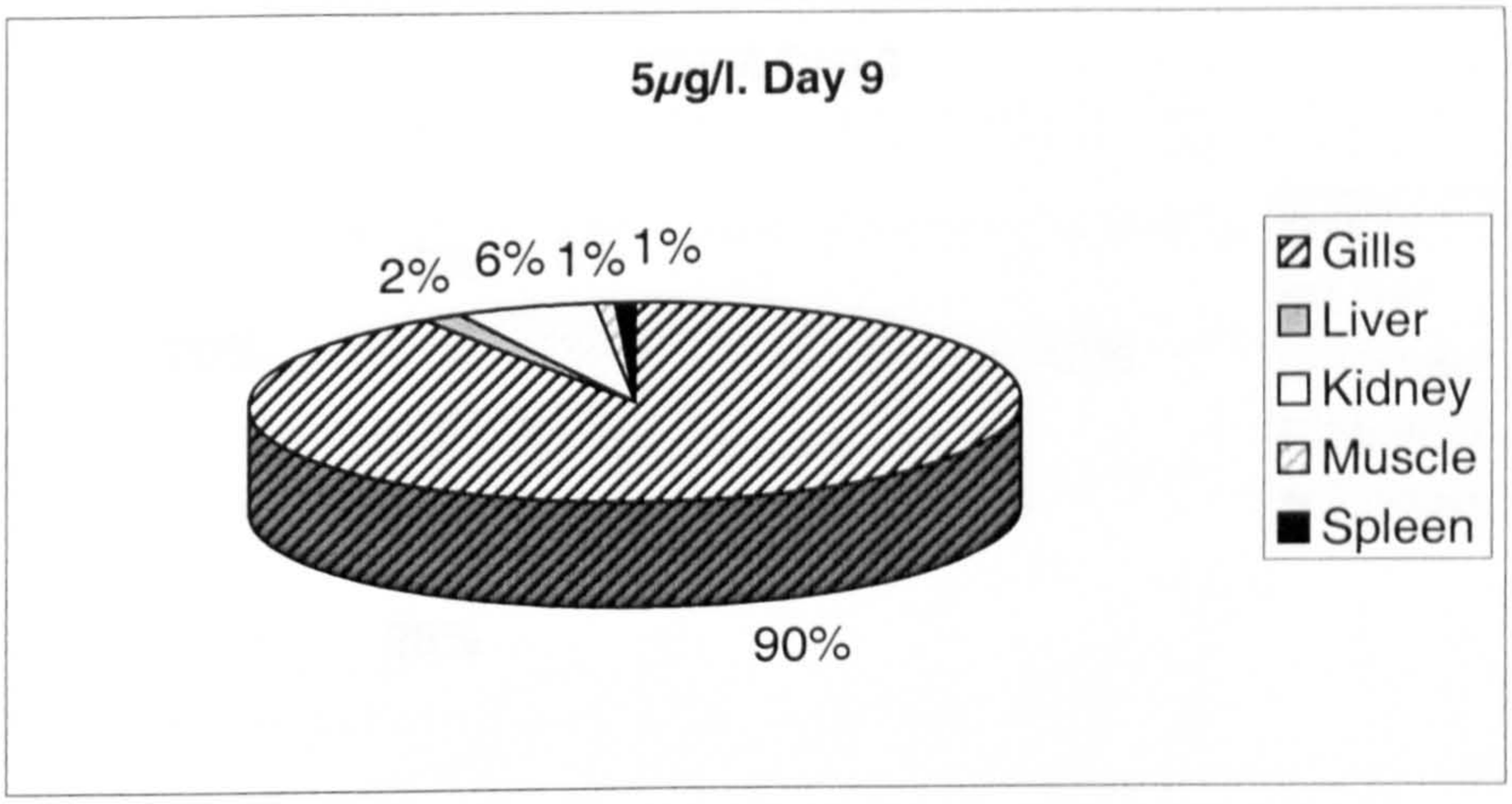


Fig. 5.11. Piecharts showing the proportion of cadmium found in each tissue of control carp at (a) 9 days, (b) 21 days and (c) 29 days post-start of the trial. For values refer to Fig. 5.10a-e.

a)



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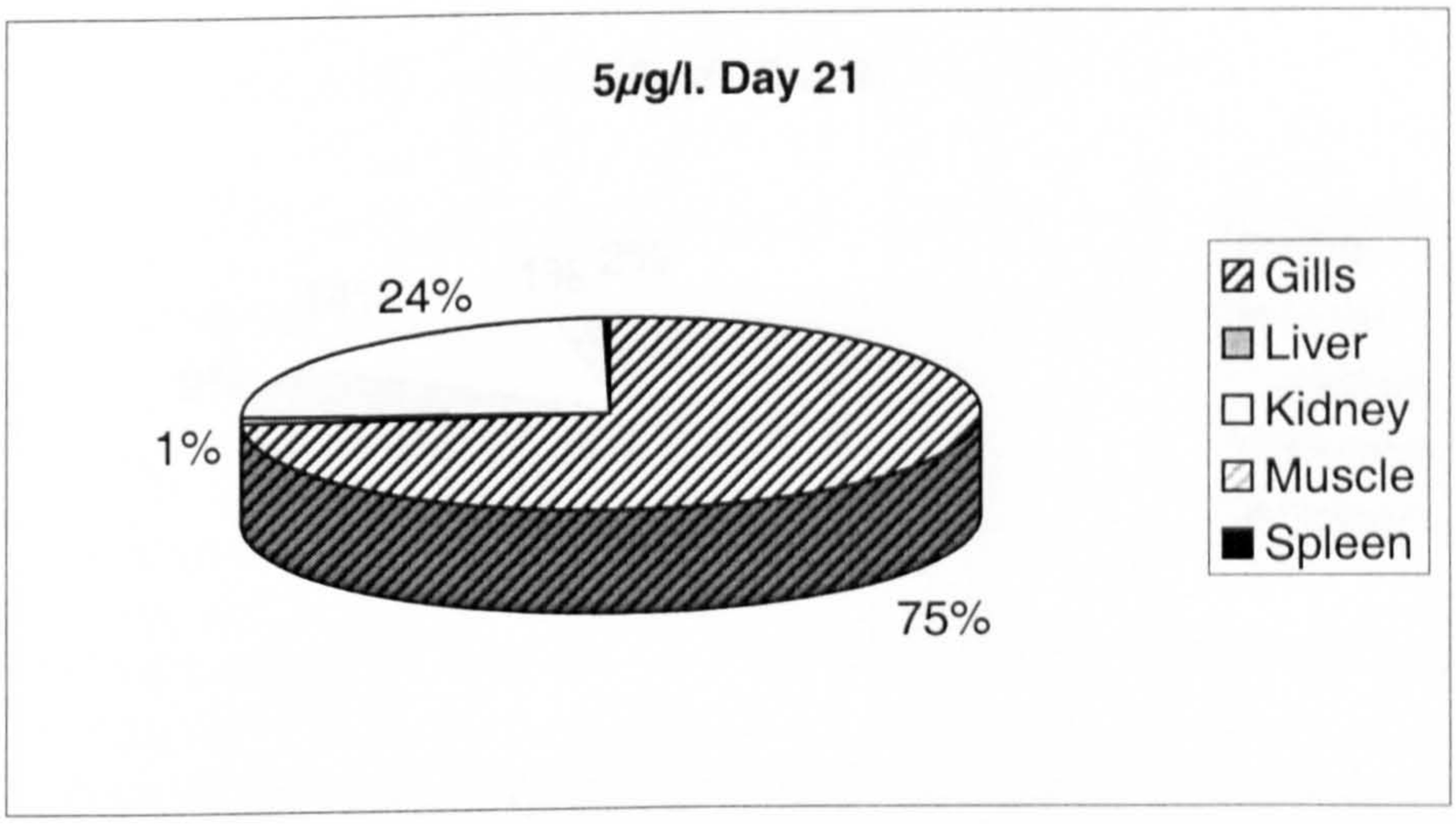

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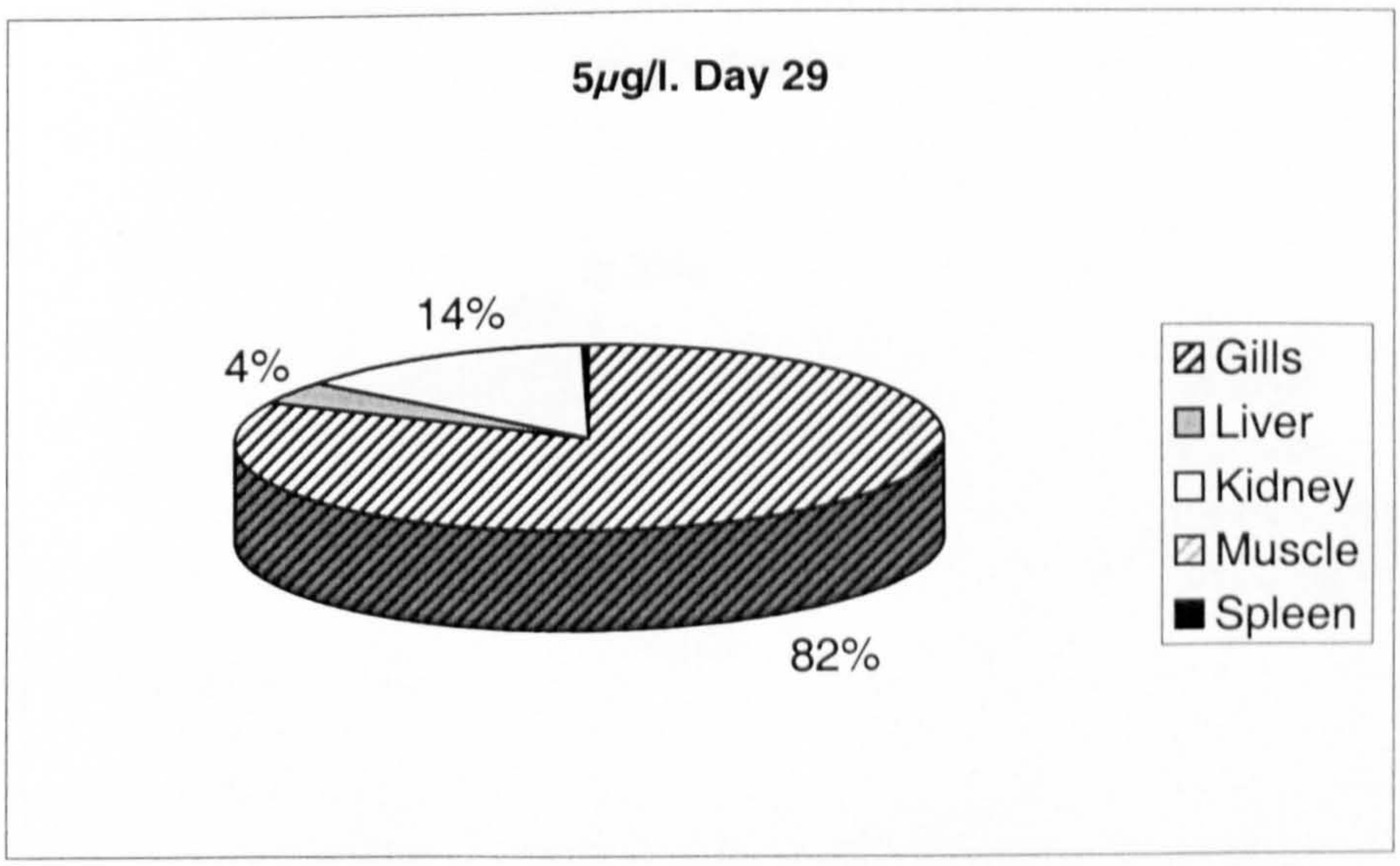
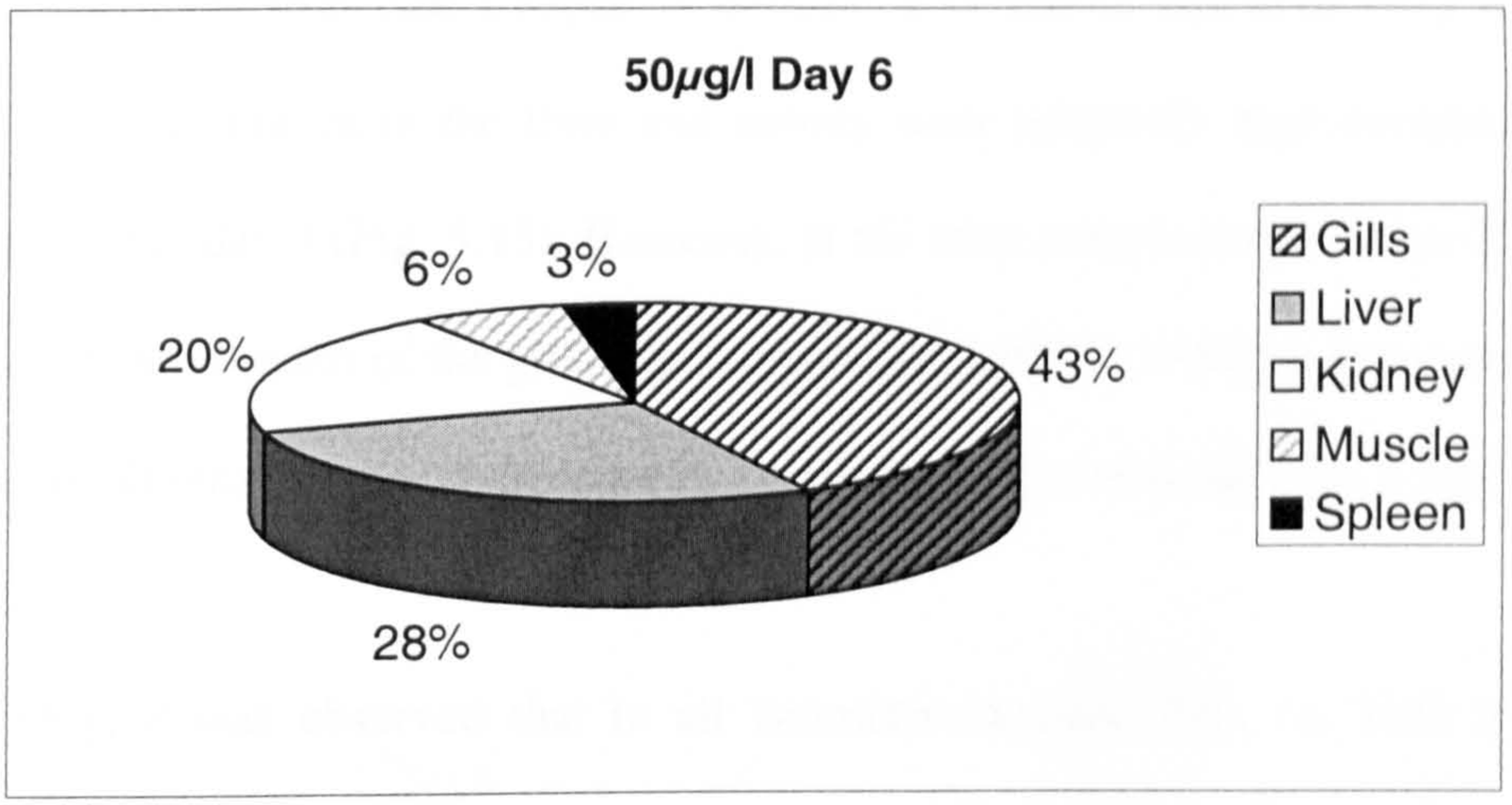
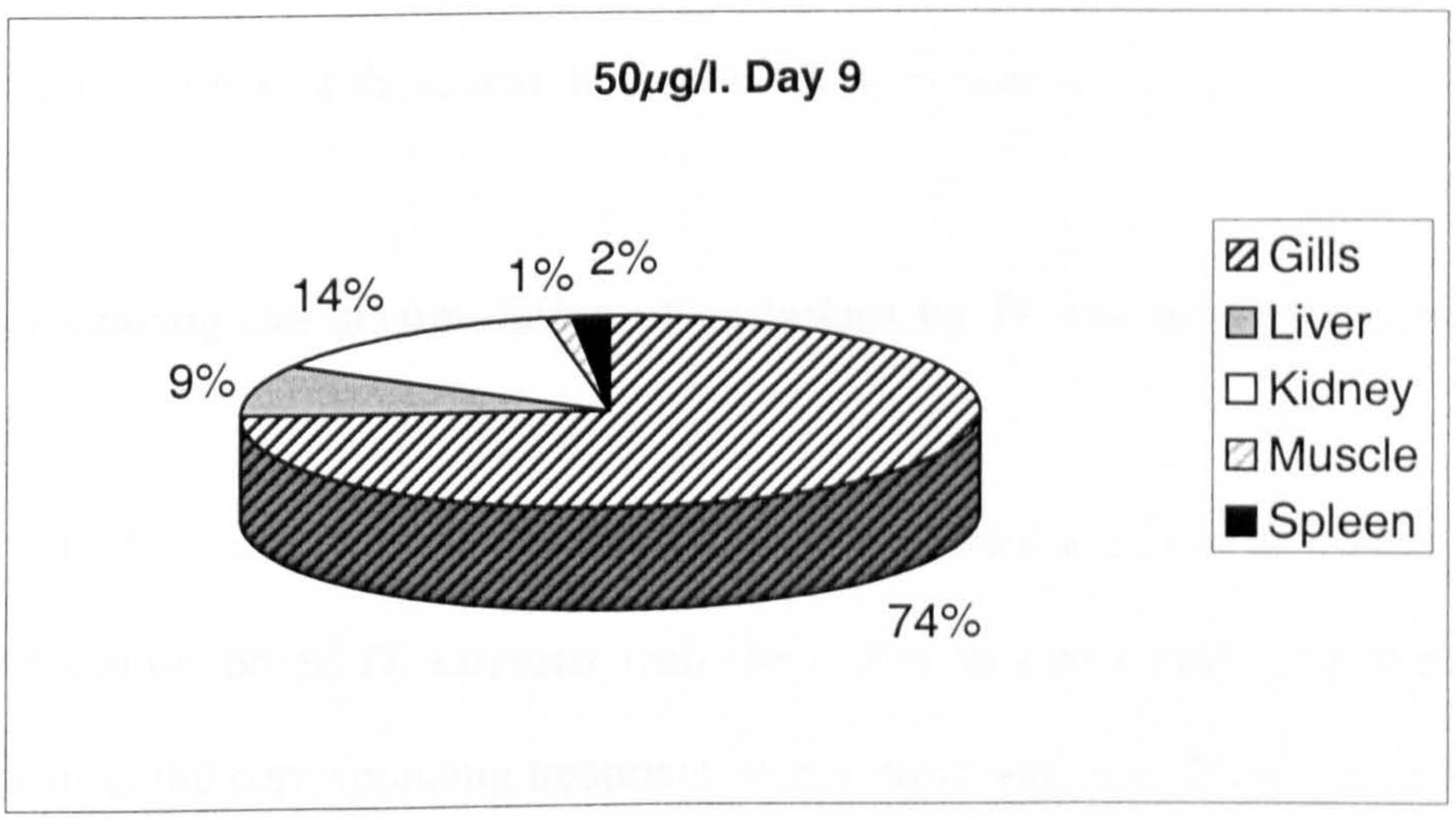


Fig. 5.12. Piecharts showing the proportion of cadmium found in each tissue of 5μg/l cadmium-exposed carp at (a) 9 days, (b) 21 days and (c) 29 days post-start of the trial. For values refer to Fig. 5.10a-e.

a)



b)



c)

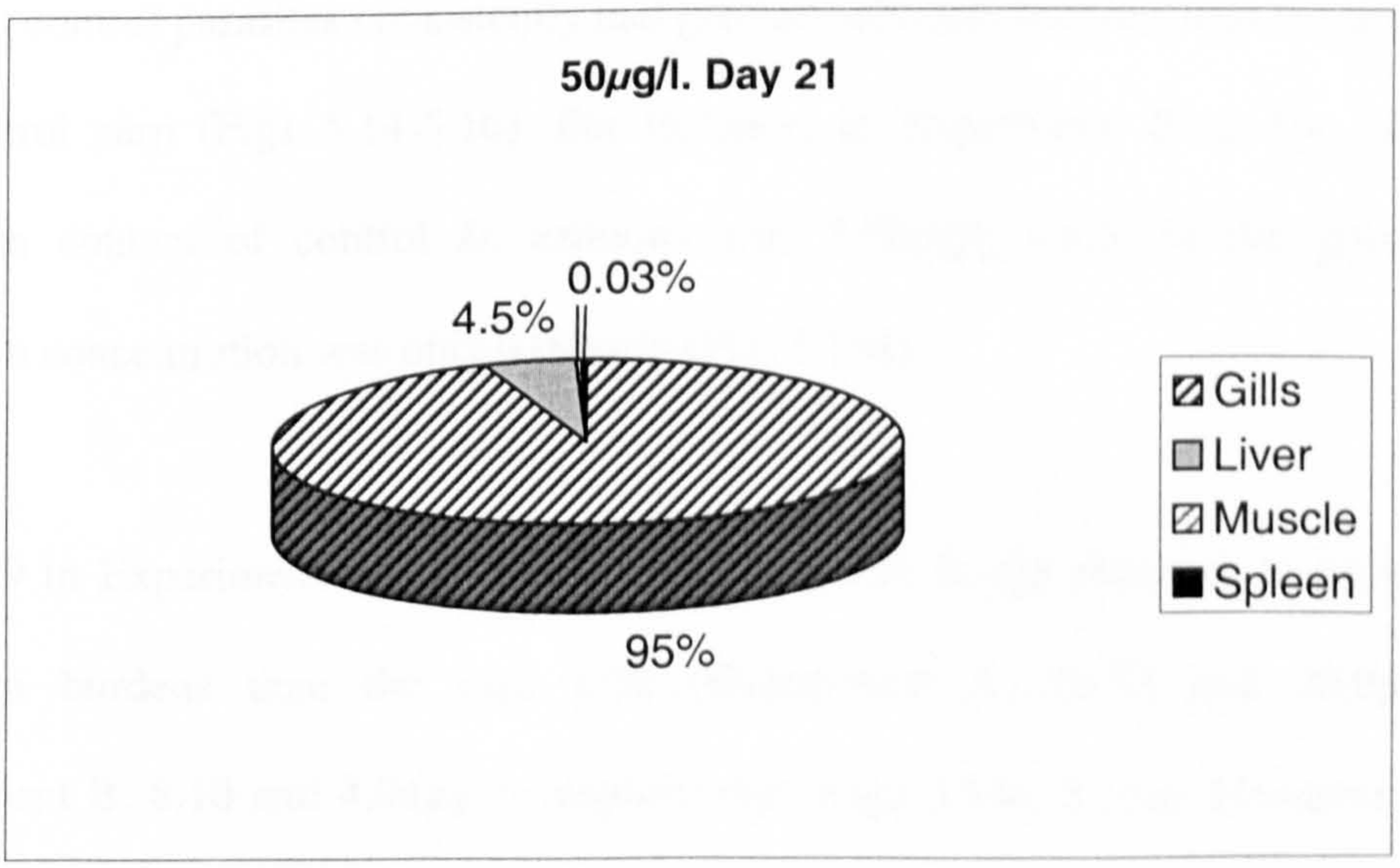


Fig. 5.13. Piecharts showing the proportion of cadmium found in each tissue of 50μg/l cadmium-exposed carp at (a) 6 days, (b) 9 days and (c) 21 days post-start of the trial. There are no day 29 data. For values refer to Fig. 5.10a-e.

early stages of the trial (see Chapter 4, section 4.4) and at this time (day 6) the proportions of cadmium in the liver and kidney were relatively high compared to the 5μg/l carp at day 9 (Fig. 5.13). However, at the later sample points (days 21 and 29) the cadmium burden of the gills was the greatest (>95%), with the proportion of cadmium in all other tissues decreasing in a time dependent manner (Fig. 5.13).

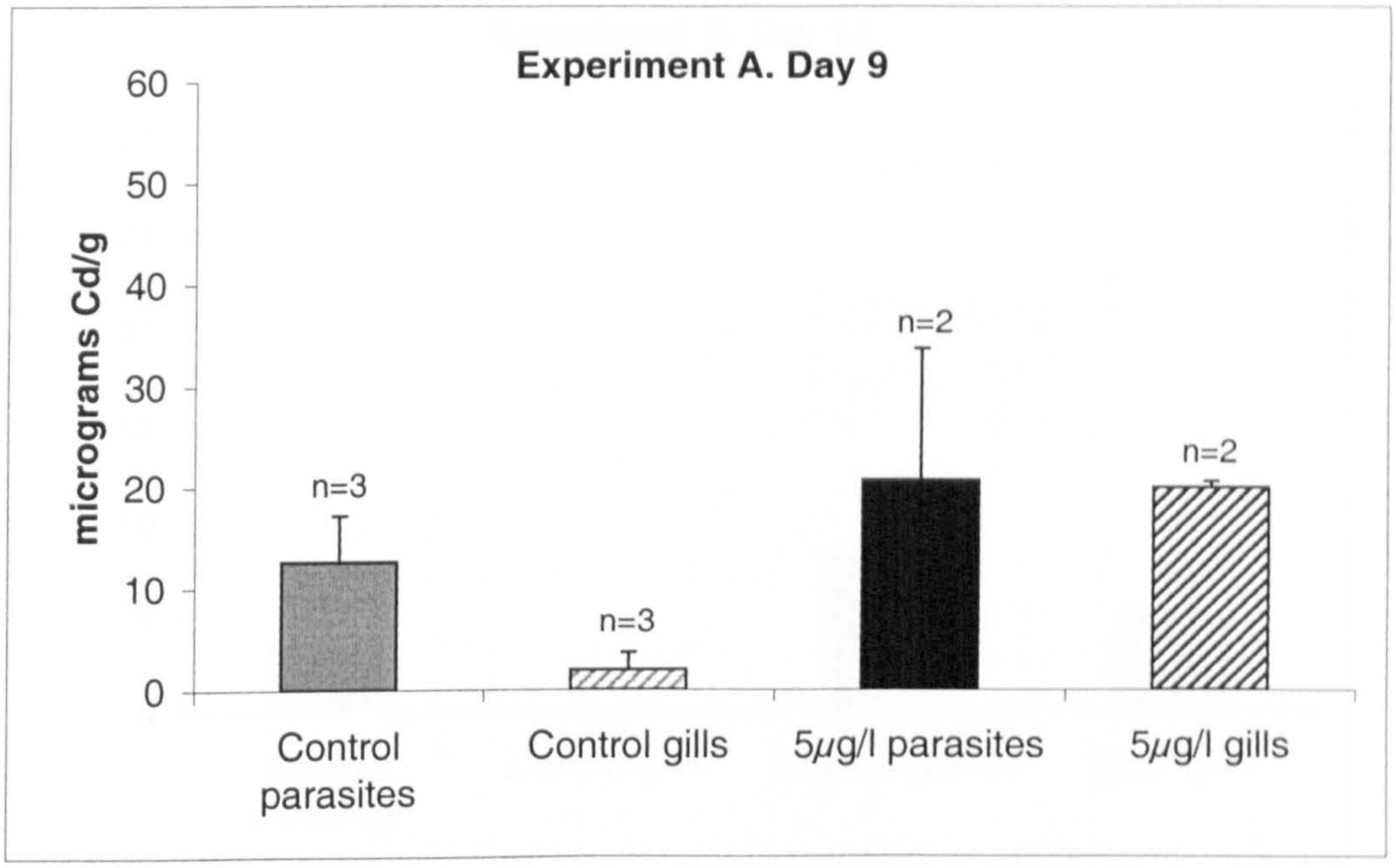
Interestingly, it was observed that in all cadmium-exposed fish, *i.e.* both 5 and 50μg/l, the gall-bladders were enlarged in comparison to those of the control fish. The cadmium content of these was, however, not determined.

5.3.5. Comparing the accumulation of cadmium by *D. extensus* and common carp gills

Figures 5.14-5.16 compare the concentration of cadmium detected in control and 5μg/l cadmium-exposed *D. extensus* with the cadmium concentration detected in carp gills from the corresponding treatment. A common feature in Experiments A-C was that control parasites consistently had greater cadmium burdens than the gills of the control carp (Figs 5.14-5.16). For instance, in Experiment B at day 14 the cadmium content of control *D. extensus* was 3.66μg/g while in the gills the cadmium concentration was only 0.04μg/g (Fig. 5.15a).

At day 9 in Experiment A and day 14 in Experiment B, the parasites had greater cadmium burdens than the carp gills (Experiment A, 20.73 and 20.0μg/g, Experiment B, 8.10 and 4.94μg/g, respectively) (Figs 5.14a, 5.15a). However, the pattern in accumulation observed by the parasites in these 2 experiments (A and B)

a)



b)

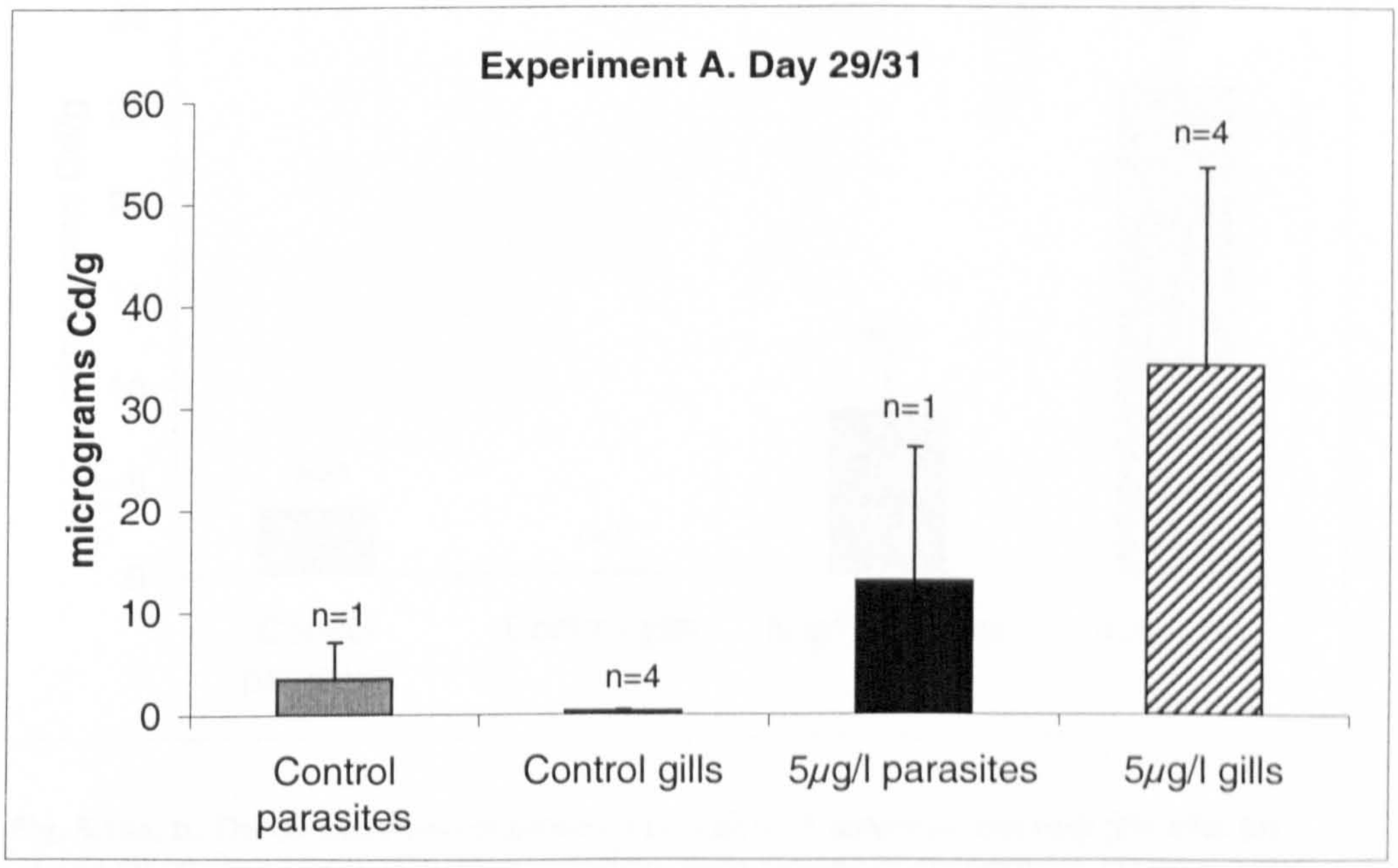
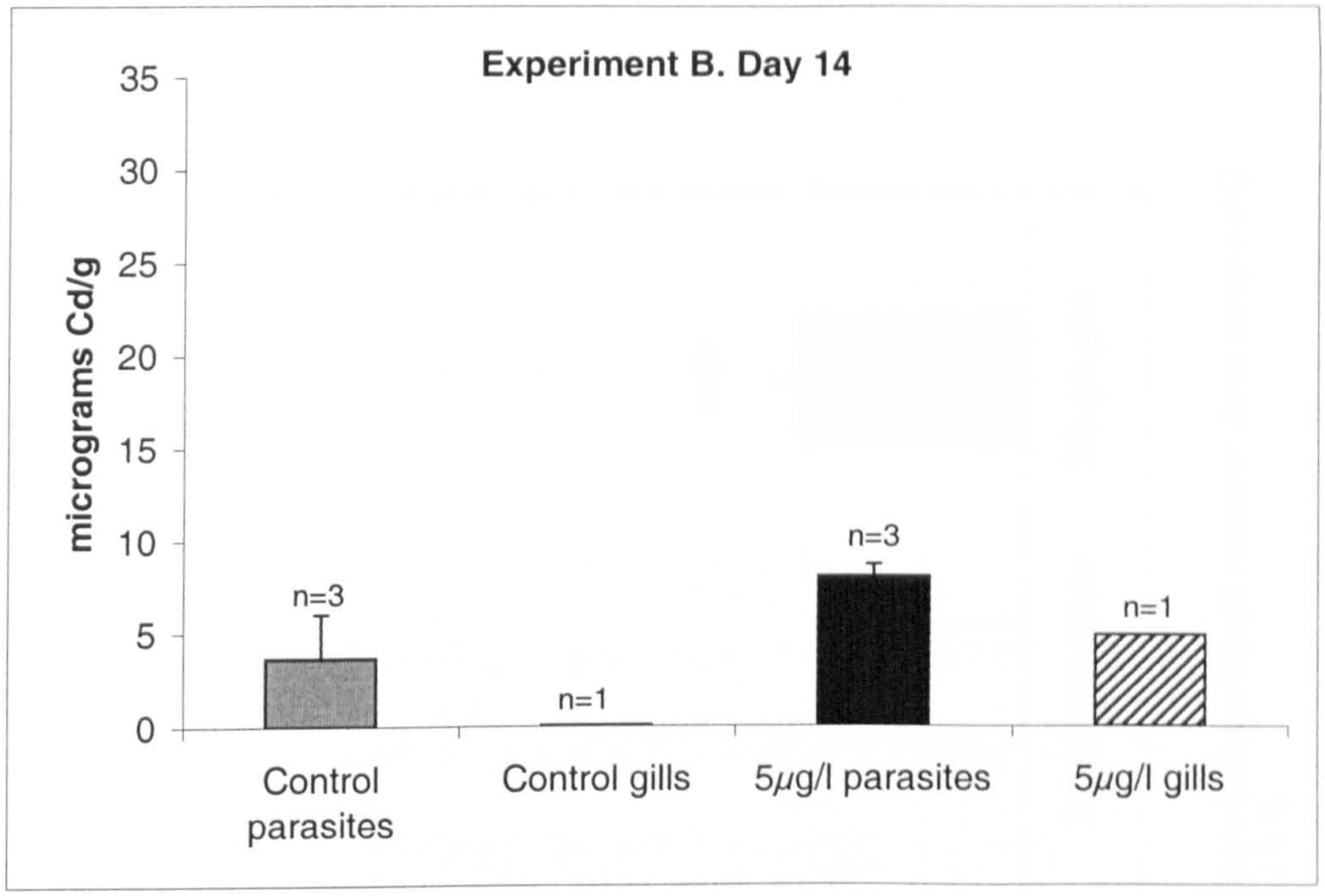


Fig. 5.14a, b. The concentration of cadmium (± S.E.) in *D. extensus* and carp gills after (a) 9 and (b) 29/31 days exposure to 5µg/l cadmium. Each sample of *D. extensus* consists of 50 adult parasites. For the source of *D. extensus* and carp see section 5.2.1.1 A.

a)



b)

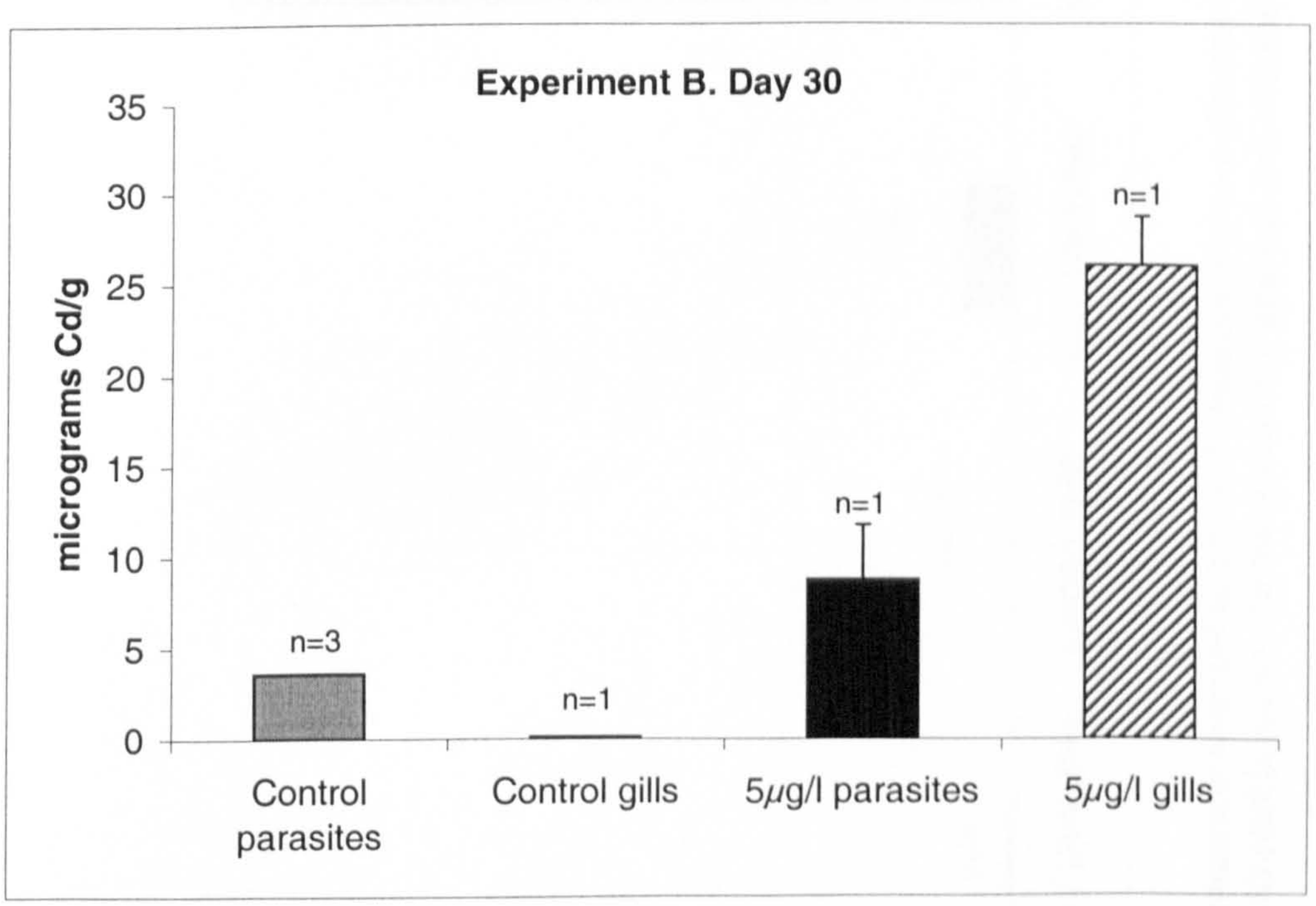


Fig. 5.15a, b. The concentration of cadmium (± S.E) in *D. extensus* and carp gills after (a) 14 and (b) 30 days exposure to 5µg/l cadmium. Each sample of *D. extensus* consists of 50 adult parasites. For the source of *D. extensus* and carp see section 5.2.1.1 B.

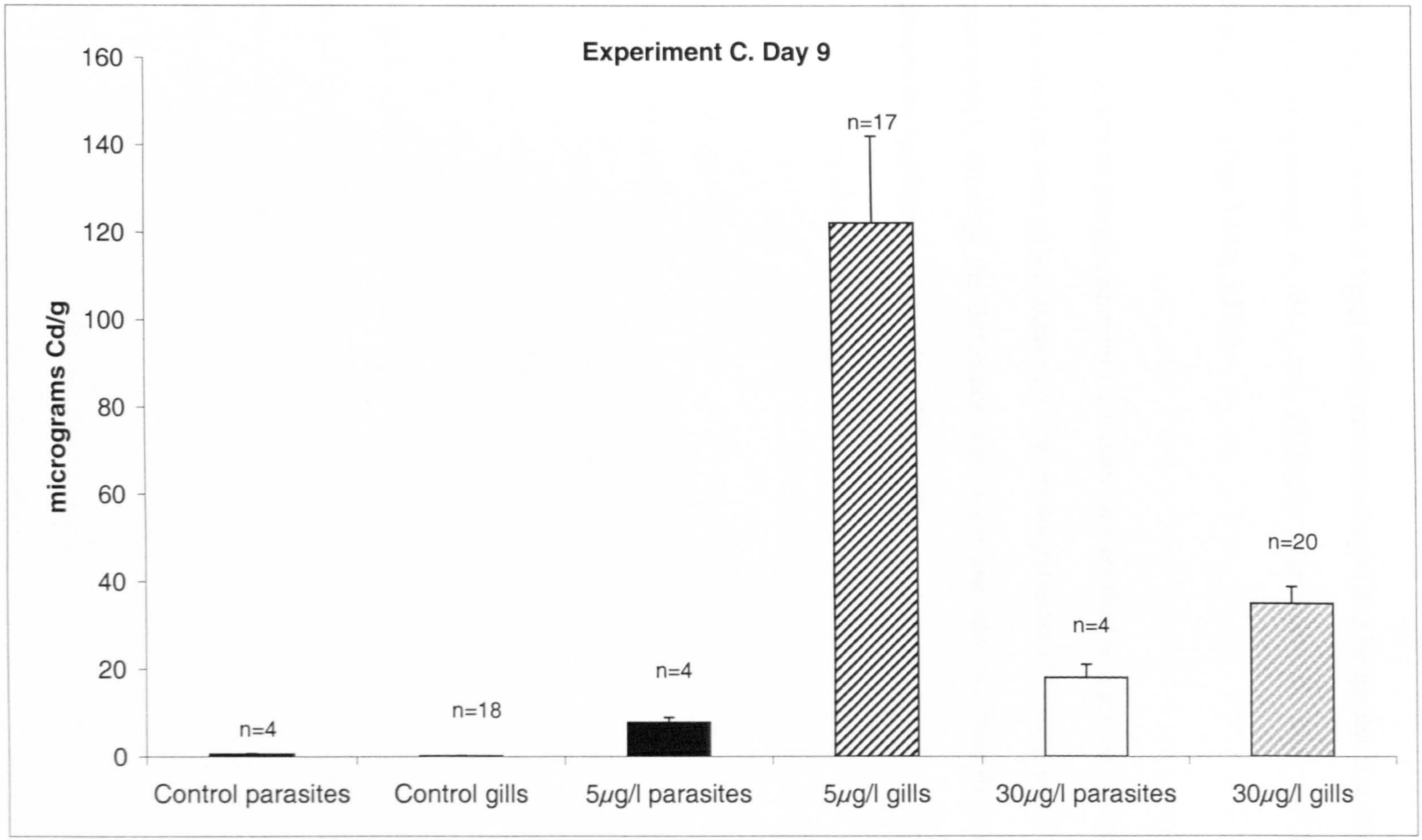


Fig. 5.16. The concentration of cadmium (± S.E.) in *D. extensus* and carp gills after 9 days exposure to 5 and 30µg/l cadmium. Each sample of *D. extensus* consists of 50 adult parasites. For the source of *D. extensus* and carp see section 5.2.1.1 C.

was not repeated in Experiment C at day 9. Here, 5μg/l carp gills had a much higher cadmium burden than that in *D. extensus* (122.0 and 7.8μg/g, respectively) (Fig. 5.16). By the end of Experiments A and B, however, the cadmium concentration of the carp gills exposed to 5μg/l cadmium was observed to be greater than that in the parasites (Experiment A, 34.1 and 13.0μg/g, Experiment B, 26.0 and 8.7μg/g, respectively) (Figs 5.14b, 5.15b).

Exposing carp to 30μg/l cadmium for 9 days also resulted in a greater accumulation of cadmium in their gills compared to that in the parasites (35.0μg/g and 17.9μg/g, respectively), although the difference was much less obvious than that observed between the 5μg/l carp and parasites (Fig. 5.16).

5.4. Discussion

All three experiments in the current trial demonstrated that *D. extensus* are able to accumulate cadmium and that accumulation increases significantly with exposure concentration. These findings correlate with the work of Sures and co-workers, who found that acanthocephalans and cestodes were able to take up and sequester cadmium from their environment (Sures & Taraschewski, 1995; Sures *et al.*, 1997b). The concentrations of cadmium found in *D. extensus* that had been exposed to 5μg/l cadmium was, however, greater than that recorded in adult *Pomphorhynchus laevis* (see Sures & Taraschewski, 1995), approximately 8.0μg/g compared to 4.27μg/g. The difference in the cadmium burden between the two species could be attributed to the cadmium concentrations of the respective waters, with *D. extensus* exposed to approximately 50 times more cadmium than *P. laevis* (ca. 8.0 and 0.16μg/l, respectively). However, as the control *D. extensus* accumulated approximately as much cadmium as *P. laevis* (ca. 4.78 and 4.27μg/g, respectively) when the concentrations of the metal in the two water sources were similar, this suggests that another factor such as microhabitat may influence cadmium uptake. Microhabitat has been known to influence the accumulation of lead by *Acanthocephalus lucii* (see Sures & Taraschewski, 1995) and *P. laevis* (see Sures & Siddall, 2001), and it is possible that the position of *D. extensus* on the gills allows it to take up cadmium from both the water and from the host, while the intestinal position of the acanthocephalans exposes them only to cadmium taken in by the host. However, as the fish intestine has been recorded to contain very high levels of cadmium (see Kraal *et al.*, 1995), the difference in the ability of *D. extensus* and *P. laevis* to sequester cadmium is most likely to be due to species specific differences in their accumulation abilities.

The mechanisms of metal uptake in aquatic invertebrates, principally marine crustaceans, have been thoroughly researched and reported by Rainbow and co-workers (Rainbow, 1990, 1993, 1995, 1996, 1997a,b, 2002; Rainbow, Amiard-Triquet, Amiard, Smith & Langston, 2000). The general principles of metal uptake from solution also apply to freshwater invertebrates (Rainbow *pers. comm.*) and will thus be discussed with regard to *D. extensus* and cadmium. While it is not possible to conclude which method of cadmium uptake applies to *D. extensus*, several possible hypotheses can be suggested.

Only the bioavailable form of a dissolved metal can be taken up from solution by aquatic invertebrates, and this form is generally considered to be the free (hydrated) metal ion (Rainbow, 1995). The first possible method of cadmium uptake by *D. extensus* from solution is the passive process of facilitated diffusion (Rainbow, 1990, 1995, 1996). Facilitated diffusion involves the cadmium ion being transported across the membrane of the organism by a carrier protein (Rainbow, 1995). The cadmium ion then passes down a gradient of metal binding ligands (Rainbow, 1990, 1995, 1996) and becomes bound to proteins (Rainbow, 1996).

The second possible method of entry of dissolved cadmium in to *D. extensus* is via an energy-dependent calcium (Ca^{2+}) ion pump (Rainbow, 1995, 1997b). Calcium and cadmium ions are of similar size, with ionic radii of 114 picometres (pm) and 109pm, respectively, and thus some cadmium may be taken up via the calcium pumps (Rainbow, 1995). Accumulation of cadmium is thus responsive to changes in the Ca^{2+} concentration and the greater the activity of the calcium pumps the greater the amount of cadmium that could enter by this route (Rainbow, 1993). In the

present trial there was no significant difference in the concentration of calcium ions (where analysed) between water from any treatment. Thus, the observed differences in cadmium concentration between control and 5μg/l cadmium-exposed *D. extensus* are unlikely to be due to differing rates of metal uptake caused by the varying activity of the calcium pumps.

While one or both of the above uptake methods may account in part for the accumulation of cadmium by *D. extensus*, uptake may also occur from the parasites feeding on contaminated gill tissue. Fish mucus is known to act as a strong complexing agent for cadmium (Pärt & Lock, 1983) and the grazing of mucus and epithelial cells from the gills by *D. extensus* could thus result in their exposure to and acquisition of more cadmium. *D. extensus* attach to the gill filaments and these have been shown in the current trial to accumulate the greatest concentration of cadmium (at 5μg/l) when compared to the gill rakers. The higher cadmium concentration in the filaments may be attributed to their greater blood supply, to the presence of the mucus found attached to the filaments or to their greater surface area compared to the rakers.

Invertebrates can be divided into two categories, net accumulators or regulators, depending on their method of metal uptake (Rainbow, 1990). Net accumulation occurs when excretion of the metal does not equal the uptake of the metal, while regulation has been defined by Rainbow (1990) as being " *strictly the maintenance of an approximately constant body metal concentration over a wide range of ambient metal bioavailabilites*". Evidence from the current trials lends itself to the idea that *D. extensus* is a net accumulator of cadmium as the concentration of

cadmium accumulated by *D. extensus* increased with increasing exposure concentration (Experiment C), resulting in significantly greater body burdens of cadmium after exposure to 30μg/l cadmium compared to the controls. The apparent plateau in accumulation reached over time by *D. extensus* exposed to 5μg/l cadmium would seem, however, to discount this hypothesis. However, it is more than probable that between the time points in the plateau, net accumulation was occurring slowly but was just not rapid enough to make a significant alteration to the overall cadmium concentration in the allotted time. To verify the accumulation ability of *D. extensus,* it would, thus, be useful in future experiments to assess the cadmium concentration of the dactylogyrids after longer than 30 days exposure to cadmium. However, this would be dependent on maintaining a healthy number of *D. extensus* on carp for a prolonged period. For this to occur the problems of lack of recruitment need to be addressed (see Chapter 3 section 3.4).

A particularly interesting observation made during the current trial was the relatively high concentration of cadmium in control *D. extensus* compared to the 5μg/l cadmium-exposed parasites. Analysis of *D. extensus* directly after their arrival from the fish farm showed cadmium concentrations to be much lower than that in the control dactylogyrids after 14 days exposure to experimental conditions. However, in this particular experiment (Exp. B), the concentrations of cadmium in pond water from the fish farm and in the water from the experimental system were the same (0.06 $\pm$ 0.02 and 0.06 $\pm$ 0.01μg/l, respectively). The similarity in cadmium concentrations between the two water sources excludes the possibility that the plastic tanks of the experimental system may have been leaching cadmium and that the water in the control tanks was being contaminated in some manner. It should be

noted here that the cadmium concentration of the pond water represents only a single point in time and the concentration may vary depending on the dynamics of the water flow at the fish farm. The carp used in Experiment B arrived from the fish farm in an emaciated state and had perhaps therefore not been fed for several months, a method used to maintain the stock at a particular size for sale. Cadmium is a ubiquitous heavy metal and can be found in low concentrations in many organisms and environments and subsequently feedstuffs. Analysis of the cadmium content in the commercial pelleted food used to feed the carp during these trials gave a value of $0.1 \pm 0.005 \mu g/g$. While this value is low, it is possible that it was substantial enough to build up in the tissues of the carp and thus in the dactylogyrids feeding off them, resulting in the higher cadmium concentration in the experimental dactylogyrids compared to those sampled directly from the farm. As cadmium concentrations in the muscle and kidney of control carp in Experiment B increased with time, this is indeed a probable hypothesis. In order to explore this hypothesis further, tissue samples would need to be taken from carp directly on their arrival from the farm and the cadmium concentrations compared to those in tissue samples taken from fish after exposure to the experimental system.

Siriwardena *et al.* (1995) reported that metal uptake by aquatic organisms involves a two-step process whereby metal becomes rapidly adsorbed to or bound to the cell surface, followed by the slow penetration by some metal ions into the interior. The adsorbed fraction never enters the body but can contribute to the total body concentration of the metal, particularly in small invertebrates where the surface area to volume ratio is high (Rainbow, 1990). In the present study it was hoped to determine only the amount of cadmium that had been accumulated and stored

internally by *D. extensus* and thus the samples were rinsed in the chelating agent EDTA to remove the externally adsorbed fraction of cadmium from the parasites (Siriwardena *et al.*, 1995). However, in contrast to the findings of Siriwardena *et al.* (1995), where EDTA-rinsing significantly reduced the concentration of cadmium in cadmium-exposed tilapia sac-fry, alcohol fixed *D. extensus* exposed to 5μg/l cadmium and subsequently rinsed in EDTA had greater cadmium concentrations (μg/g) than the non-EDTA-rinsed samples in the same experiment (Experiment A). It is possible that, as the sample sizes of both rinsed and unrinsed parasites (n=3 and n=1, respectively) were so small, a true representation of the use of EDTA could not be achieved in this instance. Alternatively, as non-EDTA-rinsed parasites in Experiment A had similar cadmium concentrations to the EDTA-rinsed parasites in Experiments B and C it is possible that the amount of cadmium adsorbed by *D. extensus* is negligible with regards to the whole body concentration of the metal and thus EDTA-rinsing is relatively ineffective. To accurately assess the effectiveness of EDTA in removing externally bound cadmium from *D. extensus*, it would be necessary to repeat the experiment, ensuring that as many samples as possible could be obtained and processed.

Interestingly, the highest concentrations of cadmium in both control and 5μg/l *D. extensus* were recorded in Experiment A in the alcohol-preserved specimens where, after 9 days exposure to experimental conditions, cadmium levels in these two groups of parasites were respectively 8 and 3 times greater than those recorded for the same treatments in later experiments. This anomalous result cannot be attributed to either a greater cadmium concentration in the water or in the carp gills during this experiment, as these values were similar throughout the trials. However, the Ca^{2+}

ion concentration was not determined in this experiment and it is possible that the difference in cadmium accumulation could be attributed to differences in the Ca^{2+} concentration. Rainbow (1996) commented on the inherent variability that can occur between samples when ambient metal bioavailability is the same and how this can increase the problems of interpreting intraspecific metal concentrations. However, while this is a very probable cause of the variability, coupled with the sample sizes in this experiment (Experiment A) being the lowest of all experiments, the possibility that storage in alcohol may have affected the cadmium concentrations in some manner cannot be excluded. While the exact manner in which alcohol may have affected the cadmium concentrations of these parasites is not known, it is known, through atomic adsorption analysis of alcohol samples, that the alcohol contained $0.12 \pm 0.001 \mu g$ Cd/l (n=2). Whether the cadmium in the alcohol would be capable of penetrating the preserved *D. extensus* and/or binding to the parasites is unknown, but future trials could aim to investigate this possibility by fixing control parasites in alcohol contaminated with differing concentrations of cadmium and subsequently analysing them using atomic adsorption spectrometry.

The elemental profile of *D. extensus*, generated by ICP-MS, has been demonstrated in this current trial for the first time. Of particular interest with regard to the profile is the greater concentration of all elements in cadmium-exposed parasites compared to the controls. However, as there were no statistical differences in the concentration of these elements in parasites from different treatments, the slight variations between treatments should be interpreted with caution. However, it is possible that synergism between metals, whereby the presence of one metal (cadmium) enhances the bioaccumulation of another (Rainbow *et al.*, 2000), may be responsible for this

observed phenomenon. While the elemental composition of *D. extensus* is of great interest, the focus of this chapter was to assess the ability of this parasite to accumulate cadmium. The ICP-MS analysis confirms the ability of *D. extensus* to accumulate cadmium and verifies the cadmium concentrations detected by atomic adsorption spectrometry (AAS), (*i.e.* ICPMS 5μg/l parasites = 5.46 ± 0.7μgCd/g, 30μg/l parasites = 16.46 ± 2.32μgCd/g compared to AAS 5μg/l parasites = 7.76 ± 1.13μgCd/g, 30μg/l parasites = 17.91 ± 2.97μgCd/g).

In summary, preliminary investigations show that *D. extensus* is capable of accumulating cadmium to relatively high concentrations. These findings are particularly interesting with regard to how cadmium may affect the biology of *D. extensus*. In terms of their use as a potential bioindicator, the main focus of fish parasite-heavy metal studies to date, the small size of the parasite and the large number required to gain one reading on the atomic adsorption spectrometer make it an inappropriate indicator species. Future experiments may benefit from using a different monogenean-host system with larger parasites that may help to overcome the problems with sample sizes. Further work should include a TEM study of cadmium-exposed *D. extensus* to determine if accumulation of the metal causes damage to particular organs of the parasite, particularly the reproductive organs. Such a study may help to elucidate the findings of the impact of cadmium on the egg production of this parasite species recorded in Chapter 3.

In reviewing the literature associated with the accumulation of cadmium by fish, it became apparent that different units are used to express the concentration of cadmium/unit weight. All units in the following discussion are presented as found in

each paper. The concentrations of cadmium in the present study are expressed as μg/g, sometimes expressed by other authors as the equivalent, mg/kg.

Accumulation has been defined in The Penguin English Dictionary as "*an increase caused by repeated or continuous addition*". Most of the papers reviewed for this chapter refer to particular fish organs that have accumulated the greatest concentration of cadmium in comparison to other organs and tissues. It is important to highlight that, while some organs do accumulate cadmium, the accumulation in all organs might not be permanent as cadmium storage can be transitory (detailed below). However, in keeping with the reviewed literature, the term accumulation will be used in this discussion but the possible transient nature of cadmium storage in some organs will be alluded to.

During the current trial, carp exposed to both 5 and 50μg/l cadmium accumulated more cadmium (often significantly more) than the control fish. However, cadmium concentrations in control fish organs were, in general, above the detection limit of the atomic adsorption spectrometer (0.5μg/l), showing that even low levels of the metal present in the water or feed can be bioaccumulated. The accumulation of low concentrations of cadmium (0.16μg/l) from riverine water has been demonstrated by *Perca fluviatilis*, with the liver and intestine accumulating 1.19 and 0.22μg/g, respectively (Sures & Taraschewski, 1995). Conversely, *Leuciscus cephalus* (Sures & Taraschewski, 1995), *Tinca tinca* and *Scopthalmus maximus* (see Sures *et al.*, 1997b) all failed to accumulate cadmium to levels above that found in their surrounding environment.

Studies by Giles (1988) and Suresh *et al.* (1993) both recorded the gills of fish as accumulating the greatest concentrations of cadmium compared to the kidney, liver and muscle. The findings of these two authors are consistent with the observations made in Experiments A, B and D of the current trial, where the gills also accumulated the greatest concentration of cadmium of all tissues investigated. Giles (1988) did, however, find that, after 180 days exposure to 6.4μg/l cadmium, the cadmium content of the kidney of *Oncorhynchus mykiss* was approximately equivalent to that in the gills, which had reached a plateau in cadmium concentration after an initial, rapid increase in the first 10 days of exposure. Interestingly, a similar plateau in cadmium accumulation was observed in the carp in the current trial, although this was not reached until day 21 (Experiment D). The observation by Suresh *et al.* (1993), that cadmium was accumulated in greater concentrations in the gills of *C. carpio,* was only seen in the fingerlings exposed to 3.42 mg/l; in the fry exposed to 0.86 mg/l, cadmium concentrations were greatest in the muscle. This suggests that age may indeed play a part in the pattern of cadmium accumulation in fish. In contrast to the findings of the current trial, De Smet & Blust (2001) recorded 2.4 times more cadmium in the kidney of carp compared to the gills after 29 days exposure to 4μM (ca. 22.5μg/l) cadmium. It would thus appear that age, species and size of the fish might all impact on the accumulation of cadmium.

Surprisingly, Experiments C and D of the current trial showed that the concentration of cadmium was greater in the gills of fish exposed to 5μg/l cadmium compared to the gills of the 30 and 50μg/l cadmium-exposed carp. One hypothesis for this observation could be attributed to the different effects of the two cadmium concentrations on the kidney function of the carp. Cadmium-induced renal damage

in mammals results in large quantities of cadmium being excreted, in association with metallothioneins, via the urine (Nordberg, 1978 reviewed by Giles, 1988). In undamaged kidneys, however, cadmium or the cadmium/metallothionein complexes can be reabsorbed from the glomerular filtrate, limiting the excretion of metal by the kidneys (Giles, 1988). In these instances, Giles (1988) has suggested that elimination of cadmium probably occurs over permeable body surfaces, such as the gills. Thus, it is probable that the kidneys of the carp exposed to 50μg/l cadmium had been damaged by this concentration of cadmium, resulting in the excretion of large amounts of cadmium in the urine. Indeed, Mohan (1990) observed structural damage to the renal glomeruli of the kidney after 15 days exposure to 50μg/l cadmium as well as necrosis and degeneration of the cells in the kidney tubules. However, Giles (1984), on exposing *O. mykiss* to 3.6 and 6.4μg/l cadmium for 178 days, found little impairment of renal function in the fish. Thus, the carp exposed to 5μg/l cadmium in the current trial were unlikely to have suffered renal damage and thus the cadmium is more likely to have been eliminated via the gills. This hypothesis would explain the greater concentration of cadmium in the gills of the 5μg/l carp compared to the 50μg/l and the greater proportion of cadmium in the liver and kidneys of the 50μg/l carp. To test this hypothesis, in future, the cadmium concentration of the urine of both cadmium-exposed groups of carp could be determined and the histopathology of the kidneys of carp from all treatments could be assessed for signs of damage.

The particularly high concentration of cadmium in the gills of 5μg/l cadmium-exposed carp (122μg/l) in Experiment C at 9 days, is approximately 3.6, 4.7 and 2.4 times greater than that in the gills of carp from the same treatment in Experiments

A, B and D, respectively. The reasons for this high value are not known, but it is possible that, despite all carp being purchased at the same size, the fish used in this experiment were of a different age or health status to those used in the other experiments and thus potentially at a different stage of development with regard to their detoxification abilities.

The only experiment (Experiment D) in the current trial to simultaneously investigate cadmium accumulation in carp kidney and liver recorded higher levels of the metal in the former organ compared to the latter after exposure to both 5 and 50μg/l cadmium. Similar observations to the above have been recorded by others (see Giles, 1988; Suresh *et al.*, 1993; Brown, Thomas, Shurben, Solbe, Kay & Vryer, 1986; Brown, Shurben, Miller & Crane, 1994; Kraal *et al.*, 1995; De Conto Cinier *et al.*, 1997,1999 and De Smet & Blust, 2001). In carp exposed to 50μg/l cadmium, the concentration of this metal in the liver was initially high (in comparison to later liver cadmium concentrations) before decreasing over time. This decrease in the cadmium concentration in the liver over time was accompanied by an increase in its concentration in the kidney and gills. Data for cadmium concentrations in the kidney of the 50μg/l fish were not, unfortunately, obtained after day 9 of the trial. However, it is possible that the kidney concentrations of cadmium would have continued to increase over time, as recorded by both De Conto Cinier *et al.* (1999) and Giles (1988). Interestingly, Kito *et al.* (1986) recorded an increase in cadmium binding protein (metallothionein Cd-MT) in the kidneys, liver, gills and gastrointestinal tract of carp exposed to cadmium and De Smet & Blust (2001) also recorded elevated levels of the Cd-MT in the gills and kidney of cadmium-exposed carp. It is possible that in the current trial,

metallothionein production in the gills and kidney increased over time and this could explain the high cadmium accumulation recorded in these organs. As the liver also produces metallothioneins to bind cadmium, it would be expected that the cadmium burdens of the carp liver should also have increased. This did occur in the carp exposed to 5μg/l cadmium, while the opposite effect was observed in the liver in the 50μg/l cadmium-exposed carp. It is possible that the higher level of cadmium (50μg/l) resulted in the carp needing to excrete cadmium rapidly so that cadmium bound to metallothionein in the liver was translocated to other organs for excretion. Suresh *et al.* (1993) suggested that while the liver is the initial site for cadmium detoxification, higher levels of cadmium may cross the finite line for intracellular storage and result in structural abnormalities in the liver and the redistribution of this metal to other areas of the body. However, in the carp exposed to the lower cadmium concentration (5μg/l), the accumulation of cadmium by the liver may have been within the capability of the liver to detoxify the metal, resulting in the fish being able to adapt to the conditions without any serious adverse effects.

During an experiment exposing carp to 450μg/l cadmium, De Conto Cinier *et al.* (1997) reported that the carp had developed cadmium-sequestering detoxifying systems that made the metal harmless to the fish, resulting in their displaying normal behaviour throughout the trial. Several of the carp exposed to the highest level of cadmium (50μg/l) in Experiment D of the current trial did not display normal behaviour during the trial and displayed extended periods of hyperactivity (see Chapter 4, section 4.3.2). The amount of cadmium that these fish were exposed to may have exceeded their ability to synthesise enough of these detoxifying metallothioneins, leading to the metal becoming toxic to the fish (George & Olsson,

1993) and disrupting the detoxifying and accumulating pathways. However, as some of the carp did not display abnormal behaviour, it suggests that there is heterogeneity in the ability of carp to cope with cadmium exposure and thus heterogeneity in their abilities to detoxify this metal.

All test carp (5 and 50μg/l cadmium-exposed) in the current trial exhibited greatly swollen gall-bladders in comparison to the control carp. Sures & Siddall (1998) suggested that the bile can form organo-metallic complexes with lead, and it is possible that similar complexes can be formed with cadmium. Kraal *et al.* (1995) suggested that the gut of carp acts as a sink for cadmium or that cadmium may be excreted into the gut via the bile. Analysis of the gall-bladder of sticklebacks exposed to 0.1 and 1.0 mg/l cadmium by Woodworth & Pascoe (1983) revealed a cadmium concentration similar to that of the gills and the kidney. Thus, the swollen gall-bladders of the cadmium-exposed carp may have been a function of cadmium being excreted with the bile and from here passing into the gut. In future studies, an analysis of the bile and the intestine would determine to what extent they are involved in excretion of cadmium by carp exposed to 5 and 50μg/l cadmium.

With regard to cadmium accumulation in fish muscle, the most important consideration is that to human health. The permitted level of cadmium in food fish muscle is 0.05 mg/kg (μg/g) as set by EC Regulation 466/2001. Control carp muscle consistently had lower cadmium concentrations than the permitted value, ranging from 0.007 to 0.04μg/g. However, carp exposed to the maximum permissible level of cadmium in drinking water (5μg/l) had consistently higher cadmium burdens than that permitted, ranging from 0.05 to 0.25μg/g. As carp are bred for human

consumption (De Conto Cinier *et al.,* 1997), the accumulation of cadmium to these concentrations is of great interest and possible concern. The ability of a range of food fish species to sequester cadmium when exposed to only the maximum permitted level of cadmium should be investigated. It is worth noting, however, that the carp used in these trials were well below the size that would be sold for human consumption. It is possible, as recorded by Suresh *et al.* (1993), that as the carp get larger (fry to fingerlings) less metal becomes stored in the muscle, with more being detoxified and sequestered in other organs. Changes in the cadmium content with increasing age and size of fish appear, however, not to have been studied to any great extent.

Cadmium concentrations in the spleen of carp in the current trial were comparable to the concentrations observed in the muscle (< 0.05µg/g). This figure is similar to that observed by Thomas, Cryer, Solbe & Kay (1983) on exposing rainbow trout to 9µg/l cadmium for two weeks. Stickleback spleen was found, however, to have a concentration of cadmium four times greater than that of the muscle (Woodworth & Pascoe, 1983). The presence of cadmium in the spleen could be attributed to the breakdown of erythrocytes to which the cadmium had been bound.

Cadmium levels in blood plasma have been recorded to be 30 times greater in cadmium-exposed fish compared to their respective controls after 29 days exposure to 20µM (ca. 112µg/l) cadmium (De Smet & Blust, 2001). In samples of whole blood from carp (Experiment A section 5.2.1.1), the cadmium concentration was found to be 10 times greater in the cadmium-exposed fish (5µg/l) than in those of the controls after 29/31 days. In contrast to the findings of De Smet & Blust (2001),

cadmium concentrations in blood from carp in the current trial decreased over time, possibly as detoxification systems became "triggered" and the cadmium became sequestered in the body organs.

Direct comparisons between the cadmium-accumulating ability of carp and *D. extensus* are hard to make, but have been carried out to compare with the findings of other authors who have drawn comparisons between metal accumulation by hosts and their parasites. The results from this current trial show that control *D. extensus* appeared to accumulate more cadmium than is found in the control carp gills. As with acanthocephalans capable of bioaccumulating cadmium to levels above that found in their hosts (Sures & Taraschewski, 1995), it is possible that *D. extensus* can also bioaccumulate cadmium to a level greater than that found in the carp.

With the exception of Experiment C, where the cadmium level in the gills was considerably higher than that in the gills of carp from the other experiments, parasites exposed to 5μg/l cadmium for 9 days also had greater body burdens of cadmium than levels recorded in the gills of the cadmium-exposed carp.

However, the 5μg/l cadmium-exposed parasites had less cadmium than the carp gills by day 29, as did the 30μg/l parasites at day 9. It is possible that, at increasing exposure durations and concentrations, the ability of carp to detoxify and sequester cadmium exceeds that of the parasite, resulting in the fish tissues having greater burdens of cadmium than the parasites. A further hypothesis is that the accumulation of cadmium by carp is much more rapid than that of the parasites, so

changes in the concentration of cadmium in the gills are more obvious between samples.

In conclusion, the ability of dactylogyrids to accumulate heavy metals is in its infancy due to the constraints of the small size of the parasites and the difficulties in collecting large enough numbers of them for analysis. However, ectoparasites, being in direct contact with both the surrounding environment and the fish host, provide an interesting model for further research. From the work in the current trial, it is apparent that there are several avenues for future research, in addition to those already mentioned. Firstly, it would be interesting to investigate the uptake of cadmium by both uninfected and infected carp tissues to determine whether the presence of dactylogyrids impacts on the final concentrations of metal in the host. Despite the accumulation of cadmium in both uninfected and infected carp being analysed in the current trial, the results cannot be compared due to differences in fish size and age and thus possible differences in their abilities to process and store cadmium. However, such a trial would allow a whole range of elements to be determined in carp and parasites, by ICP-MS analysis, and would show whether competition for elements was occurring between host and parasite. Secondly, as previously stated, a similar experiment run with a different monogenean-host system, where the parasites are larger would be useful and may solve some of the problems associated with collecting sufficient material for a large number of replicate samples to be analysed.