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

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By

Phoebe Kate Louise Carter

Institute of Aquaculture
University of Stirling
Stirling
FK9 4LA

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Chapter 6

The impact of cadmium on the population dynamics of *Gyrodactylus* spp.

6.1. Introduction

Gyrodactylids are viviparous monogeneans, ectoparasitic on the skin and gills of teleost fish. The parasite has a direct life-cycle with the offspring attaching directly to the host and containing a well-developed embryo *in utero*. As many as three generations of embryos, at various developmental stages, can be present within one individual gyrodactylid and the parent is able to give birth to its offspring within one day of its own birth (Scott, 1982), offering the potential for rapid population growth. This rapid population growth of gyrodactylids, coupled with the ease at which they can be maintained in the laboratory, means that they have been the focus of several experimental studies on both short and long term population dynamics (Scott, 1982; Scott & Anderson, 1984; Scott & Robinson, 1984; Richards & Chubb, 1998), on their reproductive biology (Harris, 1989; Harris, Jansen & Bakke, 1994, Cable & Harris, 2002) and on host susceptibility (Madhavi & Anderson, 1985; Bakke, Jensen & Kennedy, 1991).

Being ectoparasitic, gyrodactylids are in direct contact with both their host and the surrounding environment, and comparisons of their population size on fish from polluted and "clean" areas were used by Koskivaara (1992) to provide information on the effect of adverse environmental conditions on the host. Hemmingsen & MacKenzie (2001) stated that as a general rule, the number of ectoparasites on fish increases with increasing levels of pollution. The review of the literature that

follows concerns studies that frequently deal with several parasite species; however, for the purpose of this review, unless otherwise stated, specific focus will be given to the gyrodactylids. Literature referring specifically to heavy metals and gyrodactylids is scarce and thus the effect of a variety of polluting agents on these parasites will be discussed.

The effects of water-soluble fractions of crude oil on *Gyrodactylus* sp. parasitising the gills of cod (*Gadus morhua* L.) have been recorded by Khan & Kiceniuk (1988). After experimentally exposing *G. morhua* to 30, 80 and 500ppb crude oil extracts, they found no significant difference in the mean number of gyrodactylids compared to the control fish. However, some of the *G. morhua* exposed to the crude oil fractions were retained in unpolluted water for a further 16 weeks and, following this period, all the fish were infected with *Gyrodactylus* sp. compared to only 45% of the control fish. The authors suggested that the oil had caused branchial hyperplasia in the *G. morhua* gills, resulting in a habitat conducive to gyrodactylid survival and reproduction. While branchial hyperplasia may have led to the increased levels of gyrodactylids, they made no reference to the possibility that alterations to host defence mechanisms, caused by the oil fractions, could also have led to the higher level of parasitisation. Skinner (1982), on the other hand, suggested that the increased numbers of monogenean gill parasites (*Neodiplectanum wenningeri* Mizelle & Blatz, 1941 (now *Diplectanum wenningeri*), *Ancyrocephalus parvus* Linton, 1940 and *Ancyrocephalus* sp.) which he noted on fish taken from Biscayne Bay, Florida, polluted with ammonia, trace metals and pesticides, was due to gill damage that resulted in a stress response and the subsequent weakening of the hosts' immune responses. A slight increase in the number of *Gyrodactylus* sp. above

those levels observed on control fish has also been recorded on roach (*Rutilus rutilus* L.) exposed to bleached kraft mill effluent (BKME) from pulp and paper mills in Finland (Bagge & Valtonen, 1996). Khan & Thulin (1991), reviewing the work of Bagge & Valtonen (1996), postulated that the slightly elevated number of gyrodactylids could have been caused by the BKME weakening the immunological responses of the fish.

Marcogliese, Nagler & Cyr (1998) and Moles & Wade (2001) have carried out more recent studies involving the exposure of fish to oil and the subsequent determination of their parasite intensities. Exposing *Hippoglossoides platessoides* (Fabricius) to sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) resulted in a greater abundance of *Gyrodactylus* sp. compared to abundances on fish from the reference site (Marcogliese *et al.*, 1998). Moles & Wade (2001) exposed the sand lance (*Ammodytes hexapterus* Pallas) to crude oil-laden sediments and determined the intensity of parasite infection alongside an assessment of the host's phagocytic function. The results from this trial were interesting, with the highest level of oil contamination (61μg/g total hydrocarbons) resulting in statistically reduced phagocytosis and superoxide production by kidney phagocytes, which was linked with the highest mean abundance of *Gyrodactylus* sp. In contrast, lower levels of hydrocarbons (28μg/g total hydrocarbons) significantly increased phagocytosis and superoxide production with a resulting drop in the mean abundance of gyrodactylids to a level below that of the controls (Moles & Wade, 2001).

A study by El-Naggar, Hagras, Ogawa, Hussien & El-Naggar (2000) looked at the monogeneans of *Oreochromis niloticus* (L.) and *Tilapia zilli* (Gervais) in waters polluted with the heavy metals Cd^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+} and Cr^{2+}. The concentrations of all heavy metals were below the permitted values. El-Naggar *et al.* (2000) recorded a positive correlation between the mean intensity of *Gyrodactylus cichlidarum* Paperna, 1968 on both fish species and the concentration of iron in the water column, while the concentration of zinc negatively correlated to the mean intensity of *Gyrodactylus cichlidarum*.

The study by El-Naggar *et al.* (2000) is the only field investigation to specifically define the type (although not exact concentrations) of heavy metal pollutants found at the sample sites from where the fish were removed for parasitological assessment. Zharikova (1993) stated that, in the polluted area of the Volga River where she was studying, there were elevated levels of heavy metals, but, with the exception of copper, found at 1-5 times greater concentration than the maximum allowed concentration, she did not list the type or concentration of the metals present. However, in this area of the Volga River, also contaminated by hydrocarbons, the numbers of *G. elegans* Nordmann, 1832 were lower when compared to the numbers on fish from the non-polluted reference site (Zharikova, 1993). Another feature of note from the study by Zharikova (1993) was the finding that some specimens of *Diplozoon paradoxum* von Nordmann, 1932, from the polluted section of the Volga River, were found with morphological abnormalities to their attachment clamps. Instead of the normal 4:4 ratio of clamps, *D. paradoxum* were found with ratios of 4:2, 4:3 and 3:2. Kuperman (1992) also observed

abnormalities in the clamps of *D. paradoxum*, with ratios of 4:3, 4:1, 4:0 and 3:3 being recorded.

As with the studies detailed above, Gelnar and co-workers did not state the exact type or concentration of pollution present in the River Morava. For example, Gelnar, Koubková, Plášková & Jurajda (1994) stated that the river is a suitable area to explore the relationships between pollution and parasitism, as it is often seriously affected by human industrial and agricultural activities, while Gelnar, Šebelová, Dušek, Koubková, Jurajda & Zahrádková, (1997) and Dušek, Gelnar & Sebelova (1998) stated that the pollution is organic, originating from industrial and municipal waste waters. Whatever the exact type of pollution, Gelnar *et al.* (1994) found that in polluted areas 72% of the metazoan parasite species were monogeneans compared to only 53% in the unpolluted areas of the river. These figures were based on 135 metazoan parasite species found over a four-year period on 30 fish species (n = 404). Dušek *et al.* (1998) looked specifically at the gyrodactylid community of chub (*Leuciscus cephalus* L.), and pollution appeared to have little impact on the community structure, with an equal distribution of gyrodactylid species found on fish from both polluted and unpolluted sites. The authors suggested that the gyrodactylid community was relatively resistant to the effect of pollution as a result of the high reproductive potential, direct life-cycle and short life-span of the parasites (Dušek *et al.*, 1998).

The studies detailed above have all been concerned with using the number of gyrodactylids and other parasites to assess the pollution status of particular areas and the impact of the pollutants on host health. One of the most interesting studies

concerning gyrodactylids and metals comes from the completely different perspective of using aluminium sulphate as a chemotherapeutant against infections of *Gyrodactylus salaris* Malmberg, 1957 (Soleng, Poltto, Alstad, & Bakke, 1999). Atlantic salmon (*Salmo salar* L.) infected with *G. salaris* were exposed to 50, 90 and 200μg/l aluminium sulphate at a variety of pH levels. Irrespective of water pH, the number of *G. salaris* fell as the concentration of aluminium increased, suggesting that the metal may act as an effective chemotheraputant (Soleng *et al.*, 1999). The authors commented that the study did not investigate whether the rapid elimination of the gyrodactylids from the host was the result of the direct effect of the aluminium on the parasite or whether it was due to an indirect effect of the aluminium acting on the host. However, it was concluded that, whatever the mode of elimination, the gyrodactylids were unable to survive exposure to aluminium as detached parasites (from salmon exposed to 52 +μg/l aluminium) were torpid and failed to resume normal activity after being transferred to optimal water conditions

6.1.1. Aims of the present study

Poulin (1992) stated that it is impossible in field studies to isolate and quantify the effects of an individual pollutant and that any differences arising in parasite number between polluted and unpolluted areas could be due to a range of other biological and chemical factors that differ between the two areas. With this is mind, considering the degree of contradictory evidence that exists regarding the impact of pollutants on gyrodactylid populations and the paucity of information pertaining to heavy metals, the aim of this chapter was to determine the effect of a single heavy metal, cadmium, on the population dynamics of *Gyrodactylus* spp. under controlled experimental conditions. As a baseline, the maximum permissible level of cadmium

(5μg/l) was used and, due to the ease of maintaining both fish and parasite in the laboratory and the high reproductive potential of the parasite, the *Poecilia reticulata-Gyrodactylus* spp. model system was chosen. In light of the findings of apparent pollutant-induced abnormalities in *D. paradoxum* by Kuperman (1992) and Zharikova (1993), a further aim of this chapter was to determine whether prolonged exposure to 5μg/l cadmium would induce morphological abnormalities to the attachment complex (opisthaptor) of *G. turnbulli*.

6.2. Materials and methods

6.2.1. Experiment 1 - The impact of 5µg/l cadmium on the population growth of *Gyrodactylus bullatarudis* parasitising female guppies (*Poecilia reticulata*)

6.2.1.1. Source and maintenance of guppies

Eighty female guppies (ca. 4 cm) were purchased from a commercial aquarist in the east of Scotland. Only female guppies were selected from the aquarist, as on the date of collection several mortalities were observed in the male stock tanks, and, in light of this, it was decided that it was unwise to purchase these fish. The guppies were maintained for 1 week prior to the start of the trial, in well aerated, 20 litre static tanks heated to 23°C with a Visi-therm aquarium heater (100W) and were fed Aquarian Tropical Fish Flakes® daily. Fifty percent of the water in the tank was changed daily. Heavily pregnant females were separated from the other fish and were not used in the trials.

On arrival from the aquarist, 5 fish were examined under a dissecting microscope to determine if they were parasitised by gyrodactylids or other parasite species. The method for examining the fish followed that of Harris (1986b) (detailed in section 6.2.1.6). The fish were found to be parasite-free, but, as a precaution, 7 days prior to starting the trial the fish were treated with 100ppm of formalin for 45 min. Before adding fish to the experimental system, a further 5 fish were screened and found to be free from parasitic infection.

6.2.1.2. Establishing and maintaining a stock population of *G. bullatarudis*.

Two male guppies were brought from a local pet shop and, on examination of their ectoparasite fauna, were found to be parasitised by *G. bullatarudis*. The guppies were placed in a small, lidded, plastic aquarium (internal dimensions 21.1 × 13.4 cm) containing 2 litres of water maintained at 23°C using a Visi-therm (40W) aquarium heater. Naïve guppies, from an in-house bred stock were added regularly (2 guppies/week) to the tank containing the 2 infected guppies, to ensure maintenance of the gyrodactylid population by transmission to the uninfected individuals. Fifty percent of the water was changed in each tank daily using fresh, dechlorinated water heated to 23°C.

6.2.1.3. Infecting the experimental guppies

Twenty-one, formalin-treated guppies were divided equally between 7 different 250 ml circular, glass containers (3 fish/container) filled with 250 ml of dechlorinated water. The glass containers were placed in a large plastic tank (internal dimensions 70 × 52 × 45 cm) filled with water to a depth of 4 cm. The water was heated to 23°C using a Visi-therm (100W) heater. To start the infections in each experimental tank, 3 guppies, each infected with 3 gyrodactylids, were to be used. Thus, setting up 7 glass containers ensured that, in case of any mortalities or in the event of any guppy not becoming infected, there would be spare fish from which to choose.

A small hole was drilled through the plastic lid of each glass container and a length of airline tubing passed through. The aeration to each pot was maintained at a low level ensuring that the fish were not buffeted against the sides of the pots.

Into each 250 ml container, 1 infected guppy (see section 6.2.1.2) was added and all guppies were then left for 24 h. The small confines of the containers ensured that parasite transmission from the infected to the naïve guppies was likely.

After 24 h the guppies were examined (see section 6.2.1.6 for methodology) and the number of parasites present on each fish counted.

6.2.1.4. Experimental tanks

Three control tanks and 3 cadmium-exposed tanks were chosen at random from the 12 polyethylene tanks (35 × 28 cm internally) of the flow through system in the tropical aquarium (ambient temperature 20-24°C during the winter) (see Figure 2.1, Chapter 2, General materials and methods). Into each of these tanks, 1 small plastic tank (internal dimensions of 21.1 × 13.4 cm) was placed.

The small tanks were filled with exactly 2 litres of ambient, dechlorinated water and aerated with a 1" air-stone fitted with a lid to prevent any guppies escaping. The tanks were labelled as control (C1-C3) or test (T1-T3).

6.2.1.5. Starting the trial

Due to time constraints, all guppies used for this trial were moved to the experimental system on the day the trial started. However, they had been acclimated to the experimental temperature for 1 week prior to the start of the trial. All guppies were fed to satiation daily.

Each replicate pair of tanks (*i.e.* one control and one test) was started a day apart. Thus, the numbers of fish to be sampled on each day were minimised, ensuring that all fish were given adequate time for sampling and all parasite counts were as accurate as possible.

Guppies infected with gyrodactylids (section 6.2.1.3) were examined as detailed in section 6.2.1.6 and their parasite burden determined. A total of 9 parasites were required for each experimental tank using 3 guppies each infected with 3 parasites (as in section 6.2.1.3). In some cases, however, where guppies were infected with fewer than 3 parasites, more guppies infected with fewer parasites were added to some of the tanks. The total number of guppies in each tank was made up to 10 using the formalin-treated parasite free fish.

Thus, in control and test tank 1 (C1, T1), there were 3 parasitised guppies and 7 unparasitised guppies; in C2, T2 there were 4 parasitised guppies and 6 unparasitised guppies; in C3 there were 2 parasitised and 8 unparasitised guppies; and in T3 there were 3 parasitised and 7 unparasitised guppies.

Two millitres of a 5000μg/l cadmium sulphate stock solution was added to the test tanks at the start of the trial to give a nominal concentration of 5μg/l cadmium.

Complete water changes were made to each tank daily. The guppies were carefully removed from the tanks and placed in a 1 litre plastic beaker containing water from the tanks from which the fish had been removed. Separate beakers were used for control and test tanks to avoid contaminating the control tanks with cadmium. Two litres of fresh, dechlorinated water was then added to each tank and cadmium sulphate stock solution added to the test tanks. The guppies were then returned to the tanks.

6.2.1.6. Sampling regime

All guppies in each tank were sampled at 5-day intervals (*i.e.* days 5, 10, 15, 20, 25 post-start) following Harris' (1986b) protocol. A 5 cm Petri dish was tilted on its side and fixed to the lid of the dish using Blu Tack®. Harris (1986b) held the Petri dish rather than fixing it in place as detailed above. A small volume of water, sufficient for the guppy to remain in an upright position, was placed in the tilted Petri dish and the guppy added to this water. The dish was then placed under a dissecting microscope and the fish examined at ×3 magnification. Extra light was provided by a gooseneck fibre optic lamp (Olympus Highlight 3000).

Each guppy was examined along both sides and the parasites present in each area of the body counted. The number of parasites on the head (incorporating the eyes, mouth and opercula), the fins (dorsal, pectoral/pelvic, caudal and anal), the caudal peduncle and the body surface (*i.e.* all areas other than those listed *e.g.* flanks, dorsal surface) were

recorded. The fins were examined by carefully manipulating them with a blunt seeker, allowing both sides to be observed.

Any parasites present on the ventral surface of the fish were not counted during sampling, as this involved moving the petri dish in such a manner that the fish became very stressed. By excluding the ventral surface of the fish in counts, the time each fish had to spend in a small volume of water was minimised.

At the end of the trial, the guppies were killed by concussion, followed by severing the spinal nerve cord and pithing the brain. This process was carried out in a water-filled, 5 cm Petri dish so that any parasites that had been situated on the head or close to where the nerve cord was severed would be collected in the dish and could be counted. Some guppies were removed from the experimental tanks and killed during the trial when the parasite burden became so great that it impaired normal swimming. When this occurred, the total number of gyrodactylids present on the guppies at this point was recorded. Guppies that were killed at 2 days or less prior to the next sample point had their parasite data incorporated with that of the forthcoming sample.

At the end of the experiment the gyrodactylid species was confirmed by comparing specimens prepared using ammonium picrate gylcerine with the descriptions and drawings of Harris (1986a). Sixty individual parasites were examined in total and were selected at random from 3 guppies in each tank. All specimens were found to be *G. bullatarudis*.

6.2.2. Experiment 2 - The impact of 5µg/l cadmium on the population growth of *Gyrodactylus turnbulli* Harris, 1986 parasitising both male and female guppies

6.2.2.1 Source and maintenance of guppies

Batches of male and female guppies (1-4 cm) were procured from a commercial aquarist in the east of Scotland and maintained in 20 litre static tanks at 22-24°C. Fifty percent of the water in each tank was changed daily from a source of heated, dechlorinated water. Fish were fed daily (*ad libitum*) on Aquarian Tropical Fish Flakes®.

At both 10 and 7 days prior to the start of the trial, the fish were treated with formalin at 100ppm for 45 min. The formalin treatment was to ensure that there were no gyrodactylids present on the guppies so that experimental infections only would be present and monitored. After the second treatment, 5 guppies were lightly anaesthetised with 0.02% tricaine methanesulfonate MS222 (Sigma Aldrich) and examined under a dissecting microscope and were found to be free from ectoparasites.

6.2.2.2. Establishing and maintaining a stock population of *G. turnbulli*

In this trial, a single species population of *G. turnbulli* was established from a single parasite individual. An infected guppy was killed and kept in a 5 cm Petri dish of water for 30 min, as this was found to facilitate the transfer of gyrodactylids to the uninfected naïve fish. A scale supporting a single worm was removed from the infected fish and held gently against the anal fins of a naïve, anaesthetised fish. The scale was held in place until the parasite moved onto and attached to the new host.

The newly infected fish was placed in a small plastic tank (internal dimensions of 21.1 × 13.4 cm) containing 2 litres of water maintained at 22-24°C. A naïve fish was then screened under light anaesthesia to determine whether it was parasite-free and was then added to the tank to allow the 2 fish to mix in anticipation that the gyrodactylid infection would establish and transmit naturally to all fish within the tank. The infection was monitored regularly with further naïve fish added to the tank every 10 days or when an infected fish was removed due to a high gyrodactylid burden.

Individual parasites were randomly selected from guppies in the tank and mounted on slides in ammonium picrate glycerine to confirm that a single species infection had been established. The marginal hooks and hamuli of the parasites were then studied at ×100 and the species compared to the descriptions and drawings of Harris (1986a). All parasite populations in the trial were seeded from this single species infection of *G. turnbulli*.

6.2.2.3. Infecting the experimental guppies

Two fish per experimental tank were infected with 3 parasites each to seed each individual tank population. The infection of the guppies followed the procedure detailed in section 6.2.2.2. When 3 parasites had moved from the scale onto the anal fins of the living fish and were attached, the fish was placed into clean, aerated water and allowed to recover. The procedure was repeated with the second guppy and, once both were infected and had recovered, they were transferred to the experimental tanks.

6.2.2.4. Experimental tanks.

For each replicate, 2 small tanks measuring 21.1 × 13.4 cm were placed inside a larger tank measuring 35 × 28 cm (see Fig. 2.1). Three replicate tanks were set up, one control and one test tank in each, separated by plastic sheeting to prevent contamination of the control tanks with cadmium from the test tank. The small static tanks were filled with 3 litres of dechlorinated water and aerated using a 1" air stone. The replicate tanks in this trial were placed in the same large tank (see Fig. 2.1) to reduce the chance of differences in temperature that had occurred in Experiment 1 when individual small tanks were placed in separate large tanks.

Three days prior to the start of the experiment, 13 formalin-treated guppies were randomly placed into each tank to acclimate. Based on the numbers of each sex available, a male to female ratio of 9:6 was chosen for each tank. However, after random allocation of fish to each tank, control tank 2 (C2) and test tank 3 (T3) had a male to female ratio of 10:5, while control tank 3 (C3) had a ratio of 11:4. Half of all fish in each tank were individually identifiable by their colour pattern, allowing infections to be tracked on individuals over the course of the experiment.

6.2.2.5. Starting the trial

Replicate pairs of tanks were started on 3 consecutive days to allow for the limitation that only 30 fish could be screened in a day. On day 1 of the trial, the 2 infected fish (section 6.2.2.3) were added to the tanks and left for 1 h to acclimate. After 1 h, the

cadmium exposure experiment was started by adding 3 ml of a 5,000μg/l cadmium sulphate stock solution to the test tank, giving a nominal concentration of 5μg/l.

Complete water changes were made in each tank daily. Fish were carefully netted from the tank and placed in a 1 litre beaker containing 500 ml of the tank water from which they had been removed. The remaining water in the tank was either used for water quality analysis or was disposed of. Three litres of fresh, dechlorinated water were then measured into the tanks using a 1 litre plastic measuring cylinder. Cadmium sulphate stock solution was added to the test tanks and thoroughly mixed before the fish were replaced. Separate beakers and nets were used for control and test tanks to avoid any contamination of the control fish with cadmium.

6.2.2.6. Sampling regime

During the trial, each tank of fish was screened at 6-day intervals for a total period of 60 days. A 100 ml volume of 0.02% MS222 anaesthetic was made up in a small beaker using the water from each tank being sampled. This ensured that the parasites were not subjected to a change in pH, which preliminary trials (not presented) had shown to result in high parasite mortalities. A recovery tank was set up and half-filled (1.5 litre) with the water taken from the tank that was being sampled. The recovery tanks were well aerated with a 1" airstone.

Individual fish were placed in the anaesthetic solution and removed as soon as they showed reduced mobility (reduced swimming speed and unbalanced swimming). The

fish were then transferred to a 5 cm Petri dish containing a small volume of the anaesthetic solution. Fish were screened at ×3 using an Olympus dissecting microscope with additional lighting provided from a gooseneck fibre-optic lamp (Olympus Highlight 3000). The number of parasites present and their position on the guppy were counted and recorded at each sample. The fish were carefully turned over during the screening process to ensure that all parasites present were counted, and the number of parasites on the head, pectoral fins, dorsal fin, caudal fin, ventral fins (anal and pelvic), caudal peduncle, ventral surface and the dorsal body surface (*i.e.* all areas other than those listed) were recorded. Anaesthetising the guppies ensured that the ventral surface of the guppies could be thoroughly examined. During the second sample (day 10), the body (nose to caudal peduncle) and tail length of each guppy was measured and recorded.

Following screening, the fish were placed in the recovery tank and monitored until normal swimming was resumed. The bottom of the beaker containing the anaesthetic was screened after each fish had been anaesthetised and any detached parasites counted and recorded.

During the experiment it became necessary to remove some guppies from the tanks when their parasite load impaired their normal swimming ability. The number of parasites on these guppies was counted and the fish were then killed by an overdose of anaesthetic.

6.2.3. Statistical analysis

Raw data from both Experiments 1 and 2 (6.2.1 and 6.2.2) were analysed for normality and homogeneity of variance. Both data-sets were neither normally distributed nor homogenous as a result of aggregation (overdispersion) with regard to the parasite population. Log-transformation was thus used to normalise the data and make the variance independent of the mean.

6.2.4. Experiment 3 - The impact of 5μg/l cadmium on the morphology of the *G. turnbulli* attachment complex (opisthaptor)

6.2.4.1. Source of guppies

The guppies used in the morphometrics trial were derived from an in-house bred population and routine screening showed them to be gyrodactylid free before the start of the trial.

6.2.4.2. Experimental tanks

Two small, plastic aquaria (21.1 × 13.4cm) were placed adjacent to each other in a 20 litre plastic tank. Four litres of water was added to the large 20 litre tank and a Visi-therm heater (100W) set at 21°C added. The water level in the 20 litre tank was checked daily and topped up when necessary to prevent evaporation. Each of the 2 small aquaria was filled with 2 litres of fresh dechlorinated water heated to 21°C. One tank was a control and the other, its test replicate, was exposed to 5μg/l cadmium. The water in both tanks was replaced daily.

6.2.4.3. Experimental fish

Four naïve guppies were selected at random from the population bred in-house and were lightly anaesthetised (0.02% MS222) and examined at ×3 using a dissecting microscope to verify that they were free from gyrodactylid infection. Each guppy was then infected with 1 individual *G. turnbulli* (as in section 6.2.2.3) taken from the population established as in section 6.2.2.2. Two fish were then placed into each of the 2 small aquaria (see section 6.2.3.2) and left to acclimate for 3 days.

6.2.4.4. Starting the trial

After 3 days, the 2 fish from each experimental tank were lightly anaesthetised using MS222 and examined under a dissecting microscope to ensure that the *G. turnbulli* infection had become established. Once the infection status was determined the trial was started by the addition an appropriate volume of cadmium sulphate stock solution to the test tank giving a nominal concentration of 5μg/l.

Naïve guppies (checked and found to gyrodactylid free) were added to both tanks every 10 days and each time a fish was removed and sacrificed to obtain parasites. Addition of naïve fish to the tanks ensured maintenance of the parasite population.

Complete water changes were made to each tank daily and cadmium sulphate stock solution was added to the test tank after each water change to maintain the 5μg/l exposure concentration.

6.2.4.5. Sampling regime

At 2, 4 and 6 weeks post-cadmium exposure, 1 fish from the control tank and 1 from the 5μg/l cadmium-exposed tank was removed and killed by overdose of anaesthetic. Individual gyrodactylids (n = 15) were removed from the fish using fine pointed mounted needles and were placed on slides. Small, glass coverslips were placed on top of each specimen and a small volume of ammonium picrate glycerine fed under the coverslip, acting as a fixative.

The gyrodactylid specimens were examined at ×100 and their species checked against the descriptions in Harris (1986a). All specimens were identified as *G. turnbulli*. Image analysis was used to take 10 point-to-point measurements from a hamulus, a marginal hook and the ventral bar of 10 control parasites from 2 weeks post-start of the trial, and 10 test parasites from 6 weeks post-start of the trial (see Figure 6.1). The image analysis programme used was KS300 ver. 3.0 (1997 Carl Zeiss Vision GmbH; Imaging Associates) and was linked to a JVC KY-F3OB 3-CDD camera, interfacing on an Olympus BH2 compound microscope.

Based on univariate statistics, there were no statistical differences to separate the control and cadmium-exposed *G. turnbulli* hooks under study, and it was therefore necessary to consider all the morphological variables simultaneously. This was achieved by Principal Component Analysis (PCA). The method for PCA follows that detailed in Shinn *et al.* (1996) using Statistica ver. 6.0 (Statsoft Inc 1984-2001, Tulsa,

USA). The surface area of both hamuli from each specimen was also calculated using image analysis and the data compared using a parametric unpaired t-test.

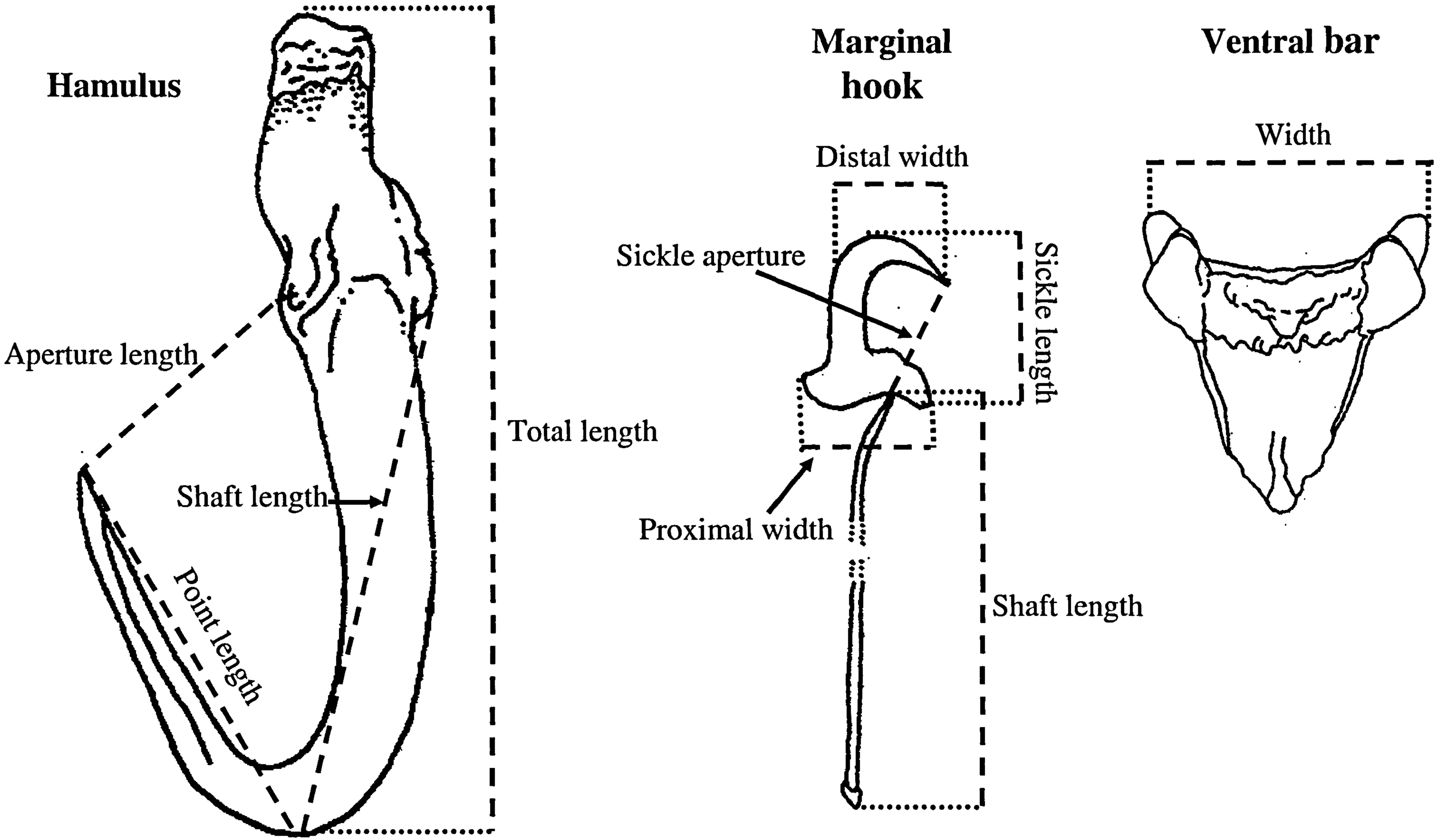


Fig. 6.1. Schematic illustration of the morphometric features measured on *Gyrodactylus turnbulli*. Diagrams adapted from Shinn (unpublished).

6.3. Results

6.3.1. Water quality

Table 6.1. Summary of the water quality data from Experiments 1-3 giving mean values ± S.E. (n = number of water samples analysed). Significant differences are shown in bold.

Experiment 1	**Controls**			**5µg/l cadmium**			**Statistical significance P**
	C1	**C2**	**C3**	**T1**	**T2**	**T3**	
Cadmium concentration (µg/l)	0.08 ± 0.05 (n=5)	0.04 ± 0.003 (n=6)	0.05 ± 0.02 (n=6)	4.47 ± 0.19 (n=5)	4.24 ± 0.43 (n=9)	4.07 ± 0.19 (n=5)	Controls = 0.91 5µg/l = 0.43
Temperature (°C)	23.0 ± 0.15 (n=20)	23.5 ± 0.08 (n=30)	23.9 ± 0.08 (n=20)	23.5 ± 0.15 (n=20)	23.7 ± 0.06 (n=30)	23.8 ± 0.09 (n=17)	**0.0001** C3,T2,T3 > C1, C2
Alkalinity (meq/l)	0.6 ± 0.01 (n=4)	0.5 ± 0.002 (n=5)	0.5 ± 0.002 (n=5)	0.5 ± 0.005 (n=3)	0.5 ± 0.003 (n=6)	0.6 ± 0.009 (n=3)	0.78
pH	7.6 ± 0.09 (n=5)	7.6 ± 0.11 (n=6)	7.7 ± 0.11 (n=5)	7.5 ± 0.09 (n=4)	7.6 ± 0.15 (n=6)	7.9 ± 0.03 (n=3)	0.26
Total hardness (ppm $CaCO_3$)	37.0 ± 4.34 (n=5)	31.3 ± 1.11 (n=6)	36.0 ± 1.12 (n=5)	30.8 ± 2.20 (n=4)	32.1 ± 1.18 (n=7)	33.7 ± 1.76 (n=3)	**0.03** C3 > C2
Dissolved oxygen (mg/l)	7.61 ± 0.09 (n=3)	7.64 ± 0.11 (n=3)	7.87 ± 0.19 (n=3)	7.87 ± 0.19 (n=3)	8.38 ± 0.09 (n=3)	8.13 ± 0.20 (n=3)	Too few data
Experiment 2	**C1**	**C2**	**C3**	**T1**	**T2**	**T3**	**Statistical significance P**
Cadmium concentration (µg/l) (n=14)	0.31 ± 0.16	0.34 ± 0.23	0.52 ± 0.23	4.51 ± 0.33	4.43 ± 0.30	4.52 ± 0.30	Controls = 0.75 Tests = 0.98
Temperature (°C) (n=39)	21.5 ± 0.10	21.5 ± 0.10	21.4 ± 0.10	21.5 ± 0.10	21.5 ± 0.10	21.5 ± 0.10	0.99
Alkalinity (meq/l) (n=8)	0.4 ± 0.005	0.5 ± 0.008	0.4 ± 0.006	0.4 ± 0.006	0.5 ± 0.007	0.8 ± 0.04	0.58
pH (n=8)	6.95 ± 0.09	6.92 ± 0.10	7.00 ± 0.10	6.99 ± 0.10	6.98 ± 0.09	7.00 ± 0.08	0.99
Total hardness (ppm $CaCO_3$) (n=8)	30.6 ± 2.46	33.0 ± 3.07	33.3 ± 2.76	33.3 ± 2.93	34.3 ± 3.06	32.1 ± 2.75	0.96
Dissolved oxygen (mg/l) (n=3)	8.47 ± 0.09	8.42 ± 0.18	8.50 ± 0.15	8.43 ± 0.22	8.53 ± 0.23	8.47 ± 0.24	Too few data
Experiment 3	**Controls**			**5µg/l cadmium**			**Statistical significance P**
Cadmium concentration (µg/l) (n=4)	1.47 ± 0.83			7.13 ± 0.80			Too few values
Temperature (°C) (n=24)	21.3 ± 0.24			21.5 ± 0.17			0.45
Alkalinity (meq/l) (n=3)	0.4 ± 0.003			0.4 ± 0.002			Too few values
pH (n=3)	7.14 ± 0.16			7.21 ± 0.13			Too few values
Total hardness (ppm $CaCO_3$) (n=3)	34.7 ± 1.33			34.0 ± 2.0			Too few values

All experimental tanks used in Experiment 1 were maintained in the same manner over the duration of the trial; however, there were statistical differences in both temperature and total hardness ($P < 0.0001$ and $P = 0.03$, respectively). The difference in water hardness was between 2 control tanks, C2 and C3. Control tank 1 (C1) had a statistically lower temperature than all tanks, except for C2 and T1. However, the temperature was controlled within approximately 1°C (23-23.9°C) throughout the experiment.

In contrast to the tanks in Experiment 1, the water quality parameters of the tanks used in Experiment 2 were consistent, with no tank being statistically different from another. The water quality data collected from Experiment 3 were too few for statistical analysis, with the exception of water temperature, which was not statistically different between the control and cadmium-exposed tank ($P = 0.45$).

The cadmium concentration in all test tanks in Experiments 1 and 2 was lower than the nominal 5μg/l, while in Experiment 3 the cadmium concentration in the test tank was greater than 5μg/l. In this same experiment (Expt. 3) the cadmium concentration of the control tank was higher than in all other experiments (1.47 ± 0.83μg/l).

6.3.2. Experiment 1 - The impact of 5µg/l cadmium on the population growth of *Gyrodactylus bullatarudis* parasitising female guppies

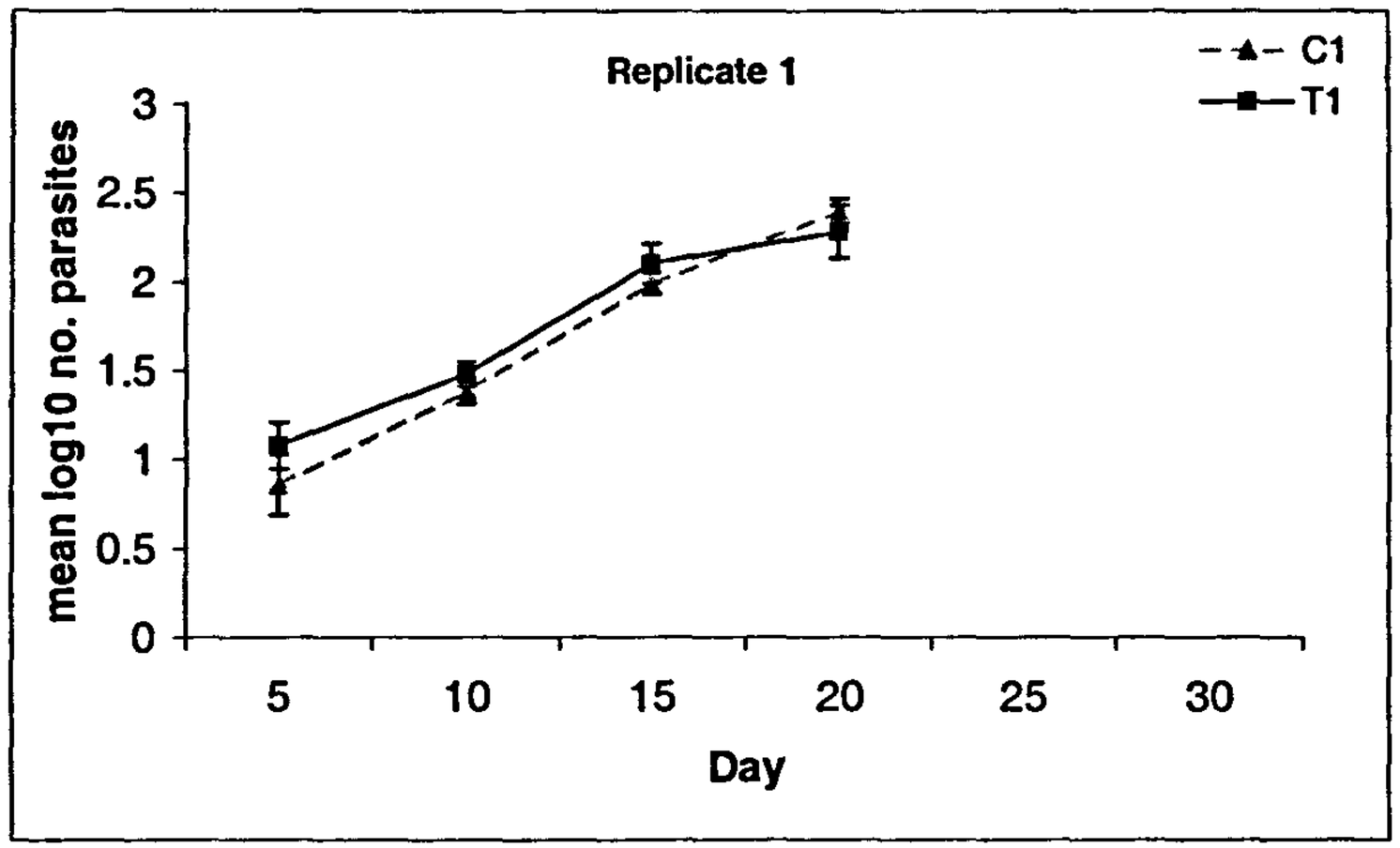
6.3.2.1. Population growth of *G. bullatarudis*

The current trial was run for a period of 20 days for replicates 1 (C1, T1) and 3 (C3, T3), after which there were no guppies remaining (see Fig. 6.6); C2, T2 were continued for 30 days.

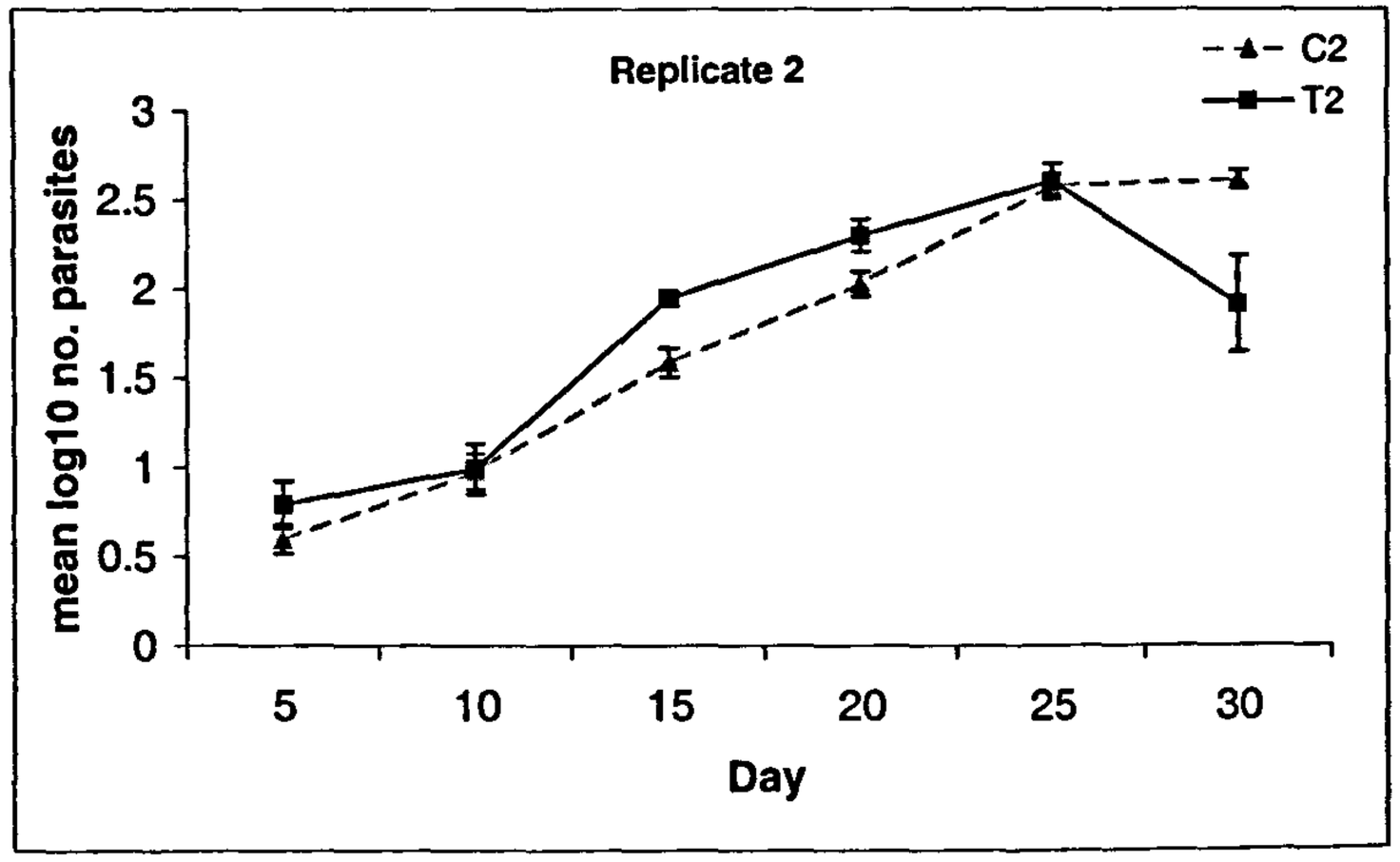
The results of monitoring a *G. bullatarudis* infection over time, on both control and cadmium-exposed guppies, showed that the mean number of parasites in both populations increased with time. The increase in the mean number of gyrodactylids on cadmium-exposed guppies was, in general, greater than that on the control guppies (Fig. 6.2a-c). However, by the end of the trial in replicate 1 (Day 20) (C1, T1) and replicate 2 (Day 30) (C2, T2), the number of gyrodactylids on the cadmium-exposed guppies had dropped below that of the controls (Fig. 6.2a-c). The temperature differences recorded between some of the tanks (see section 6.3.1) appeared to have no effect on the population growth of the parasites that was relatively consistent in all tanks.

Log-transforming the data (see section 6.2.3) resulted in a homogenous and normal distribution in all tanks, allowing data from all 3 control tanks to be pooled together and data from all 3 test tanks to be pooled. Figure 6.3 presents this pooled data, with the circled area representing data from the second pair of replicate tanks only (C2, T2), as in both other replicates there were no guppies remaining beyond day 20. At both 5 and 15 days post-start of the trial, there were statistically more gyrodactylids on the 5µg/l cadmium-exposed guppies compared to the controls (P =

a)



b)



c)

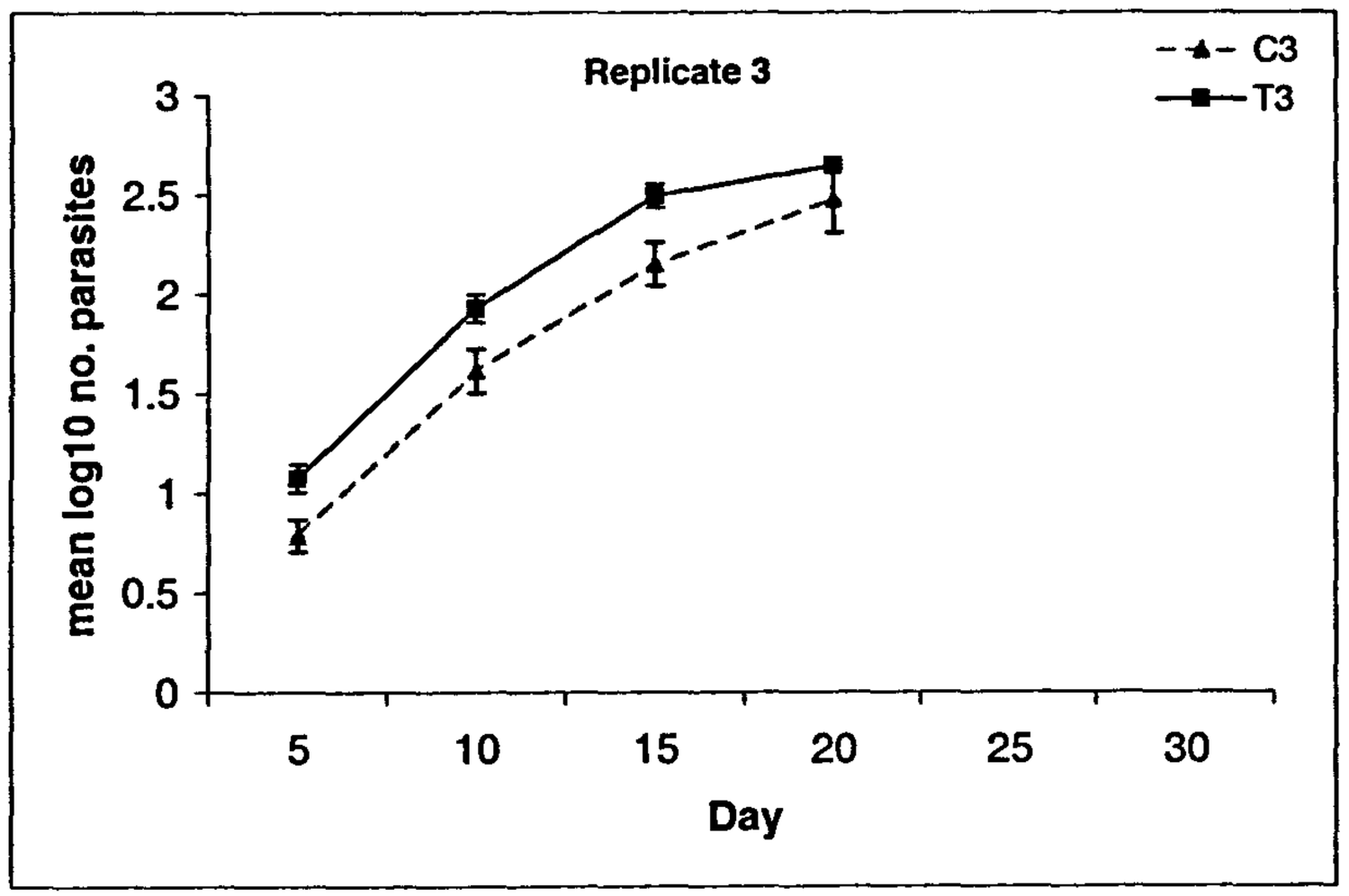


Fig. 6.2a-c. Experiment 1 - The mean (± S.E.) log transformed number of *G. bullatarudis* per guppy over time in control (C1-C3) and 5*µ*g/l cadmium-exposed (T1-T3) tanks.

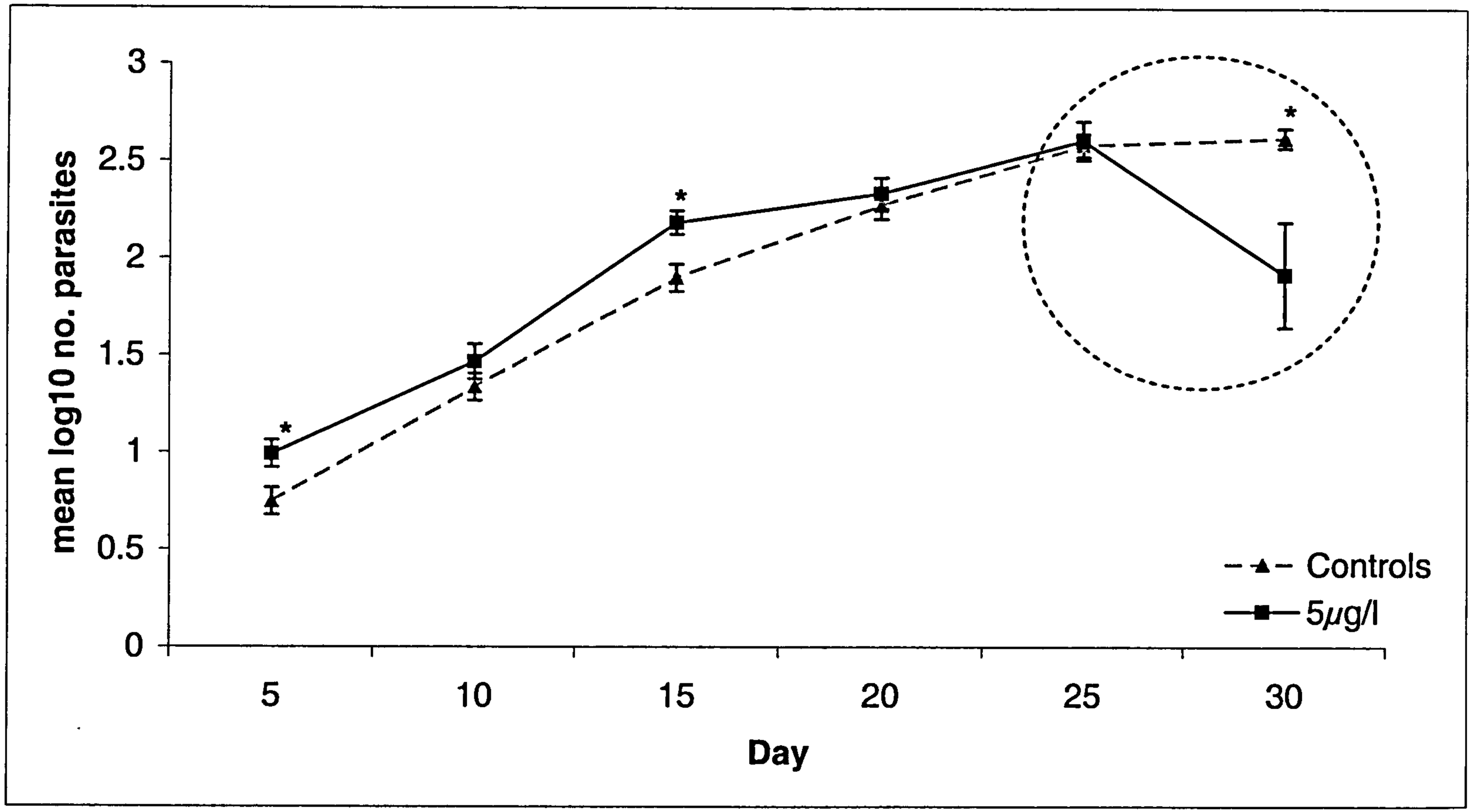


Fig. 6.3. Experiment 1 - The pooled mean (± S.E.) log transformed number of *G. bullatarudis* per guppy over time in control (C1-C3) and 5µg/l cadmium-exposed tanks (T1-T2). The circled area shows data from tanks C2 and T2 only. * Denote time points where the number of gyrodactylids was statistically different between treatments ($P<0.05$).

0.016 and P = 0.003, respectively). Comparing the mean gyrodactylid burdens on guppies from C2 and T2 at Day 30 showed that at this time the control fish harboured the statistically greater number of parasites per fish (P = 0.016) (Fig. 6.3).

6.3.2.2. The number of days to 100% parasite prevalence

As all the guppies from each tank were monitored every 5 days, it was possible to determine at which of these 5-day intervals all the fish had become infected with gyrodactylids. Figure 6.4 shows the day to 100% parasite prevalence of guppies in each tank. With the exception of T2, which reached 100% parasite prevalence on day 10 of the trial, all the other tanks had reached 100% parasite prevalence by day 5 (Fig. 6.4).

6.3.2.3. Position of the gyrodactylids on the guppies

The number of gyrodactylids present in 7 distinct regions on the guppies [*i.e.* head, incorporating the eyes, mouth and opercula), fins (dorsal, pectoral/pelvic, caudal and anal), the caudal peduncle and the body surface (*i.e.* all areas other than those listed] was recorded during the current trial and results from sample days 5-20 are presented in Figure 6.5a-d. The results presented are percentages of the total number of parasites on the guppies from all 3 control tanks (control data) or from all 3 test tanks (5μg/l cadmium data). Data from days 25 and 30 are absent, as only one pair of replicate tanks (C2, T2) still had fish, and thus these data will not be discussed here.

The pectoral fins, the caudal peduncle, the caudal fin and the body surface of the guppies were the sites where at each time point the most gyrodactylids were found

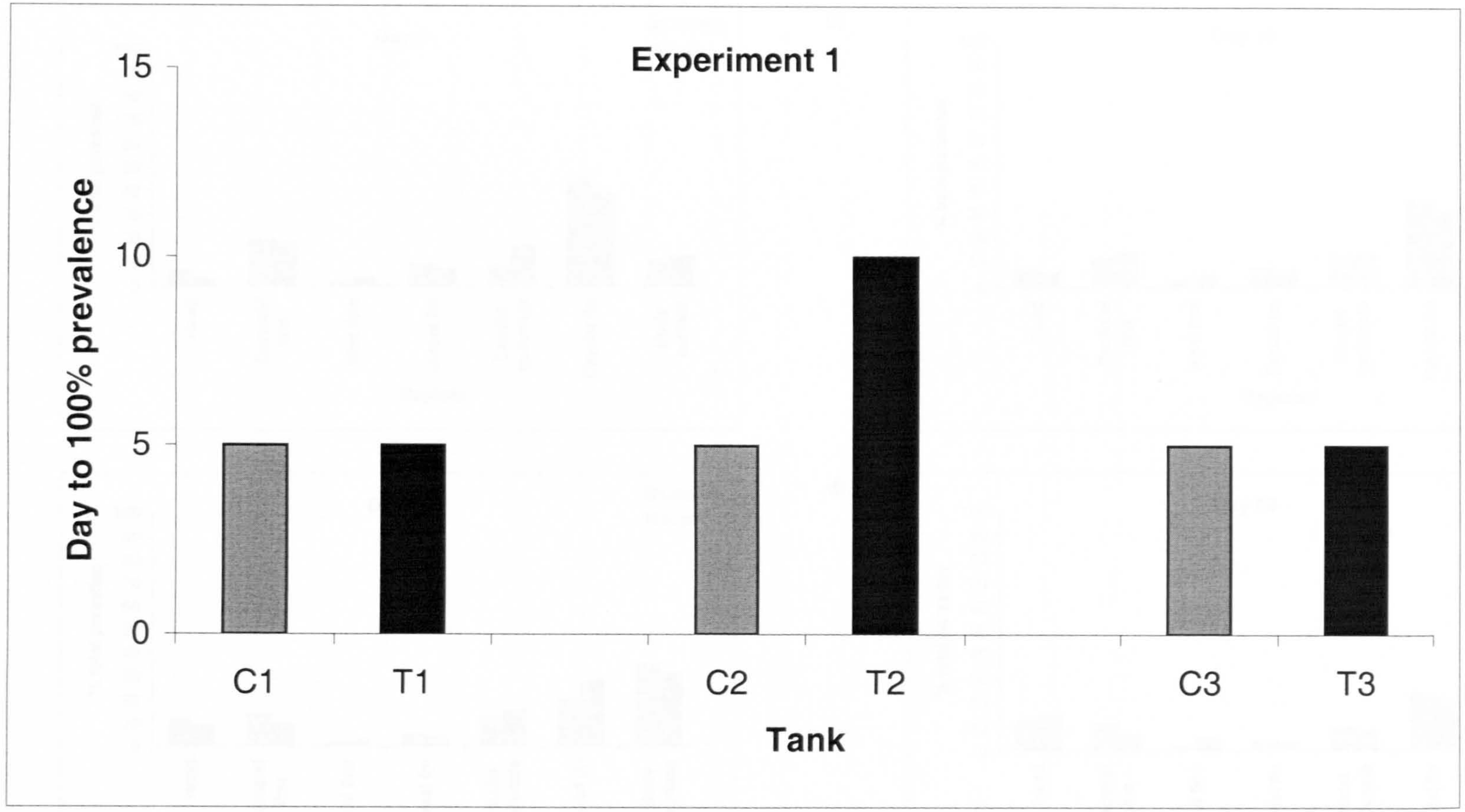
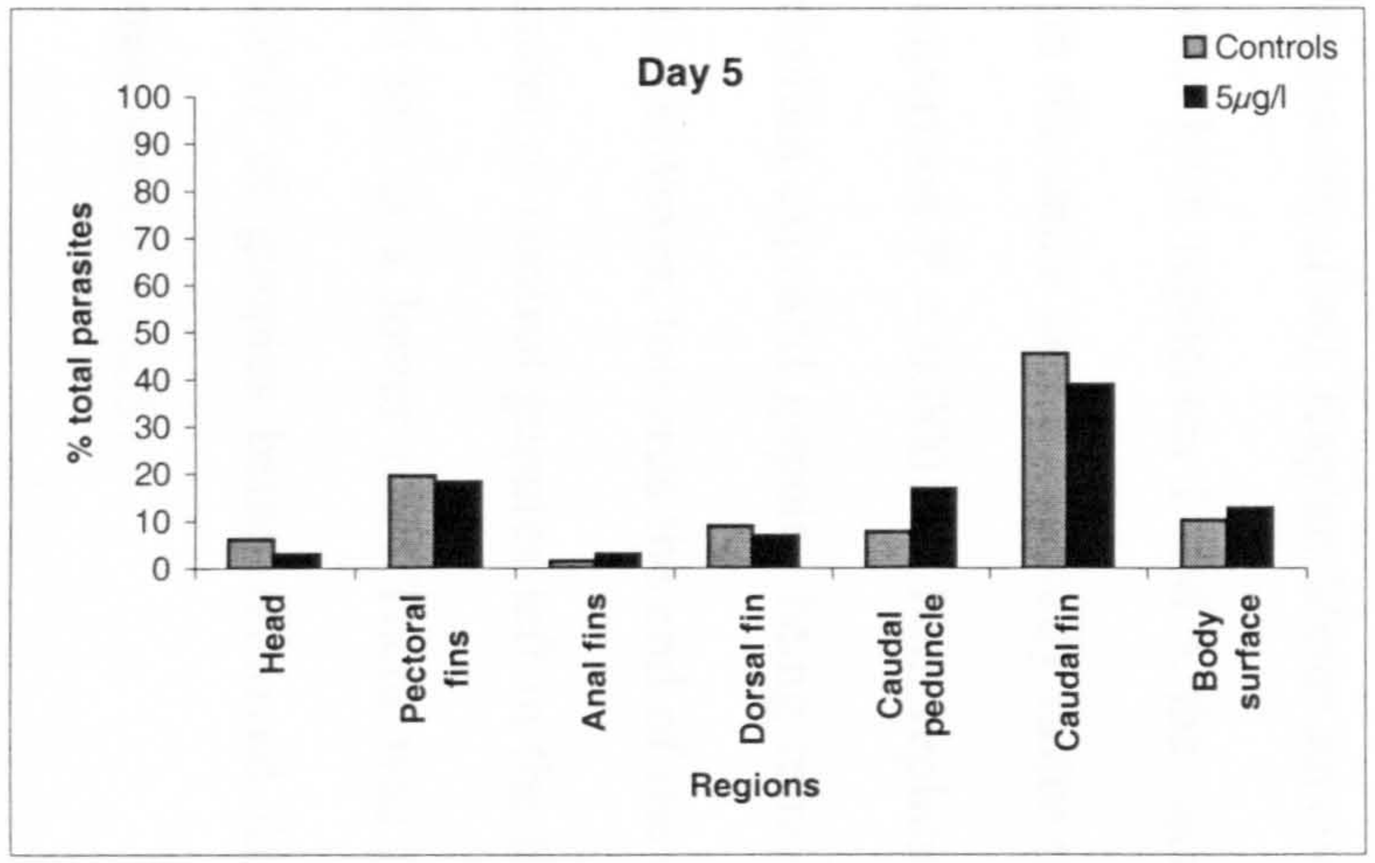
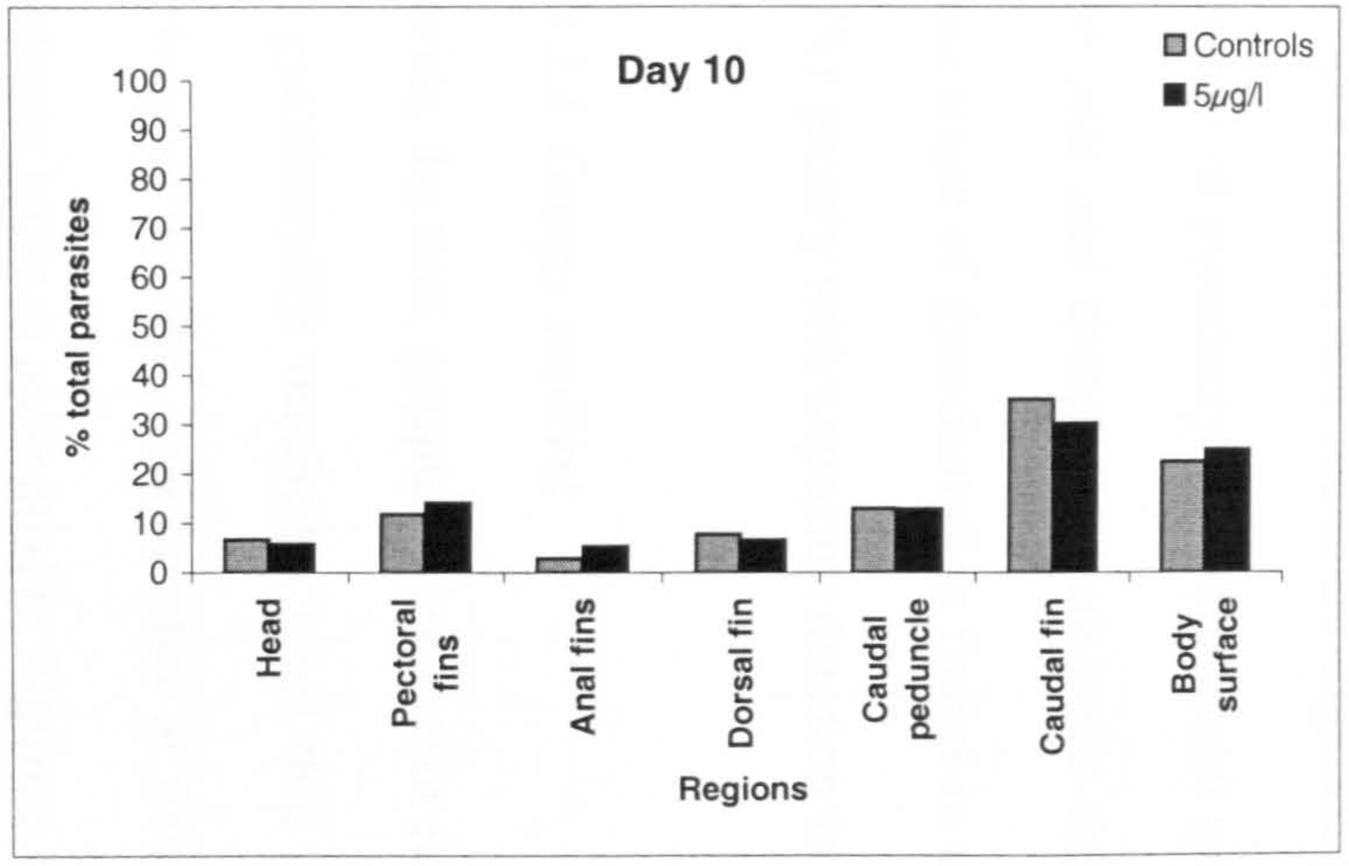


Fig. 6.4. Experiment 1 - The number of days to 100% parasite prevalence of control, C1-C3 (grey bars), and 5μg/l cadmium-exposed, T1-T3 (black bars), guppies in each tank.

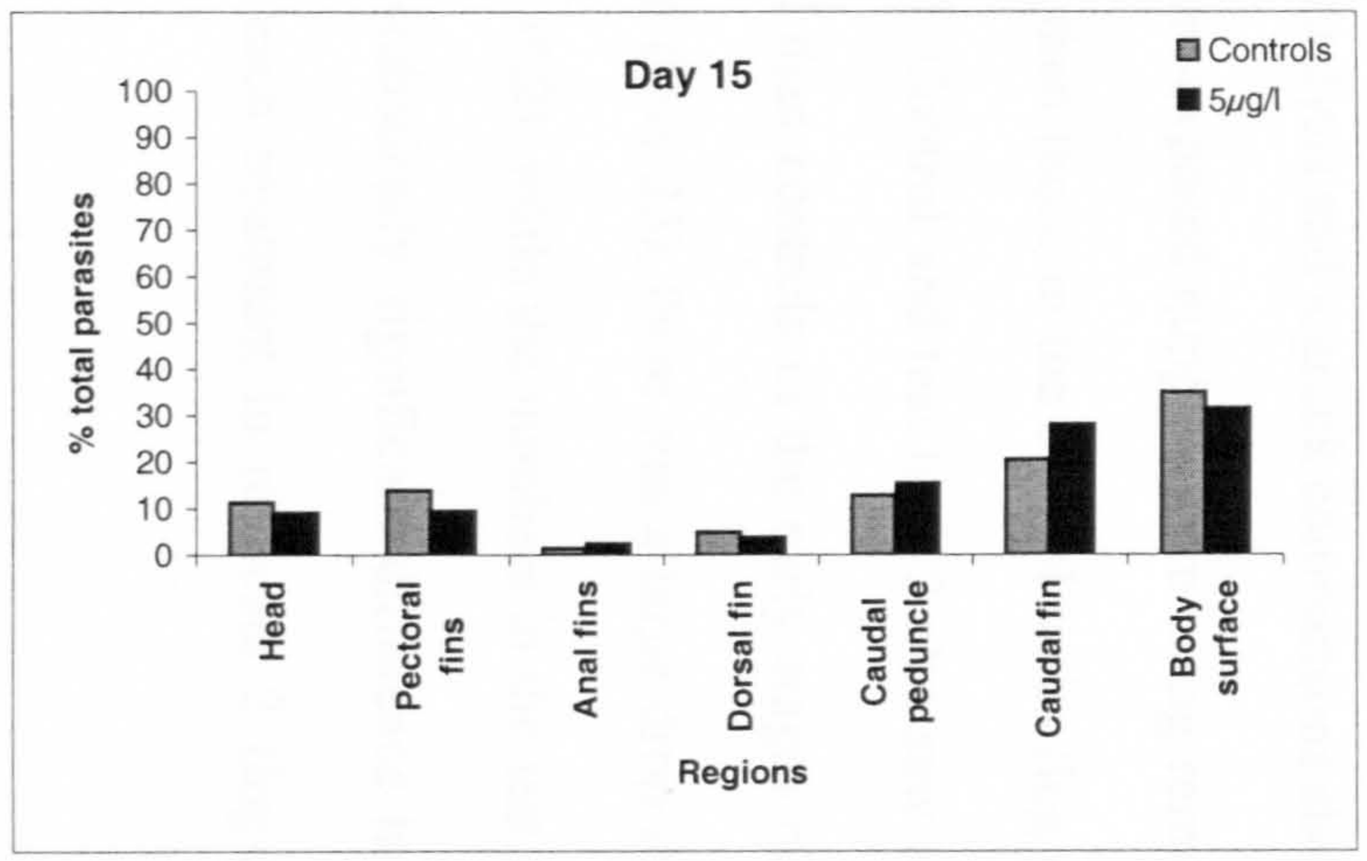
a)



b)



c)



d)

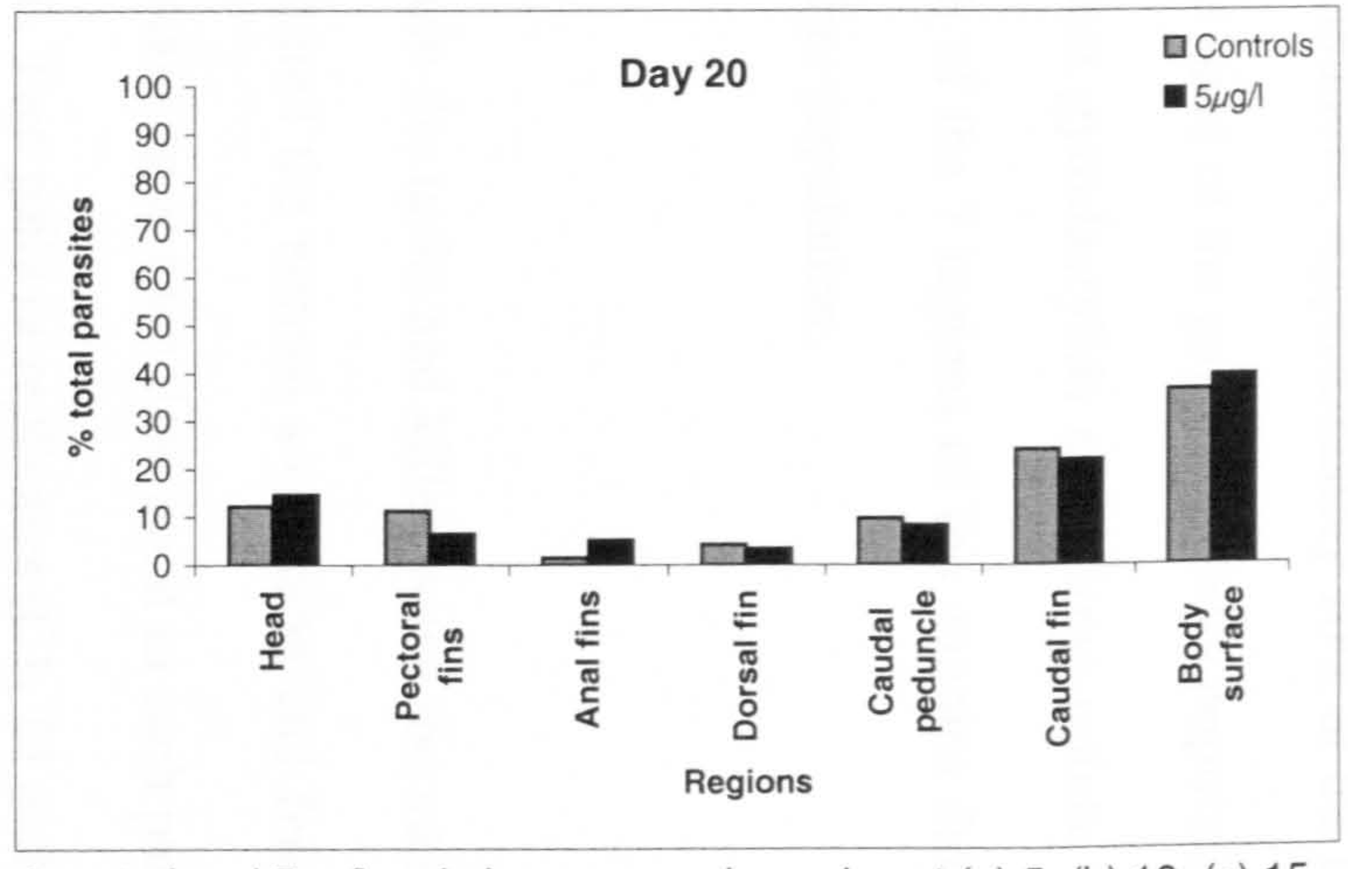


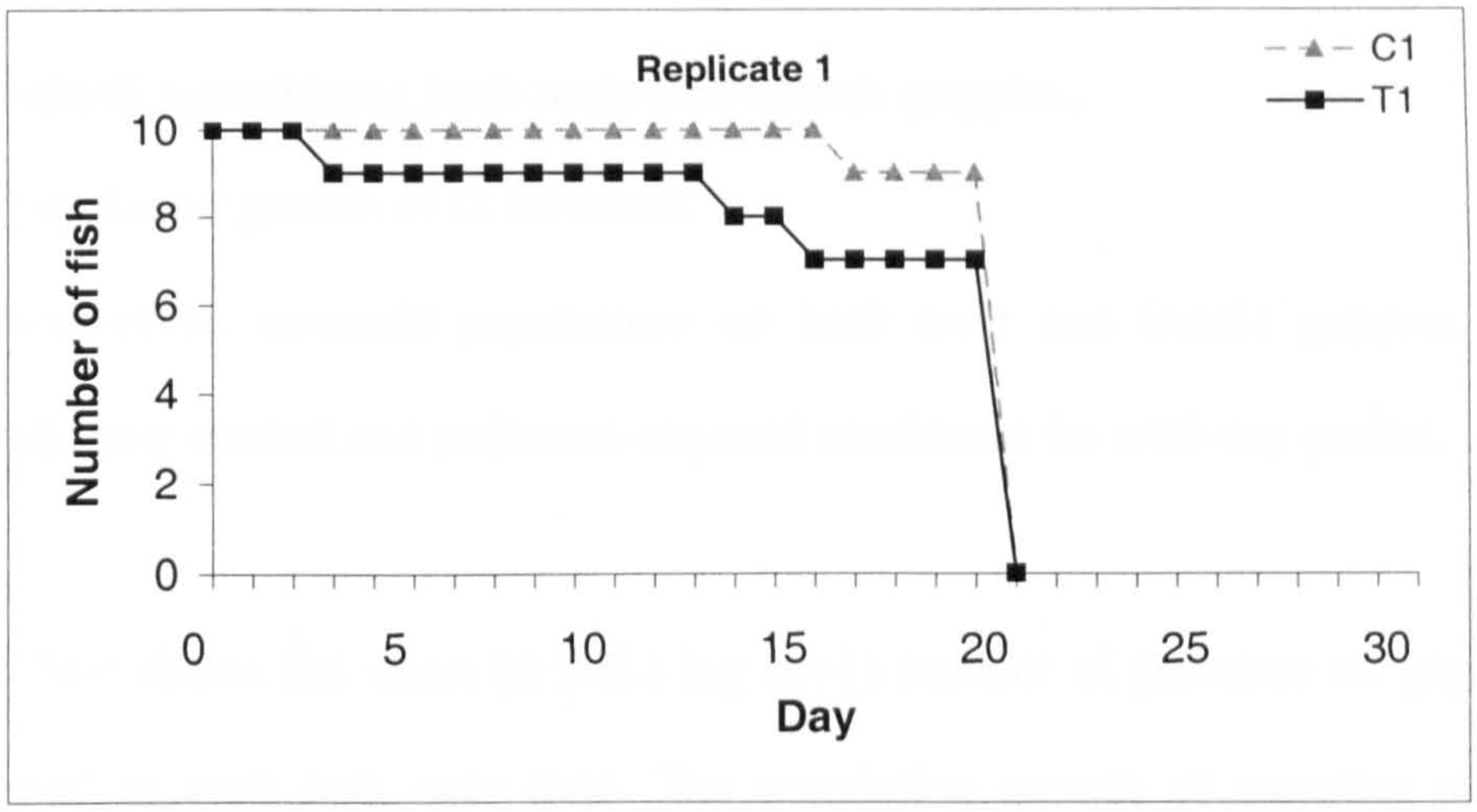
Fig. 6.5a-d. Experiment 1 - The percentage of *G. bullatarudis* present in each region of control and 5µg/l cadmium-exposed guppies at (a) 5, (b) 10, (c) 15 and (d) 20 days post-start of the trial. Control values are calculated from data pooled from all 3 control tanks (C1-C3) and test values are calculated from data pooled from all 3 tanks exposed to 5µg/l cadmium (T1-T3).

on both control and cadmium-exposed populations. Approximately 10% of the total number of gyrodactylids were found on the head of the guppies in both populations. The anal and dorsal fin harboured the least gyrodactylids at all time points. The percentage of gyrodactylids found in each of the 7 regions of the guppies did not differ greatly with exposure duration in either population.

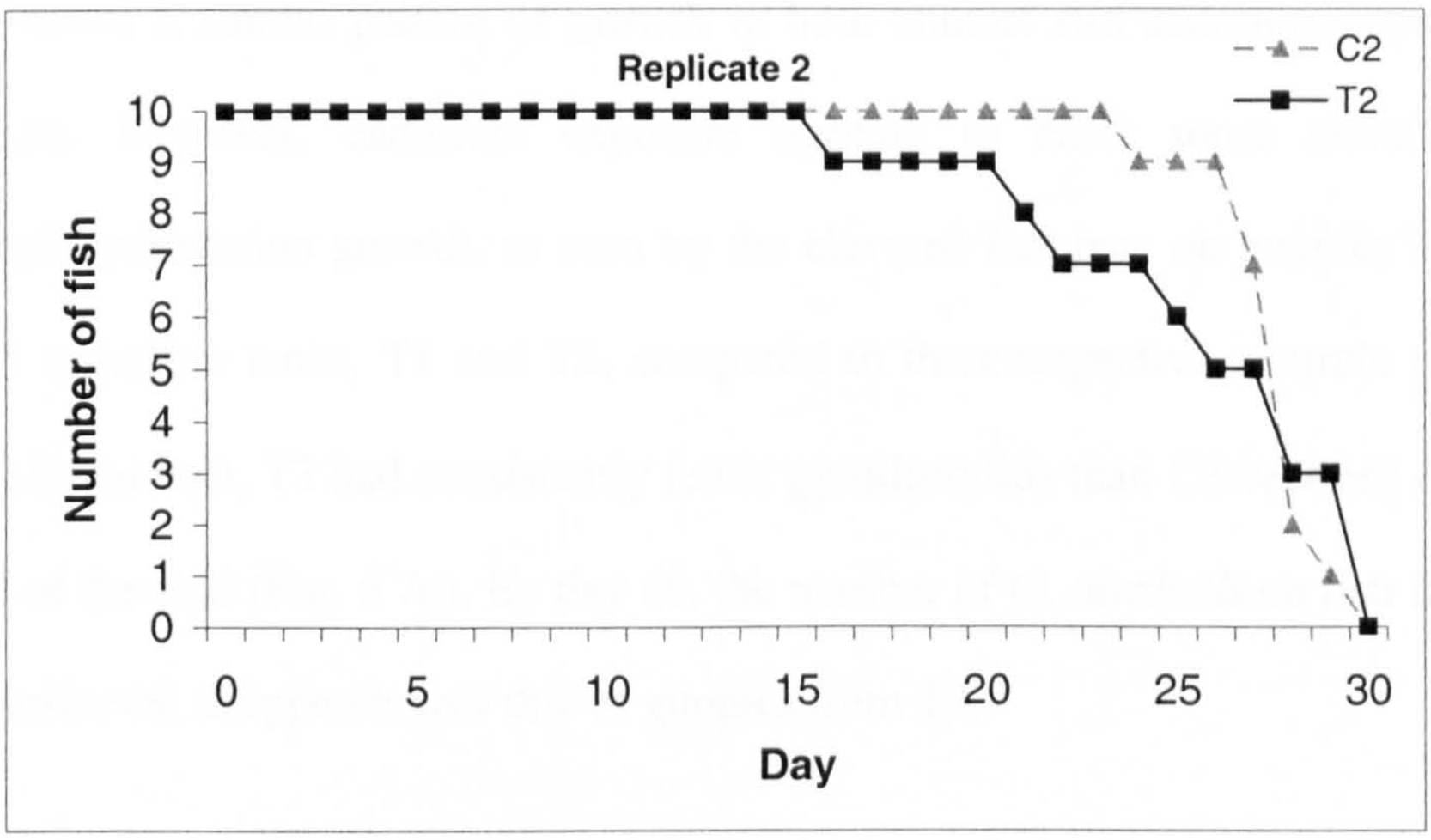
6.3.2.4. Guppy survival

During the trial, guppies were removed from the tanks and killed when the number of gyrodactylids reached a level that inhibited the normal swimming and feeding activity of the fish. The number of fish remaining at each time point in each pair of replicate tanks is presented in Figure 6.6a-c. Two pairs of replicates (C1, T1 and C3, T3) showed the same pattern, with cadmium-exposed guppies being removed from the tanks at a greater rate than the control guppies until day 20, when no further guppies remained. Kaplan-Meier survival analysis and logrank comparisons showed that in both replicates 1 and 3, the cadmium-exposed guppies were being removed from the tanks at a statistically faster rate than those in the control tanks (log rank comparison $P = 0.0001$ in both replicates). Control and test tank 2 also saw more cadmium-exposed guppies being removed than controls in the early stages of the trial. However, towards the end of the trial (Day 25), there was a large drop in the number of control guppies left in the tank (C2), while the numbers in the test tank (T2) fell at a lower rate. There was no statistically significant difference in the number of guppies being removed from each treatment in replicate 2 (log-rank comparison $P = 0.05$).

a)



b)



c)

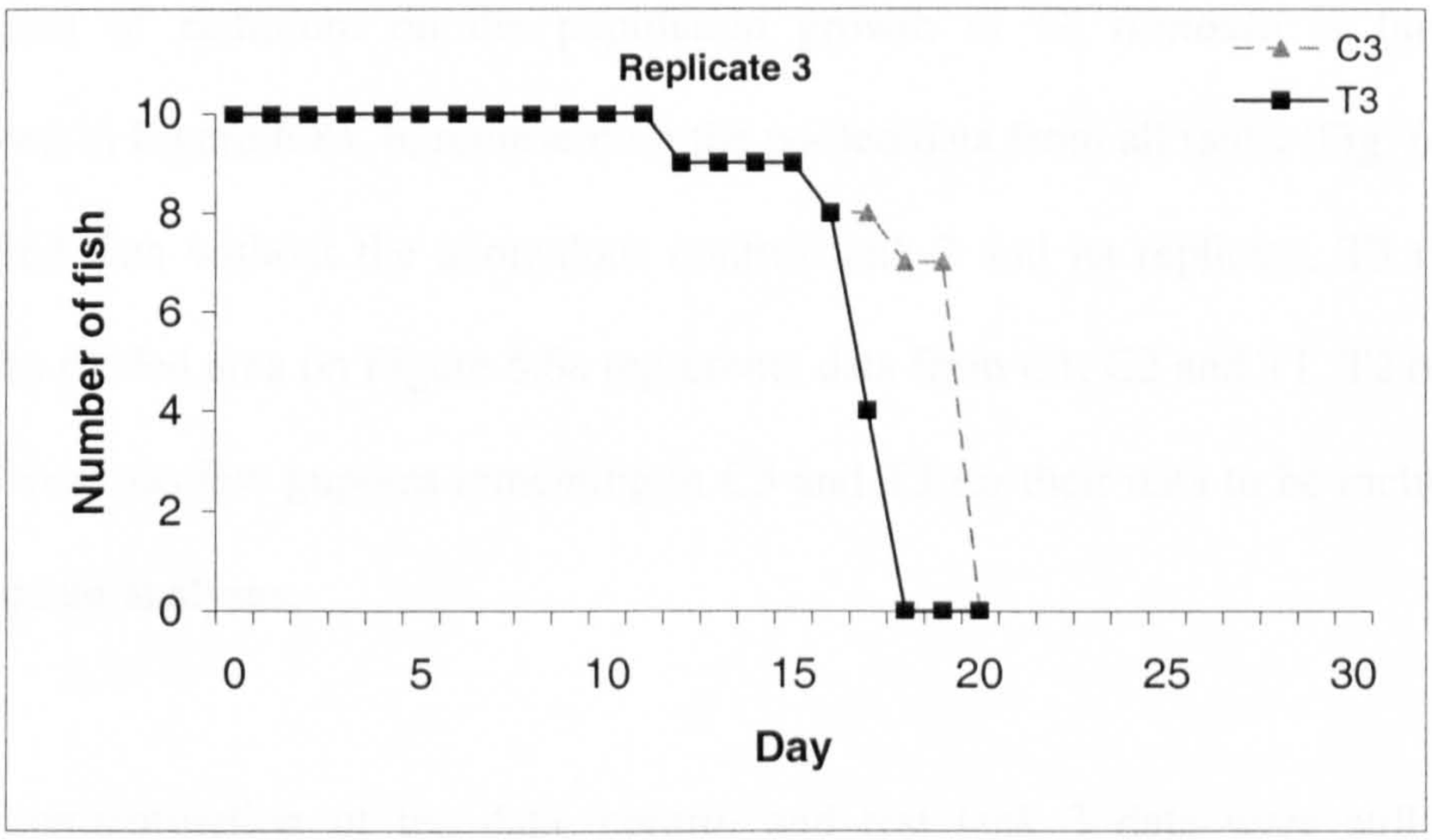


Fig. 6.6a-c. Experiment 1 - The number of guppies remaining over time in 0 and 5μg/l cadmium in each replicate: (a) replicate 1, (b) replicate 2 and (c) replicate 3.

6.3.3. Experiment 2 - The impact of 5μg/l cadmium on the population growth of *G. turnbulli* parasitising both male and female guppies.

6.3.3.1. Population growth of *G. turnbulli*

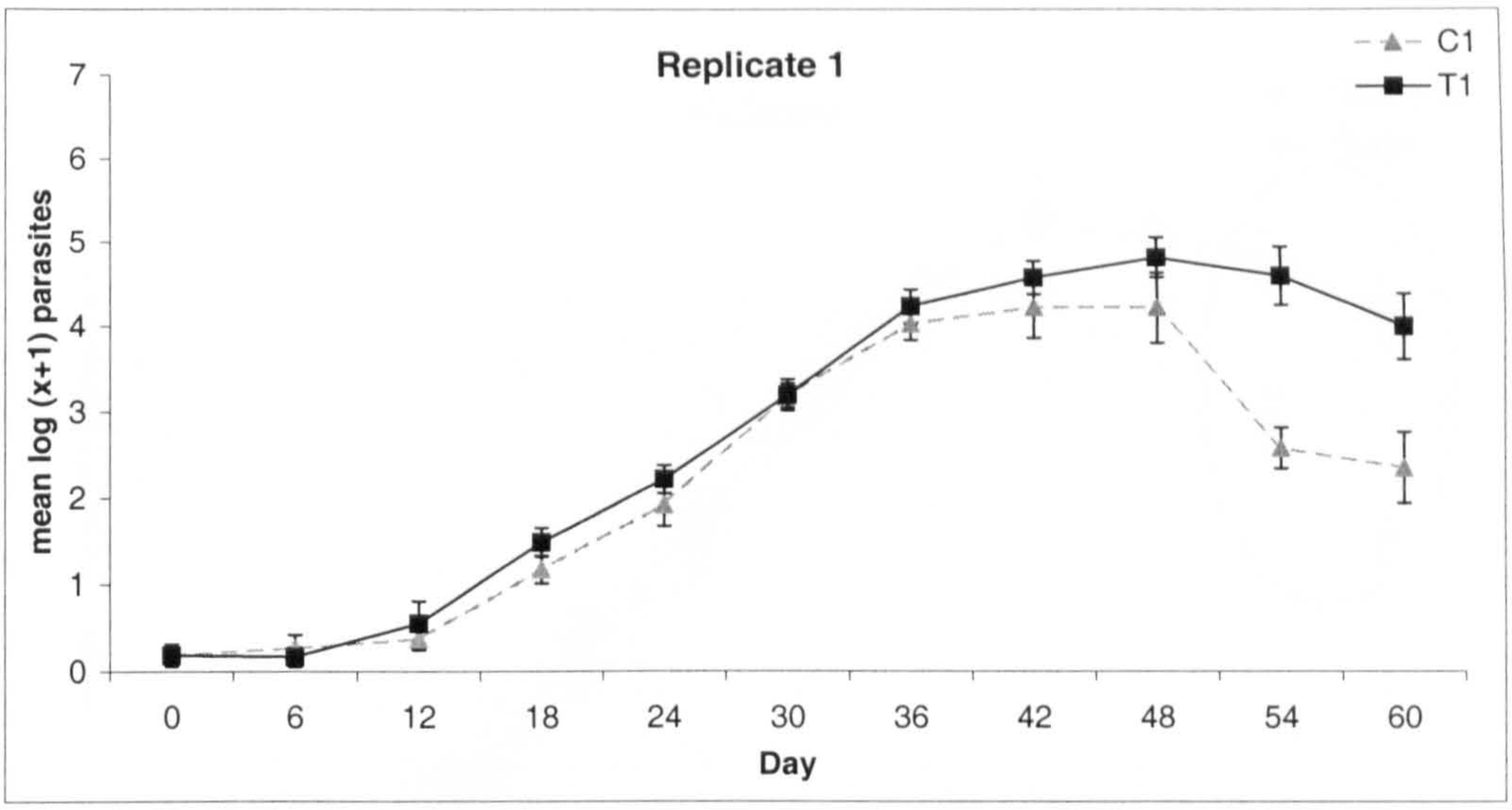
The growth of *G. turnbulli* populations on both male and female guppies was monitored under control and cadmium-exposed conditions for a 60-day period.

Figure 6.7a-c shows the mean (± S.E.) log (x+1) number of parasites on guppies (both sexes) in each tank over time. The population growth of parasites on all guppies shows a similar pattern of growth in both control and cadmium-exposed populations. However, cadmium exposure appears to exert some effect on gyrodactylid population growth, as seen by the elevated numbers on guppies from the 5μg/l cadmium tanks, T1 and T2, compared to their respective controls (Fig. 6.7a, b). In contrast, T3 had consistently fewer gyrodactylids than C3 between days 6 and 42 of the trial (Fig. 6.7c). By day 60, the number of *G. turnbulli* on fish from C3 had, however, dropped below that of guppies from T3.

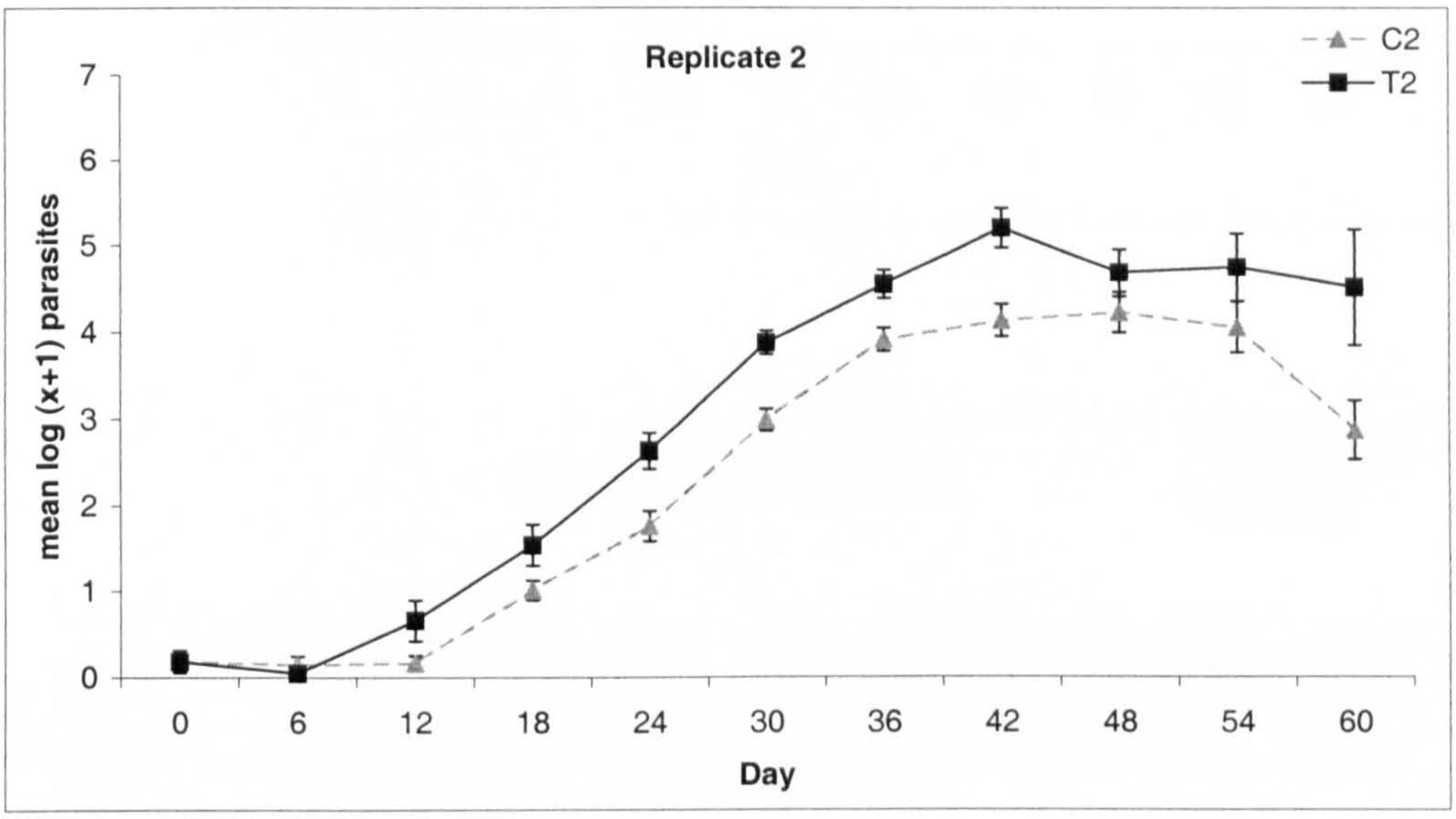
The impact of cadmium on the population growth of *G. turnbulli* is further highlighted in Figure 6.8a, b, representing the pooled data from all tanks (Fig. 6.8a) and pooled data without the anomalous control tank 3 and its replicate, T3 (Fig. 6.8b). The circled area on Figure 6.8a represents data from C1, C2 and T1, T2 only, as there were too few guppies remaining in C3 and T3 for their data to be included in subsequent analyses.

Despite transformation of the data, control and test tank 3 data were still not normally distributed at days 6-24 and day 48, and at these time-points non-

a)



b)



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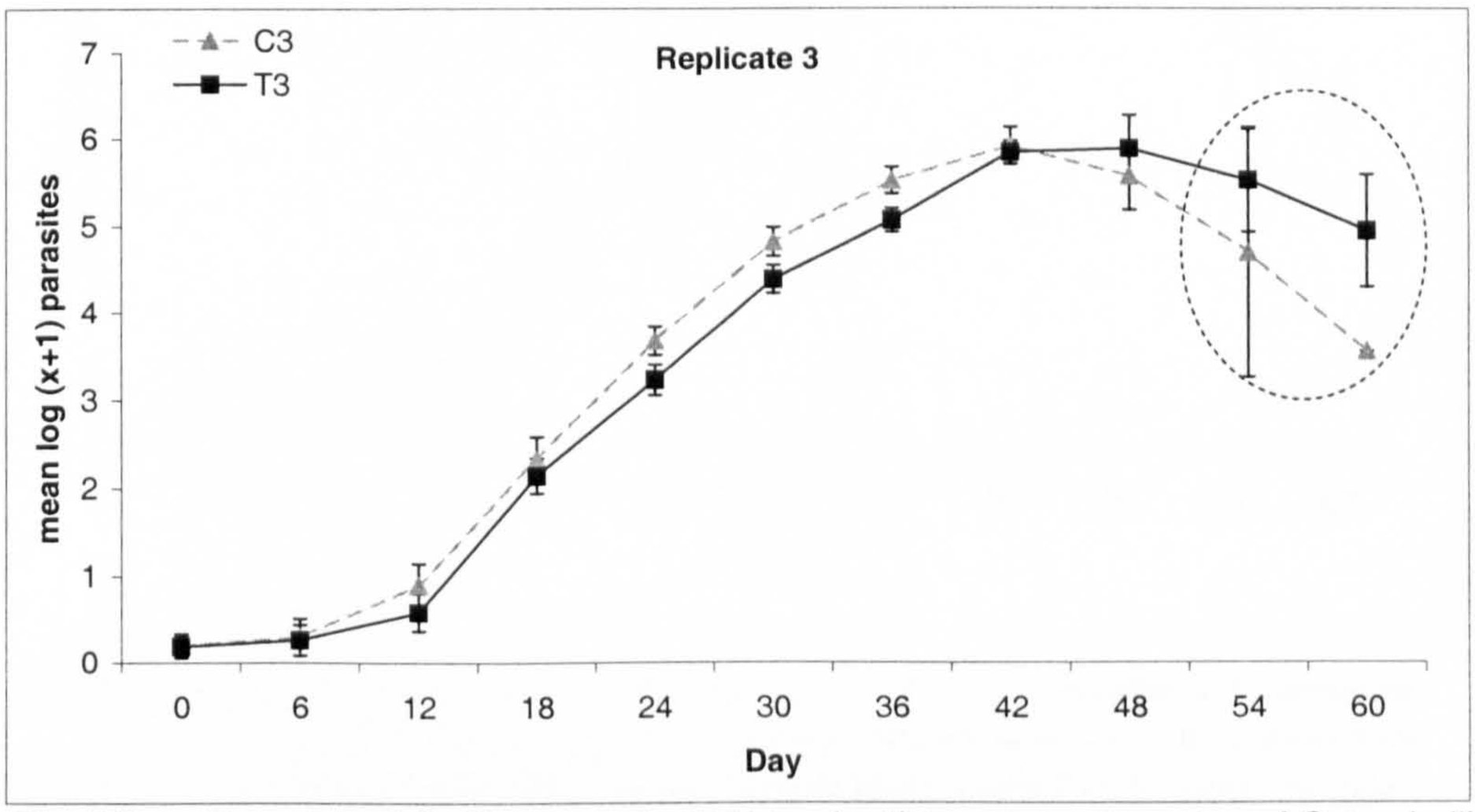
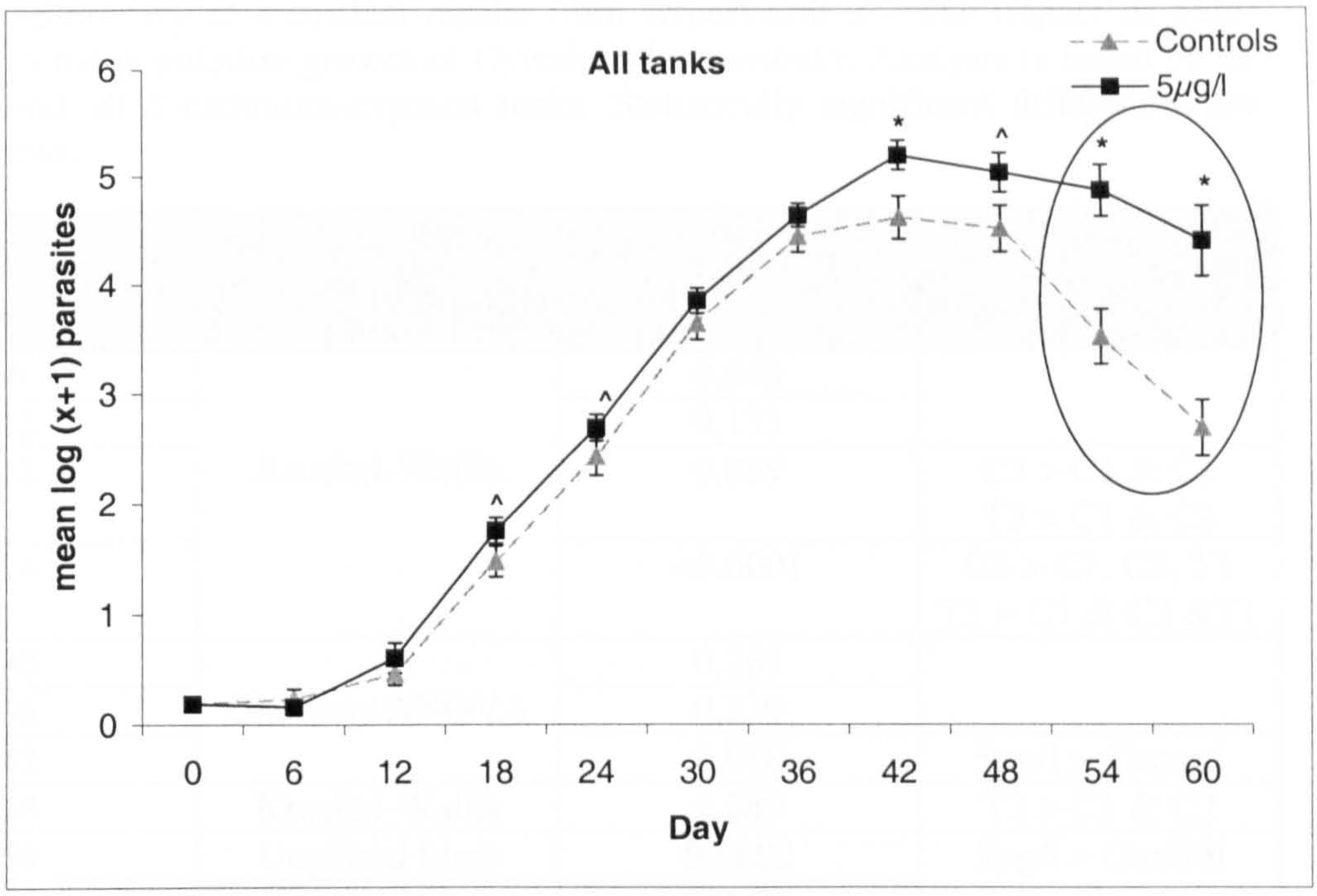


Fig. 6.7a-c. Experiment 2 - The mean (± S.E.) log (x+1) transformed number of *G. turnbulli* per guppy over time in control (C1-C3) and 5μg/l cadmium-exposed tanks (T1-T3) in, (a) replicate 1, (b) replicate 2 and (c) replicate 3. n=2 and n=1 in control tank 3 (C3) at 54 and 60 days post-start of the trial. This area is represented by the circled area on Fig. 6.7c.

a)



b)

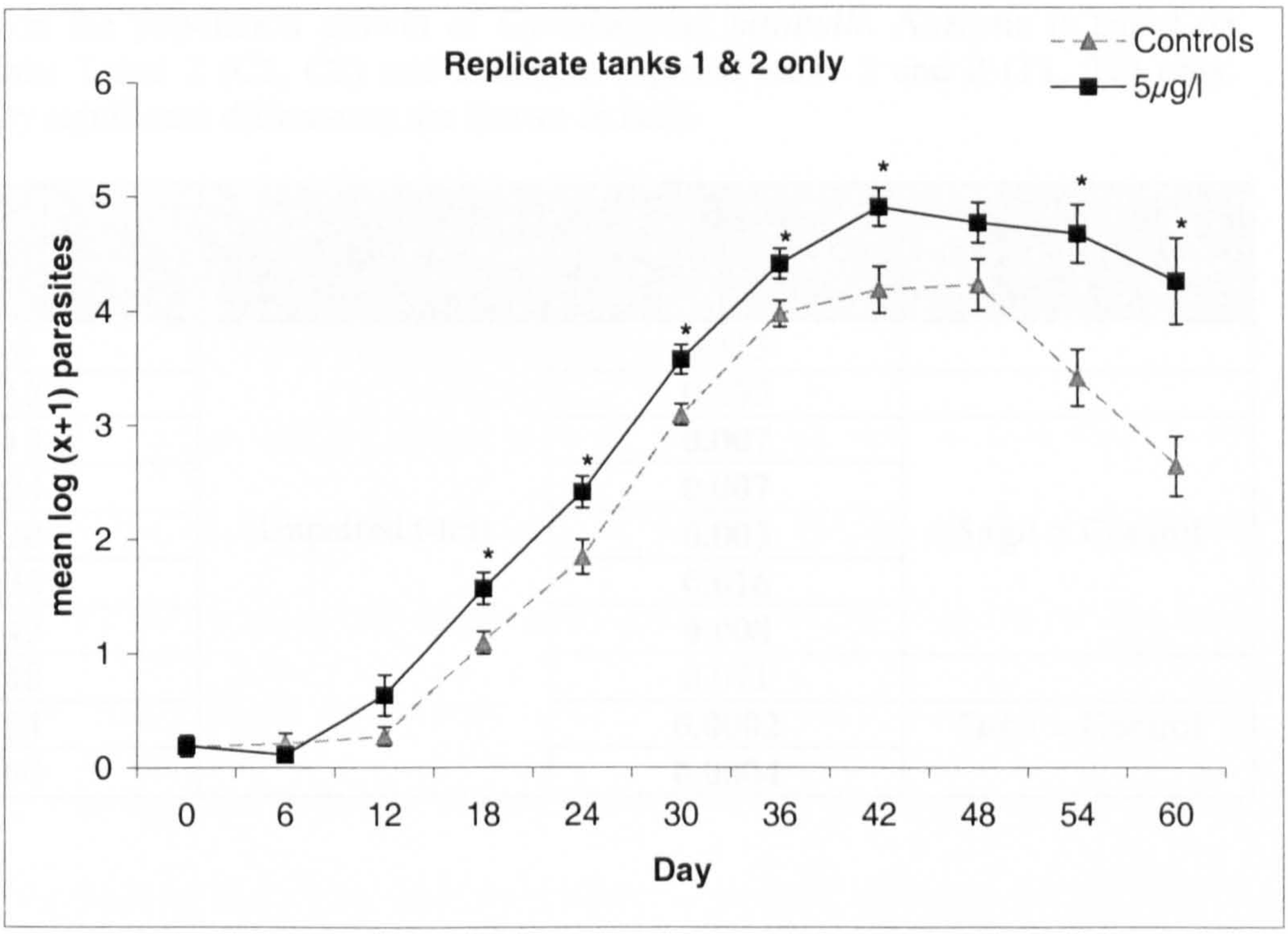


Fig. 6.8a, b. Experiment 2 - The pooled mean (±S.E.) log (x+1) transformed number of *G. turnbulli* per guppy in control and 5µg/l cadmium-exposed tanks: (a) data pooled from all 3 control (C1-C3) tanks and all three 5µg/l cadmium-exposed (T1-T3) tanks, (b) data pooled from C1, C2 only and from T1, T2 only. * and ^ denote statistically significant differences between the two populations of *G. turnbulli*. Refer to Table 6.2 for P values.

Table 6.2. Summary of statistical results from Experiment 2 - The impact of 5µg/l cadmium on the population growth of *Gyrodactylus turnbulli*. Analysis is based on all 3 control and all 3 cadmium-exposed tanks. Statistically significant differences are shown in bold.

Sample day	Statistical test applied	Statistical significance	Statistical differences between
6	Kruskal-Wallis	0.949	
12		0.133	
18		**0.039**	C3 > C1 & C2 T3 > C1 & C2
24		**<0.0001**	C3 > C1, C2, T1 T3 > C1 & C2 &T1
30	One-way ANOVA	0.265	
36		0.279	
42		**0.018**	5µg/l > Control
48	Kruskal-Wallis	**0.009**	T3 > C1 & C2
54	Unpaired t-test	**0.0002**	5µg/l > Control
60	Unpaired t-test	**0.0004**	

Table 6.3. Summary of statistical results from Experiment 2 - The impact of 5µg/l cadmium on the population growth of *Gyrodactylus turnbulli*. Analysis is based on control tanks 1 and 2 (C1, C2) and cadmium-exposed tanks 1 and 2 (T1, T2) only. Statistically significant differences are shown in bold.

Sample day	Statistical test applied	Statistical significance P	Statistical differences between
6	Unpaired t-test	0.413	
12		0.295	
18		**0.007**	5µg/l > Control
24		**0.007**	
30		**0.003**	
36		**0.016**	
42		**0.008**	
48		0.071	
54		**0.0002**	5µg/l > Control
60		**0.0004**	

parametric statistics were applied to compare all tanks together (Table 6.2). Statistical differences were found at days 18, 24 and 48 (see Table 6.2; Fig 6.8a). At days 30-42, data from all tanks were normally distributed and the variances of the mean homogeneous, and thus, the data were compared using parametric statistics. The mean number of *G. turnbulli* on 5μg/l cadmium-exposed guppies were statistically greater than the controls at days 42, 54 and 60 (P = 0.018, 0.001, 0.0002, respectively) (Fig. 6.8a; Table 6.2). However, as stated previously, the data at days 54 and 60 are based on 2 pairs of replicate tanks only (C1, T1; C2, T2).

Removing the anomalous tank C3, and its replicate T3, from the analysis resulted in cadmium-exposed populations of *G. turnbulli* being statistically greater than the controls at 7 of the 10 time points: days 18, 24, 30, 36, 42, 54 and 60 (P = 0.007; 0.007; 0.0003; 0.016; 0.008; 0.0002; and 0.0004, respectively) (Figure 6.8b; Table 6.3).

6.3.3.2. *G. turnbulli* populations on identifiable guppies

Due to their unique colour pattern at least half of all fish in each tank were individually identifiable and their infections recorded throughout the trial. Figure 6.9a-c shows the infection pattern on individual control fish over the 60 day trial and Figure 6.9d-f shows the infection pattern on individual test (5μg/l cadmium) guppies. It is apparent from both treated and control fish that there is heterogeneity in the response of fish from both treatments to parasite infection. In the control population of guppies, there was an initial increase in the number of parasites from day 12 followed by a decline in number from ca. days 42 to 48. In the test tanks (T1- T3), a similar increase in the parasite population was seen between days 12 to

42/48; however, the greater proportion of fish continued to show a rapid increase in parasite number beyond days 42/48. There were 4 times more cadmium-exposed guppies showing no evidence of a decline in the parasite population size at day 42/48 than the controls (8 and 2 guppies, respectively).

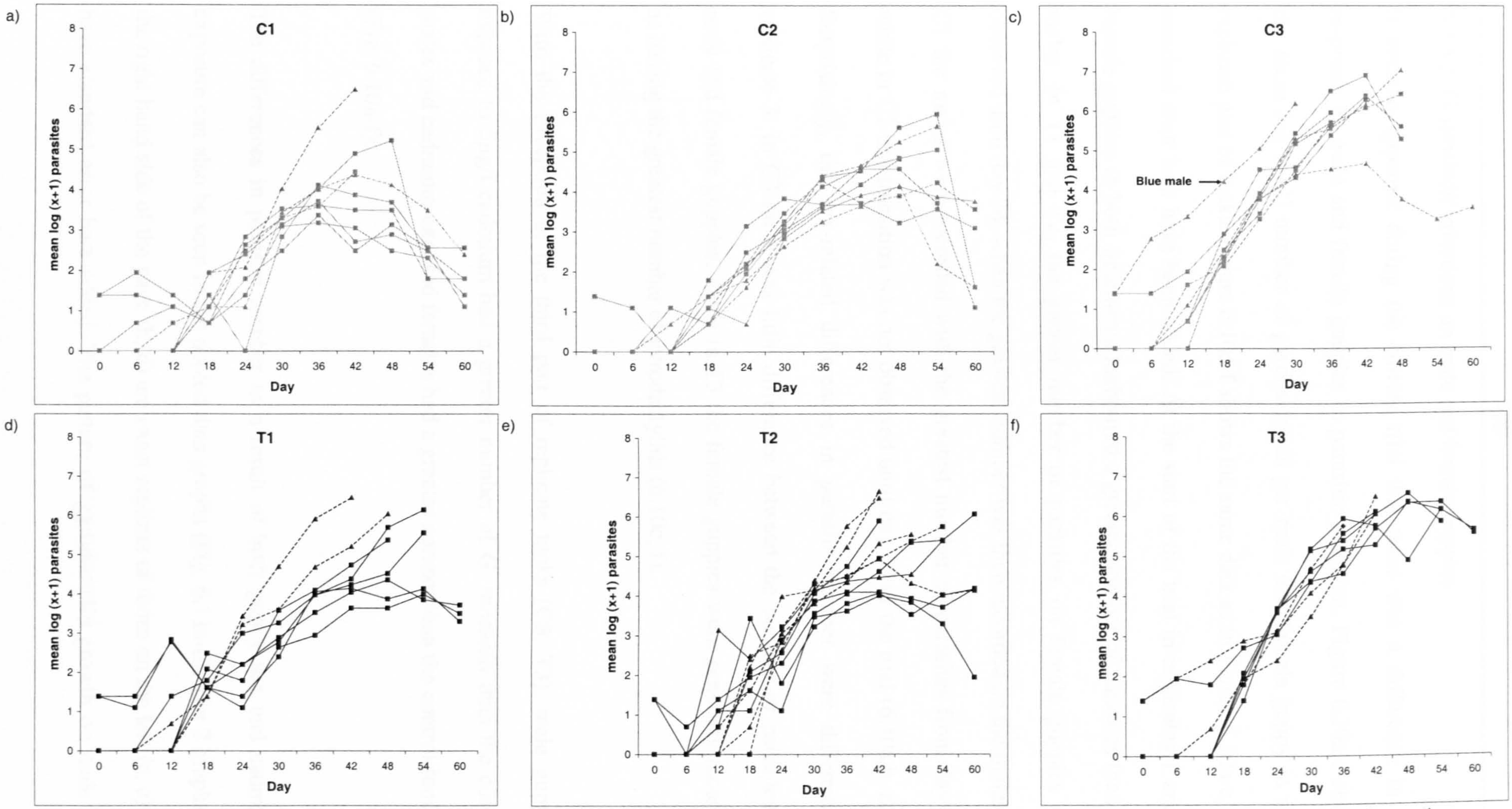


Fig. 6.9a-f. Experiment 2 - The total log (x+1) transformed number of *G. turnbulli* on individual control (a-c) and 5μg/l cadmium-exposed (d-f) guppies in each tank over time. The population growth of *G. turnbulli* could be tracked over time on individual fish due to their unique colour pattern. Dashed lines represent male guppies and the solid lines, female guppies.

6.3.3.3. *G. turnbulli* infections on male and female guppies

It became apparent during the current trial that there was a difference in the response by male and female guppies to parasitic infection. Figure 6.10a-c shows the mean log (x+1) number of gyrodactylids per male and female guppy in each replicate pair of tanks. Figure 6.10d-f shows the same data as in Figure 6.10a-c, but standard error bars have been added. At the start of the trial in replicates 1 and 2, female guppies in both treatments harboured a greater number of parasites than the males. In T1 and T2, the greater number of parasites on female guppies was observed until day 24, when the greatest number was then recorded on the males. In C1 the males were recorded with the greatest number of parasites from day 18, while in C2 this transition was not observed until day 30 of the trial (6.10a,b, d, e). Surprisingly, the sex-related differences in parasite number were different in replicate 3. In C3 there was little difference between the number of parasites on male and female guppies, while in T3 the female guppies were generally recorded as having the greatest number of gyrodactylids (6.10c, f).

With the exception of the third pair of replicate tanks (C3, T3), male guppies exposed to 5µg/l cadmium had a greater number of *G. turnbulli* than the control males and cadmium-exposed females had a greater number than the control females (Fig. 6.10a-f).

The differences in parasite number as a result of both guppy sex and cadmium exposure can also be seen in the pooled data graphs (Fig. 6.11a-d). The 2 graphs on the right hand side of the page (b, d) are exact replicas of those on the left (a, c) but have standard error bars added. The pattern of gyrodactylid growth on male and

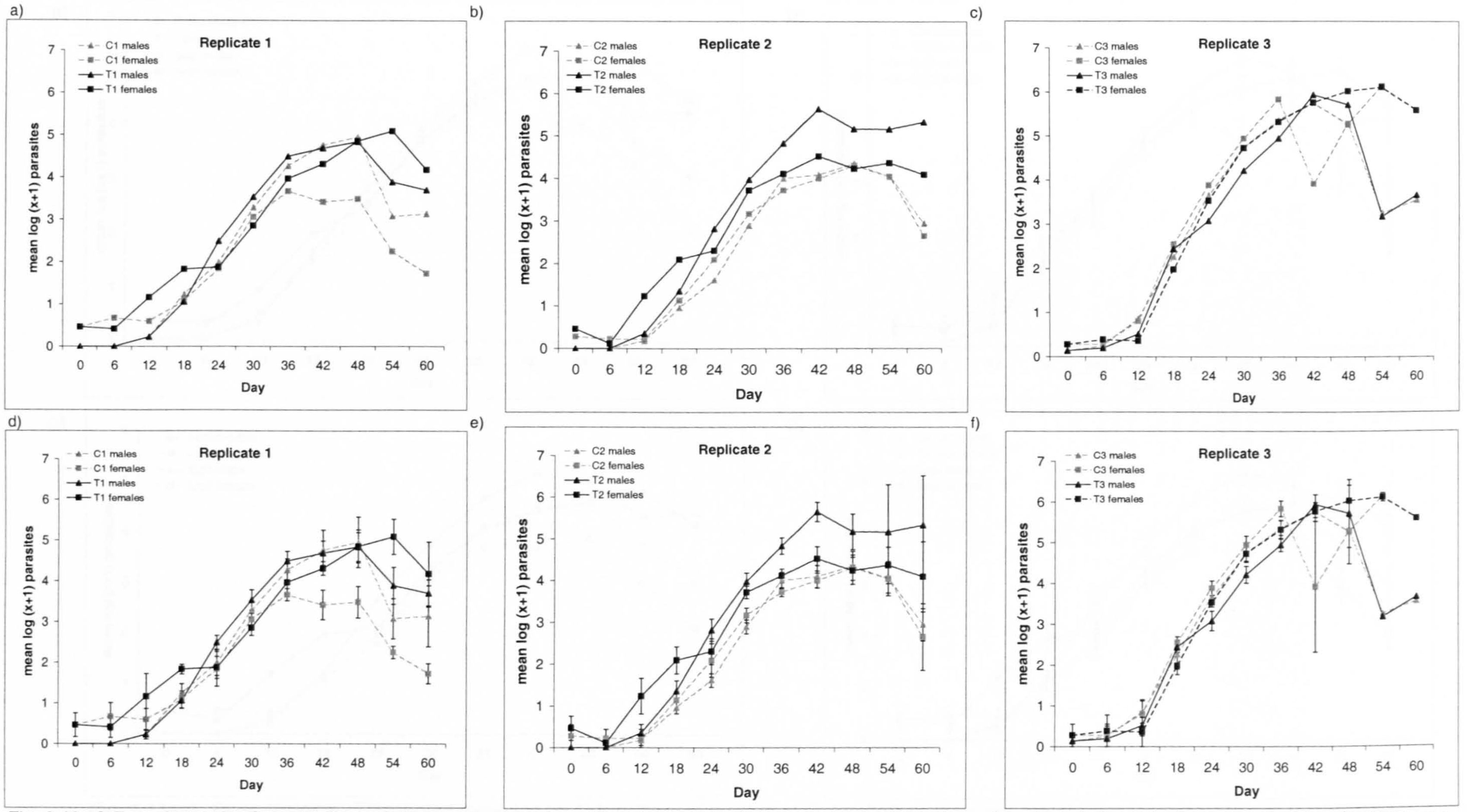
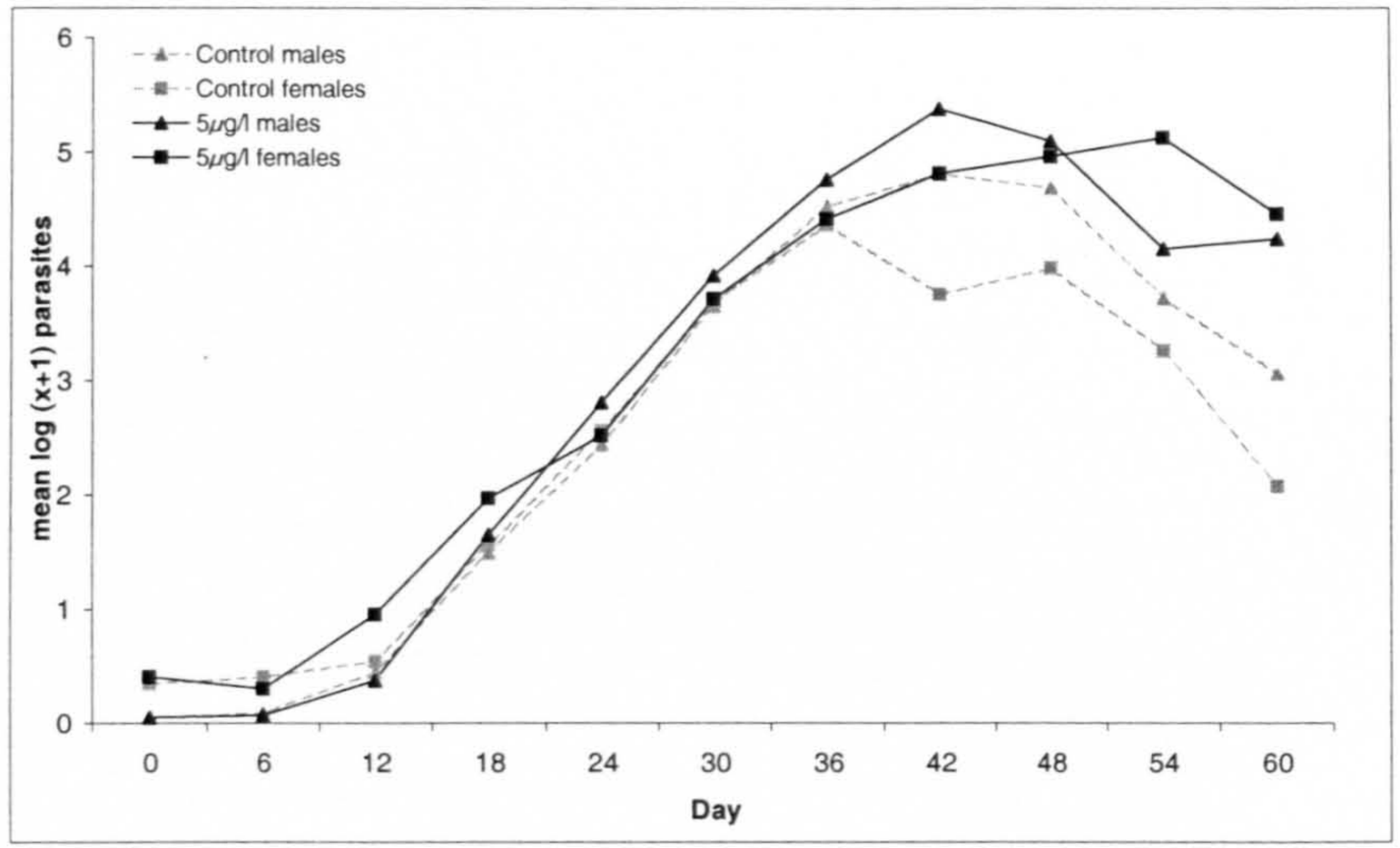
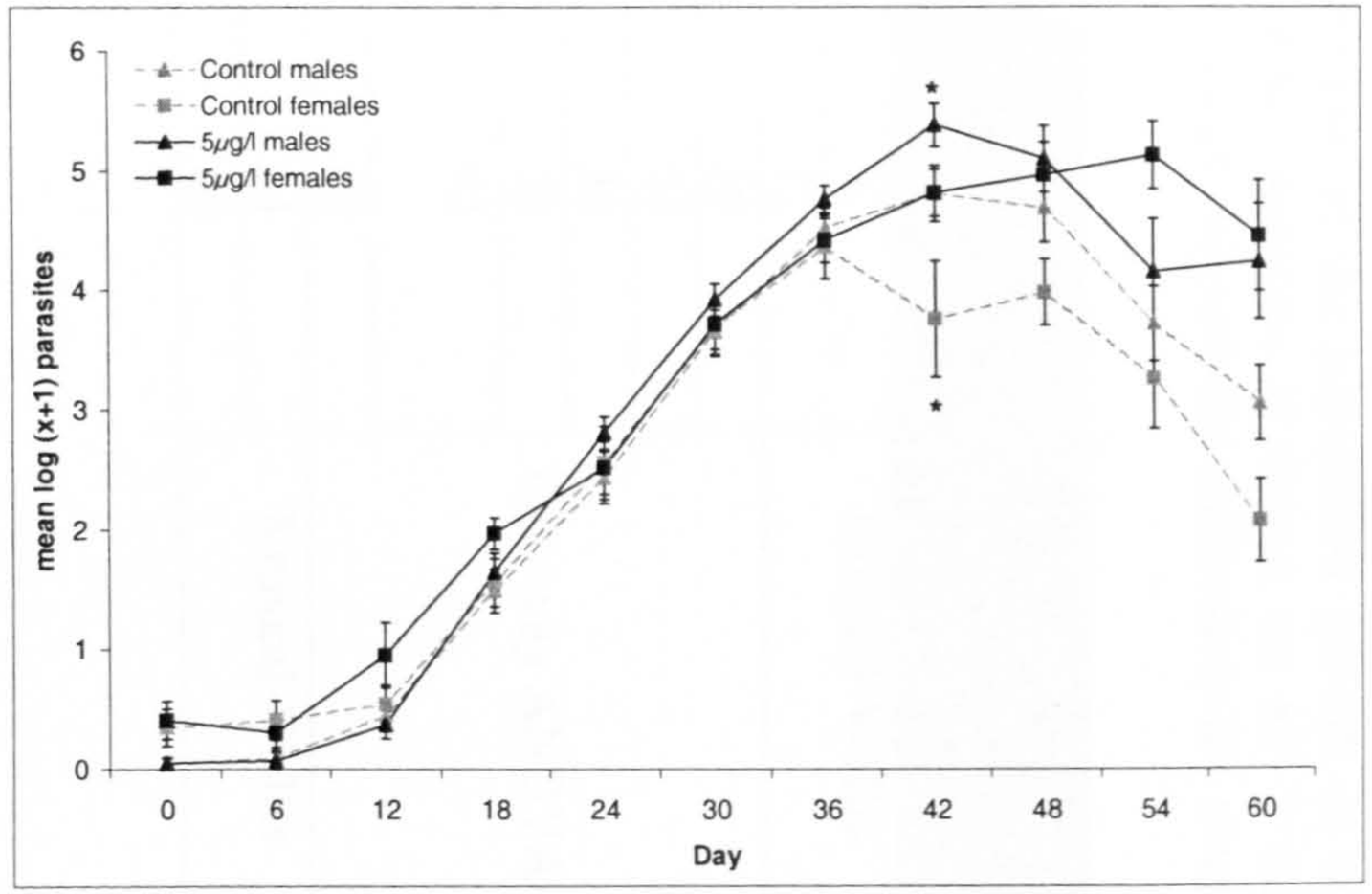


Fig. 6.10a-f. Experiment 2 - *G. turnbulli* population growth on male and female guppies. The mean log (x+1) transformed number of *G. turnbulli* per male and female guppy over time in (a, d) replicate 1, (b, e) replicate 2 and (c, f) replicate 3. The standard error bars have been left off the first set of graphs (a-c) to ensure that the pattern of population growth on both sexes is clearly visible, they are however, added on the second duplicate set (d-f).

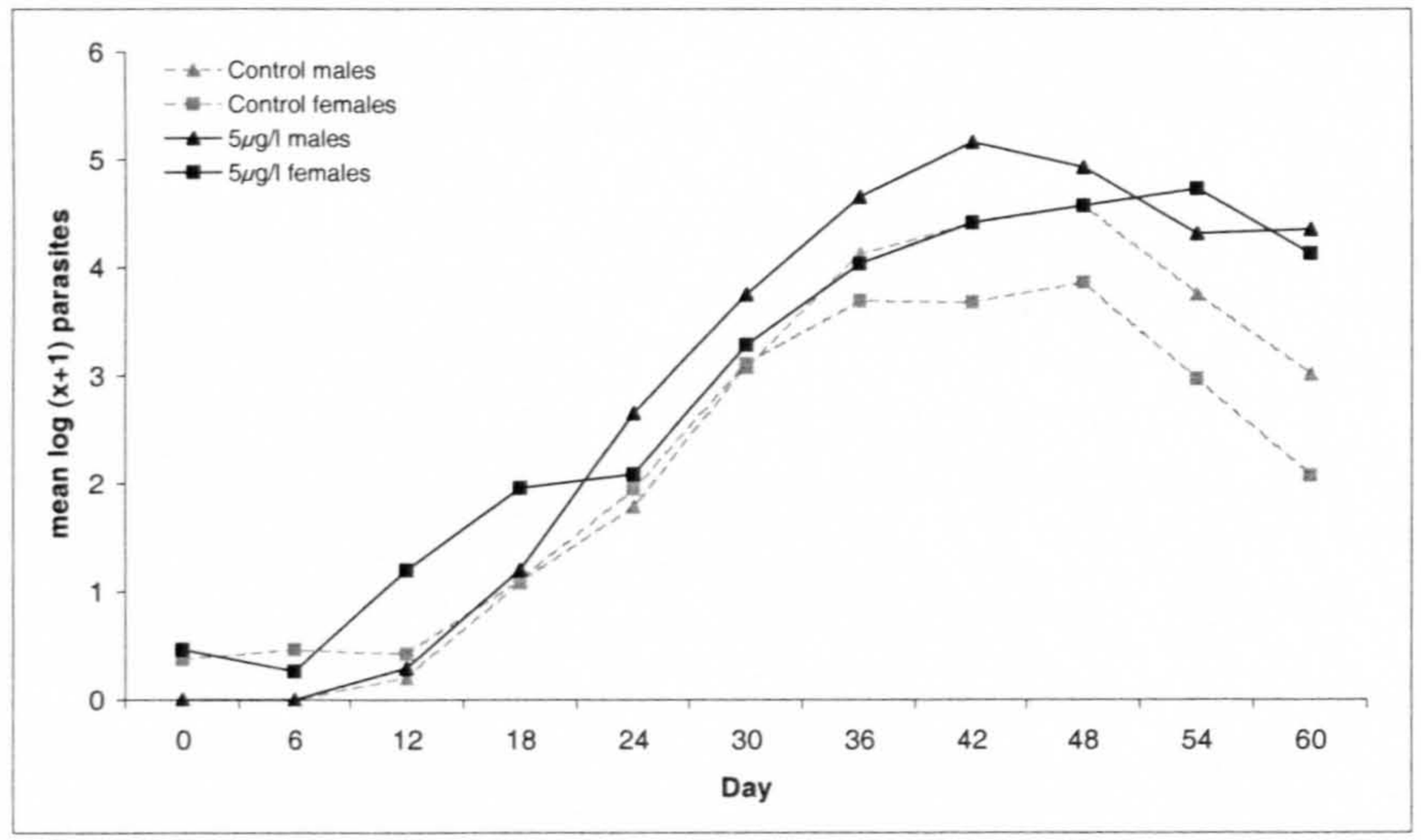
a)



b)



c)



d)

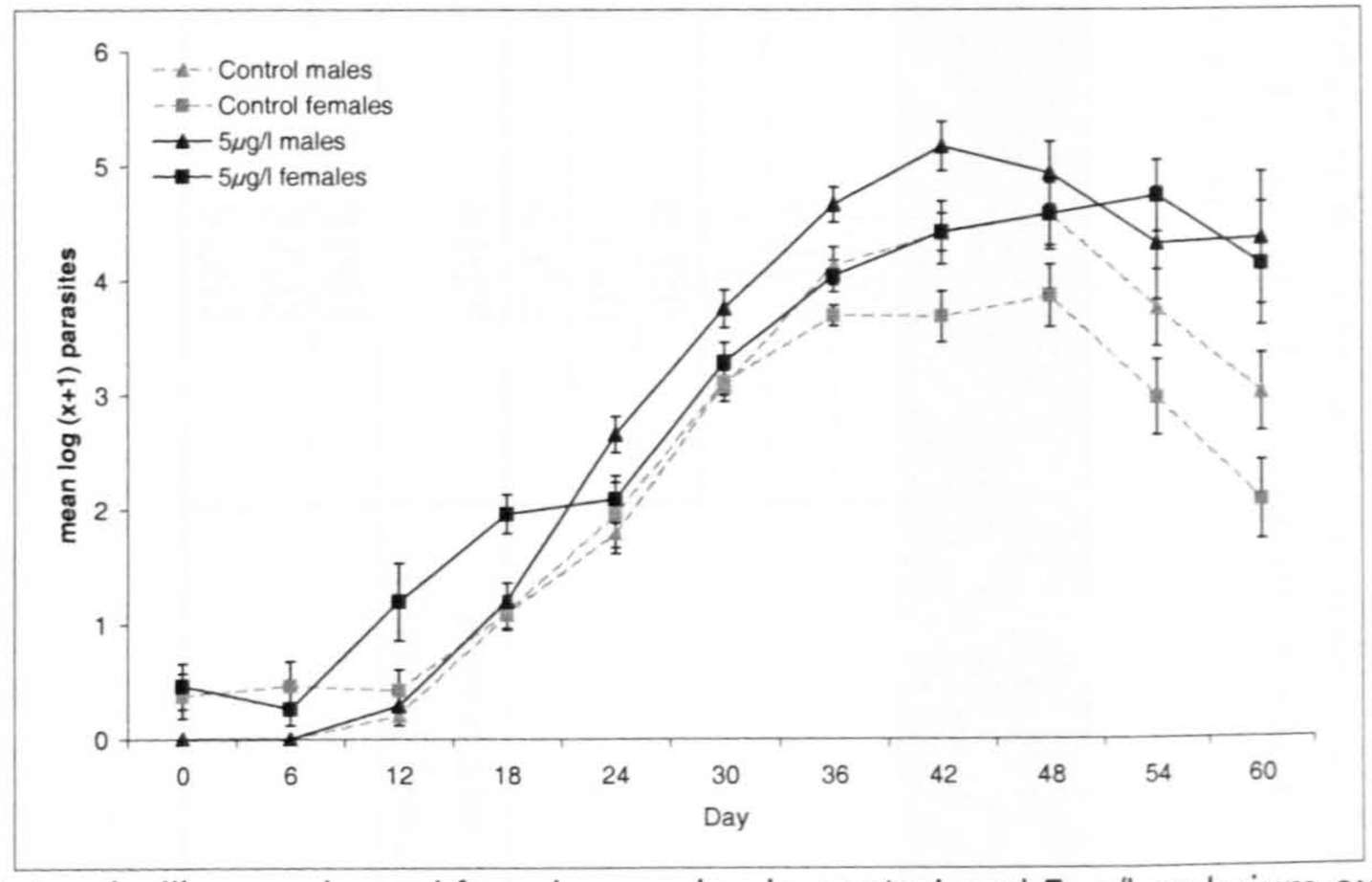


Fig. 6.11a-d. Experiment 2 - The pooled mean log (x+1) transformed number of *G. turnbulli* on male and female guppies in control and 5µg/l cadmium-exposed tanks (a) pooled data from all 3 control tanks (C1-C3) and all 3 test tanks (T1-T3), (c) pooled data from C1 and C2 only and T1 and T2 only. (b) and (d) as (a) and (c) but with error bars added (± S.E.).

Table 6.4. Summary of the statistical results from Experiment 2 - The impact of 5μg/l cadmium on the population growth of *Gyrodactylus turnbulli* on male and female guppies. Analysis is based on all 3 control and all 3 cadmium-exposed tanks. Statistically significant differences are shown in bold.

Sample day	Statistical test applied	Statistical significance P	Statistical differences between
6	Kruskal-Wallis	0.139	
12		0.102	
18	One-way ANOVA	0.303	
24		0.556	
30		0.680	
36		0.372	
42		**0.028**	Test males > Control females
48		0.064	
54	Kruskal-Wallis	0.602	
60		0.052	

Table 6.5. Summary of the statistical results from Experiment 2 - The impact of 5μg/l cadmium on the population growth of *Gyrodactylus turnbulli* on male and female guppies. Analysis based on control tanks C1 and C2 and cadmium-exposed tanks T1 and T2 only. Statistically significant differences are shown in bold.

Sample day	Statistical test applied	Statistical significance P	Statistical differences between
6	One-way ANOVA	**0.004**	Control females > Control males & Test males
12		**0.001**	Test females > Test males & Control males & Control females
18		**0.0009**	Test females > Test males& Control males
24		**0.009**	Test males > Control males
30		**0.006**	Test males> Control females & Control males
36		**0.0007**	Test males > Control females & Control males
42		**0.001**	Test males > Control females
48		0.119	
54		**0.009**	Test females > Control females
60		**0.0005**	Test males > Control females Test females > Control females

female fish is similar when data from tanks C3 and T3 are included (a, b) and when data from tanks C3 and T3 are excluded (c, d). From day 24, cadmium-exposed male guppies had a greater number of parasites than their respective females, and from day 36 control males had a greater number of parasites than the control females. Cadmium exposure, generally, increased the number of parasites above the controls in both sexes (Fig. 6.11a-d).

On statistical analysis of the data from all tanks (C1-C3; T1-T3), male guppies exposed to cadmium were found to have a statistically greater number of *G. turnbulli* than the control females at day 42 of the trial ($P = 0.028$) (Fig. 6.11b; Table 6.4). Analysis of the data when replicate 3 (C3, T3) was removed resulted in statistical significances at all sample points from day 6-60, with the exception of day 48 (Fig. 6.11d; Table 6.5). At day 6, the statistically greatest number of *G. turnbulli* was found on the control female guppies when compared to the males from both populations. Between days 12 and 18 test females had the statistically greater number of parasites than male guppies from control and cadmium-exposed populations. From day 24 onwards, cadmium-exposed males harboured significantly more *G. turnbulli* than control males at days 24, 30, 36 and 60, significantly more gyrodactylids than test females at Day 60 and more than control females at all sample points (Fig. 6.11d; Table 6.5).

6.3.3.4. The number of days to 100% parasite prevalence

As there were initially only 2 infected fish in each tank, transmission of parasites from these fish to the uninfected individuals could be monitored and the number of days taken to reach 100% parasite prevalence could be determined.

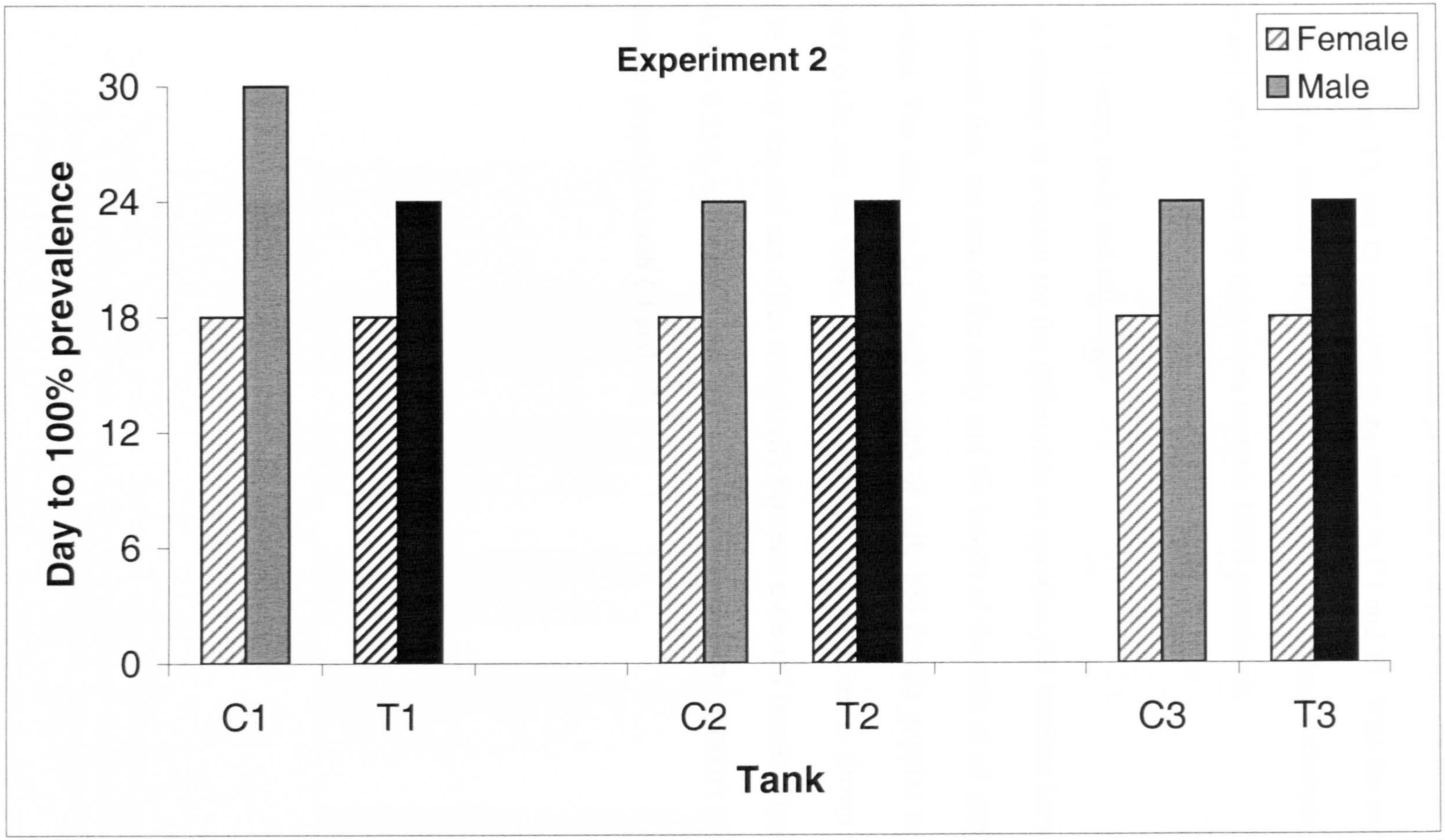


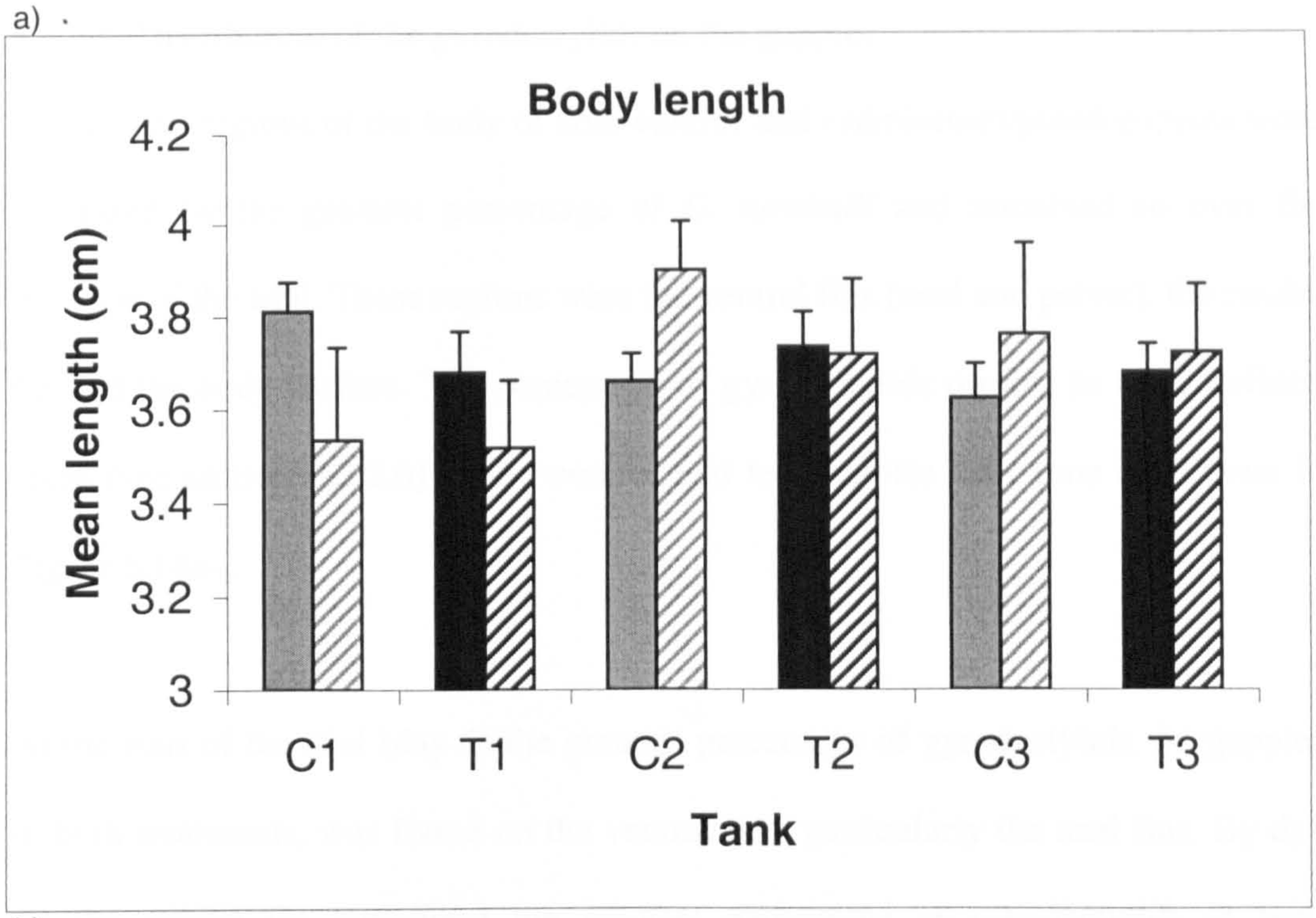
Fig. 6.12. Experiment 2 - The number of days taken to reach 100% parasite prevalence in control (C1-C3) and 5μg/l cadmium-exposed (T1-T3) guppies in each tank. Females are shown by the hatched bars, males are shown by the filled bars (Control tanks are shown in grey, 5μg/l cadmium-exposed tanks are shown in black).

Figure 6.12 shows the number of days to 100% parasite prevalence by male guppies (filled bars) and by the female guppies (hatched bars) in each tank. Female guppies were observed to reach 100% parasite prevalence 6 days before the males in C2, T2 and in C3 and T3, and 12 days before the males in C1 and T1. With the exception of T1 males that reached 100% prevalence before C1 males, cadmium exposure appeared not to affect the time taken to reach 100% prevalence.

6.3.3.5. Guppy body and tail length

In an attempt to account for the differences in gyrodactylid burdens between male and female fish, the size of the body and the length of the tails of all guppies were recorded. The mean (± S.E.) body length of male and female guppies is shown in Figure 6.13a and the mean (± S.E.) tail length of both sexes is shown in Figure 6.13b. Body size did not differ statistically between male and female guppies in any tank ($P = 0.534$), while tail length was statistically greater ($P = 0.0001$) in male than in female guppies in both C1 and C2.

a)



b)

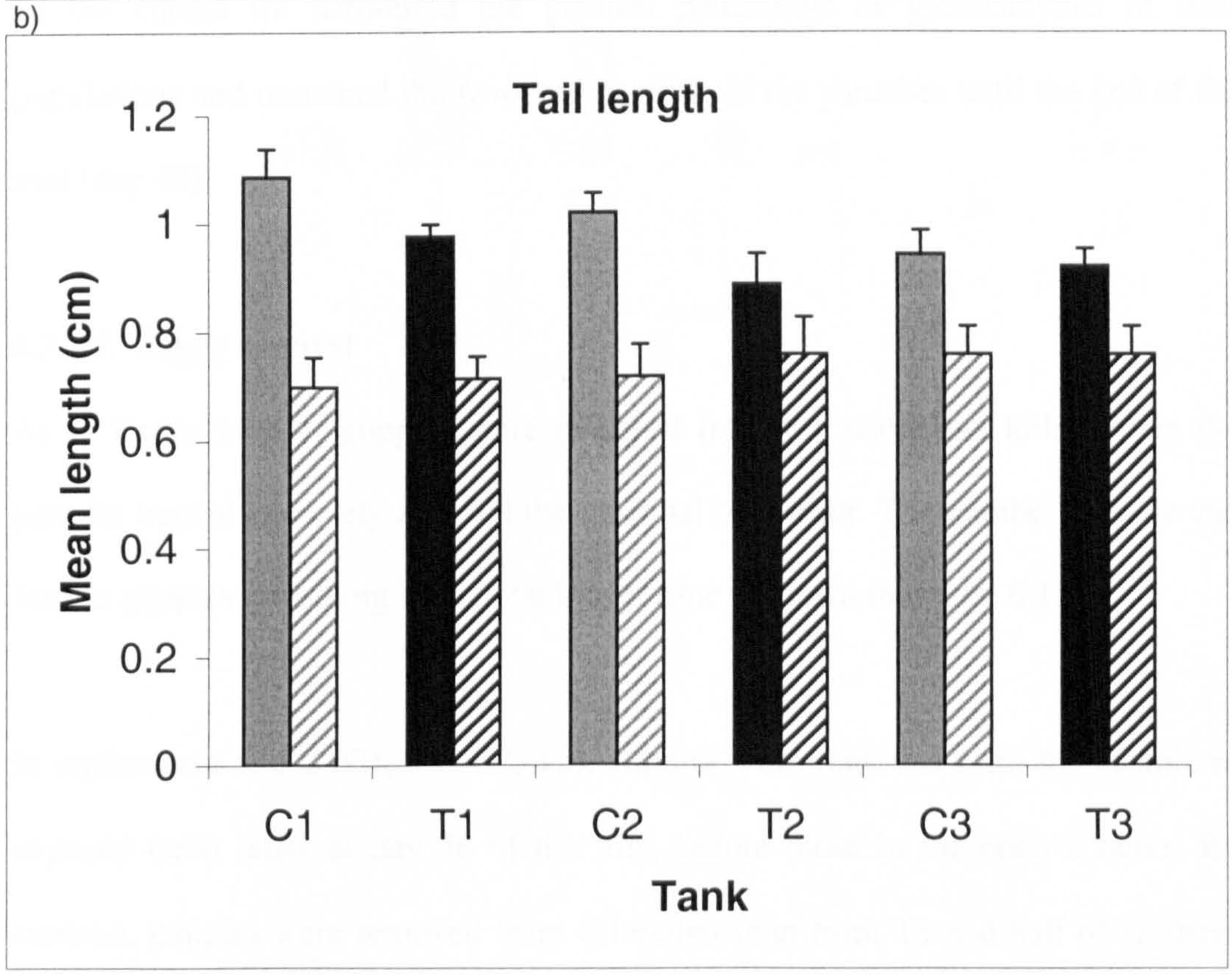


Fig. 6.13 a, b. Experiment 2 - The size of male and female guppies from control and 5μg/l cadmium-exposed tanks. (a) mean body length and (b) mean tail length. Females are shown by the hatched bars; males are shown by the filled bars. Control tanks are shown in grey, cadmium-exposed tanks are shown in black.

6.3.3.6. Distribution of the gyrodactylids on the guppies

Three main regions of the body of both control and cadmium-exposed guppies were colonised by the greatest percentage of *G. turnbulli* and remained so over the duration of the trial. These regions were the ventral fins (anal and pelvic), the caudal fin and the body surface. The percentage of gyrodactylids present in the 7 defined areas (see section 6.2.2.6) of the control and test guppies over time are shown in Figure 6.14a-j.

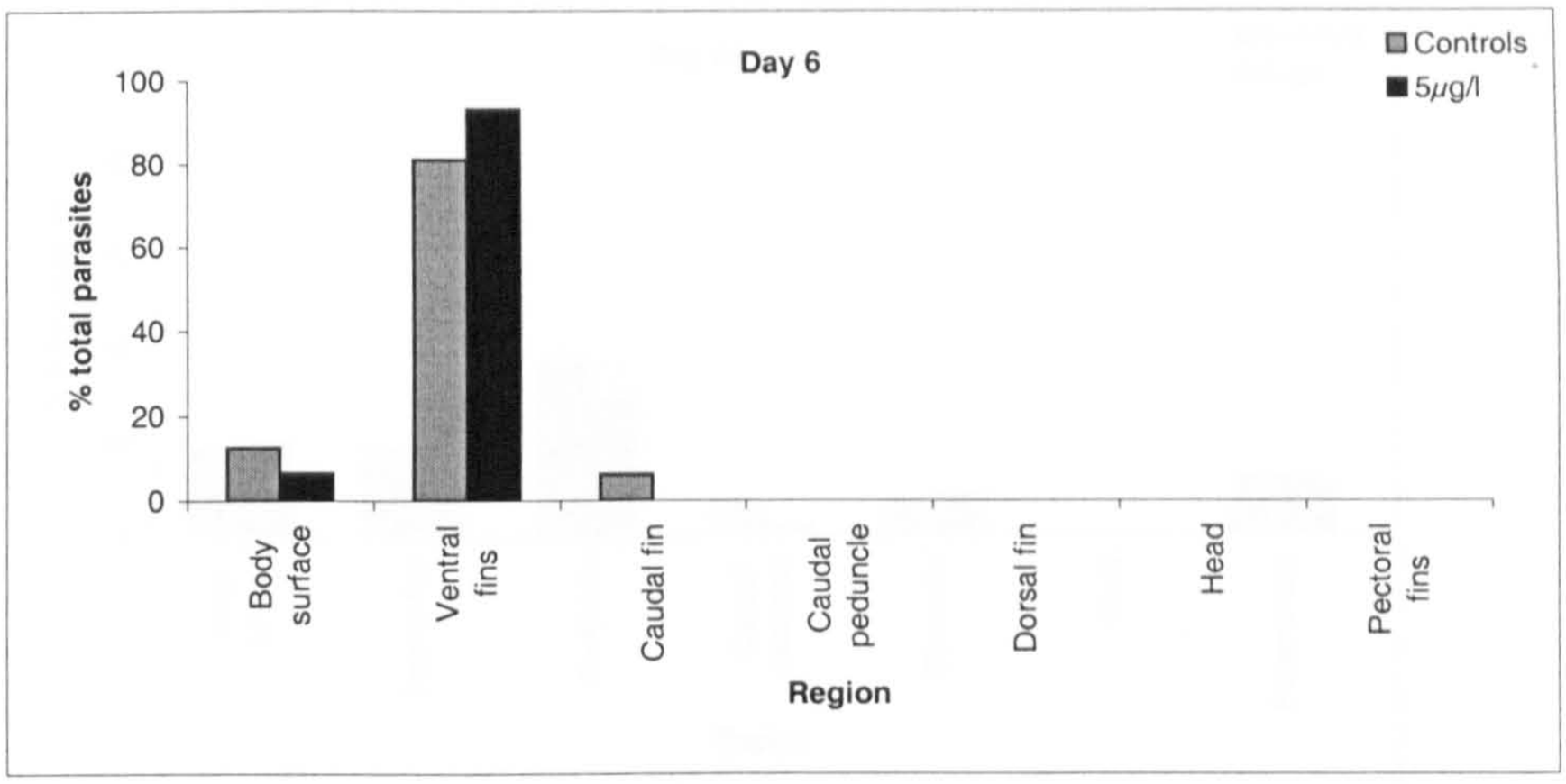
At the start of the trial (day 6) the greatest percentage of gyrodactylids, on guppies in both treatments, was found on the ventral fins, particularly the anal fins. By day 18 the caudal fin harboured the greatest percentage of gyrodactylids in both populations and remained the favoured position of the parasites until the end of the trial (day 60).

6.3.3.7. Guppy survival

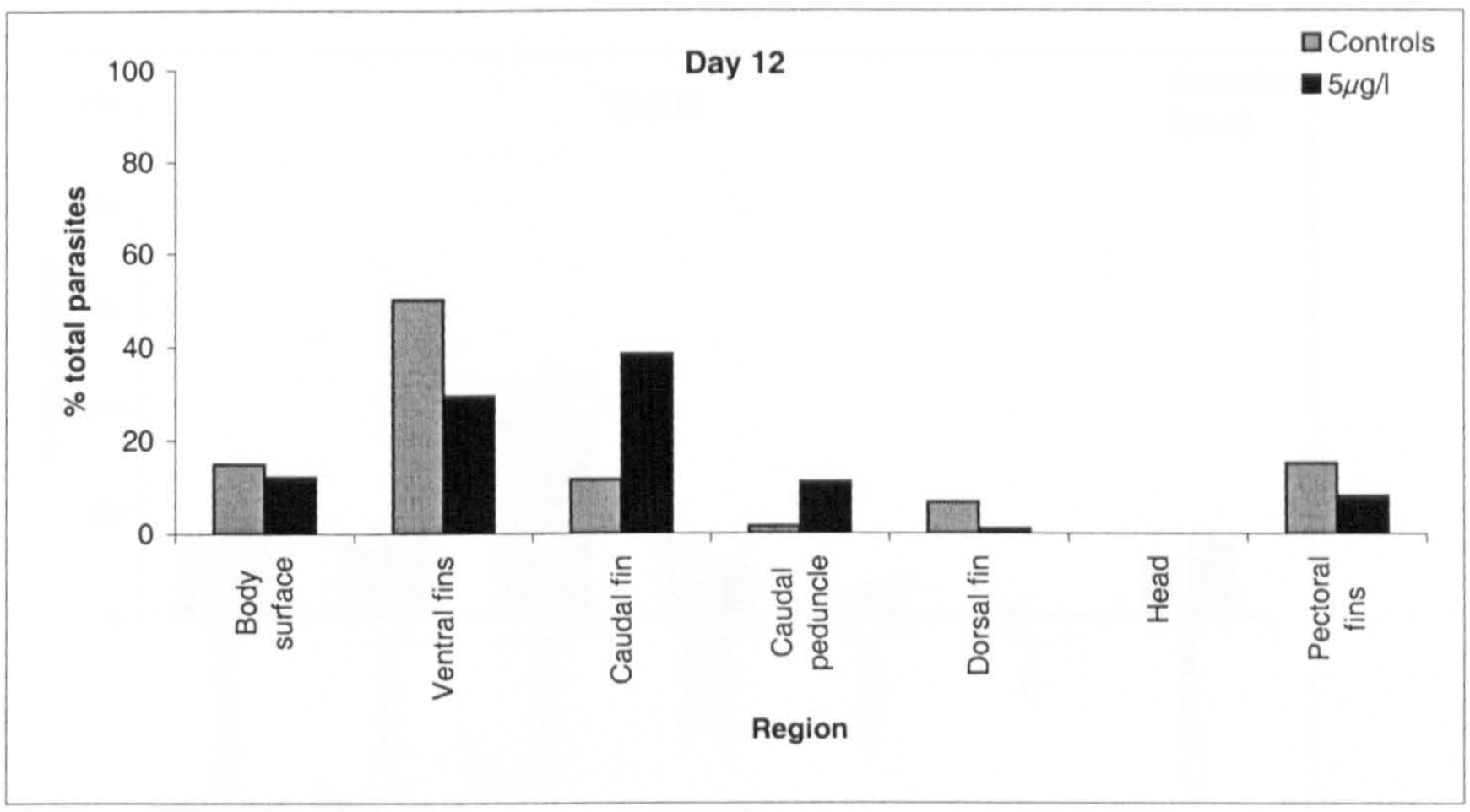
As in Experiment 1, guppies were removed from the tanks and killed when the parasite burden adversely affected their normal behaviour. The number of male and female guppies remaining in each tank over time is shown in Figure 6.15a-f.

In replicates 1 and 2 (C1, T1; C2, T2), guppies were removed from the cadmium-exposed (test) tanks at day 36 of the trial, before those in the control tanks. By contrast, guppies were removed from C3 earlier than from T3 and half of all these fish had been removed by day 36 (Fig. 6.15c, f). However, log-rank comparisons, as part of Kaplan-Meier survival analysis, showed there to be no statistically significant differences in the rate of removal of guppies from the different

a)



b)



c)

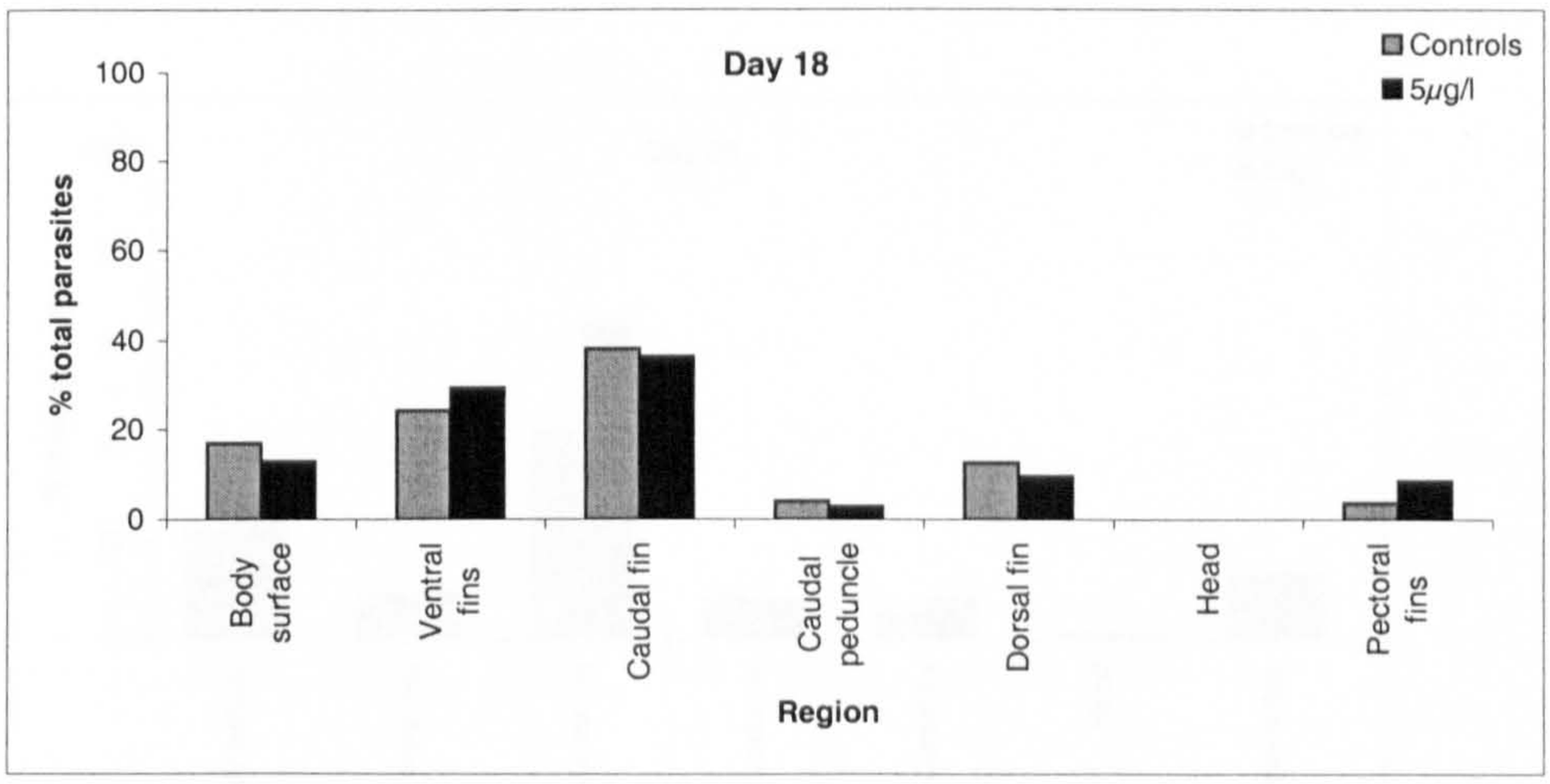
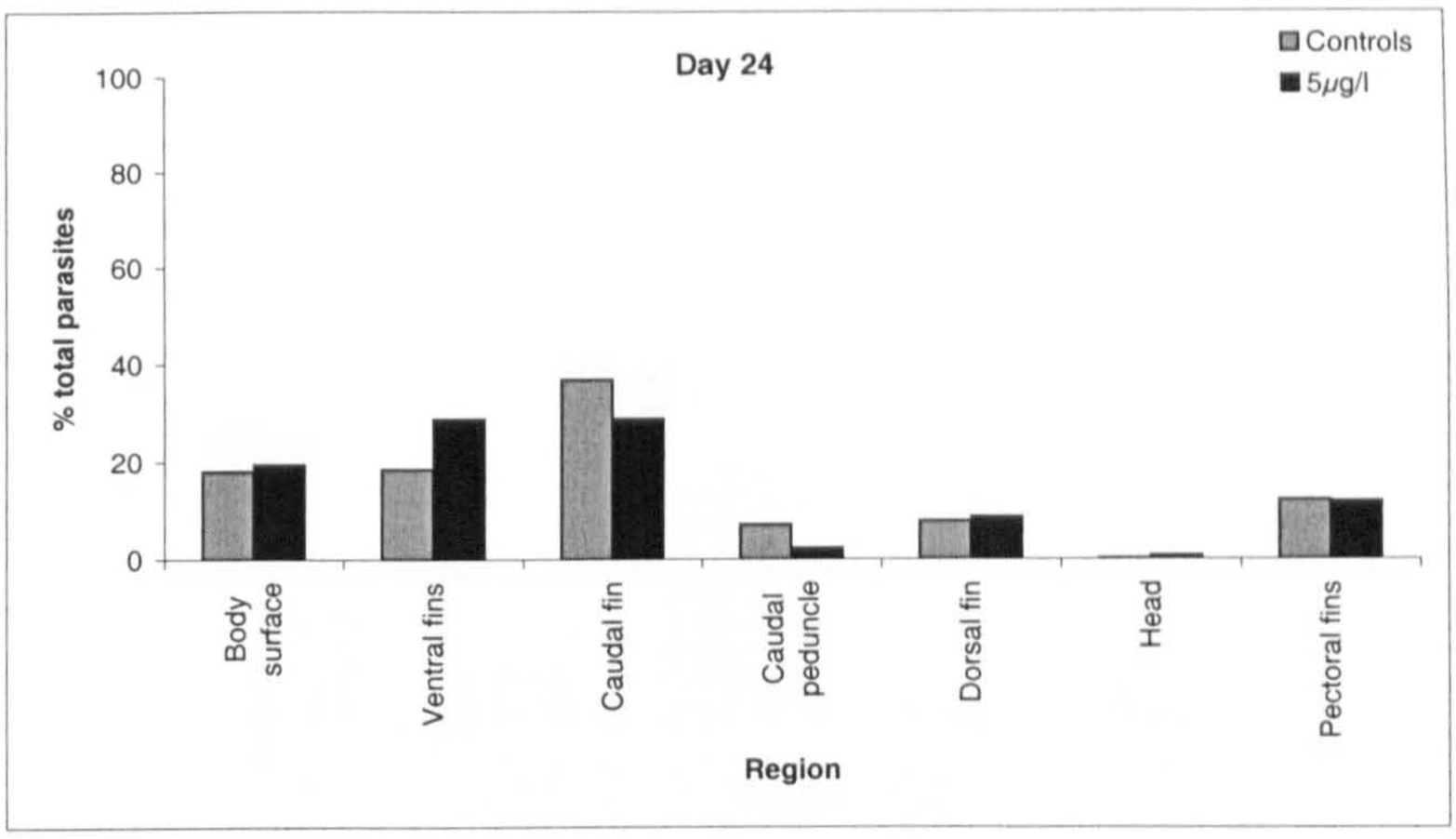
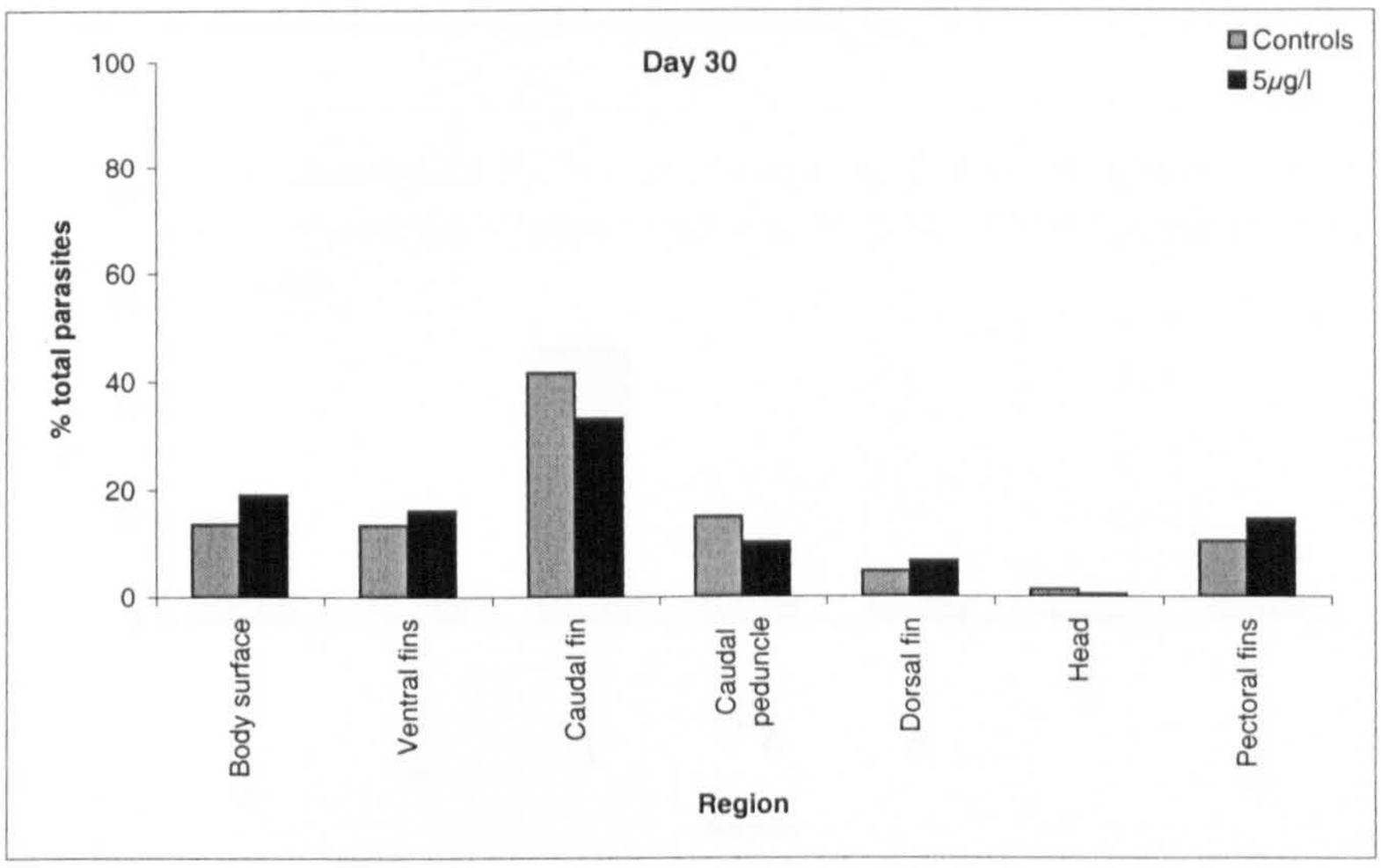


Fig. 6.14a-c. Experiment 2 - The percentage of *G. turnbulli* present in each area of control and 5µg/l cadmium-exposed guppies at (a) 6, (b) 12 and (c) 18 days post-start of the trial. All values are calculated from 3 pooled replicates.

d)



e)



f)

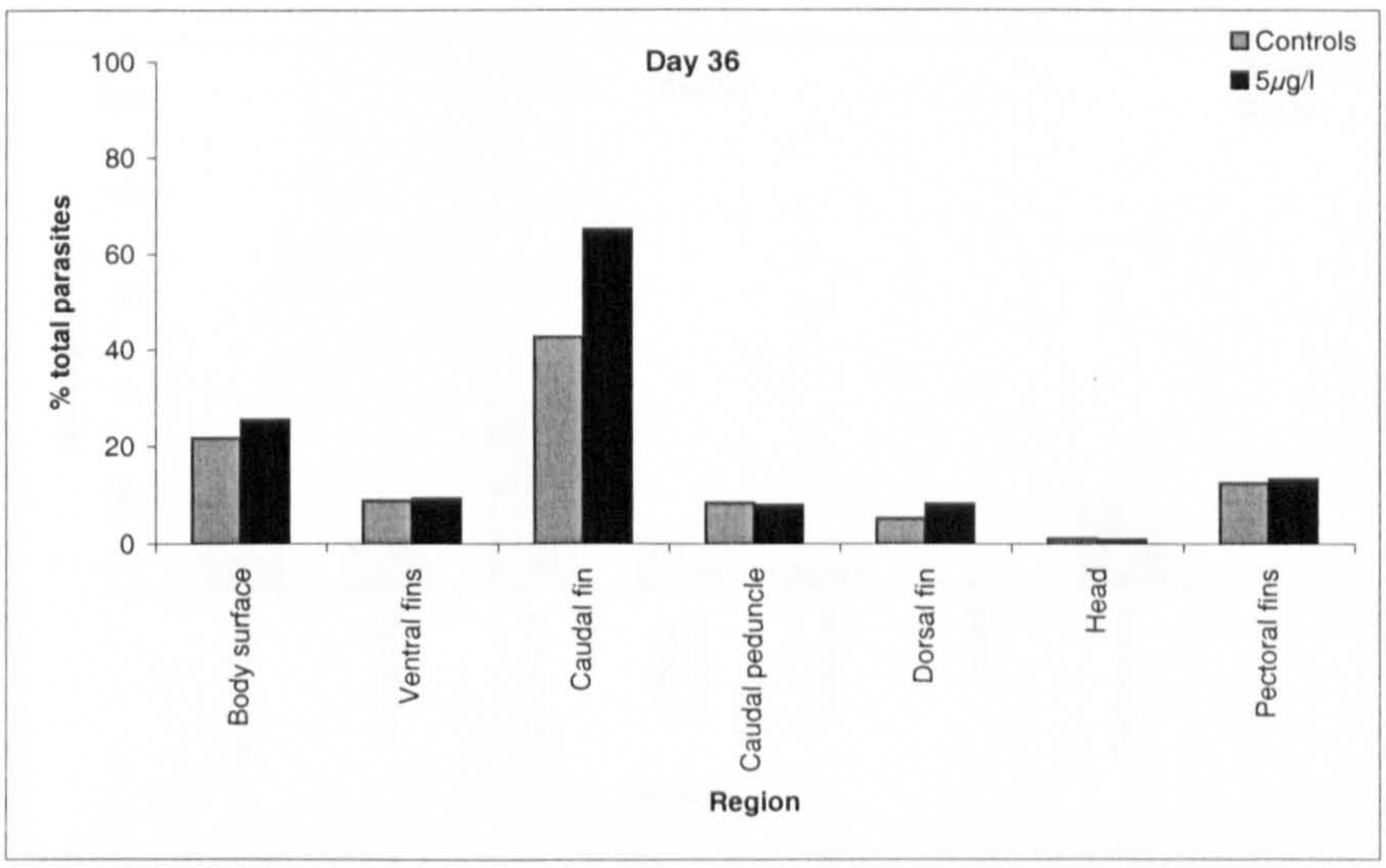
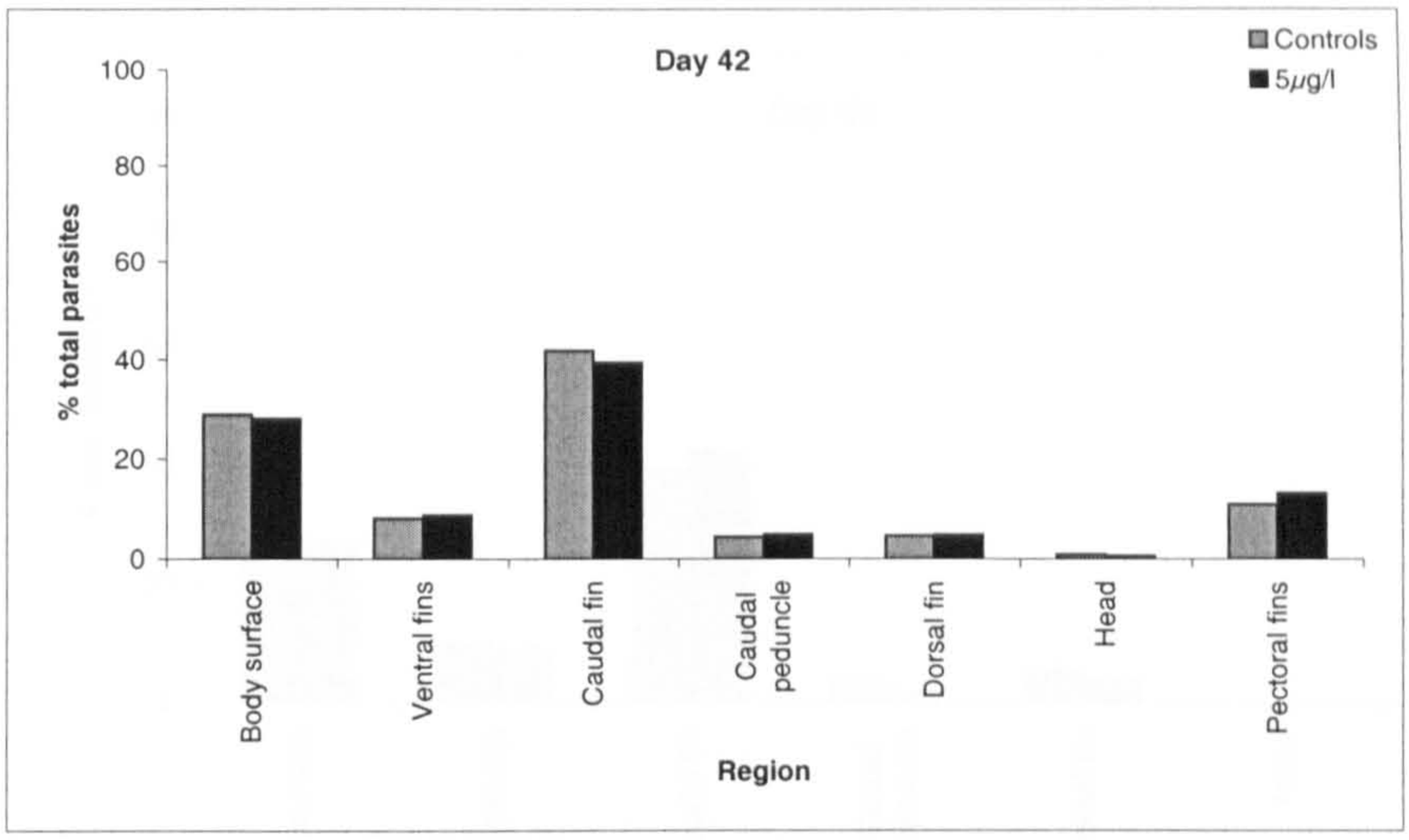
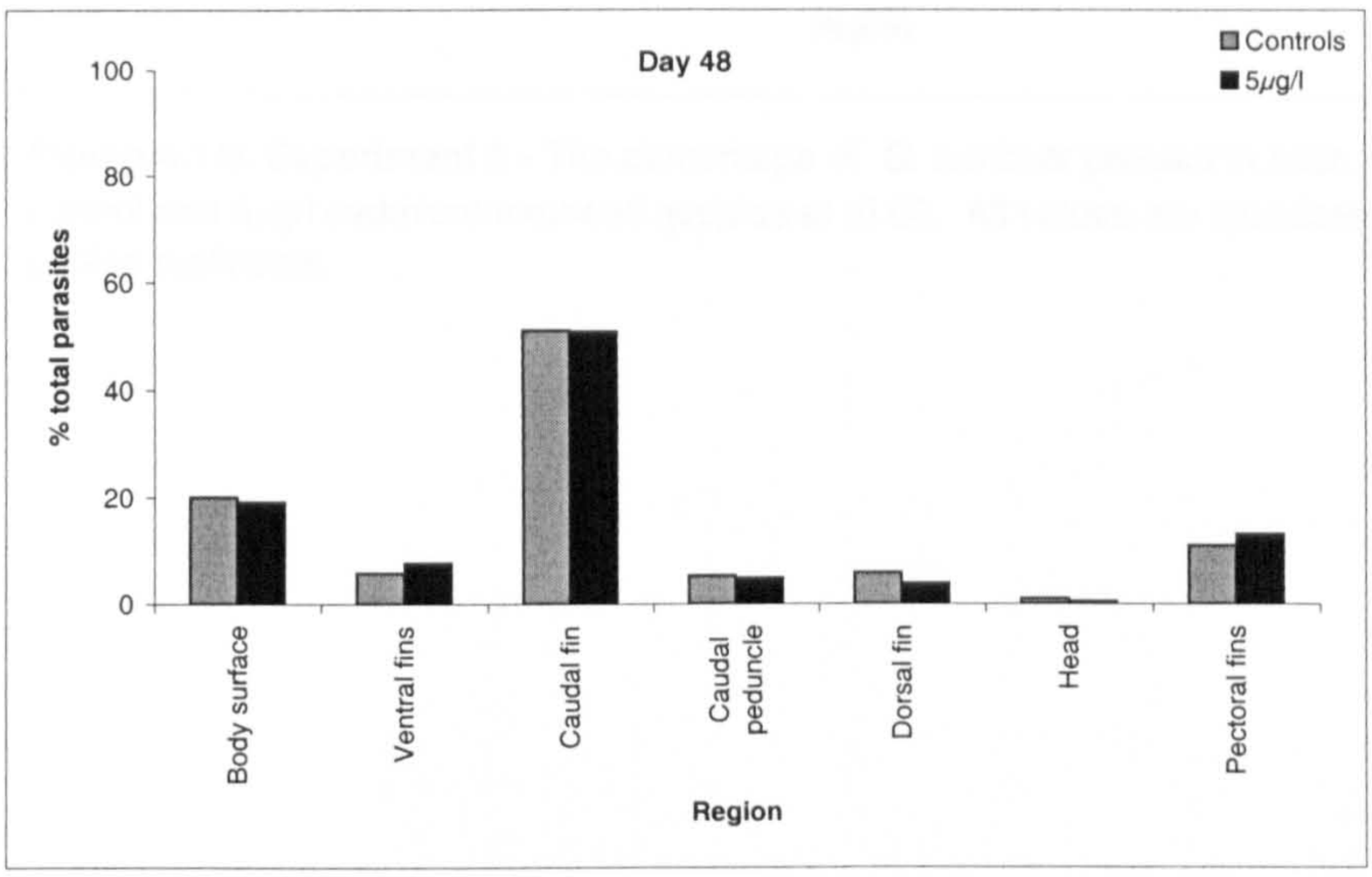


Fig. 6.14d-f. Experiment 2 - The percentage of *G. turnbulli* present in each area of control and 5µg/l cadmium-exposed guppies at (d) 24, (e) 30 and (f) 36 days post-start of the trial. All values are calculated from 3 pooled replicates.

g)



h)



i)

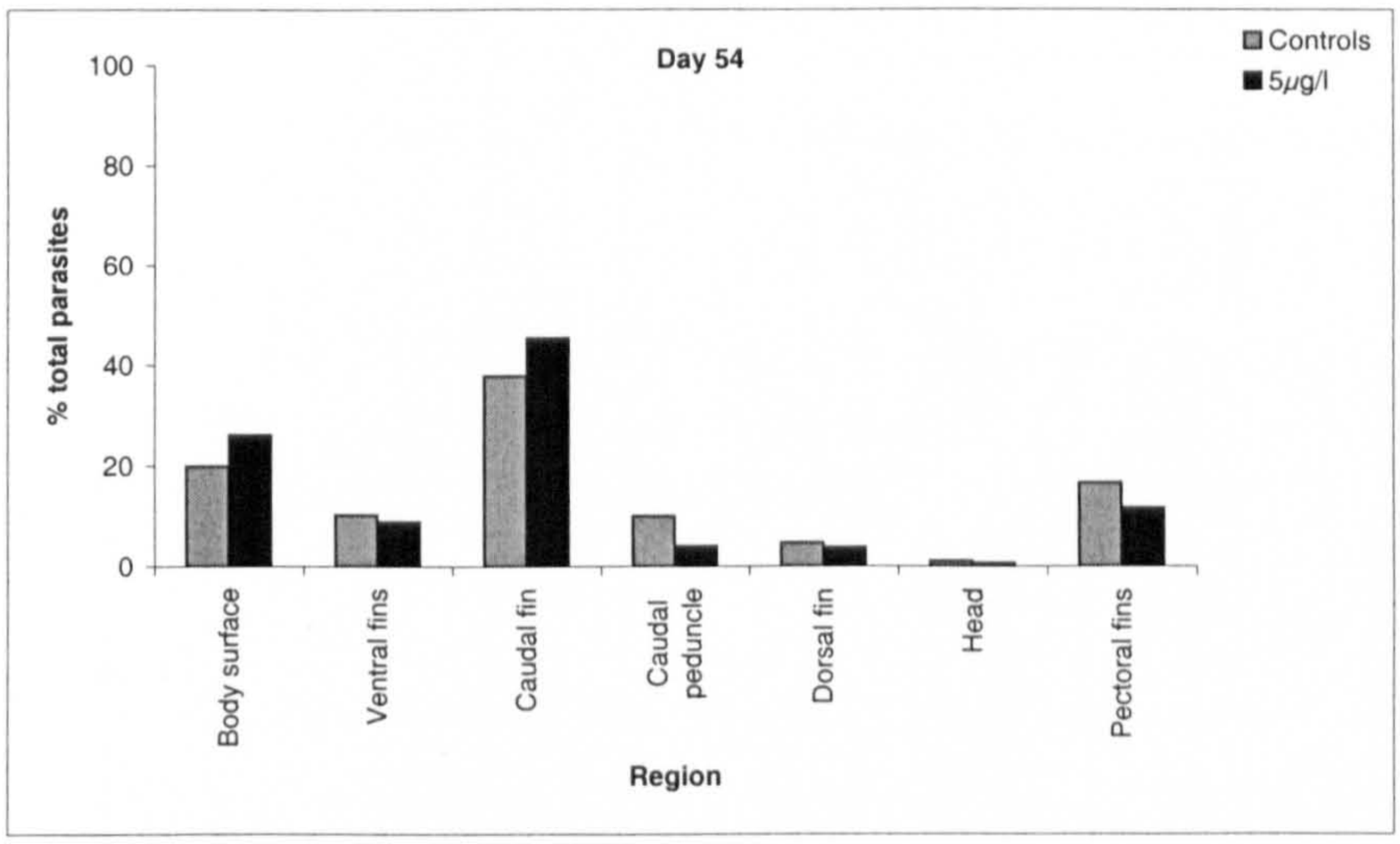


Fig. 6.14g-i. Experiment 2 - The percentage of *G. turnbulli* present in each area of control and 5μg/l cadmium-exposed guppies at (g) 42, (h) 48 and (i) 54 days post-start of the trial. All values are calculated from 3 pooled replicates.

j)

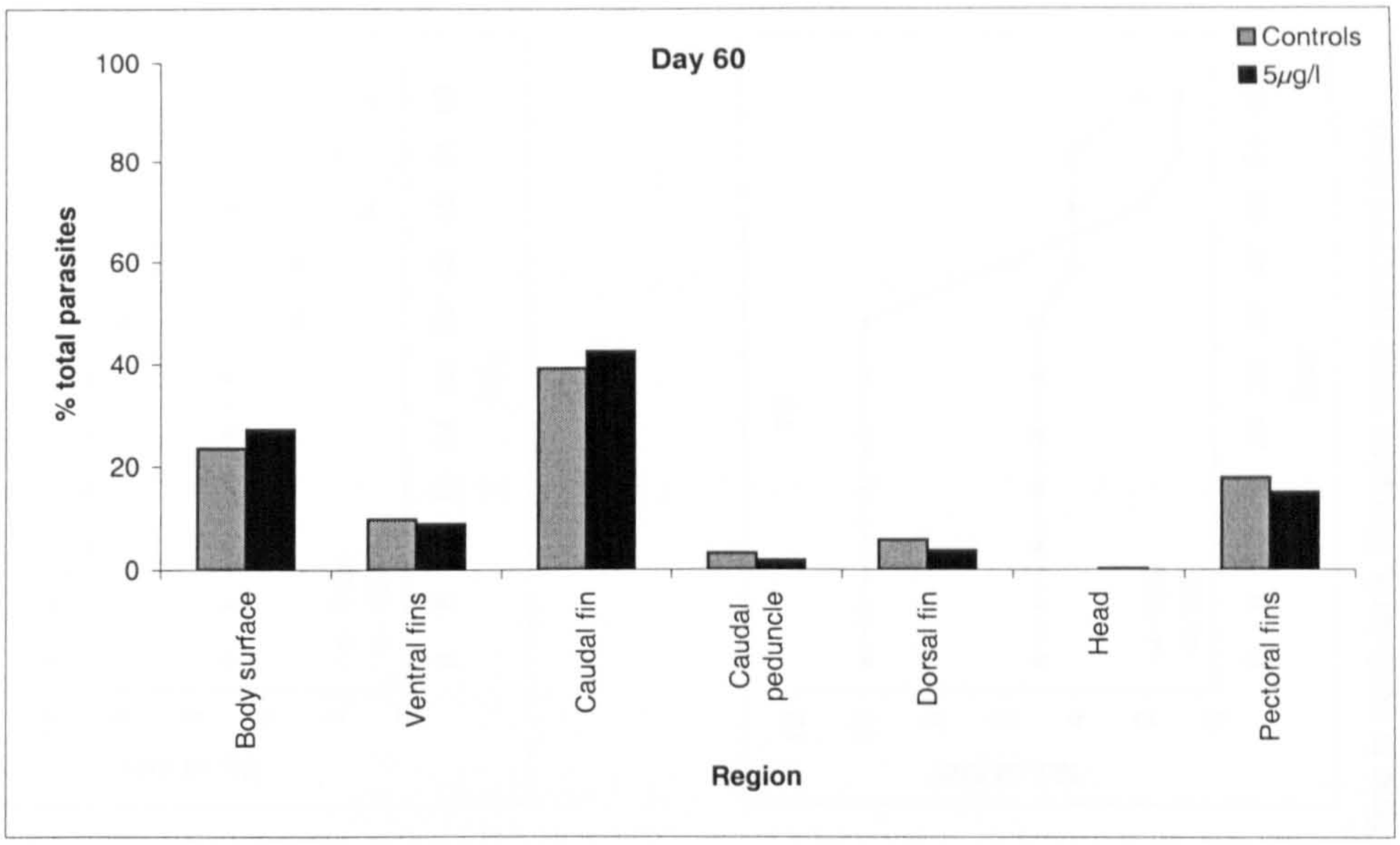
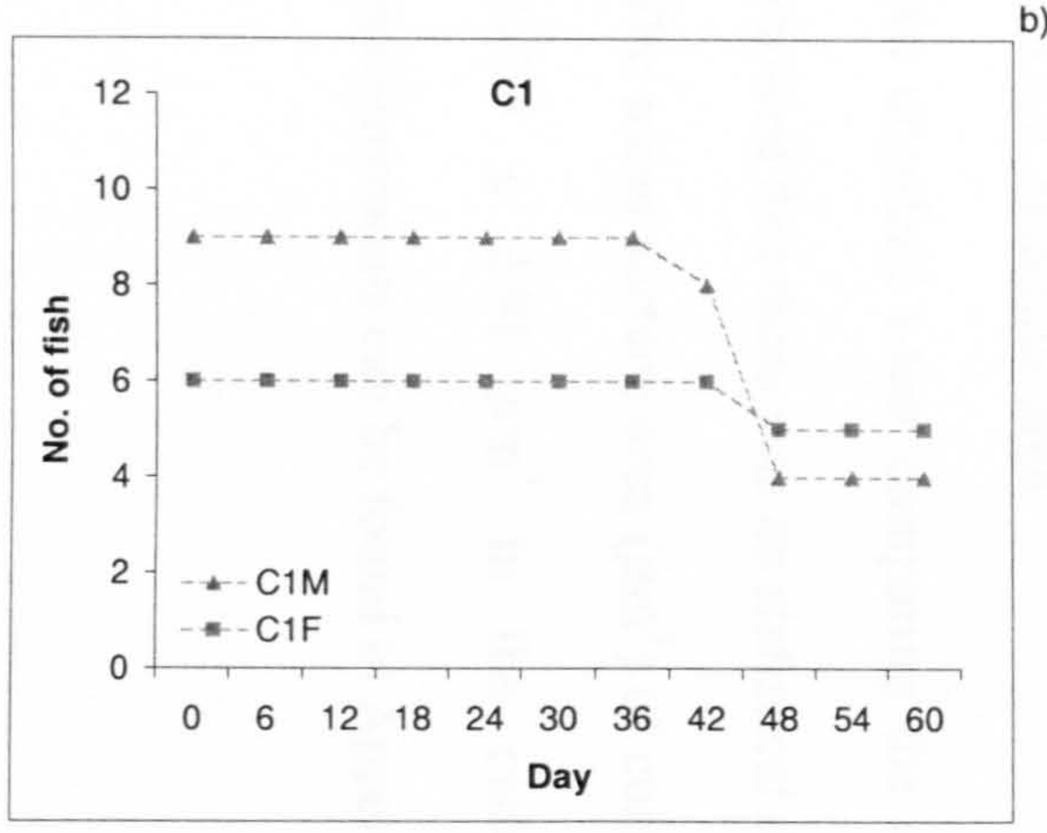
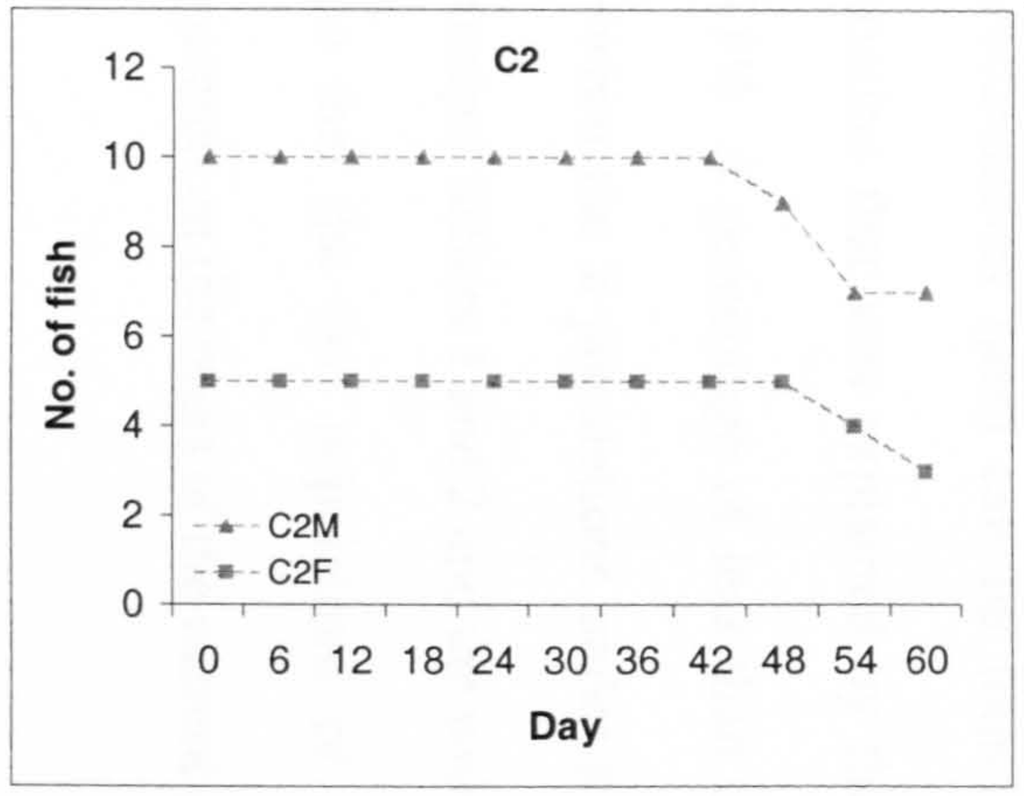


Figure 6.14j. Experiment 2 - The percentage of *G. turnbulli* present in each region of control and 5μg/l cadmium-exposed guppies at (j) 60. All values are calculated from 3 pooled replicates.

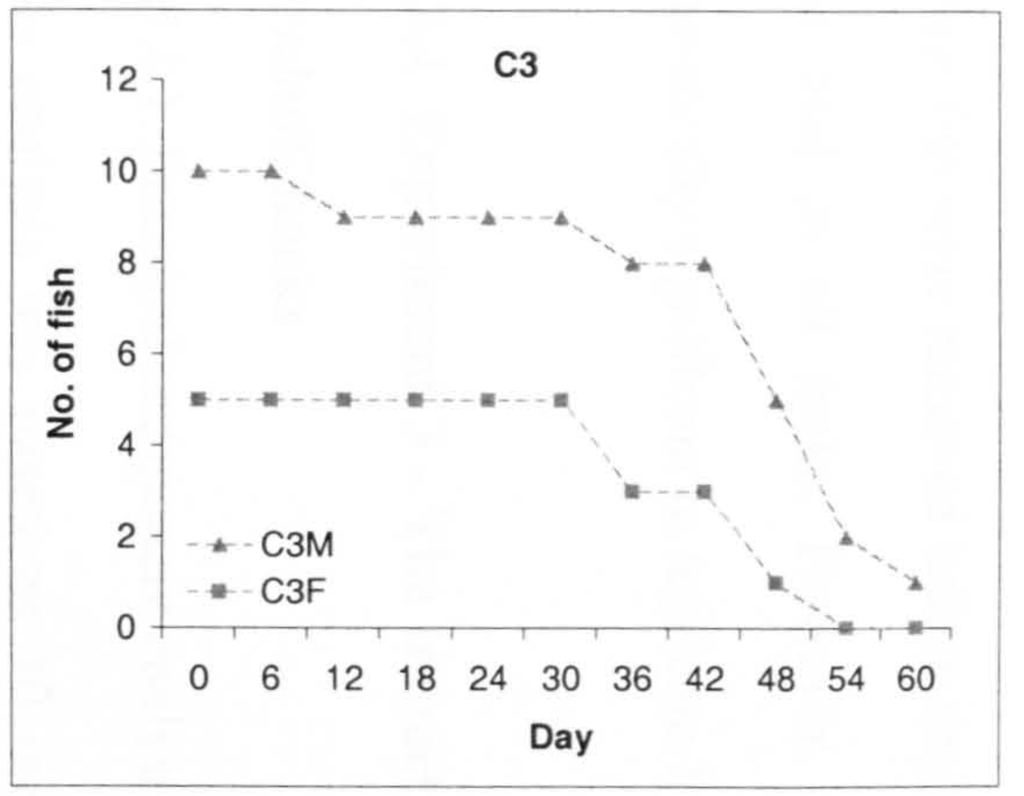
a)



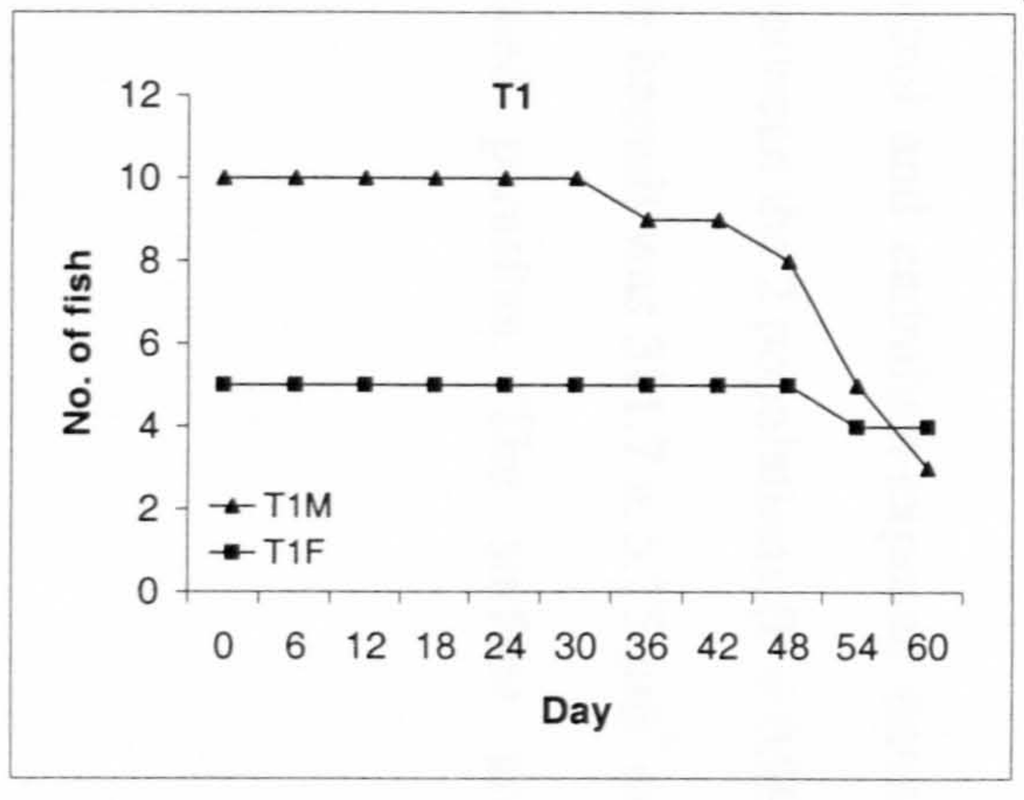
b)



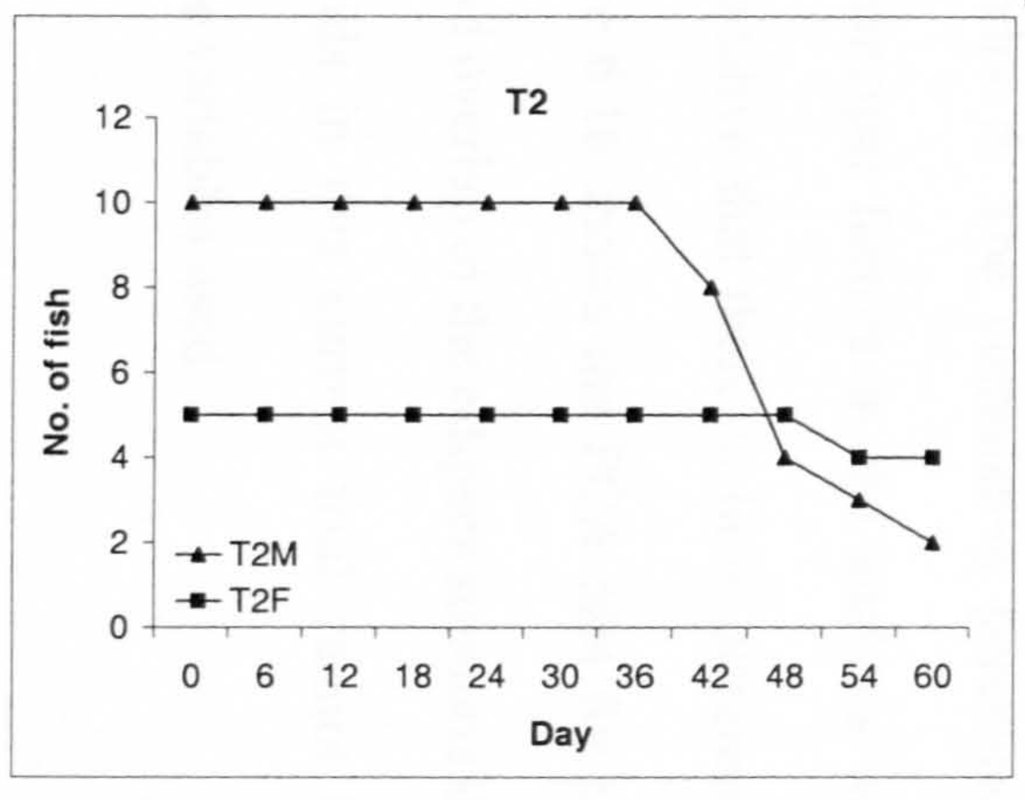
c)



d)



e)



f)

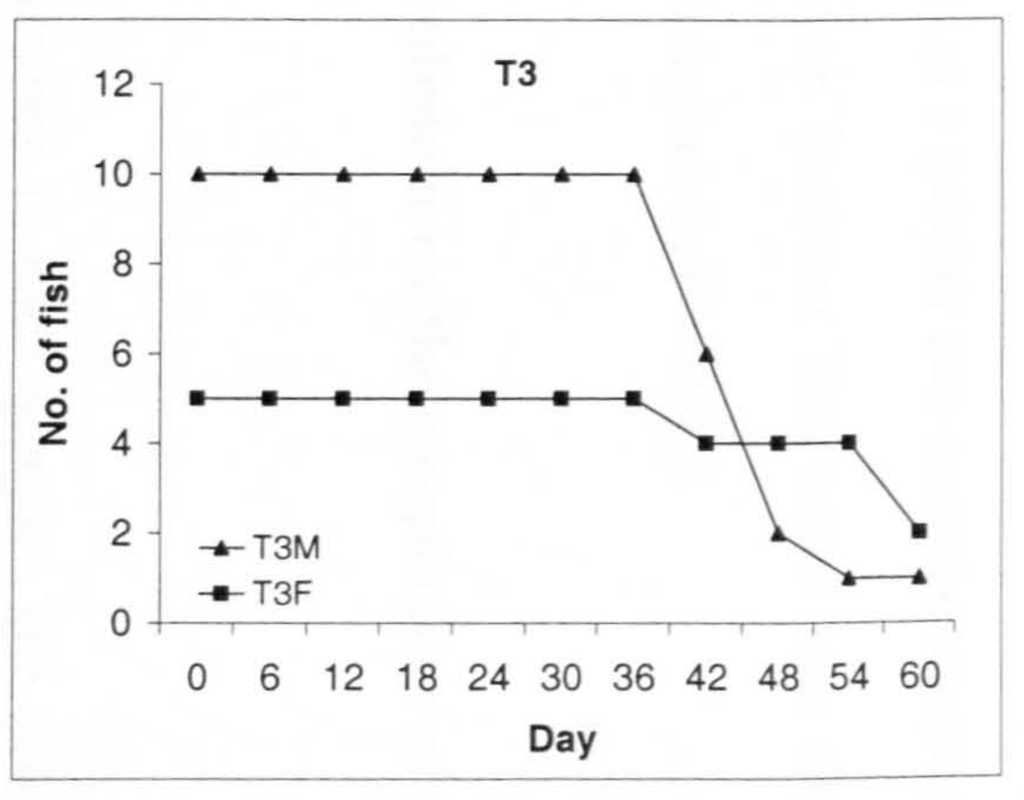


Fig. 6.15a-f. Experiment 2 - The number of male and female guppies in 0 and 5μg/l cadmium remaining over time in each tank. Guppies were removed from tanks when their parasite burden impeded normal swimming behaviour.

treatments in any replicate (P = 0.79, 0.29, 0.20 in replicates 1-3, respectively). Male fish were removed before any females and at a quicker rate over the course of the trial in all tanks. However, the difference between the sexes was only statistically significant in replicate 1 (log-rank comparison P = 0.02).

6.3.4. Experiment 3 - The impact of 5μg/l cadmium on the morphology of *G. turnbulli* hooks

6.3.4.1. Principal component analysis

The analysis was based on 10 individual parasites in each sample. The actual measurements (μm) can be found in Appendix 5. The cumulative percentage variation that was explained by the first 3 principal factors in the analysis was 54.9%. A percentage of less than 60% is indicative that there is little difference between the 2 populations under study. Figure 6.16 shows the PCA plot for the principal factors 1 and 2 and the wide spread and overlap of the ellipses supports the idea that the two populations of gyrodactylids in this current trial cannot be separated on the basis of the current data and the variables used.

6.3.4.2. Hamulus area

An unpaired t-test comparing the area of control and cadmium-exposed hamuli showed that there was no statistical difference between the 2 populations (P = 0.99). The mean surface area (μm^3) of control parasite hamuli was 311.7 ± 5.75 μm^3 and 311.7 ± 2.91 μm^3 in the cadmium-exposed parasites. The surface area measurements can be found in Appendix 4.

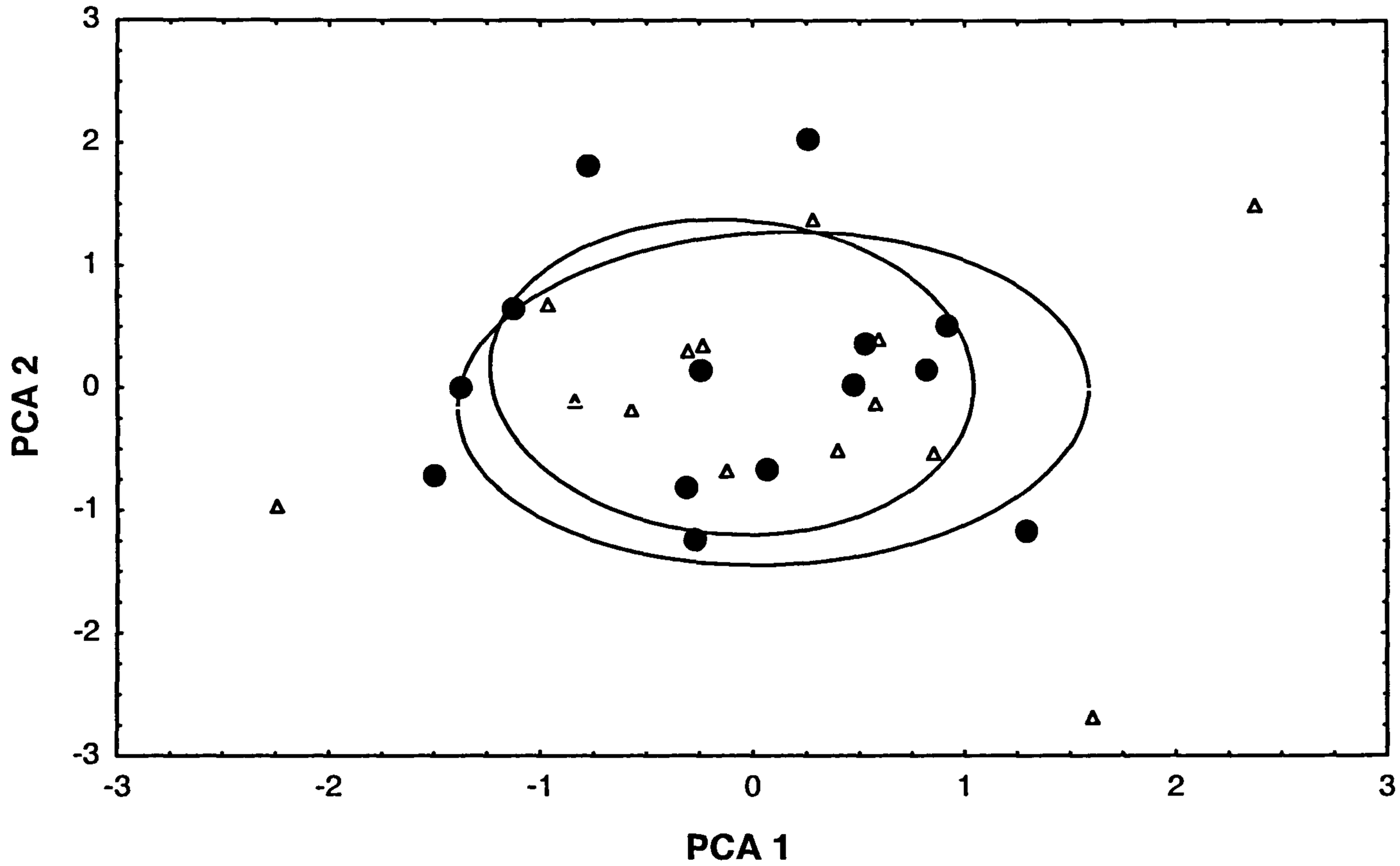


Figure 6.16. PCA plot of the *G. turnbulli* hook measurements. Triangles and dotted line represent cadmium-exposed hooks (6 weeks exposure), circles and solid line represent control hooks (2 weeks exposure). 54% of the total variation is explained by these 2 factors and the ellipses encompass 70% of the points for each group.

6.4. Discussion

The aim of this chapter was to determine, under controlled experimental conditions, how exposure to the maximum permissible concentration of cadmium (5μg/l) would affect the population dynamics of gyrodactylids parasitising guppies.

The population dynamics of both *G. bullatarudis* and *G. turnbulli* on guppies in the current trials appears to be affected by exposure to low concentrations of cadmium, resulting in statistically increased numbers of parasites compared to those on unexposed guppies. This finding is particularly interesting, given that the concentration of cadmium used in these trials was below that permitted in freshwater sources.

The time it took for all fish in each tank to become infected with gyrodactylids (100% prevalence) was independent of cadmium-exposure, and thus the differences that were observed between the numbers of parasites in the two populations occurred after the fish had become infected and were not a function of an increased transmission rate and colonisation on guppies by cadmium-exposed gyrodactylids.

While there are no other experimental studies specifically looking at the impact of heavy metals on populations of gyrodactylids, the findings of these current trials correlate with those of Khan & Kiceniuk (1988), Marcogliese *et al.* (1998) and Moles & Wade (2001), who all recorded higher infestations of gyrodactylids on a range of marine fish exposed to hydrocarbon pollution. Also those of El-Naggar *et al.* (2000), who found that the levels of iron in the water were intrinsically linked to the number of *G. cichlidarum* present on *Oreochromis niloticus* and *Tilapia zilli*. In

contrast to the findings of the present trials, Zharikova (1993) observed lower levels of *G. elegans* on *Abramis brama* (L.) taken from a section of the Volga River polluted by a range of heavy metals and hydrocarbons, and Soleng *et al.* (1999) found that the maximum permissible level of aluminium (200μg/l) eradicated rather than increased populations of *G. salaris* on Atlantic salmon.

The exact mechanisms that resulted in the increased number of gyrodactylids on cadmium-exposed guppies were not elucidated in these experiments. Exposure to cadmium could have impacted directly on the parasite population or indirectly by acting on the immune responses of the guppies. It has been suggested by Marcogliese *et al.* (1998) and Moles & Wade (2001) that the observed increases in *Gyrodactylus* spp. on pollutant-exposed fish was the result of pollutant-induced immunosuppression in the host, resulting in a proliferation of the parasite population. The study by Moles & Wade (2001) incorporated an assessment of the impact of the hydrocarbon pollution on the macrophage function (phagocytosis and superoxide production) of *Ammodytes hexapterus* Pallas, along with an assessment of the level of *Gyrodactylus* spp. infection. The highest concentration of hydrocarbons was observed to immunosuppress the host (reduced phagocytosis and superoxide production), leading to high burdens of gyrodactylids, while lower levels of pollution enhanced the immune responses of the host and resulted in low burdens of gyrodactylids (Moles & Wade, 2001). It is possible that cadmium-exposure in the current trial affected some aspect of the guppy immune response resulting in the greater burden of gyrodactylids. The effect of cadmium on the host responses is an important factor, deserving considerable attention, and will be discussed in the following chapter (Chapter 7).

Experiment 1 of the current trial, acted as a preliminary assessment of the effect of cadmium on the population dynamics of gyrodactylids using *G. bullatarudis*. Once it had been ascertained that 5μg/l cadmium was capable of eliciting a response from the guppy-gyrodactylid model system, Experiment 2 was carried out using *G. turnbulli* due to the large amount of available literature regarding the population dynamics of this species. It is this second experiment (Experiment 2) that offers some of the most interesting results for discussion.

Perhaps the first thing to note in Experiment 2 is the anomalous results shown by control tank 3 (C3). In contrast to the other control tanks in the trial, C3 had a greater number of parasites than its cadmium-exposed replicate T3 and the number of *G. turnbulli* in both these tanks (C3, T3) increased at a greater rate than the other two replicates (C1, T1; C2, T2). There appear to be two possible explanations. Firstly, in all tanks, except C3, the first day that guppies were removed on the basis of welfare issues (*i.e.* when high parasite burdens inhibiting swimming and feeding) was day 36 and that by this time, in control tank 3 (C3), 50% of the fish had already been removed. The guppies in this tank showed signs of a bacterial or viral infection, which may have resulted in their having a lowered immune response and thus less control over their gyrodactylid infection than the fish in the other tanks. One male guppy suffered from a swollen belly (ascites) coupled with scale protrusion, indicative of a bacterial or viral infection, the clinical signs of which are commonly referred to as dropsy. The result was that the guppy was dead by day 9 of the trial. No other fish in C3 died during the trial, but scale protrusion was observed in several other guppies in this tank over the duration of the trial. Unfortunately, as the gyrodactylid infections on all other guppies in the tank were being monitored

every 6 days, it was not possible to kill one of these guppies and send it for post mortem analysis. In future experiments, it would be advantageous to add extra guppies to each tank so that, in the event of a possible disease outbreak, there would be guppies available to be post-mortemed without disrupting the experiment.

Secondly, one of the guppies from C3 that was initially infected with three gyrodactylids was a large, bright blue male, who appeared more dominant than the other males in the tank, particularly with regard to courting the female guppies. Combes (2001) discussed the implications of brightness in male organisms with regard to parasitism. Brightly coloured males have the advantage of increasing their chances of attracting a mate but the disadvantage of the energy costs of building and maintaining this secondary male characteristic (Combes, 2001). The androgens involved in developing these secondary sexual characteristic immunosuppress the male, which can result in its being less resistant to, and more likely to become infected by parasites. The rapid increase in number of parasites on the bright blue male guppy in C3 could be attributed to the costs it incurred in maintaining its bright colouration, which subsequently led to some degree of immunosuppression.

The more rapid increase in parasite number on guppies from both C3 and T3, compared to the other two replicate pairs of tanks, could also be attributed to their being the last tanks to be started in the trial, as each tank was started on consecutive days. While this meant that they were subject to two extra days of acclimation to their new conditions, they were also subjected to two extra days of water changes compared to replicate 1 (C1, T1) and 1 extra day compared to replicate 2 (C2, T2). During water changes, all guppies in each tank were removed and placed in a

separate vessel containing the same water from which they had been removed. While the removal of fish was carried out as carefully as possible, there is no doubt that it would have been a stressful procedure that could have affected the immune status of the guppies. This may have resulted in the guppies that were subjected to more water changes being more immunocompromised than those subjected to fewer changes, leading to a greater prevalence of gyrodactylids. For example, lifting carp from their tanks for 30 seconds, three times a day, has the effect, as recorded by Jacobs (1995), of increasing the number of dactylogyrids on the gills from only 2/fish to 4.8/fish after 28 days of the experimental stressing. It is thus possible that the extra days of netting, which the guppies from C3/T3 were subjected to, could have increased the number of *G. turnbulli* on these fish. Infecting the two guppies required to start the trial in both C3 and T3 had taken longer than with the other two replicates, with the gyrodactylids proving more difficult to transfer from their dead host. Thus, the guppies being infected were anaesthetised and recovered several times before the infection process was completed, and the added stress of such an operation may have altered their subsequent resistance to gyrodactylid infection. Unfortunately, because there were not enough guppies available, these two fish had to be used, although it would have been preferred to infect new guppies subjected to a shorter period of anaesthesia. Due to the anomalous results of C3, and to a lesser extent T3, and the variety of factors possibly contributing to the peculiarity of the data, it was considered best to analyse the data-sets both with and without the third replicate (C3, T3).

At least half of the guppies in each tank in Experiment 2 could be individually identified by their colour pattern, enabling the gyrodactylid infection on individual

fish to be monitored over the 60-day trial. This gave rise to two further interesting observations. Firstly, in both control and cadmium-exposed populations of guppies, there was heterogeneity in the response of the individual fish to the gyrodactylid infections, particularly in the cadmium-exposed population. Such heterogeneity has been recorded in guppies infected with *G. bullatarudis* (see Madhavi & Anderson, 1985) and with *G. turnbulli* (see Scott, 1982; Scott & Anderson, 1984), and in *Oncorhynchus mykiss* and *Salmo salar* parr of the Norwegian Lone and the Baltic Neva strains, all infected with *G. salaris* (see Bakke, 1991; Bakke *et al.*, 1991). In this latter study, the two salmon species differed in their resistance to *G. salaris* infection as well as showing marked heterogeneity among individuals of the same species. The Norwegian Lone strain had little control over the growth of *G. salaris* populations, with the numbers of parasites continuing to increase steeply over time, while *G. salaris* infections on the Baltic Neva salmon increased up to about day 20, when the number of parasites fell, reaching low numbers on all fish by day 30 (Bakke, 1991).

Scott (1982) attributed the heterogeneity, which she observed in the response of the guppies to gyrodactylid infection, to stochastic elements in the experimental system, with chance playing a large part in determining the number of resistant and susceptible guppies in a small population. In a further study, it was suggested that the heterogeneity was due to one or more factors, including host behaviour, host genetics and the past or current experience of infection of each guppy (immune status) (Scott & Anderson, 1984). The greater heterogeneity seen in the response of cadmium-exposed guppies to gyrodactylid infection could be the function of different susceptibilities of the fish to cadmium exposure, with some guppies being

more resistant to exposure than others. Varying immune condition, caused by the cadmium, could have led to varying susceptibilities to gyrodactylid infection among the guppies.

The second interesting feature to note from looking at the population growth of *G. turnbulli* on identifiable fish was that the population growth of these parasites was, generally, similar on guppies from both populations up until day 42. Up to this point, both populations had shown a rapid increase in parasite number between days 12 and 36, followed by a decline in number at about days 42-48. However, at around day 42, the majority of fish in the control tanks began to show a decrease in the number of parasites, an observation also made by Richards & Chubb (1998), who found that the mean intensity of *G. turnbulli* on guppies increased until day 50, when it began to fall, followed by persistence of very low levels of infection on a few individuals in the population only. A greater proportion of the guppies in the cadmium-exposed tanks, however, continued to show an increase in the number of gyrodactylids beyond day 42. It is possible that cadmium was affecting the immunity of these guppies and altering some aspects of their immune response, compared to the controls, leading to proliferation of the gyrodactylid population. As previously stated, determining the impact of cadmium on guppy immune responses may help to elucidate the findings from this current trial.

The history of the guppies used in these experiments was unknown. In future trials, it may be advantageous to use only naïve guppies, so that none of the fish have a past experience of infection that could affect both the heterogeneity in the population and the overall pattern of gyrodactylid population growth. It would also

be interesting to determine the threshold concentration of cadmium that would cause a decline in the parasite population in future trials. Future work should also include a trial which simultaneously investigates parasite population growth and host responses. A study of the effect of cadmium on the birth and death rates of individual *G. turnbulli*, following a method similar to that of Scott (1982), would also be of interest and may help to elucidate the reasons behind the larger population size of cadmium-exposed gyrodactylids. For instance, an increased birth rate caused by cadmium may explain the more rapid build up in numbers of gyrodactylids on the cadmium-exposed guppies. A study of the death rates would make it clear whether cadmium was detrimentally affecting the parasites and if in response to this they increased their reproductive rate in compensation.

A particularly interesting feature of infecting mixed sex guppies with *G. turnbulli* came to light during Experiment 2, with male and female fish exhibiting different susceptibilities to gyrodactylid infection. The following discussion is based on pooled data collected from the first two pairs of replicates only (*i.e.* C1, T1; C2, T2). The third pair of tanks (C3, T3) showed opposing results to the other replicates; the reasons for this are unclear, but it is possibly due to some of the factors discussed above, such as the extra days the guppies were subjected to water changes and the health status of the guppies.

At the beginning of the trial, female guppies from both populations had a statistically greater mean number of gyrodactylids compared to the male guppies and reached 100% prevalence earlier in the trial. The reason for the female guppies having a greater number of *G. turnbulli* in the early stages of the trial may simply be

because, in three of the tanks (C1, T1, T2), both the guppies initially infected with parasites were female and in the remaining three tanks one guppy of the initially infected pair was female (C2, C3, T3). Thus, potentially, more gyrodactylids would have been present on the female guppies at the start of the trial. Indeed, female guppies in all tanks reached 100% prevalence earlier (all by day 18) than the males and, while this may merely be a function of there being fewer females compared to male guppies, it would still contribute to a greater number of parasites being recorded on the female fish in the early stages of the trial. To clarify these findings, it would be interesting to individually infect a number of male and female guppies (n=20) with one gyrodactylid each and monitor the infection rate on each fish over time. This would determine on which sex the infection becomes established first.

Many reports of sex-related differences in the intensity and prevalence of a range of parasite species infections have shown that male hosts are more likely to have the greater prevalence and intensity of infection than female hosts. The findings of the current trial show that, from day 18 in the cadmium-exposed guppy population and from day 30 in the control population, male guppies had a greater number of gyrodactylids than their respective females.

Increased levels of parasites on male fish have been observed in *Gadhus morhua* infected with the acanthocephalan *Echinorhynchus gadi* (Müller, 1776) (see Hemmingsen & MacKenzie, 2001); in *Salmo trutta* infected with *Ichthyopthirius multifilis, Gyrodactylus* sp. and *Scyphidia* sp. (see Pickering & Christie, 1980); in *Crenilabrus melops* (L.) infected with the copepod *Leposphilus labrei* Hesse, 1886; in *Raja* sp. infected with the monogenean *Calicotyle kroyeri* Diesing, 1850 (see

Williams, 1965) and in *Salmo trutta* infected with the monogenean *Discocotyle sagitta* (Leuckart, 1842) (see Paling, 1965). Several explanations for the increased level of parasites on male fish of a variety of species have been suggested, including immunosuppression caused by testosterone production (Hemmingsen & MacKenzie, 2001), male fish being larger than females (Donnelly & Reynolds, 1994) and males being more attractive to the infective stages of parasites (Paling, 1965). The immune status of guppies was not investigated in this current trial but is the focus of Chapter 7, and this may help elucidate the differences in gyrodactylid burdens between male and female guppies. The difference in size between male and female guppies was, however, investigated and, while body size did not differ between the sexes, tail length was statistically greater in males in two of the control tanks. However, as tail length did not differ between the sexes in all the tanks, it is unlikely to be the sole reason for the observed differences in parasite load between the male and female guppies.

The difference in intensity of infection of *G. turnbulli* observed on male and female guppies could have been due to the male fish being more "stressed" than the females, due to a male-biased operational sex-ratio (OSR) in each tank. The OSR has been defined by Jirotkul (1999) as the number of sexually active males to the sum of sexually active males and females. A male biased OSR, *i.e.* more males present, results in more male-male competition, as there are fewer females available with whom to mate (Jirotkul, 1999). The possibility that competitive stress caused by competition for females resulted in the proliferation of gyrodactylids on the male fish cannot be excluded, particularly as Poulin & Vickery (1996) and López (1999) have stated that parasitised females may opt to breed quickly with any available

male rather than invest a lot of energy in long and costly mate-assessment rituals. Having a small number of females that are willing to mate indiscriminately may have intensified the male-male competition between the guppies and affected their immune functions, resulting in high parasite burdens.

It is possible that male-male competition coupled with immune response changes caused by testosterone production could account in some way for the differences in *G. turnbulli* infections seen on male and female guppies. As both male and female guppies exposed to cadmium had higher parasite burdens than the control males and females, it affirms that cadmium impacts on the population growth of *G. turnbulli.* Male guppies exposed to cadmium had the greatest number of gyrodactylids of all fish, and it is thus possible that cadmium exposure exacerbates the already potentially stressful environment that the male guppies occupy.

Future work into the sex-related differences in gyrodactylid burden on guppies should involve a study of the population growth of the parasites on single sex populations of guppies. By keeping male and female guppies separate, it may be possible to elucidate whether the differences in parasite load observed in this trial exist all the time or whether it is merely a function of the competitive circumstances that the male guppies are faced with in the presence of females. Altering the OSR by adjusting the ratio of sexually active male and female guppies and looking at the population growth of *G. turnbulli* under these different OSRs would allow some interpretation of how male-male competition contributes to the observations made in this current study.

Richards & Chubb (1998) observed that the greatest percentage of *G. bullatarudis* is located on the head and opercula of adult guppies and the greatest percentage of *G. turnbulli* occur on the caudal fin and caudal peduncle. The *G. bullatarudis* infection in this trial, while colonising the head of guppies, was however, found in greater abundance on the caudal fin. It is possible that *G. bullatarudis* were in high numbers on the underside of the head, which, due to the method of examination used, could not be counted, thus giving a false representation of their distribution. Because of the difficulties in examining the ventral surface of guppies using the method of Harris (1986b), it was decided that for Experiment 2 the fish should be lightly anaesthetised to enable a full examination of the body to take place. For the first six days of Experiment 2, the greater number of *G. turnbulli* were found on the ventral fins where the infection was initiated. Further on in the trial, the greatest number of *G. turnbulli* were found on the caudal fin. This suggests that, when the numbers of parasites increase in the area of initial colonisation due to rapid reproduction, competition for space for attachment intensifies and the gyrodactylids move to colonise new regions of the host. It is also possible that the initial site for infection, the anal fins, became the primary site for a localised host response, which was strengthened over time, causing the parasites to move to a new area to avoid damage to themselves. Richards & Chubb (1998) considered the movement of gyrodactylids over the external surface of the guppy, avoiding areas of localised host responses, as a mechanism that allows low-level, persistent infections to remain on adult fish. As the favoured areas of colonisation by gyrodactylids were the same on both control and cadmium-exposed guppies, it would appear that exposure to cadmium has little effect on the distribution pattern of the parasites on their hosts.

Kuperman (1992) and Zharikova (1993) both recorded specimens of the monogenean *Diplozoon paradoxum* from areas of the Volga River system that were polluted with heavy metals and hydrocarbons, with abnormal ratios in the number of their attachment clamps. Instead of the normal 4:4 ratio of clamps, specimens were found with ratios of 4:2, 4:3 and 3:2 (Zharikova, 1993) and ratios of 4:3, 4:1, 4:0 and 3:3 (Kuperman, 1992). It was hoped that, by exposing *G. turnbulli* populations to 5μg/l cadmium (actual 7.13 ± 0.8μg/l) for a six-week period, any morphological abnormalities in the hooks of the opisthaptor would be revealed. It was expected that the hooks of *G. turnbulli* would have been susceptible to changes caused by cadmium, as they are high in sulphur (Lyons, 1966) to which cadmium has a high affinity (Rainbow, 1997b). The resulting absence of any morphological abnormalities could be attributed to several possible factors. Primarily, the concentration of cadmium used in the current trial may have been too low to elicit any abnormalities in the hook formation of the gyrodactylids and, secondly, the trial may not have been run for long enough for any changes to occur in the successive generations of parasites. For instance, there is no way of knowing how long the abnormal *D. paradoxum* had been exposed to the pollution and they may have been descended from parasites that had been exposed to the polluted water over many generations. It is also possible that one heavy metal alone cannot cause abnormalities to the structure of gyrodactylids and that a variety of heavy metals and other pollutants, as found in the Volga River, are required for abnormalities to occur. The changes to the clamps of *D. paradoxum*, not being generated under controlled experimental conditions, could, however, have been the result of a range of unknown physical, chemical and biological factors found in the surrounding environment. Thus, in order to prove that pollution was the cause of the

morphological abnormalities in *D. paradoxum*, experimental studies should be carried out.

Further attempts to elicit morphological changes to the hooks of gyrodactylids could involve longer-term trials using higher concentrations of cadmium and could expose the parasites to a mixture of heavy metals and other pollutants at known concentrations.

In conclusion, levels of cadmium, below the permitted maximum, appear to impact significantly on the population dynamics of *G. turnbulli* parasitising guppies. The current trial has also demonstrated that the response of male and female guppies, to infections of *G. turnbulli*, differs. The following chapter will investigate some aspects of the guppy immune response to elucidate the interactions between this host and *G. turnbulli* in the presence of cadmium.

Chapter 7

The impact of cadmium on selected aspects of the guppy immune system

7.1. Introduction

Information regarding the immunology of guppies is scarce, with only one study by Takahashi & Kawahara (1987) being found. These authors immunised adult female guppies with formalin-killed bacteria, *Aeromonas hydrophila*, and observed the production of agglutinating antibodies in these fish and their offspring. Between 14 and 75 days after the mothers had been immunised, the same agglutinating antibodies were found in the bodies of the newborn fry, suggesting antibody transfer from mother to foetus (Takahashi & Kawahara, 1987). Unfortunately this paper is in Japanese, with no translation available, so further details of the study are unknown.

With a paucity of information regarding the immunology of guppies, it is unsurprising that there are no studies looking at the effect of heavy metals on the immune system of these fish. One study by Khunyakari, Tare & Sharma (2001) has, however, investigated the accumulation of nickel (336.3ppm), copper (0.93ppm) and zinc (14.5ppm) by guppies and the effect that these metals had on the behaviour of the fish. Of these three metals, nickel was found to accumulate to the highest levels and to cause excessive excretion, while all three metals caused significant increases in fin movement above the controls after 48 h exposure (Khunyakari *et al.*, 2001).

Due to the small size of guppies, the immunological and haematological parameters that can be monitored are limited. Siwicki & Anderson (1993) prepared a techniques manual on measuring the immune parameters of small fish in hatchery

laboratories and this manual includes some microimmunology techniques. Siwicki, with Studnicka, Morand, Pozet & Terech-Majewska (1998) suggested a range of assays to use for immunotoxicological studies and, on the basis of these assays and those presented in the techniques manual, it was decided that the phagocytosis and respiratory burst of kidney phagocytes would be assessed, as well as the myeloperoxidase production of neutrophils. Myeloperoxidase uses H_20_2 to oxidize many organic compounds and is also capable of oxidizing chloride ions to hypochlorous acid (HOCl) that has strong antibacterial properties (Praveen, Nauseef & Goodwin, 2000).

The effect of cadmium on the phagocytosis and respiratory burst of various fish species was reviewed thoroughly in Chapter 4 (The effect of cadmium on selected aspects of the innate immune response of carp, section 4.1). In summary, the impact of this metal on phagocytosis shows varied results with Zelikoff *et al.* (1995), Sövényi & Szakolczai (1993) and Enane *et al.* (1991) all finding an enhancement in phagocytic activity, while, Sanchez-Dardon *et al.* (1999) found a suppression of activity on the exposure of fish to cadmium. Similarly contrasting results are recorded on the effect of cadmium on the respiratory burst activity of kidney macrophages. For instance, Enane *et al.* (1991) and Zelikoff *et al.* (1996, cited by Zelikoff, 1997), found that cadmium enhanced the respiratory burst, while Zelikoff *et al.* (1995) and Sanchez-Dardon *et al.* (1999) found that the respiratory burst activity was suppressed by cadmium. However, Zelikoff *et al.* (1995) did find that the spontaneous respiratory burst of *Oncorhynchus mykiss* was significantly elevated above the controls after 8 and 17 days exposure to 2μg/l cadmium. No literature relating to the effect of cadmium on myeloperoxidase production (MPO)

could be found. However, Siwicki *et al.* (1998) suggest that MPO production should be lower in fish from polluted areas when compared to those from control sites.

Since the mid-1960s, several field studies have observed differences in the parasite burdens of male and female fish, with the majority recording higher parasite numbers on male fish (Paling, 1965; Williams, 1965; Pickering & Christie, 1980; Myjak, Szostakowska, Wojciechowski, Pietkiewicz & Rokicki, 1994; Hemmingsen & MacKenzie, 2001). Sex-related differences in parasite burden have been recorded in guppies infected with G*yrodactylus turnbulli* in the current study, although these were not specifically male-biased (see Chapter 6). One suggested explanation for the differences between sexes, observed by Hemmingsen & MacKenzie (2001), was that testosterone produced by male fish was acting as an immunosuppressant, resulting in proliferation of parasites on male fish. Buchmann (1997) investigated the effect of testosterone on the population growth of *G. derjavini* Mikailov, 1975 on *O. mykiss* and found that injecting female fish (used to minimize the variation of the natural testosterone level) with testosterone caused a significant increase in parasite burden above the controls after 4 weeks. While this is an interesting observation, the author could not determine whether the results were due to the immunosuppressive effects of testosterone on the host, an enhancing effect that this sex hormone had on the parasites directly or both.

The implication that testosterone may cause immunosuppression in male fish is of great interest, but Buchmann's trial failed to determine which immune parameters may have been affected and did not compare the responses of both male and female fish. There is little information concerning the effect of pollutants on the immune

responses of male and female fish. One study has, however, looked at the effects of pollutants on the immune responses of male and female fish. Aaltonen, Jokinen, Lappivaara, Markkula, Salo, Leppänen & Lammi (2000) exposed male and female *Rutilus rutilus* L. to bleached kraft mill effluents (BKME) and measured a variety of specific and non-specific immune parameters in both sexes. The number of immunoglobulin secreting cells (ISC) in blood was found to be significantly lower in control females compared to control males, while in BKME-treated fish the ISC number was greater in the female *R. rutilus*. The authors noted, however, that the small number of female fish used in the trial hampered the comparison of results between the sexes. The same authors also observed plasma cortisol levels responding in a similar manner to the ISC number, with cortisol levels in control females being lower than that of control males, while BKME-exposed females had statistically higher levels of cortisol than the BKME-exposed male *R. rutilus*. It was suggested that sex hormones are involved in immunomodulation and sex-related factors should thus be included in immunotoxicity studies (Aaltonen *et al.*, 2000). In contrast to the above, however, the chemotaxis and respiratory burst of *R. rutilus* granulocytes did not differ between the sexes (Aaltonen *et al.*, 2001). However, Fournier, Lacroix, Voccia & Brousseau (1998) did record a difference in phagocyte function between male and female mummichogs, *Fundulus heteroclitus* (L.), exposed to BKME, with a stronger suppression in macrophage phagocytosis and respiratory burst recorded in the female fish. In this instance, the suppression of the female immune function was attributed to the high levels of oestradiol present in the polluted sites. In mammals, it is known that sex steroids influence the development and functioning of the immune system and, while the exact mechanism by which oestradiol regulates the immune system is not known, Fournier *et al.* (1998) state

that the presence of oestrogen receptors on macrophages suggests that this steroid has some regulatory role on macrophage function. Interestingly, the difference between the immune function of the male and female *F. heteroclitus* was not apparent in fish taken from the non-polluted reference sites (Fournier *et al.*, 1998).

Another aspect of the non-specific immune response that has shown sex-related differences is the alternative complement pathway as shown by Collazos, Barriga & Ortega (1994). These authors measured the alternative complement pathway titre (ACH50) in male and female *Tinca tinca* L. and correlated the results to seasonal changes. The ACH50 was found to be higher in female *T. tinca* in all seasons except autumn and was statistically higher than that of the males in the spring (Collazos *et al.*, 1994). However, no suggestions for these sex-related differences in ACH50 were proffered.

7.1.1. Aims of the present study

The principal aim of this chapter is to determine how cadmium at 5 and 20μg/l might affect selected aspects of the non-specific immune response of the guppy and to correlate the changes in immune parameters of the 5μg/l cadmium-exposed guppies with the patterns of population growth of *Gyrodactylus turnbulli* recorded under the same conditions (see Chapter 6, The impact of cadmium on the population dynamics of *Gyrodactylus* spp.). A secondary aim of this chapter, but nonetheless one of great interest, was to determine whether the various immune parameters of male and female guppies differed and if this could be used to explain the sex-related differences in gyrodactylid burdens observed in Chapter 6.

7.2. Materials and methods

7.2.1. Fish

Three hundred male guppies (3.0-4.2 cm) and 200 female guppies (3.8-5.0 cm) were procured from a commercial aquarist in the south east of England. The guppies had been maintained at the aquarist for a 3-month period prior to their delivery. On arrival, groups of 30 male guppies were placed in 9 of the tanks and the female guppies divided equally between the remaining 3 tanks of the flow-through system (for tank details see section 7.2.2). Three days before the start of the trial, 15 female guppies were placed into each of the 9 tanks holding the male guppies. Although on a larger scale, the operational sex ratio (OSR) of these tanks was the same as in Chapter 6 in order to minimise variation between the trials.

The guppies were acclimated for 12 days prior to the start of the trial and, during this time, were fed daily on Aquarian Tropical Fish Flake®. Five male and 5 female guppies were selected at random and were screened to assess their disease status. Each guppy was lightly anaesthetised using 0.02% MS222 and examined under a dissecting microscope for parasite prevalence. All 10 fish were found to be parasite-free, but, as a precaution, all guppies were treated at both 8 and 5 days before the start of the trial with 100ppm formalin. After both treatments, a further random sample of 5 male and 5 female guppies were then screened for parasites. All guppies were found to be parasite-free, were allowed to recover and were replaced in the tanks from which they had been removed.

7.2.2. Tanks

Nine tanks were selected at random from the 12-tank flow through system in the tropical aquarium (see Fig. 2.1). The outflow pipe of each tank was cut to 9 cm lengths giving a final volume of water in each tank of 9 litres. The flow rate of the incoming water in all tanks was set to 100 ml/min. As the experiment was run during winter, the incoming water was heated with a 3kW Howden immersion heater to give the desired temperature of ca. 21°C.

Three of the 9 tanks were randomly selected and labelled as controls, 3 were randomly selected for 5μg/l cadmium and the remaining 3 were allocated as 20μg/l cadmium tanks. Each replicate consisted of 1 control tank, one 5 and one 20μg/l cadmium tank.

7.2.3. Starting the trial

A set of replicate tanks was started on 3 consecutive days to ensure that there was adequate time allocated to the processing of each guppy on each sample day. Two 10 litre stock solutions of cadmium sulphate of different concentrations were made and fed via a constant volume peristaltic pump into the allotted test tanks, giving nominal concentrations of 5 and 20μg/l cadmium. Water samples were taken periodically throughout the trial to monitor cadmium concentrations, total hardness, pH and alkalinity (See Appendix 1).

7.2.4. Sampling regime

At 6, 12, 18, 24 and 30 days post-start of the trial, 5 male and 3 female guppies were removed at random from each tank and killed by an overdose of benzocaine. The guppies were then used for a series of immunological assays (see section 7.2.5).

7.2.5. Immunological methods

7.2.5.1. Differential blood counts

Once dead, the guppies were removed from the anaesthetic as quickly as possible and placed on a paper towel. The fish were blotted dry with tissue paper and the tail cut off just above the caudal peduncle using a scalpel. The severed tail of the guppy was then held on a clean, labelled slide until a small volume of blood had collected. A smear was then made and processed as in Chapter 4, section 4.2.4.4. The guppies were then placed in individual, labelled bags and kept on ice until blood smears had been made from all fish from each of the 3 tanks.

7.2.5.2. Phagocyte isolation

Each guppy was dissected and the entire kidney removed using a 5 mm wide metal spatula and fine forceps. The kidney was then disaggregated through a 100μm mesh into 1 ml of L15 (Leibovitiz) media and heparin (2μl) (cell grade) (Sigma) using the flat end of a 1 ml syringe plunger. All equipment was sterile and the whole process was carried out in a laminar flow hood. The resulting cell suspension was transferred to a labelled bijou and placed on ice until all the guppies had been processed. As the entire kidney was used, due to the small amount available, it is not possible to say that the cell suspension consisted entirely of macrophages and thus the cells used in these trials will be referred to as phagocytes.

7.2.5.3. Phagocytosis by kidney phagocytes

The following methodology was taken from Siwicki & Anderson (1993). One hundred microlitres of kidney cell suspension from each guppy was placed in 2 separate wells of a 96-well multi-well plate. A 5 mg/ml suspension of Baker's yeast in L15 was made up immediately before use and 100μl added to each well. The cell suspension and yeast solution were mixed thoroughly with a pipette. The multi-well plate was incubated at room temperature for 20 min, after which each well was thoroughly mixed again and 10μl of the mixture pipetted onto a labelled slide and a smear made. The slides were air-dried and then stained using Stain Quick (Raymond A. Lamb Ltd). Due to the small amount of kidney cell suspension available, cell numbers were low and thus each slide was read at ×100 and 50 cells counted and the number of yeast cells engulfed by each phagocyte recorded. The phagocytic index and phagocytic ratio were determined using the calculations given in Chapter 4, section 4.2.4.6.

7.2.5.4. Respiratory burst

The respiratory burst of kidney phagocytic cells was determined using the same method as that for carp (Chapter 4, section 4.2.4.7). However, as the total volume of kidney suspension was only 1 ml, 2 replicate wells were used for both the NBT only (spontaneous respiratory burst) and the NBT + PMA (stimulated respiratory burst) reactions instead of 3, and only 1 well for the lysis buffer was used instead of 2. The results presented were calculated from the wells containing NBT + PMA. The actual values for NBT and NBT+PMA production can be found in Appendix 6a-e.

7.2.5.5. Myeloperoxidase production by neutrophils

The following methodology was adapted from Siwicki & Anderson (1993). Some omissions were made in their methodology and thus the instructions that came with Sigma Kit #390-A were also consulted and the appropriate steps incorporated.

On days 6 and 30 post-start of the trial, the production of myeloperoxidase by neutrophil cells was determined. A kidney cell suspension (10μl) was placed on a clean, labelled glass slide and a smear made. The slides were air dried at room temperature and then a Sigma Kit #390-A was used in the following way. The slides were fixed for 30 sec in an ethanol/formaldehyde solution prepared by mixing 5 ml of 37% formaldehyde with 45 ml of 95% ethanol.

The slides were then rinsed in gently running tap-water for 2 min and allowed to air dry in complete darkness for 10 min. TRIZMAL 6.3 Dilute Buffer was prepared by mixing one volume of TRIZMAL 6.3 Buffer Concentrate with 9 volumes of deionized water. Dilute TRIZMAL (50 ml) was pre-warmed in a 37°C water bath and, immediately before use, 1 vial of Peroxidase Indicator Reagent (provided with Sigma Kit #390-A) and 200μl of 3% hydrogen peroxide were added and the solution mixed thoroughly.

The slides were then placed into the Peroxidase Indicator Reagent and put into the 37°C water bath for 30 min. After incubation, the slides were rinsed in gently running tap-water for 30 sec and then air-dried. Once dry the slides were stained in acid haematoxylin solution for 10 min and then rinsed in deionised water for 30 sec before being air-dried.

The neutrophils containing myeloperoxidase were stained brown-black by the procedure. One hundred cells per slide were then counted and graded. Scores of zero were given to cells showing no myeloperoxidase production (MPO), *i.e.* no stain, a score of 1 to neutrophils showing moderate MPO, *i.e.* light brown stain, and a score of 2 to cells with a high MPO, *i.e.* dark brown stain.

7.2.6. Statistical analysis

To compare the immunological parameters between treatments, all data were analysed for normality and homogeneity and on the basis of the results an appropriate statistical test was conducted. Where the data were normal and the variances homogeneous, they were pooled within treatments.

When the data were split to compare male and female guppies, the sample size for each sex was small (males n=5; females n=3). The homogeneity of the variances of the replicates was then ascertained and the data pooled accordingly, and parametric or non-parametric statistical tests were selected as appropriate.

7.3. Results

7.3.1. Water quality

The water quality results (± S.E.) from the current trial are summarised in Table 7.1. Temperature, water hardness, alkalinity and pH did not differ statistically between any of the 9 tanks in the current trial. The concentration of cadmium did not differ statistically between any tank within each treatment.

Table 7.1. Summary of the water quality data from each tank.

Parameter	Control			5µg/l cadmium			20µg/l cadmium			Statistical significance P
	C1	C2	C3	T1	T2	T3	T1	T2	T3	
Cadmium concentration (µg/l) (n = 10)	0.44 ± 0.13	0.40 ± 0.06	0.32 ± 0.04	6.78 ± 0.34	6.82 ± 0.19	7.19 ± 0.49	20.01 ± 1.83	24.28 ± 1.86	22.29 ± 1.59	One-way ANOVA Controls = 0.60 5µg/l = 0.68 20µg/l = 0.71
Temperature (°C) (n = 27)	21.1 ± 0.07	21.1 ± 0.05	21.1 ± 0.05	21.1 ± 0.06	21.1 ± 0.05	21.1 ± 0.05	21.2 ± 0.05	21.2 ± 0.06	21.1 ± 0.04	Kruskal-Wallis 0.86
pH (n = 8)	7.32 ± 0.05	7.27 ± 0.04	7.26 ± 0.05	7.28 ± 0.06	7.22 ± 0.06	7.20 ± 0.08	7.40 ± 0.13	7.27 ± 0.07	7.25 ± 0.06	One-way ANOVA 0.79
Alkalinity (meq/l) (n = 7)	0.26 ± 0.07	0.26 ± 0.08	0.26 ± 0.05	0.26 ± 0.11	0.25 ± 0.10	0.24 ± 0.08	0.28 ± 0.26	0.26 ± 0.11	0.25 ± 0.08	One-way ANOVA 0.51
Hardness (ppm $CaCO_3$) (n = 7)	27.8 ± 1.16	25.7 ± 0.57	24.5 ± 0.65	26.4 ± 0.75	27.0 ± 1.11	26.7 ± 1.01	25.4 ± 0.84	26.4 ± 0.30	27.7 ± 1.30	One-way ANOVA 0.23
Dissolved oxygen (mg/l) (n = 2)	8.70 ± 0.10	8.75 ± 0.05	8.75 ± 0.05	8.65 ± 0.05	8.70 ± 0.00	8.70 ± 0.10	8.85 ± 0.05	8.85 ± 0.05	8.80 ± 0.00	Too few data

7.3.2. Differential blood counts

Differential leucocyte counts were not undertaken due to the small amount of blood that was obtained from most of the guppies. The method used for obtaining the

blood resulted in mucus and protein being present in the blood smears which often obscured the detail of the white blood cells, making differentiation difficult.

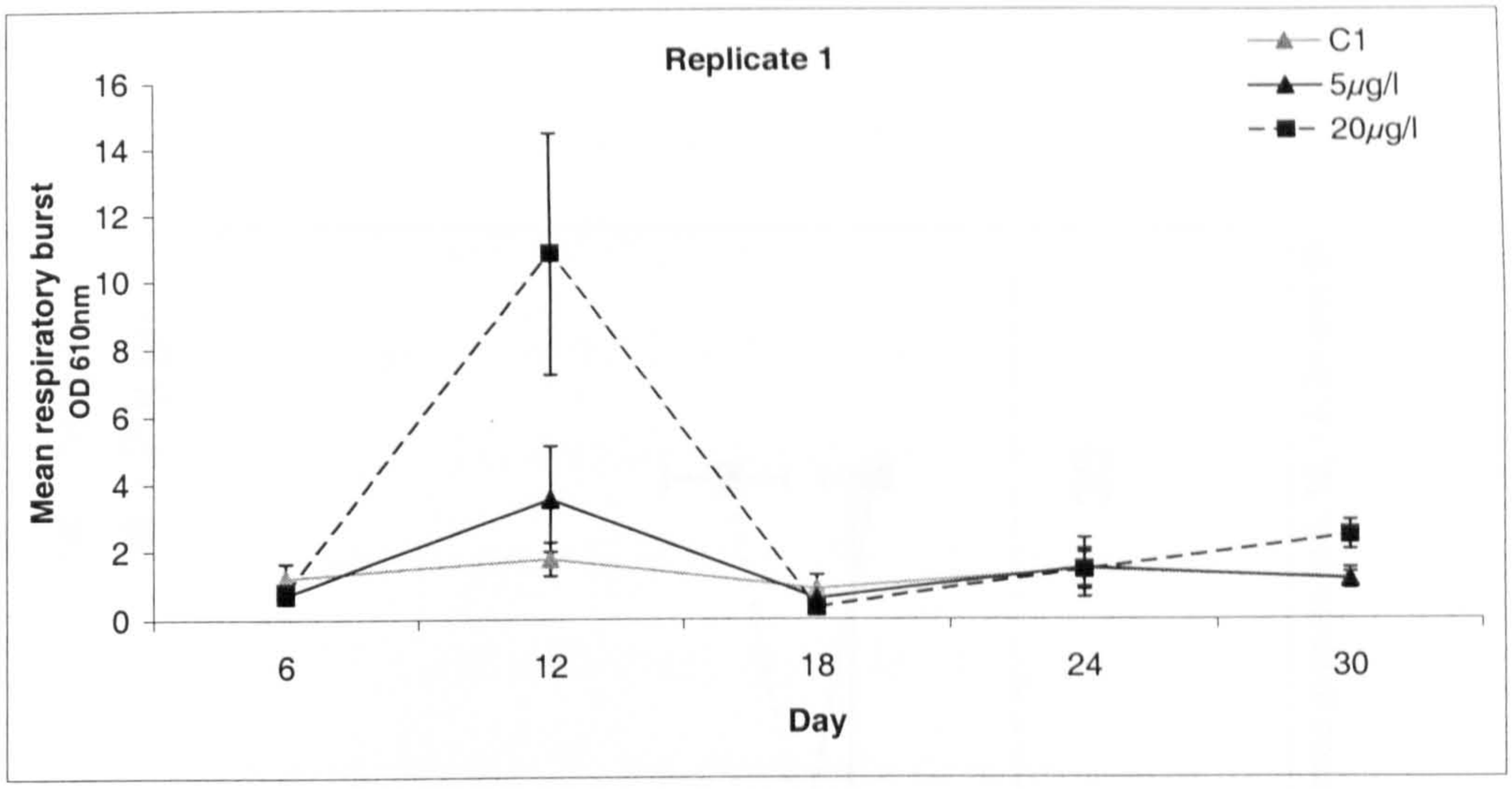
7.3.3. Respiratory burst

7.3.3.1. Respiratory burst between treatments

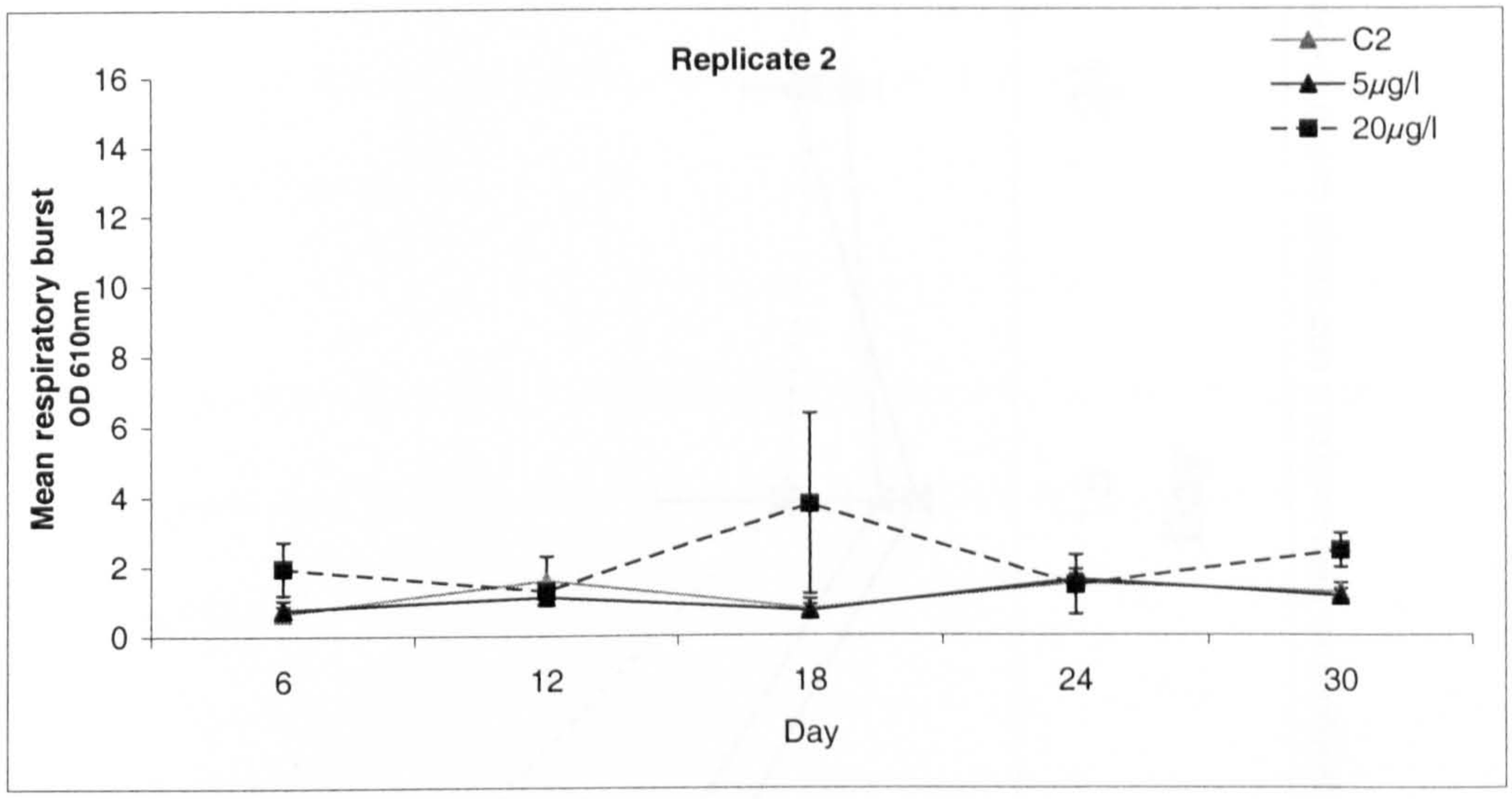
The respiratory burst of kidney phagocytes from control, 5 and 20μg/l cadmium-exposed guppies was determined every 6 days for a 30 day period and the results (mean ± S.E.) from each of the 3 replicates are shown in Figure 7.1a-c. It is clear from the 3 graphs that the respiratory burst activity of control and 5μg/l cadmium-exposed guppies followed a similar pattern over time, with the activity not differing greatly between the 2 groups at any time point in any replicate (Fig. 7.1a-c). Of note is that the respiratory burst of both the controls and 5μg/l cadmium-exposed guppies increased to varying degrees at day 12 in all replicates (Fig 7.1a-c). By contrast, the respiratory burst of guppies exposed to 20μg/l cadmium showed few similarities to the other treatments and no clear pattern in the respiratory burst activity between the 3 replicates (Fig. 7.1a-c, Table 7.2 p322). The lack of a clear pattern in activity between the 20μg/l cadmium-exposed replicates was highlighted by the statistical analysis, with the respiratory burst of replicate 3 being statistically lower than that of replicate 1 at day 12 (Fig. 7.1a, b). However, despite varying patterns in response at the beginning of the trial (Day 6-18), there was, in general, an increase in respiratory burst activity with exposure duration from day 18 onwards in all three 20μg/l cadmium-exposed replicates (Fig. 7.1a-c).

As the respiratory burst data from each tank at each time point did not conform to normality, the data could not be pooled between tanks from the same treatment for

a)



b)



c)

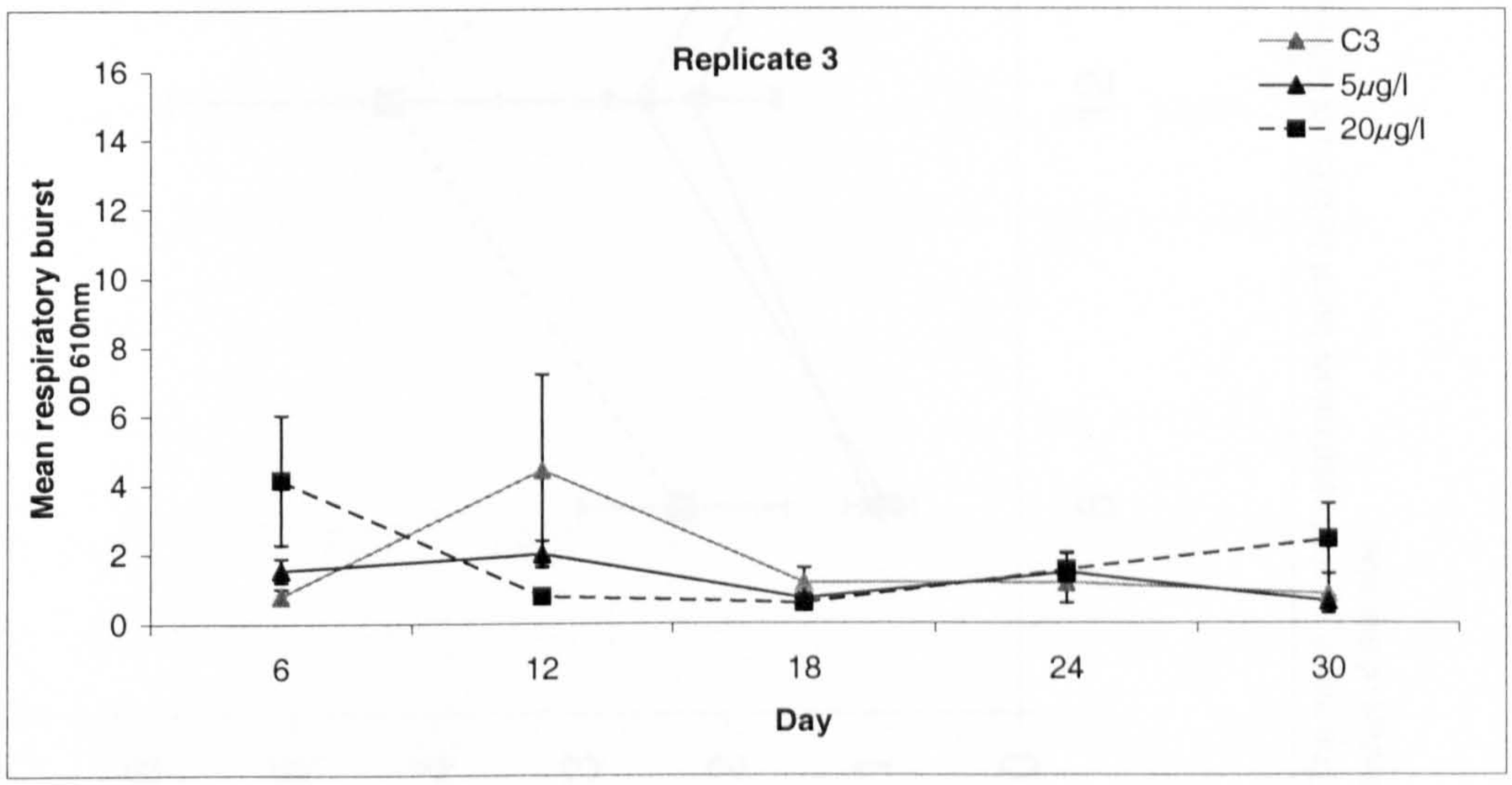


Fig. 7.1a-c. The mean respiratory burst (± S.E.) of kidney phagocytes from guppies exposed to 0, 5 and 20µg/l cadmium in (a) replicate tanks 1, (b) replicate tanks 2 and (c) replicate tanks 3.

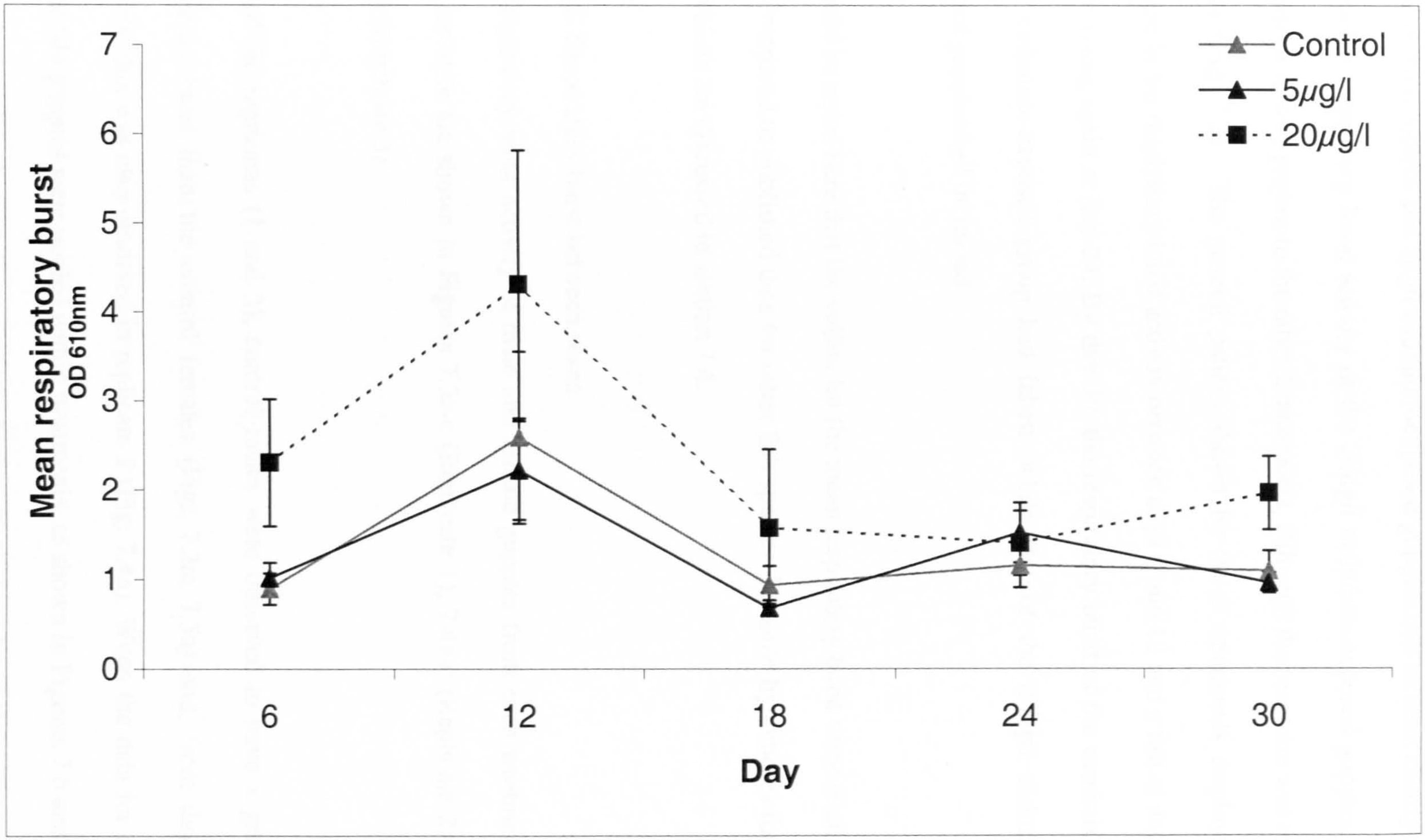


Fig. 7.2. The mean pooled respiratory burst (± S.E.) of kidney phagocytes from control, 5 and 20μg/l cadmium-exposed guppies at 6, 12, 18, 24 and 30 days post-start of the trial.

statistical analysis. However, for clarity, a graph showing the pooled data for each treatment was generated (see Fig. 7.2). Again, the similarity of the respiratory burst response from control and 5μg/l cadmium-exposed guppies can be seen clearly and, the pooled respiratory burst activity of the 20μg/l cadmium-exposed guppies also followed a similar pattern to the other 2 treatments, although the response was much greater (Fig. 7.2). The general pattern shown by the 3 treatments displays an increase in the respiratory burst activity between days 6 and 12 and a fall at day 18, before rising again at day 24. By day 30, the respiratory burst of the controls and 5μg/l cadmium-exposed group had fallen, while that of the 20μg/l cadmium-exposed guppies had increased.

It should be noted here that the values for the mean respiratory burst were relatively high compared to published data for other fish species. Possible hypotheses for the high values are discussed in section 7.4.

7.3.3.2. Respiratory burst between sexes

The respiratory burst activity of male and female guppies from each treatment in each replicate are shown in Figures 7.3a-c (Replicate 1), 7.4a-c (Replicate 2) and 7.5a-c (Replicate 3).

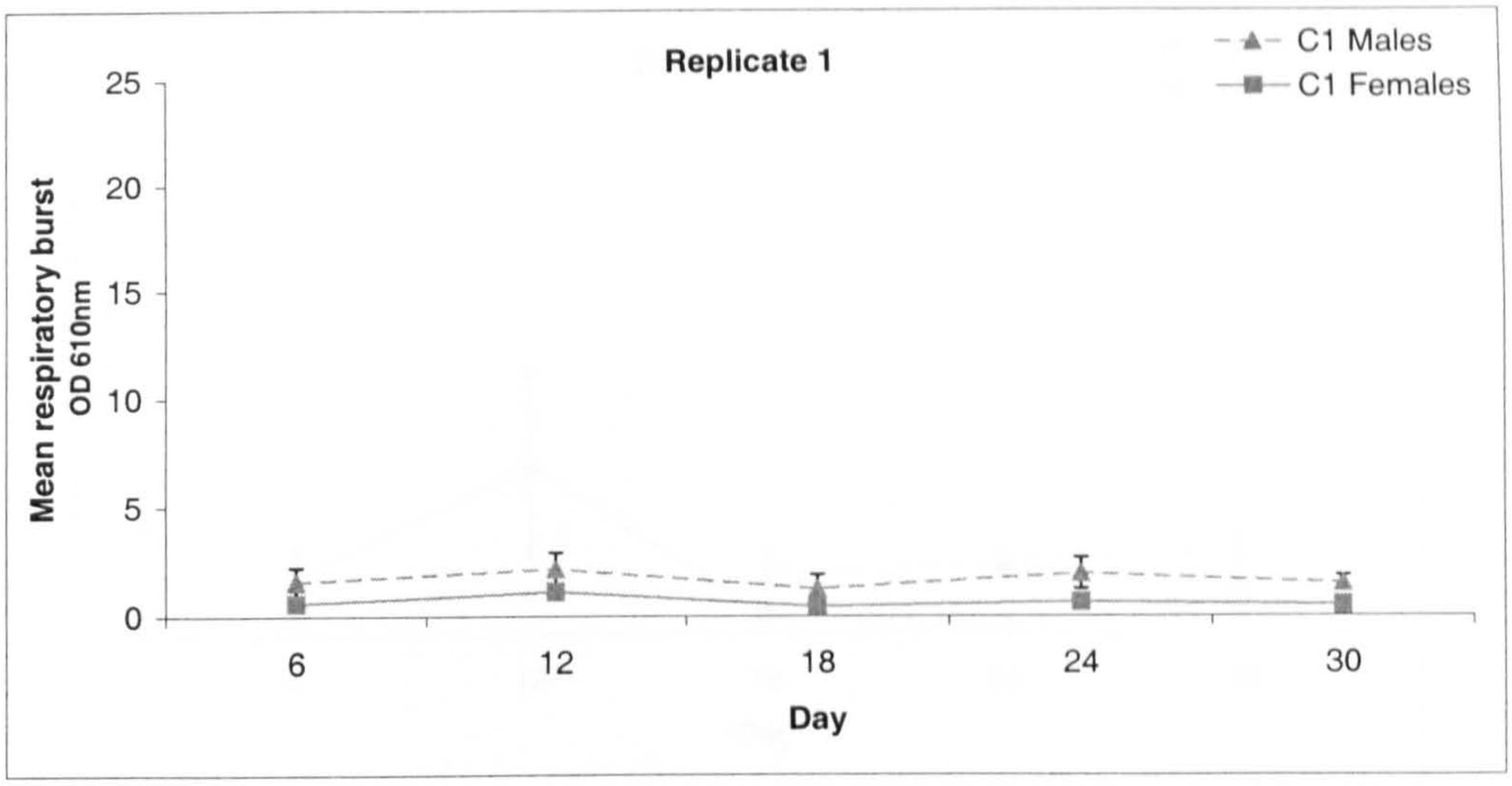
In 2 of the replicates (1 and 3), control males were observed to have a greater respiratory burst than the control females (Figs 7.3a; 7.5a) and, from day 18 onwards, this was also observed in replicate 2 (Fig. 7.4a). When the data for male and female guppies were pooled within treatments, as shown in Figures 7.6 and 7.7, the control males showed a consistently higher respiratory burst than that of the

control females. The standard error bars have been left off this graph to allow the patterns in respiratory burst to be clearly viewed. Figure 7.7 is a copy of Figure 7.6 (although on a different scale), but the standard error bars (± S.E.) have been added.

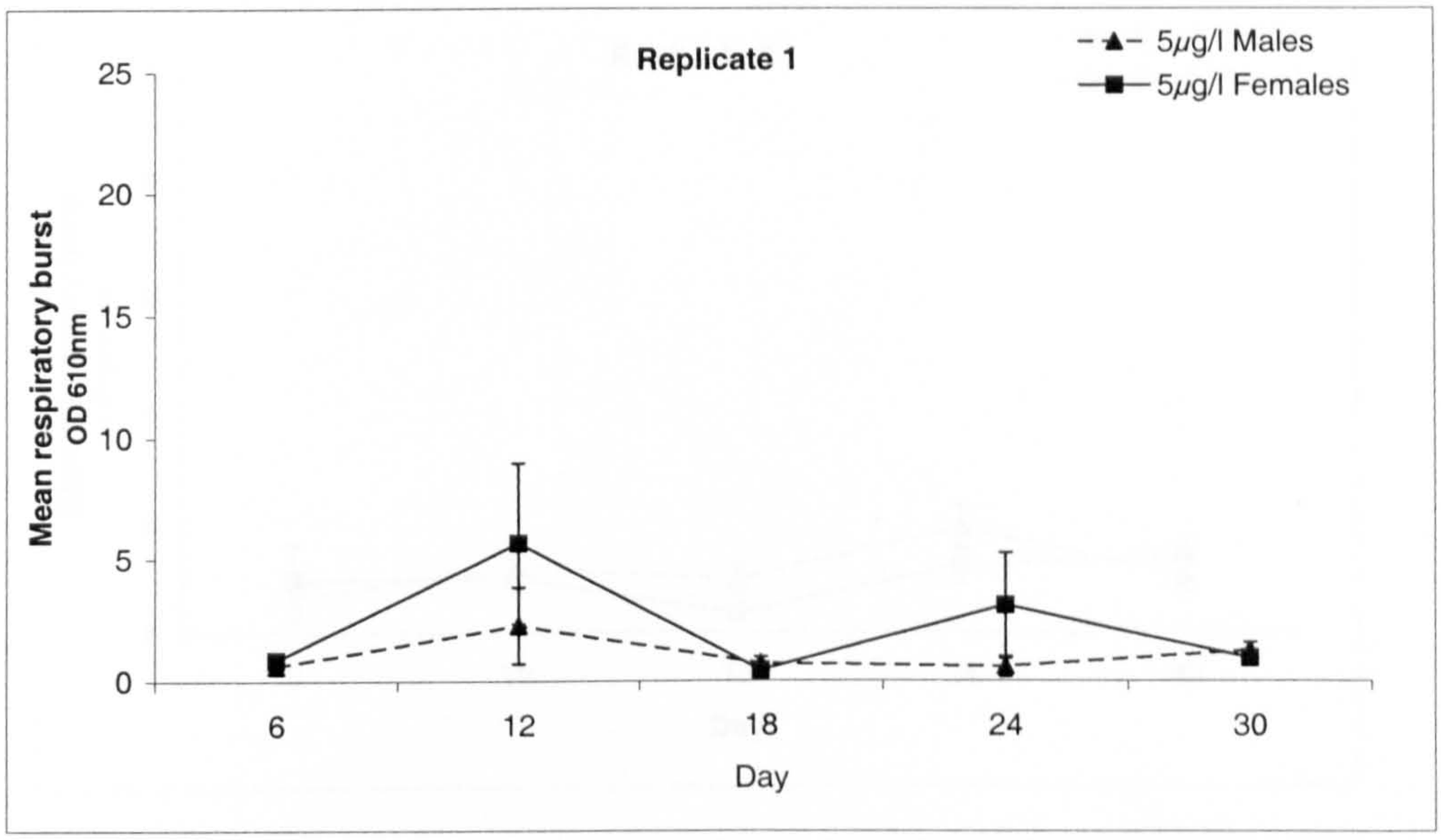
Guppies exposed to 5μg/l cadmium exhibited few consistencies between the replicates for either male or female guppies, except at day 24 where, in 2 of the replicates, female guppies had a greater respiratory burst activity than the male guppies (Figs 7.3b; 7.4b; 7.5b). In contrast to the control guppies, the 5μg/l cadmium-exposed females had a greater respiratory burst than the males at all days except days 18 and 30 (Figs 7.6; 7.7). Of particular note, is the fall in the respiratory burst activity observed in the 5μg/l cadmium-exposed females at day 18 which was also observed in the control females and dramatically so in the 20μg/l cadmium-exposed females (Figs 7.6; 7.7). At this time point, the respiratory burst of the 20μg/l cadmium-exposed females was statistically lower than that of the control and 5μg/l cadmium-exposed males ($P = 0.044$) (Table 7.5).

Figures 7.3c-7.5c show the respiratory burst activity of kidney phagocytes from male and female guppies exposed to 20μg/l cadmium. The only similarity in the respiratory burst of the kidney phagocytes between the replicates was at day 24, when in all replicates males had a greater respiratory burst activity than the females. However, looking at the pooled data in Figures 7.6 and 7.7, it is possible to see that female guppies exposed to 20μg/l cadmium had a higher phagocyte respiratory burst than the males on 3 of the sample days (days 6, 12 and 30).

a)



b)



c)

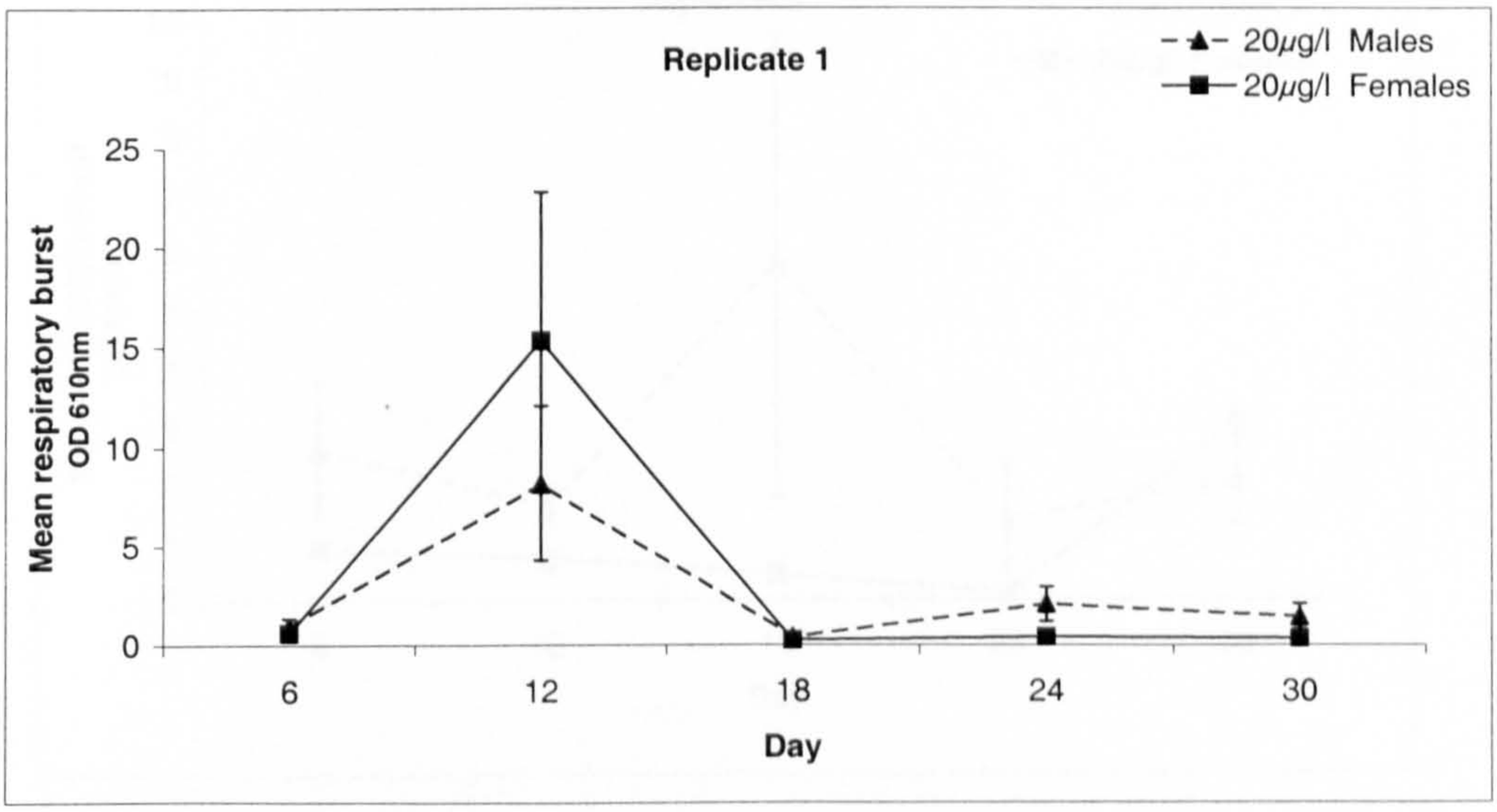
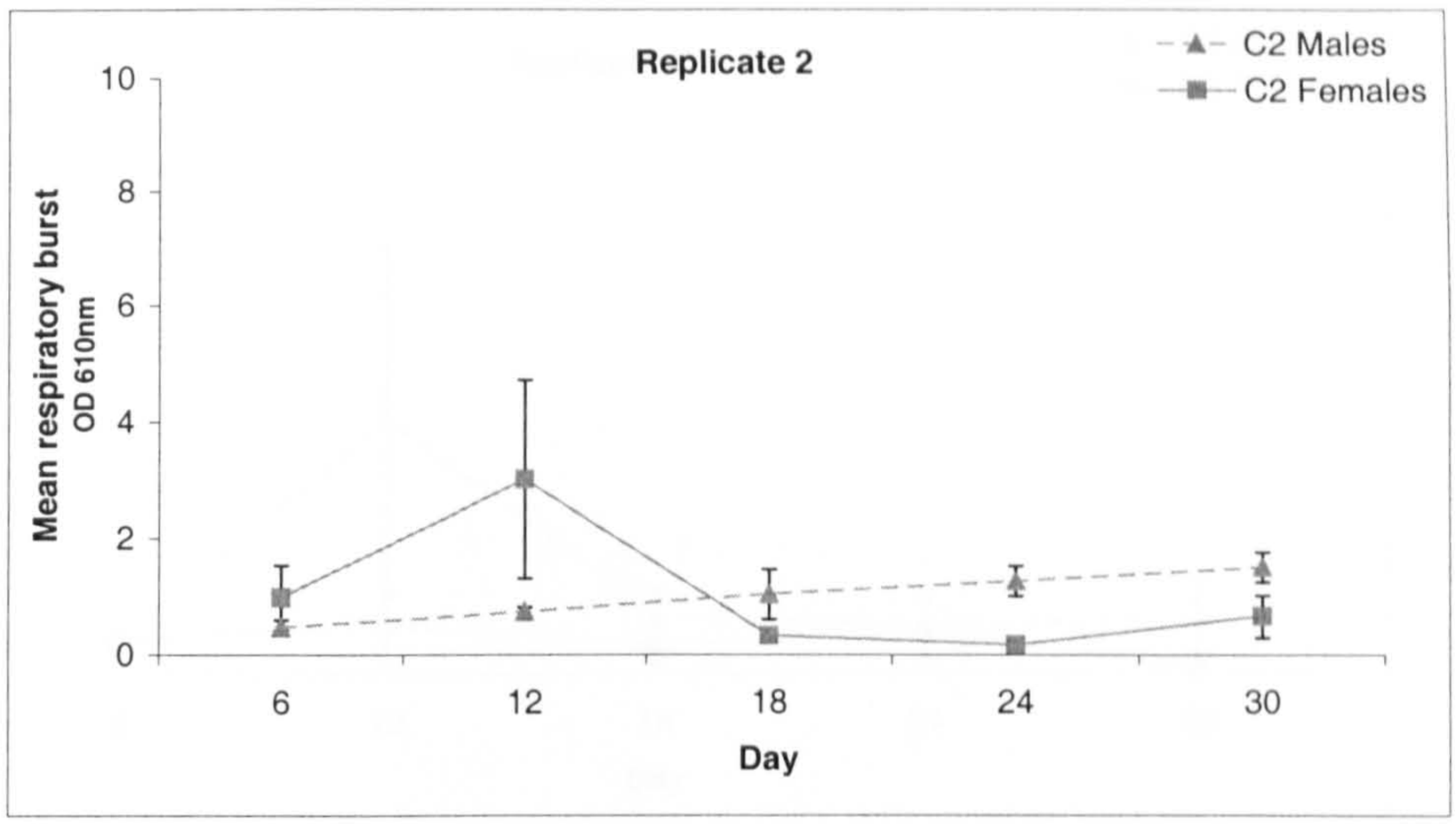
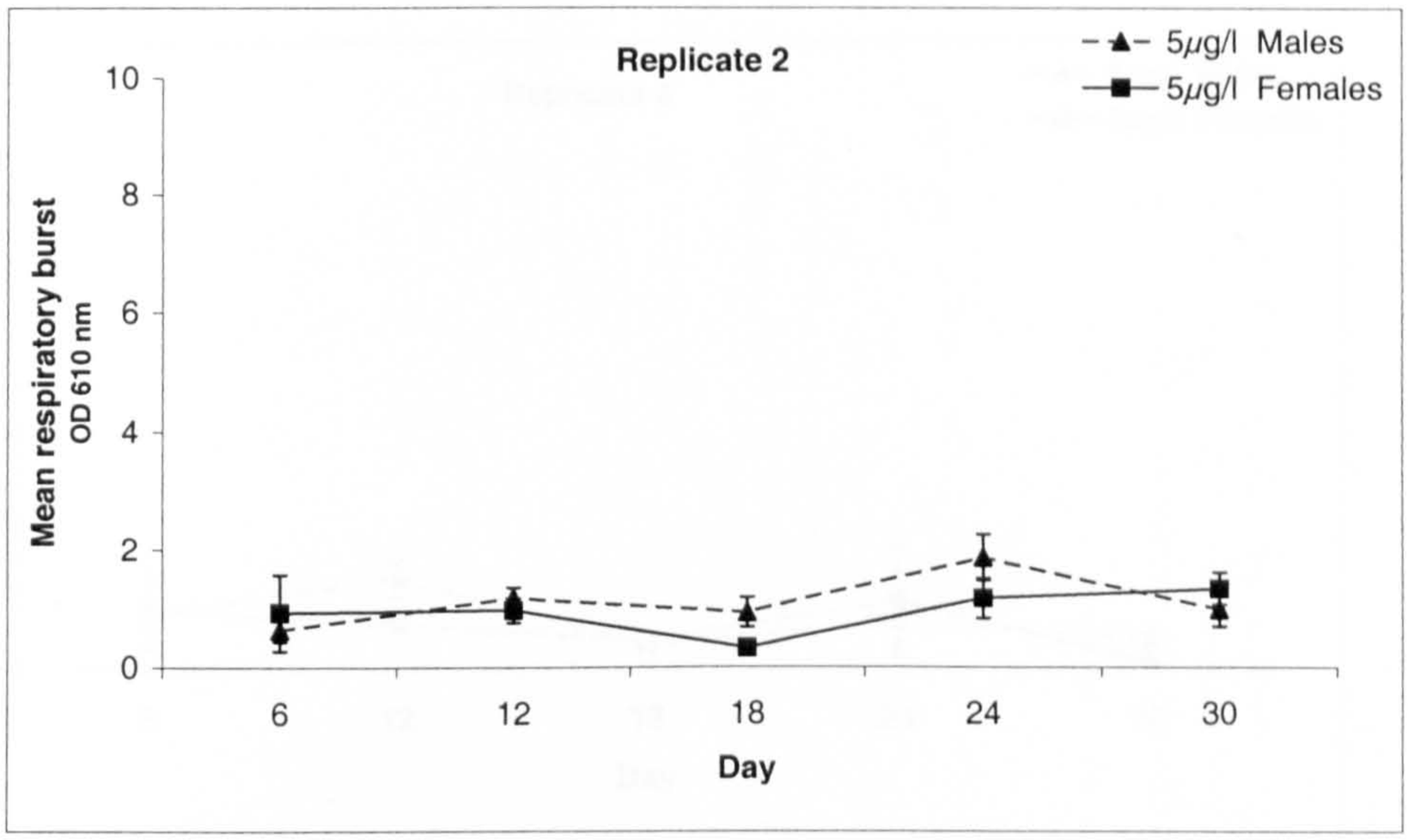


Fig. 7.3a-c. Replicate 1 - The mean respiratory burst (± S.E.) of kidney phagocytes from male and female guppies in (a) 0, (b) 5 and (c) 20µg/l cadmium.

a)



b)



c)

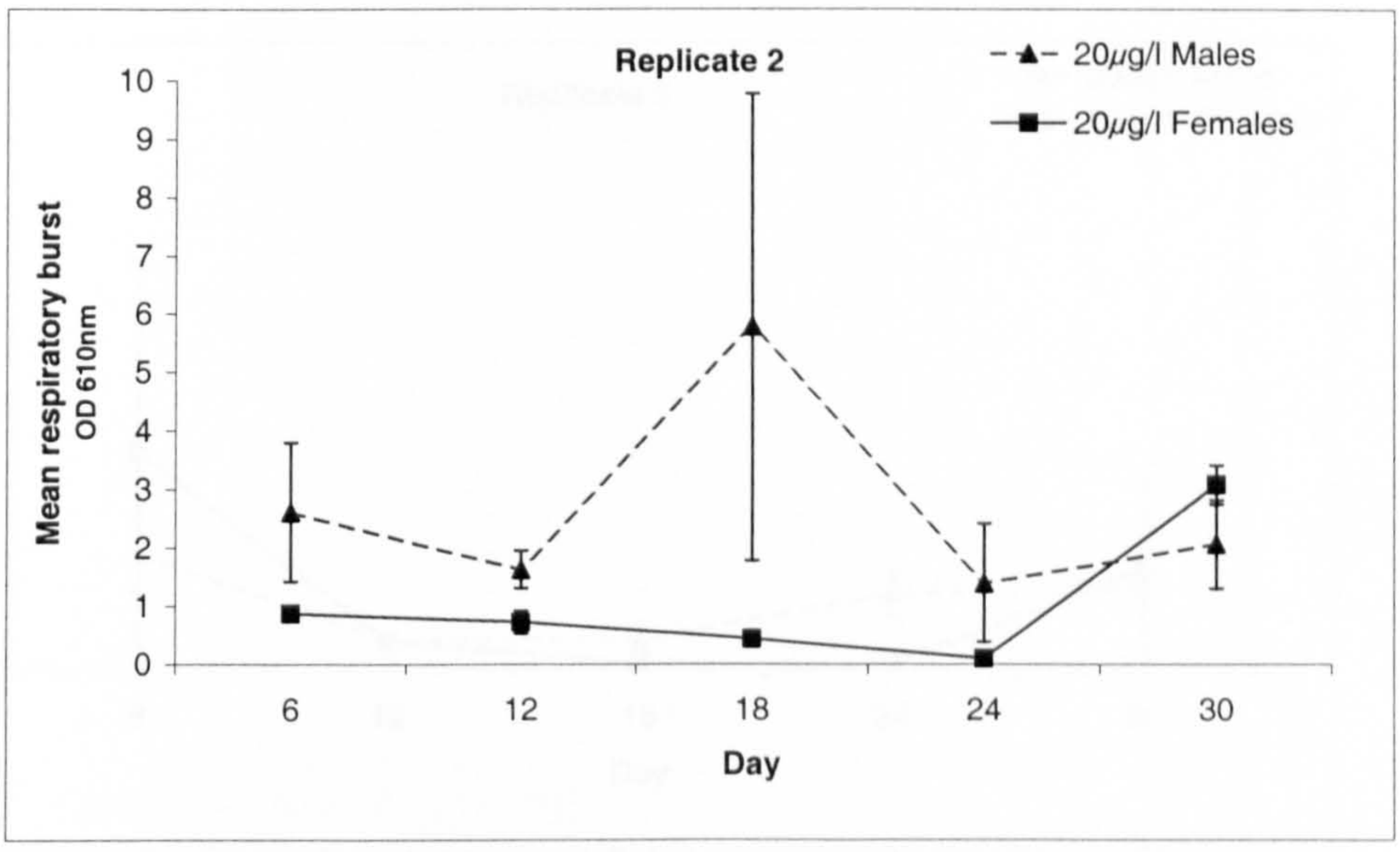
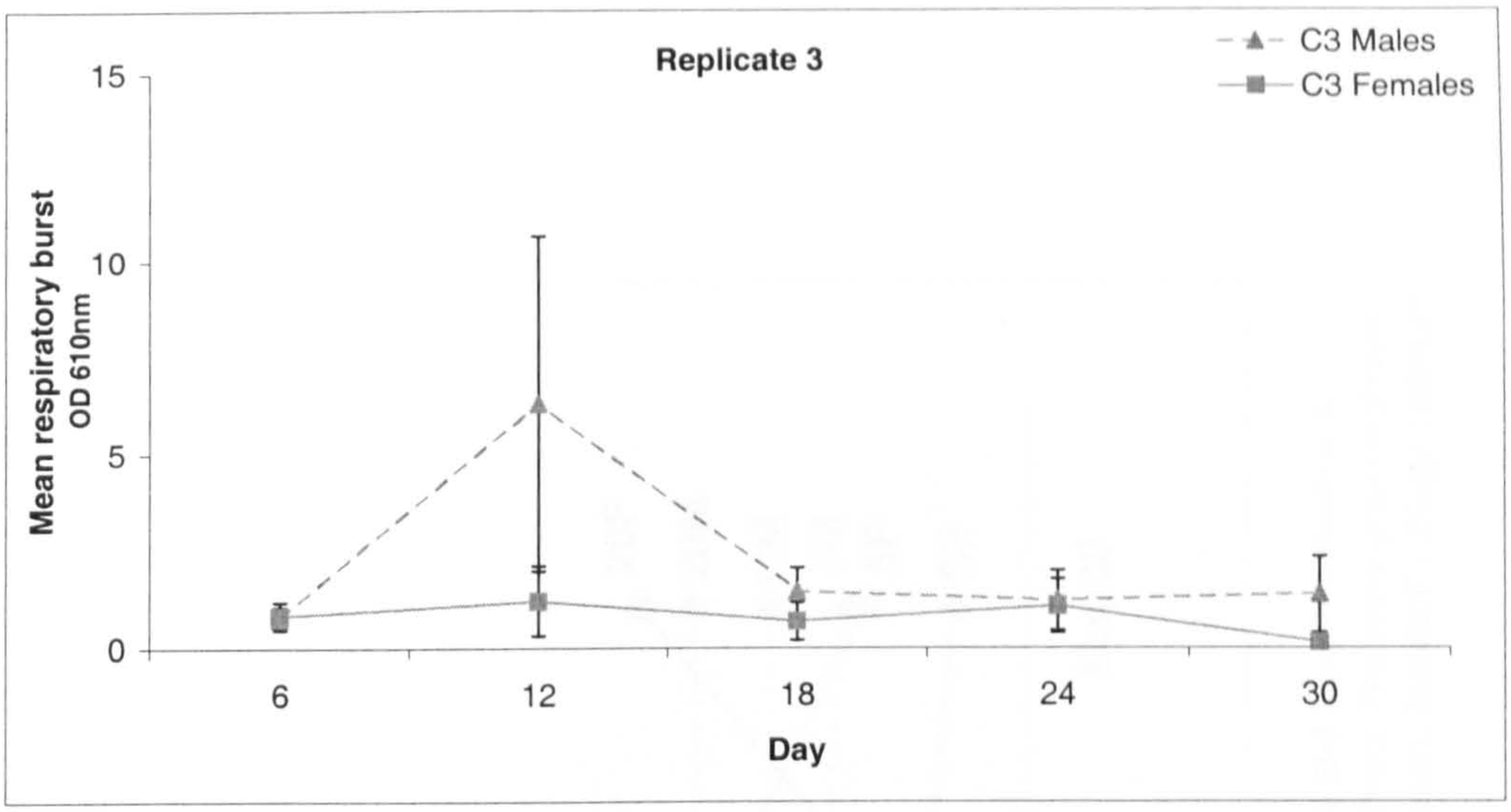
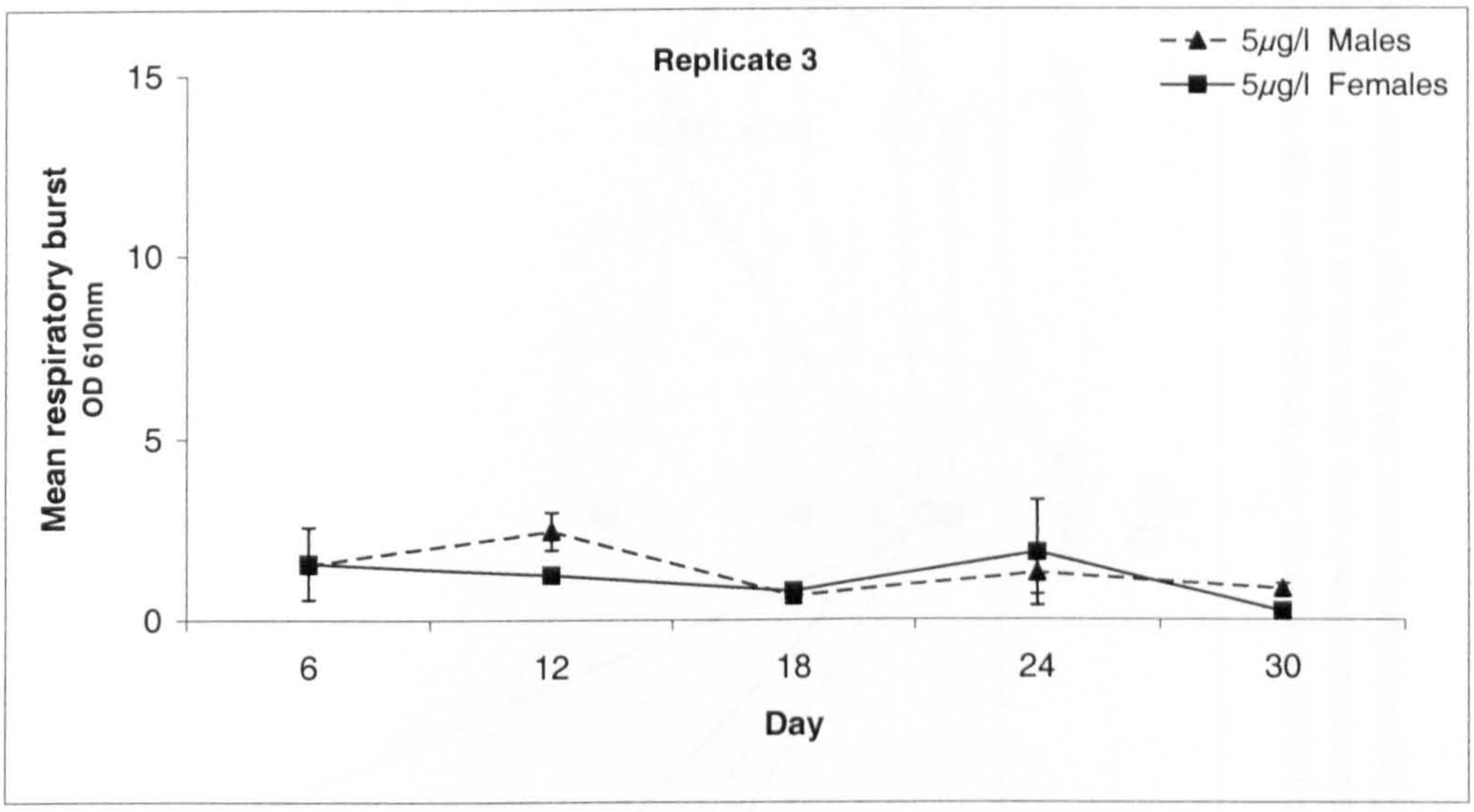


Fig. 7.4a-c. Replicate 2 - The mean respiratory burst (± S.E.) of kidney phagocytes from male and female guppies in (a) 0, (b) 5 and (c) 20μg/l cadmium.

a)



b)



c)

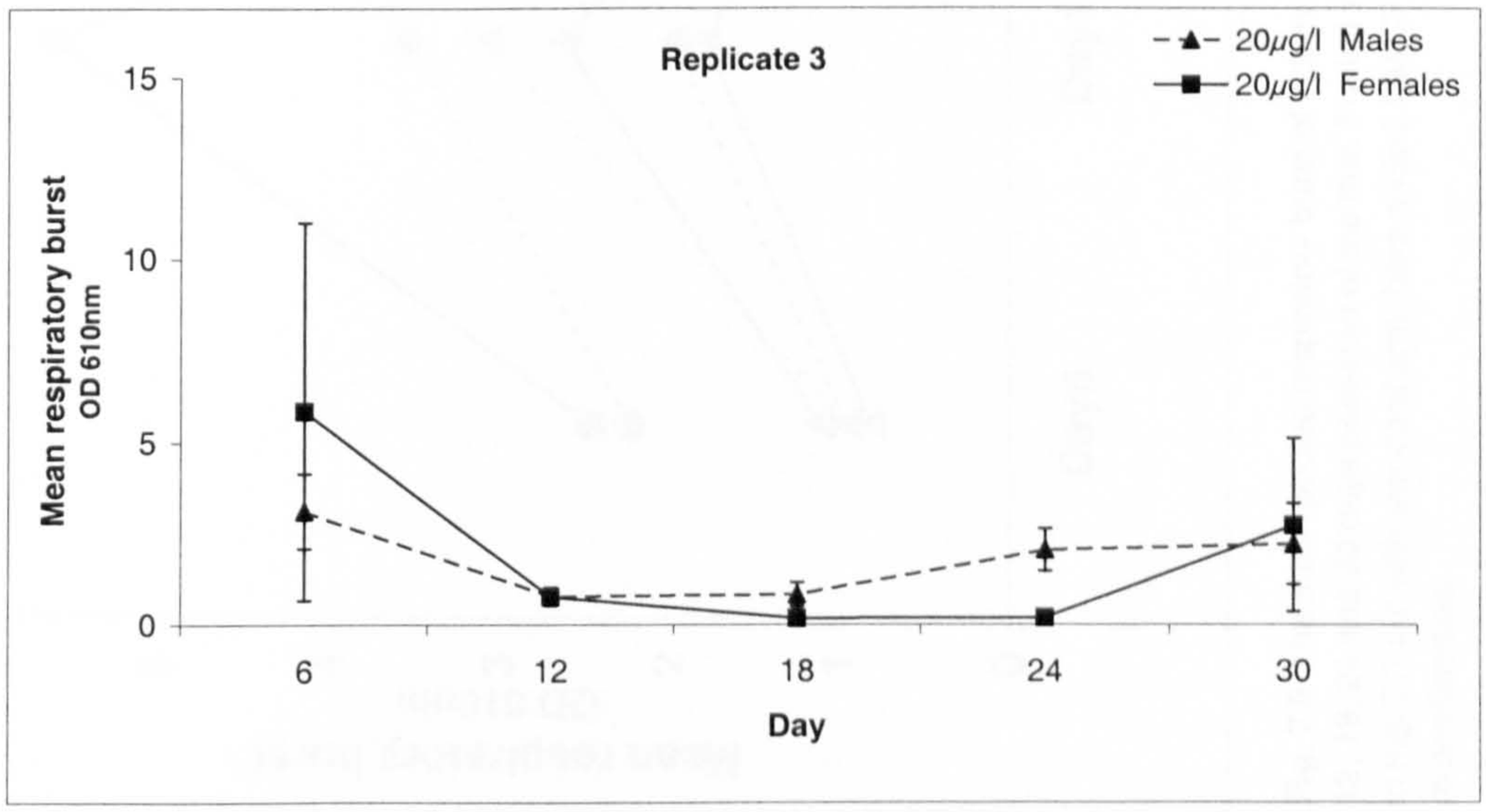


Fig. 7.5a-c. Replicate 3 - The mean respiratory burst (± S.E.) of kidney phagocytes from male and female guppies in (a) 0, (b) 5 and (c) 20µg/l cadmium.

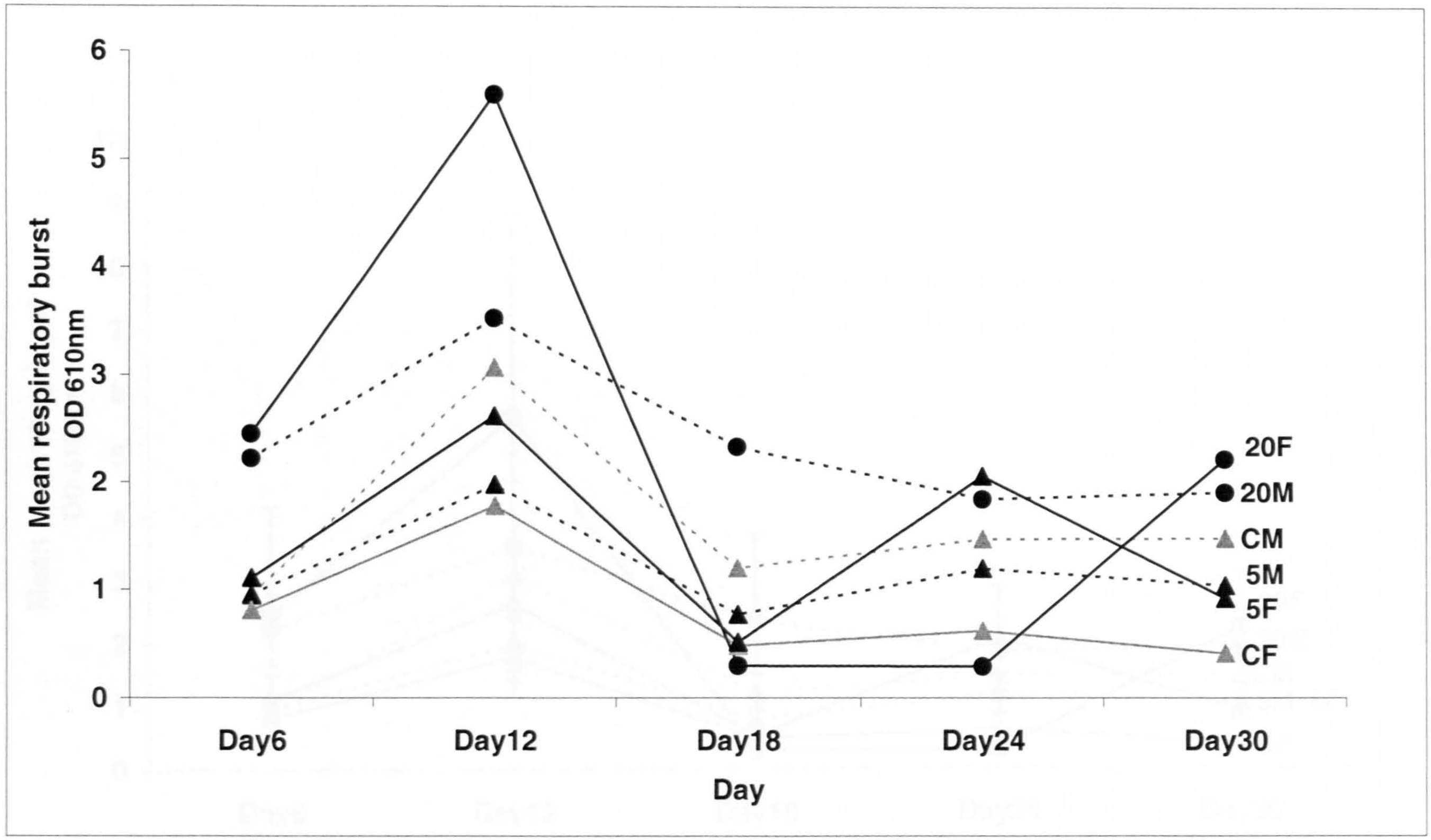


Fig. 7.6 The mean pooled respiratory burst of kidney phagocytes from male and female guppies maintained in 0, 5 and 20μg/l cadmium at 6, 12, 18, 24 and 30 days post-start of the trial. Pooled values are calculated using data from all 3 tanks in each treatment. The same graph is shown in Fig. 7.7 but with standard error bars added. CM/CF = Control males/females; 5M/5F = 5μg/l cadmium males/females; 20M/20F = 20μg/l cadmium males/females.

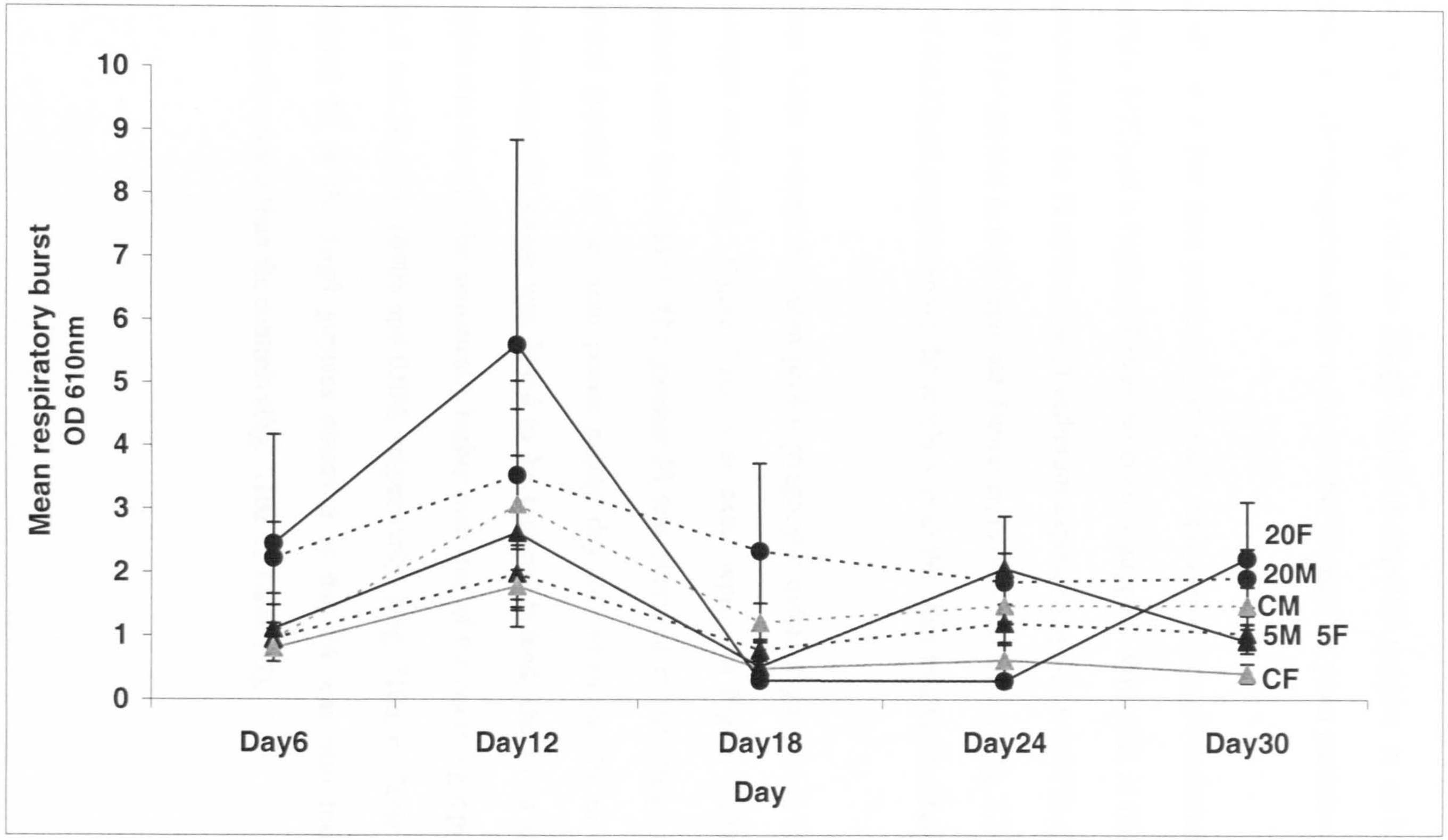


Fig. 7.7 The mean pooled respiratory burst (± S.E.) of kidney phagocytes from male and female guppies maintained in 0, 5 and 20μg/l cadmium at 6, 12, 18, 24 and 30 days post-start of the trial. Pooled values are calculated using data from all 3 tanks in each treatment.
CM/CF = Control males/females; 5M/5F = 5μg/l cadmium males/females; 20M/20F = 20μg/l cadmium males/females.

7.3.4 Phagocytosis by kidney phagocytes

7.3.4.1. Phagocytic index between treatments

Figure 7.8a-c shows the mean phagocytic index (PI) of the kidney phagocytes from the control, the 5 and the 20μg/l cadmium-exposed guppies in each of the 3 replicates. The phagocytic index is the number of engulfed yeast particles/cell.

At all but a few time points within each replicate, the 5μg/l cadmium-exposed guppies displayed a higher PI than the control guppies. With this in mind, it was expected that the PI of the 20μg/l cadmium-exposed guppies would be the highest of all 3 treatments in each replicate. However, in only replicate 3 (Fig. 7.8c) was the PI of the 20μg/l guppies above the levels of both the other treatments (0 and 5μg/l).

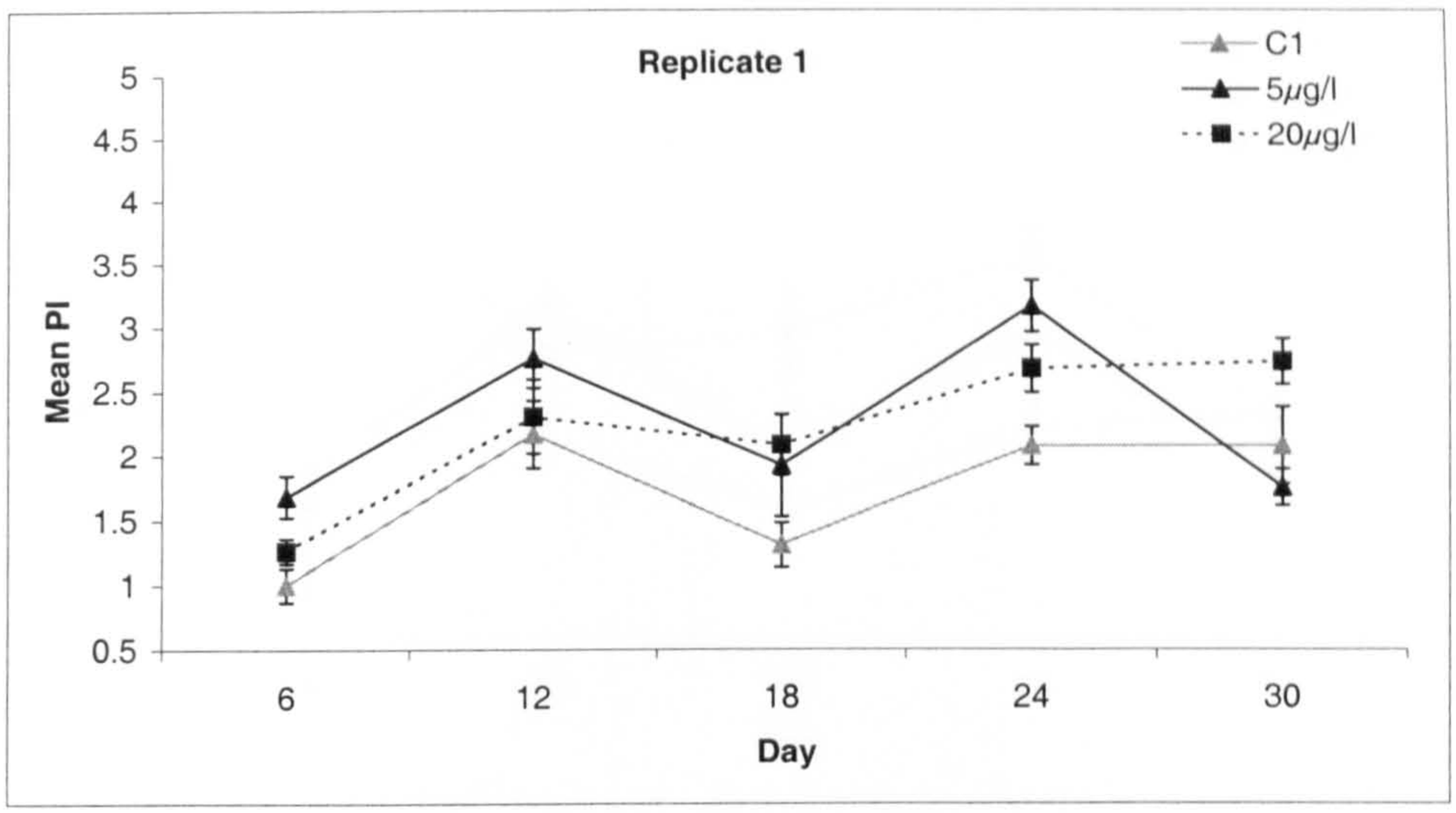
Figure 7.10a, c show the mean pooled phagocytic index of guppies in each of the treatments over time. Figure 7.10c is an exact replica of Figure 7.10a but with standard error bars added. The greatest PI was observed in the 20μg/l cadmium-exposed guppies at all time points except day 24, when the PI of the 5μg/l cadmium-exposed group was found to be highest. Indeed, the PI of the 20μg/l guppies was found to be statistically higher than that of the control guppies at both days 6 and 30 ($P = 0.0006$ and 0.008, respectively) (Fig. 710a, c; Table 7.2). The increased PI of the 5μg/l guppies observed at day 24 was also found to be statistically greater than the controls (Fig. 7.10a, c; Table 7.2).

7.3.4.2. Phagocytic ratio between treatments

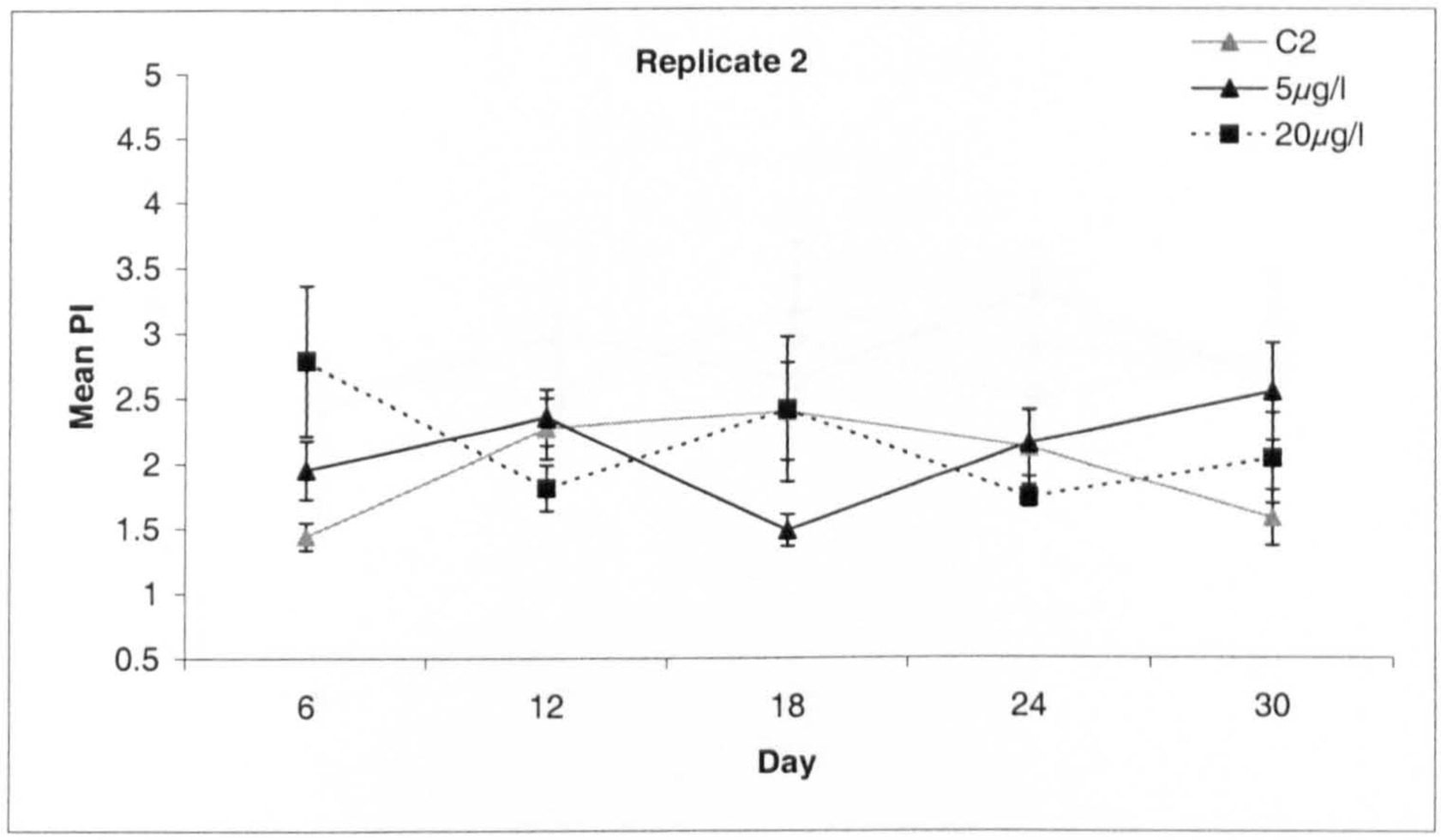
Figure 7.9a-c shows the mean phagocytic ratio (PR) of kidney phagocytes from guppies from each of the 3 treatments in each replicate. The phagocytic ratio is the proportion of phagocytes containing any internalised yeast cells.

Due to inconsistencies between the replicates, it is hard to determine a clear pattern for the PR for each treatment. However, on pooling the replicates, it becomes clear that the PR increases with increasing cadmium concentration and that this pattern is consistent over time (Fig. 7.10b, d). Figure 7.10d is an exact replica of 7.10b but with standard error (± S.E.) bars added. The greatest difference in PR between any 2 treatments was observed at day 6, with the PR of the 20μg/l guppies being statistically greater than that of the controls (P = 0.020) (Fig. 7.10b, d; Table 7.4).

a)



b)



c)

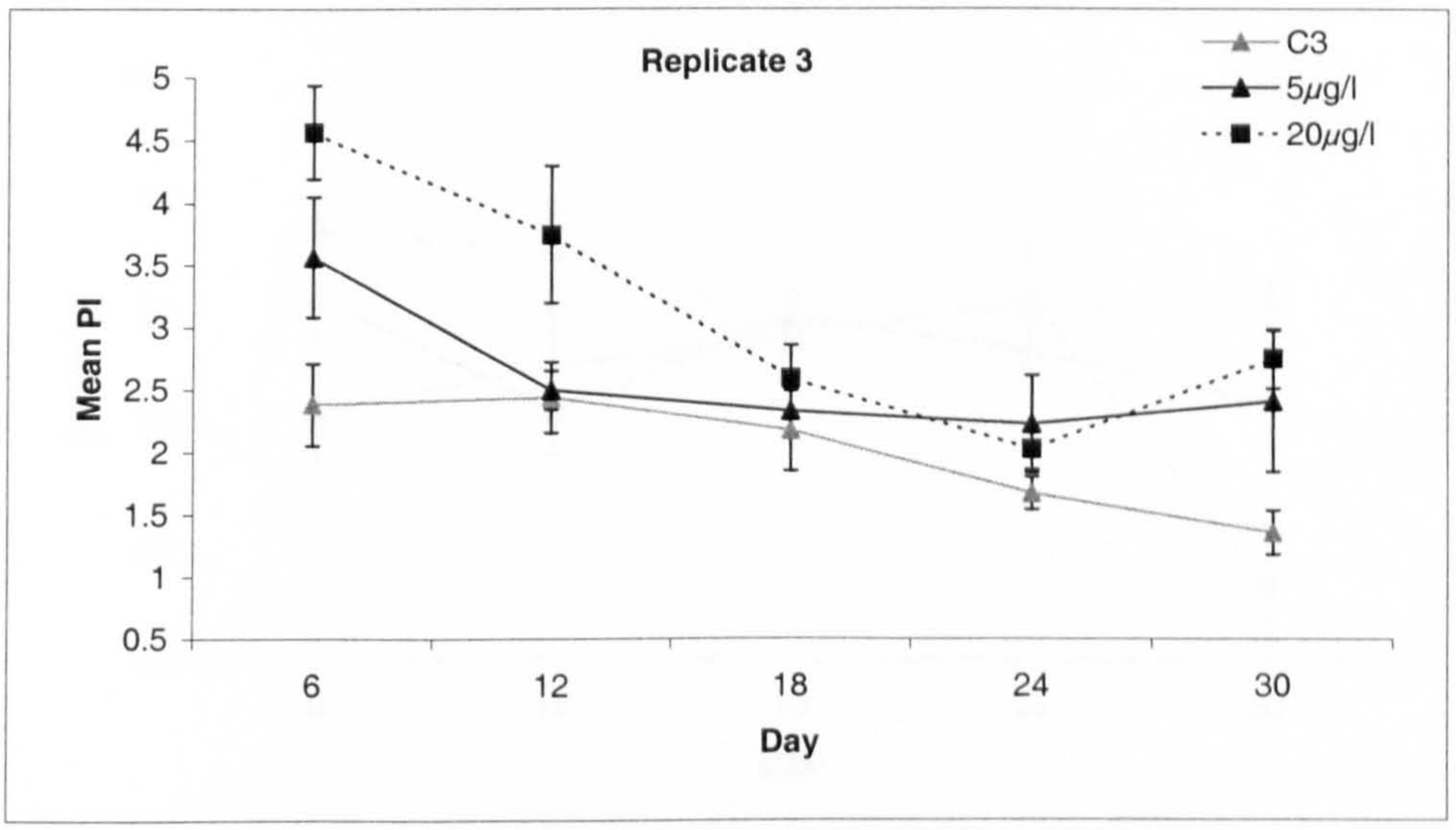
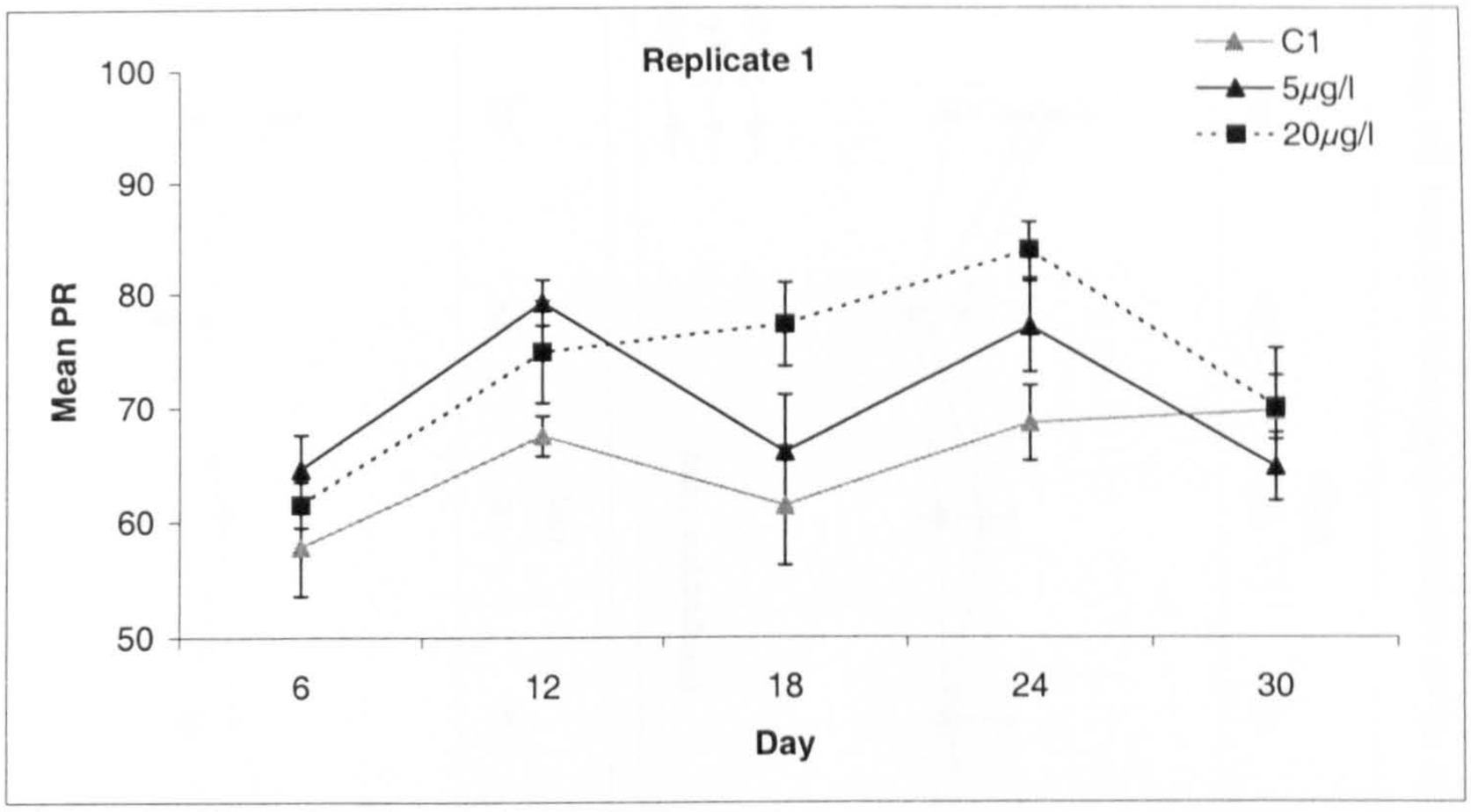


Fig. 7.8 a-c. The mean phagocytic index (PI) (± S.E.) of kidney phagocytes from guppies in control, 5 and 20µg/l cadmium treatments in (a) replicate 1, (b) replicate 2 and (c) replicate 3 over time.

a)



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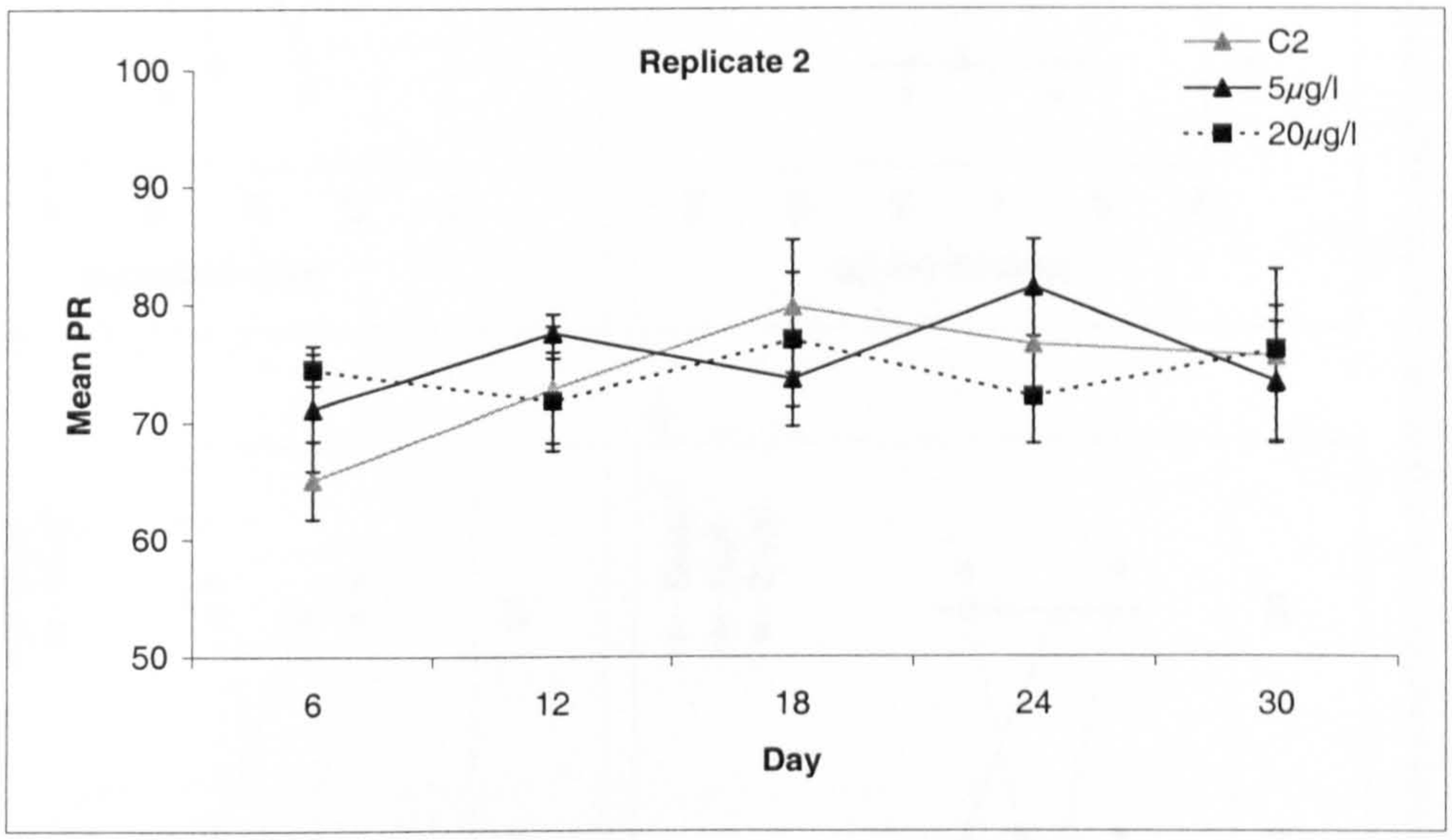


c)

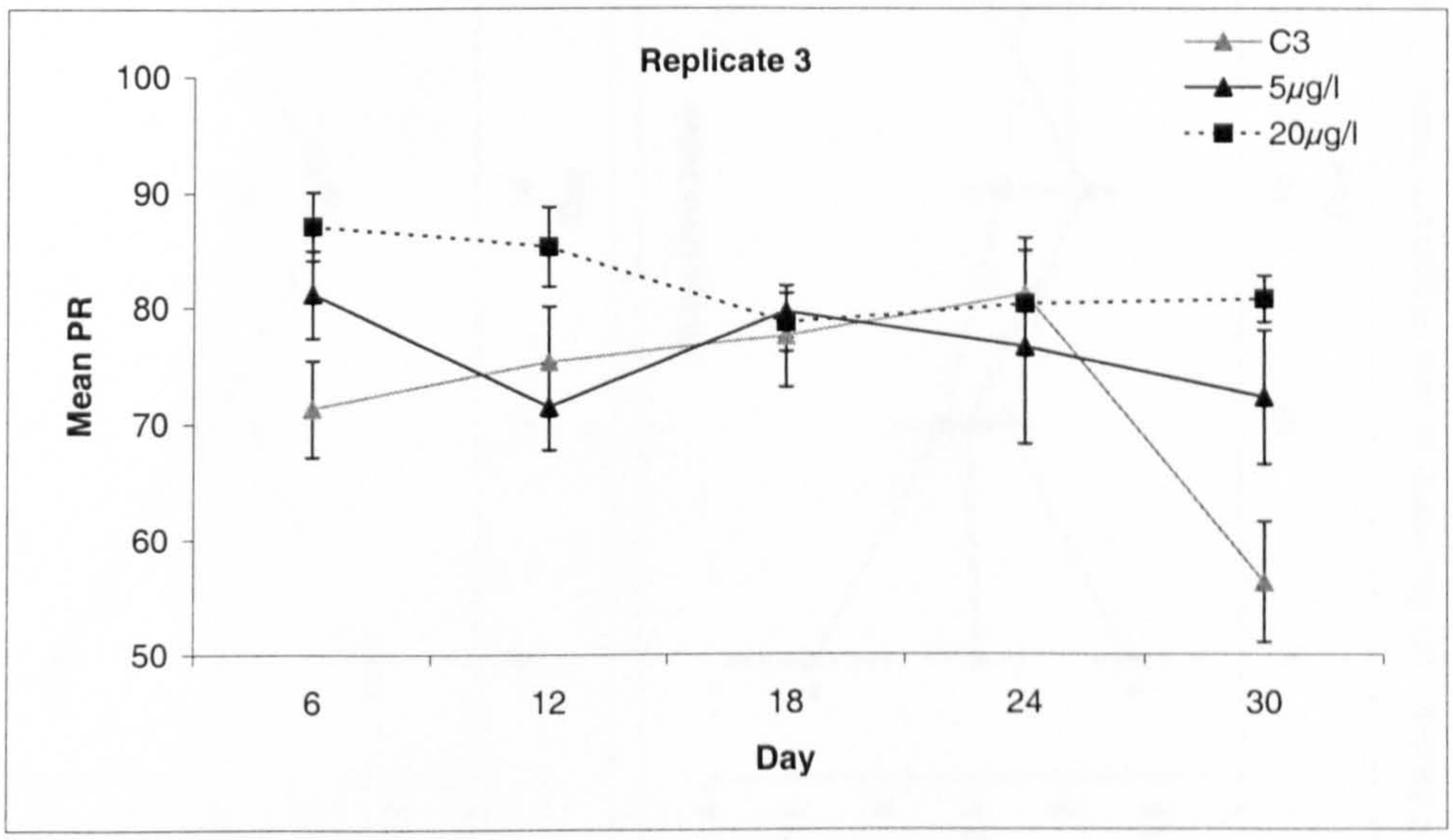
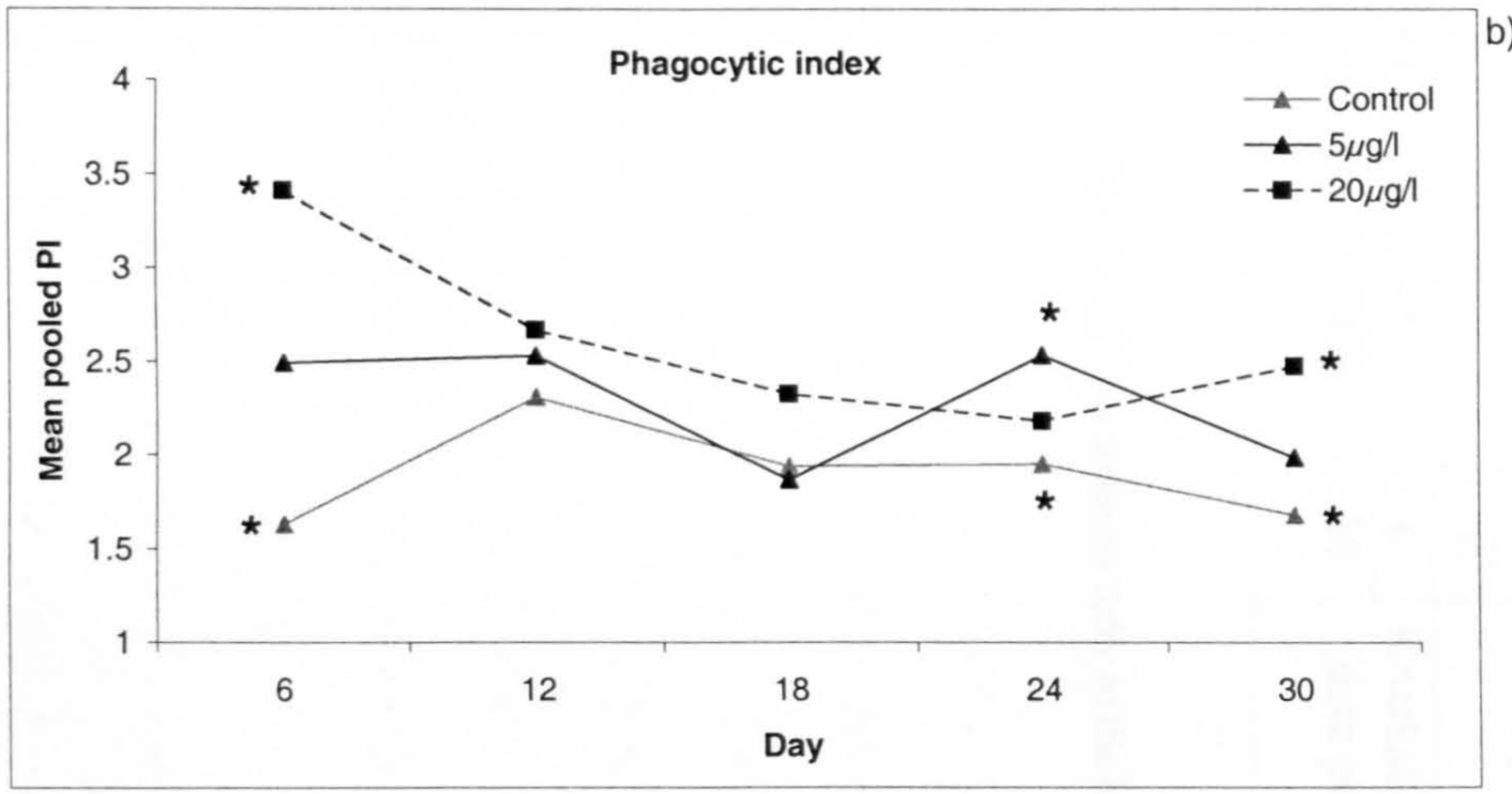


Fig. 7.9 a-c. The mean phagocytic ratio (PR) (± S.E.) of kidney phagocytes from guppies in control, 5 and 20µg/l cadmium treatments in (a) replicate 1, (b) replicate 2 and (c) replicate 3 over time.

a)



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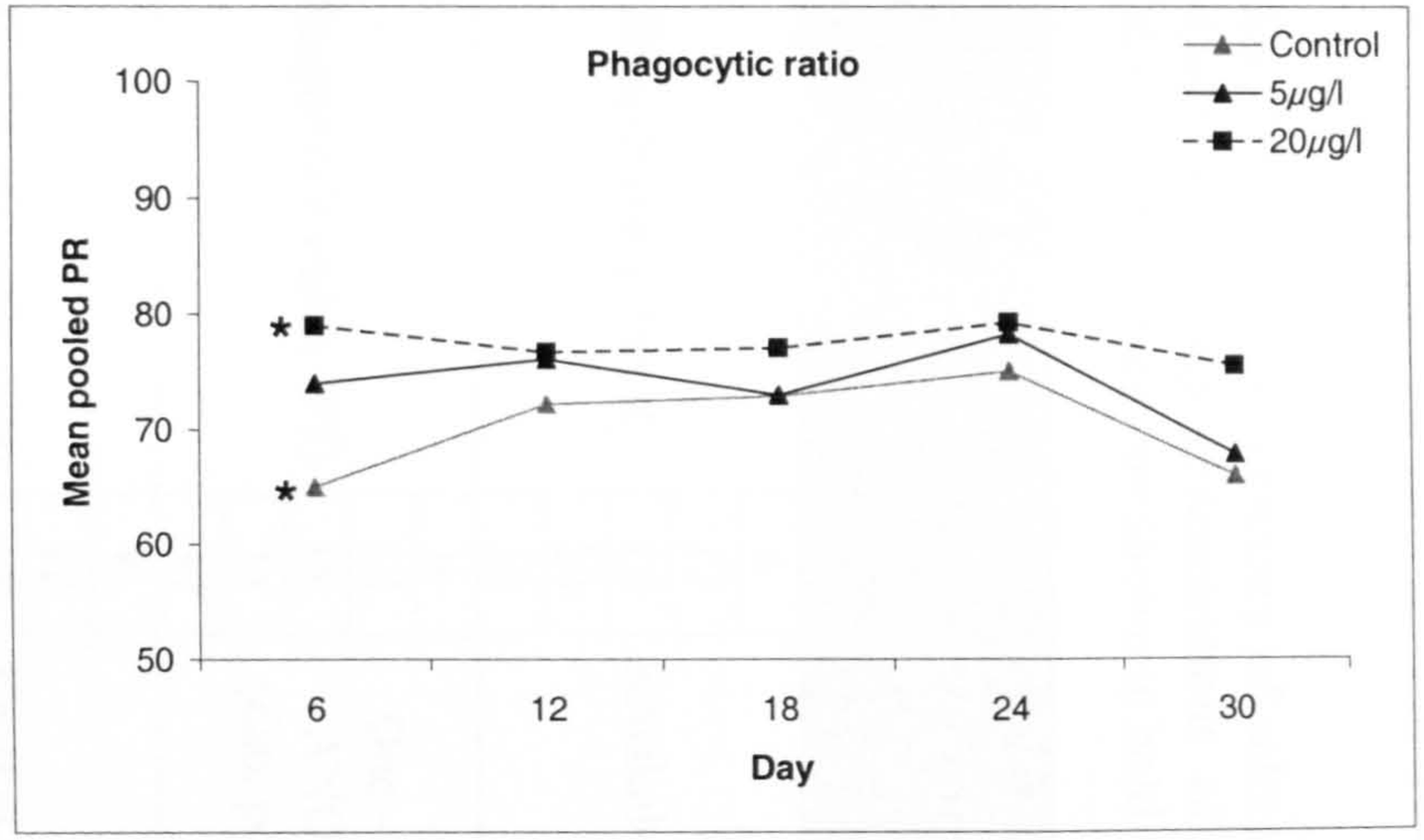
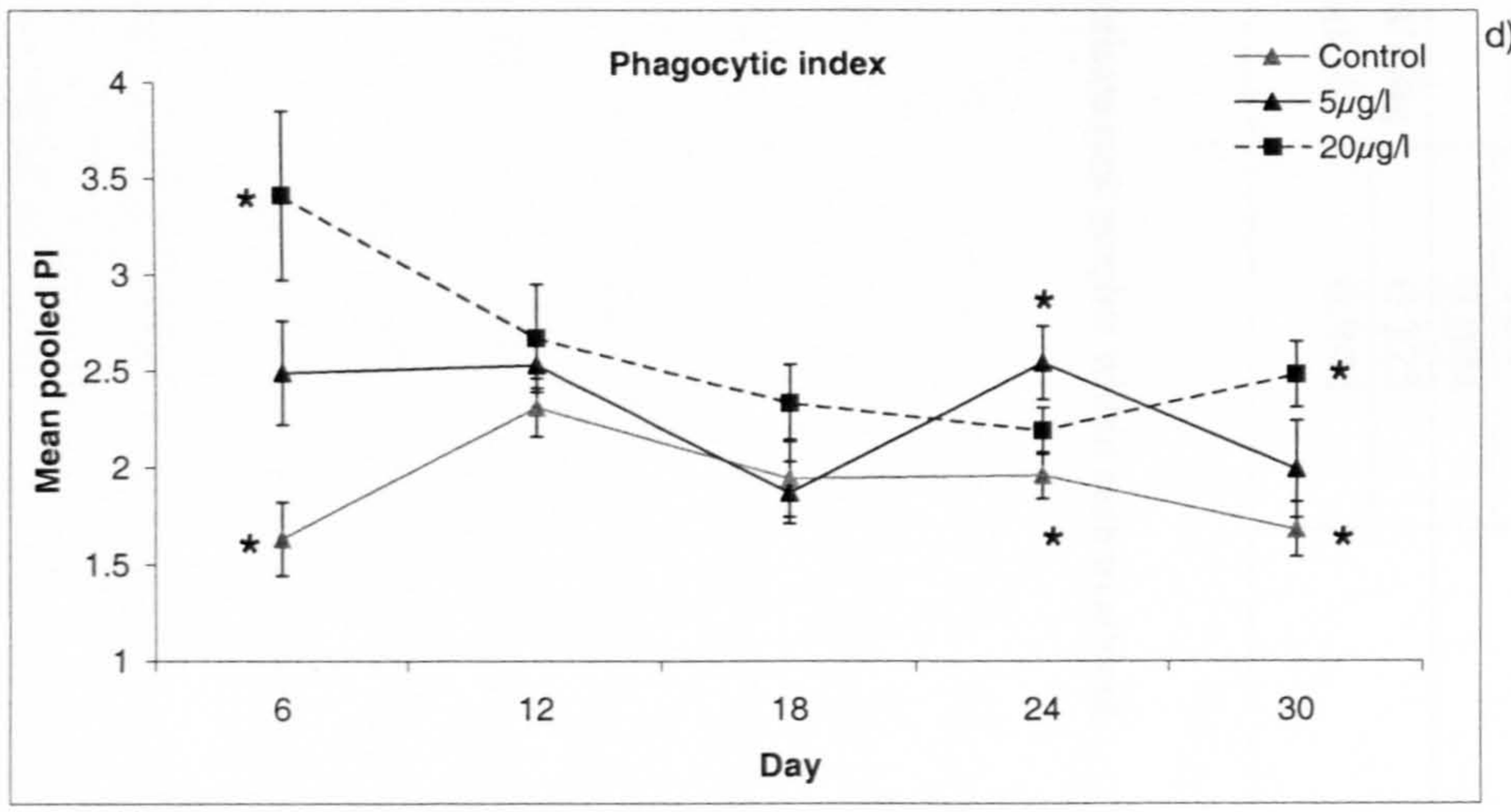


c)



d)

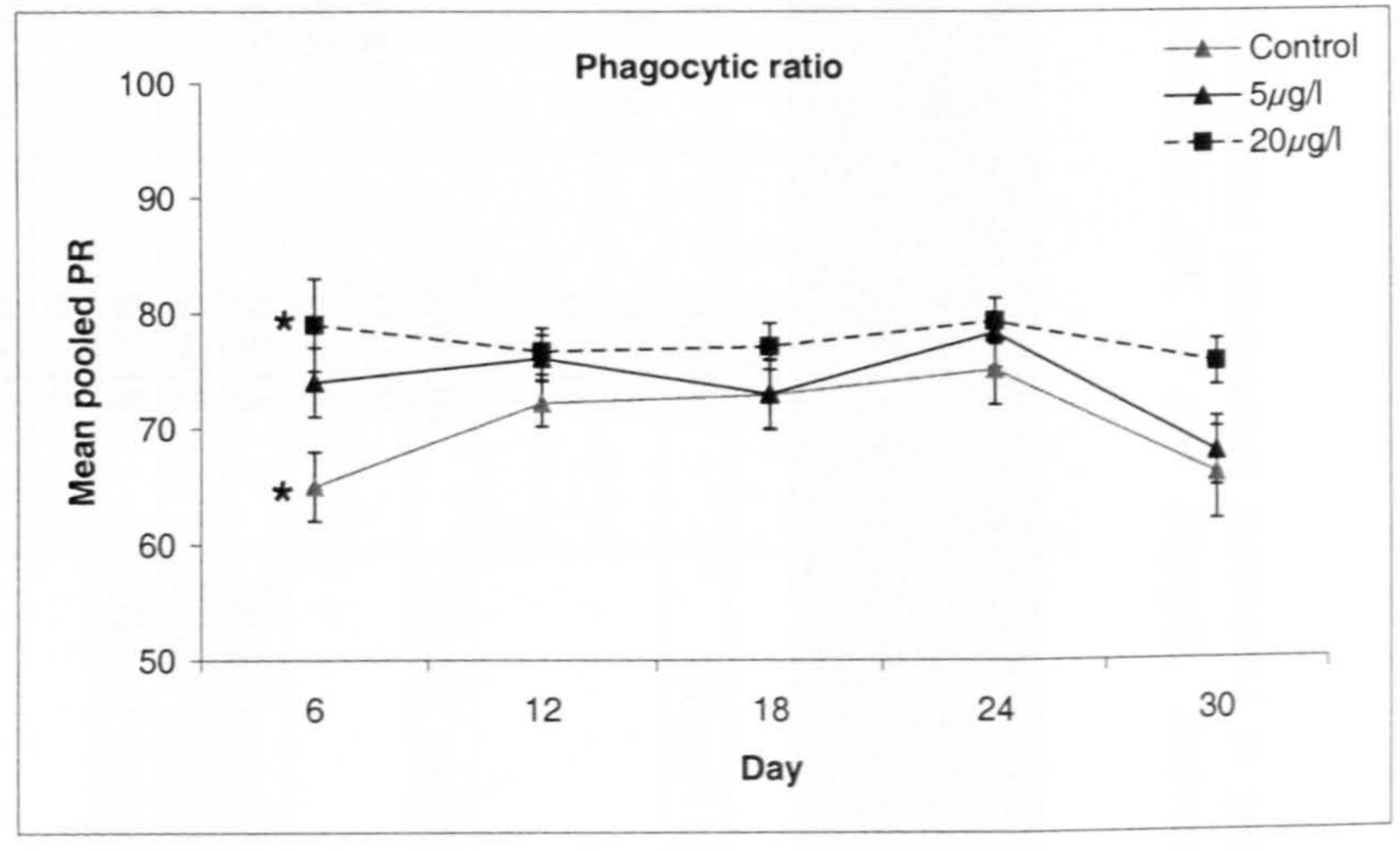


Fig. 7.10 a-d. (a) The mean pooled phagocytic index (PI) of kidney phagocytes from guppies in control, 5μg/l cadmium and 20μg/l cadmium treatments over time. (b) The mean phagocytic ratio (PR) of kidney phagocytes from guppies in control, 5μg/l cadmium and 20μg/l cadmium treatments over time. (c) and (d) as in (a) and (b) but with standard error bars added (± S.E.). Asterisks denote statistical differences between treatments.

Table 7.2. Statistical summary of selected immune parameters measured in guppies exposed to varying concentrations of cadmium (0, 5 and 20μg/l). Statistically significant differences are shown in bold.

Analysing different treatments				
Immunological parameter	**Day**	**Statistical test**	**Statistical significance P**	**Treatments between which differences lie**
Respiratory burst	6	Kruskal-Wallis	0.089	
	12		**0.048**	20μg/l (1) > 20μg/l (3)
	18		0.621	
	24		0.746	
	30		0.081	
Phagocytic index (PI)	6	One - way ANOVA (data pooled)	**0.0006**	20μg/l cadmium > Controls
	12		0.359	
	18		0.178	
	24		**0.023**	5μg/l cadmium > Controls
	30		**0.008**	20μg/l cadmium > Controls
Phagocytic ratio (PR)	6	Kruskal-Wallis (data pooled)	**0.018**	20μg/l cadmium > Controls
	12		0.196	
	18		0.528	
	24		0.387	
	30		0.094	
Myeloperoxidase activity (MPO)	6	Kruskal-Wallis (data pooled)	0.225	
	30		0.944	

Numbers in parentheses refer to the replicate tank number within each treatment.

7.3.4.3. Phagocytic index between sexes

Table 7.3 shows the mean phagocytic index (PI) of male and female guppies from each replicate in each treatment over time. Points worthy of note in Table 7.3 are that, while, in general, control female guppies have a greater PI than the males, in 2 of the replicates males have higher PIs than the females at days 6 and 30. In the 5μg/l population of guppies, males generally displayed the greatest PI, particularly noticeable at day 30 in all 3 replicates (Table 7.3). As with the control guppies, the 20μg/l cadmium-exposed female guppies were observed, in general, to have higher PI values than the males, although males in 2 of the replicates (replicates 1 and 3) had greater PIs than the females at day 6 of the trial (Table 7.3). No data could be obtained for the PI of the female 20μg/l guppies in replicate 2 at day 6, due to low cell number, and thus, it is not known whether the males in this replicate would also have had a higher PI than the females.

The mean pooled phagocytic index (PI) (± S.E.) from all males within each treatment and all females within each treatment at days 6, 12, 18, 24 and 30 is shown in Figure 7.11a-e. In contrast to the respiratory burst results, where male guppies had a greater response than the females, the mean PI of female guppies from each treatment was generally greater than that of their respective males at all time points, although the differences between the sexes in each treatment were not statistically different (Fig. 7.11a-e; Table 7.5). However, between the different treatments there were some statistical differences in the PI, with that of 20μg/l males being statistically greater than control males at day 6 ($P = 0.009$); that of the 20μg/l females being statistically greater than both control and 5μg/l males at day 18 ($P = 0.021$); that of the 5μg/l females being statistically greater than the control

males at day 24 (P = 0.049); and that of the control females being statistically lower than the 20μg/l females at day 30 (P = 0.007) (Fig. 7.11a-e; Table 7.5).

7.3.4.4. Phagocytic ratio between sexes

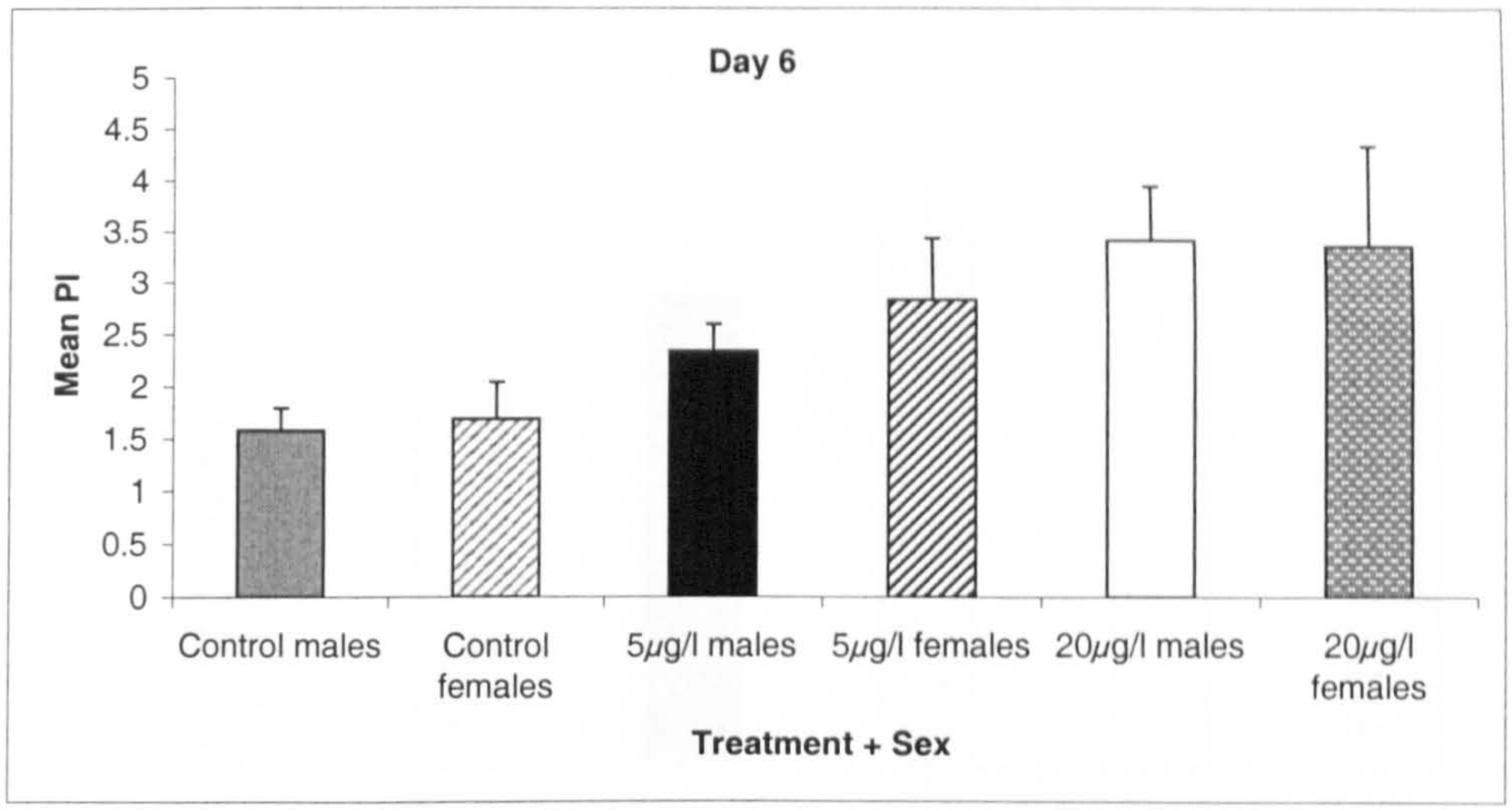
Table 7.4 is a summary of the PR in male and female guppies in all replicates of each treatment at each time point. In both the control and 20μg/l cadmium-exposed populations of guppies, females generally had higher PR values than the males. However, the control females displayed this higher PR at the beginning of the trial, while the females exposed to 20μg/l cadmium displayed the higher PR towards the end of the trial (Table 7.4). In contrast to the control and 20μg/l guppy populations, male guppies exposed to 5μg/l cadmium tended to have higher phagocytic ratios than their respective females.

Figure 7.12a-e shows the mean pooled phagocytic ratio (PR) of male and female guppies from each treatment. At days 6, 18 and 24, female guppies were, in general, observed to have a greater PR than their respective males in all treatments but, as with the results of the PI, these differences were not significant (Fig. 7.12a, c, d, Table 7.5). Conversely, at day 12, males from both cadmium treatments had a greater mean PR than their respective females, and at day 30 control and 5μg/l cadmium-exposed males had a greater PR than their respective females (Fig. 7.12e). At day 30, control females were found to have a statistically lower PR than those females that had been exposed to 20μg/l cadmium (P = 0.017) (Table 7.5).

Table 7.3. The mean phagocytic index (PI) ±S.E. of kidney phagocytes from male and female guppies in each replicate (1-3) and in each treatment (0, 5 and 20μg/l) over the duration of the trial. (n) number in parentheses represents the number of guppies for which data could be obtained.

Tank/Guppy sex		Mean phagocytic index (PI) on days				
		6	**12**	**18**	**24**	**30**
Control	**C1/Male**	1.14 ± 0.16 (n = 5)	2.20 ± 0.28 (n = 5)	1.97 ± 0.22 (n = 5)	1.71 ± 0.22 (n = 5)	1.58 ± 0.21 (n = 5)
	C2/Male	1.61 ± 0.19 (n = 2)	1.67 ± 0.14 (n = 5)	1.30 ± 0.22 (n = 4)	1.81 ± 0.10 (n =4)	2.13 ± 0.43 (n = 5)
	C3/Male	2.02 ± 0.42 (n = 5)	2.08 ± 0.07 (n = 4)	2.08 ± 0.33 (n =3)	1.78 ± 0.52 (n = 4)	1.56 ± 0.89 (n = 5)
	C1/Female	0.77 ± 0.16 (n = 3)	2.74 ± 0.57 (n = 3)	2.35 ± 0.66 (n = 2)	1.60 ± 0.10 (n = 3)	1.12 ± 0.37 (n = 2)
	C2/Female	1.33 ± 0.10 (n =3)	3.00 ± 0.19 (n = 3)	1.31 ± 0.39 (n = 3)	2.52 ± 0.14 (n = 3)	1.94 ± 0.06 (n = 2)
	C3/Female	2.99 ± 0.35 (n = 3)	3.06 ± 0.74 (n = 3)	2.80 ± 0.77 (n = 3)	2.55 ± 0.52 (n = 3)	1.59 ± 0.89 (n = 3)
5μg/l cadmium	**T1/Male**	1.60 ± 0.29 (n = 3)	2.53 ± 0.24 (n = 3)	2.32 ± 0.15 (n = 3)	2.05 ± 0.48 (n = 3)	1.84 ± 0.12 (n = 3)
	T2/Male	2.16 ± 0.32 (n = 4)	2.66 ± 0.12 (n =5)	1.78 ± 0.48 (n =5)	3.38 ± 0.18 (n = 4)	2.03 ± 0.05 (n = 5)
	T3/Male	2.94 ± 0.42 (n = 5)	2.09 ± 0.20 (n = 5)	1.53 ± 0.17 (n = 4)	1.68 ± 0.21 (n = 5)	2.95 ± 0.37 (n = 2)
	T1/Female	1.93 ± 0.21 (n = 2)	2.41 ± 0.17 (n = 3)	2.30 ± 0.62 (n = 3)	2.64 ± 0.76 (n = 3)	1.44 ± 0.44 (n = 3)
	T2/Female	1.68 ± 0.29 (n = 3)	2.94 ± 0.64 (n = 3)	2.12 ± 0.77 (n = 3)	2.82 ± 0.43 (n = 3)	1.29 ± 0.11 (n = 2)
	T3/Female	4.61 ± 0.35 (n = 3)	2.80 ± 0.74 (n =3)	1.41 ± 0.77 (n = 2)	2.77 ± 0.52 (n = 2)	1.52 ± 0.89 (n = 3)
20μg/l cadmium	**T1/Male**	1.35 ± 0.06 (n = 2)	3.5 ± 0.45 (n = 3)	2.13 ± 0.10 (n = 3)	2.00 ± 0.21 (n = 3)	2.84 ± 0.19 (n = 3)
	T2/Male	2.79 ± 0.57 (n = 2)	2.69 ± 0.29 (n = 4)	1.90 ± 0.35 (n = 4)	2.76 ± 0.17 (n = 5)	2.55 ± 0.20 (n = 5)
	T3/Male	4.51 ± 0.40 (n = 5)	2.01 ± 0.26 (n = 5)	1.77 ± 0.24 (n = 3)	1.67 ± 0.05 (n = 5)	1.96 ± 0.50 (n = 4)
	T1/Female	1.08 (n = 1)	4.12 ± 1.43 (n = 3)	3.04 ± 0.36 (n = 3)	2.06 ± 0.34 (n = 3)	2.61 ± 0.19 (n = 3)
	T2/Female	No data	1.79 ± 0.43 (n = 3)	2.38 ± 0.24 (n = 3)	2.53 ± 0.45 (n = 3)	2.97 ± 0.29 (n = 3)
	T3/Female	4.12 ± 0.86 (n = 3)	1.55 ± 0.13 (n = 3)	3.70 ± 1.3 (n = 3)	2.04 (n = 1)	2.17 ± 0.50 (n = 3)

a)



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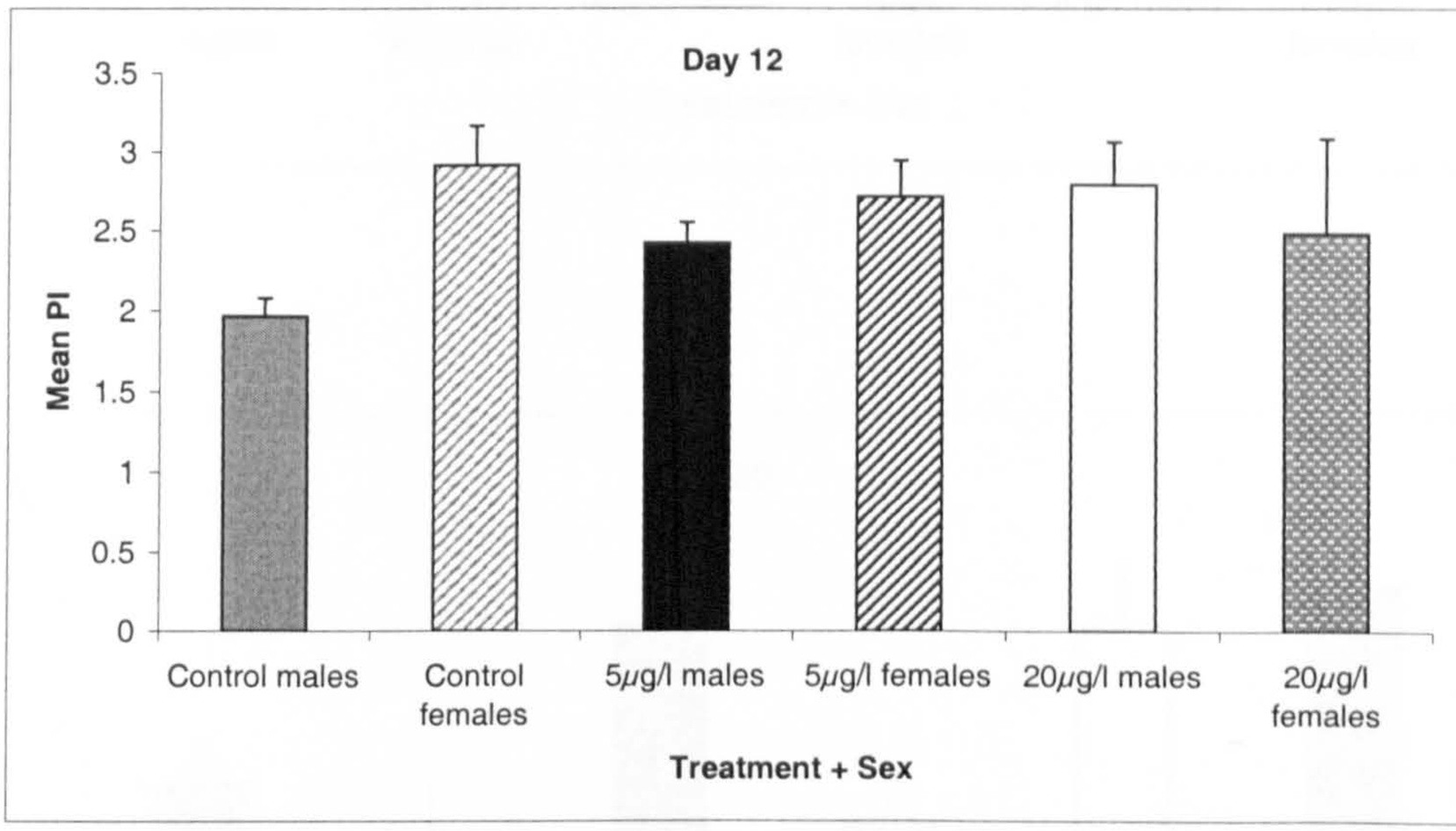

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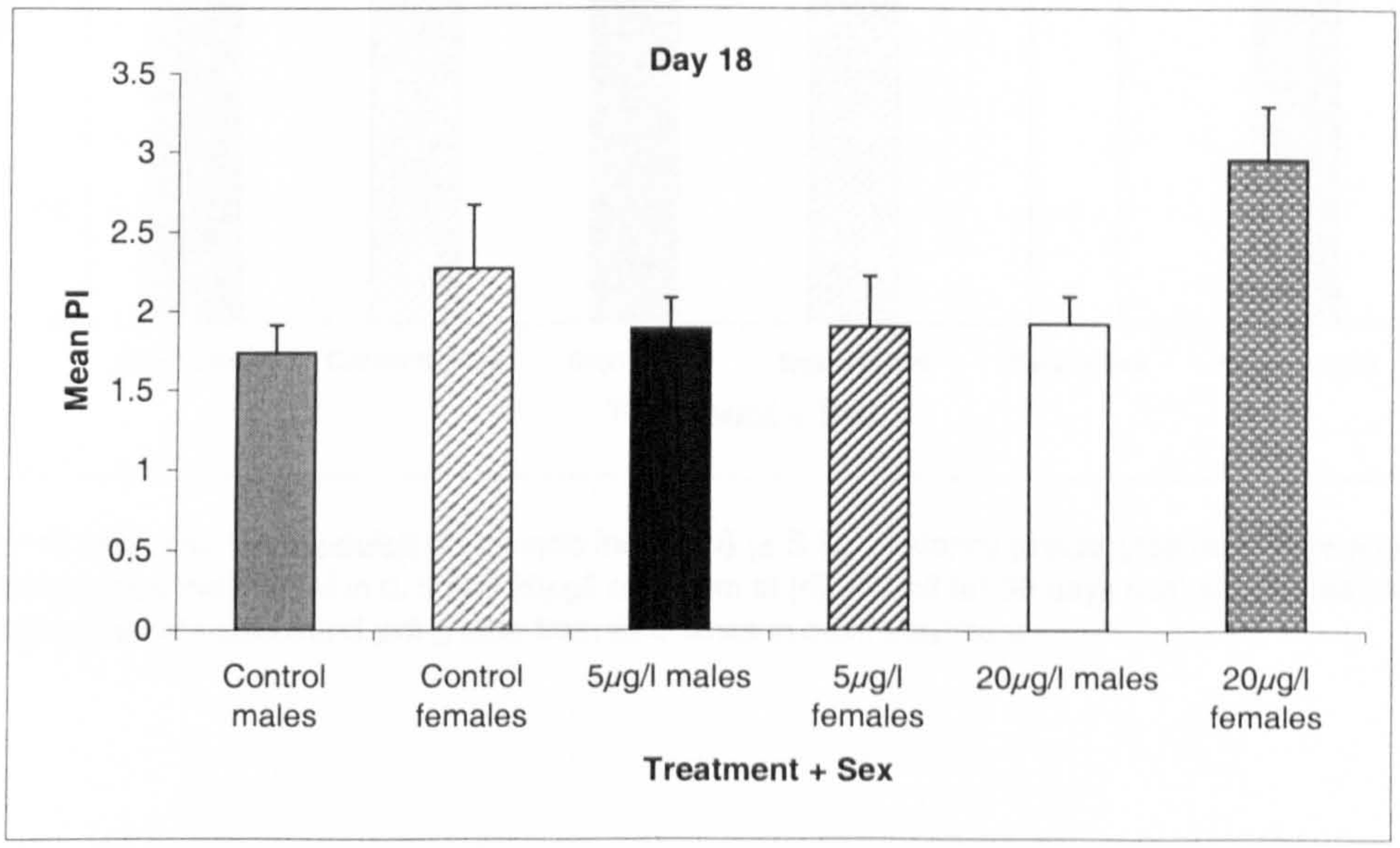
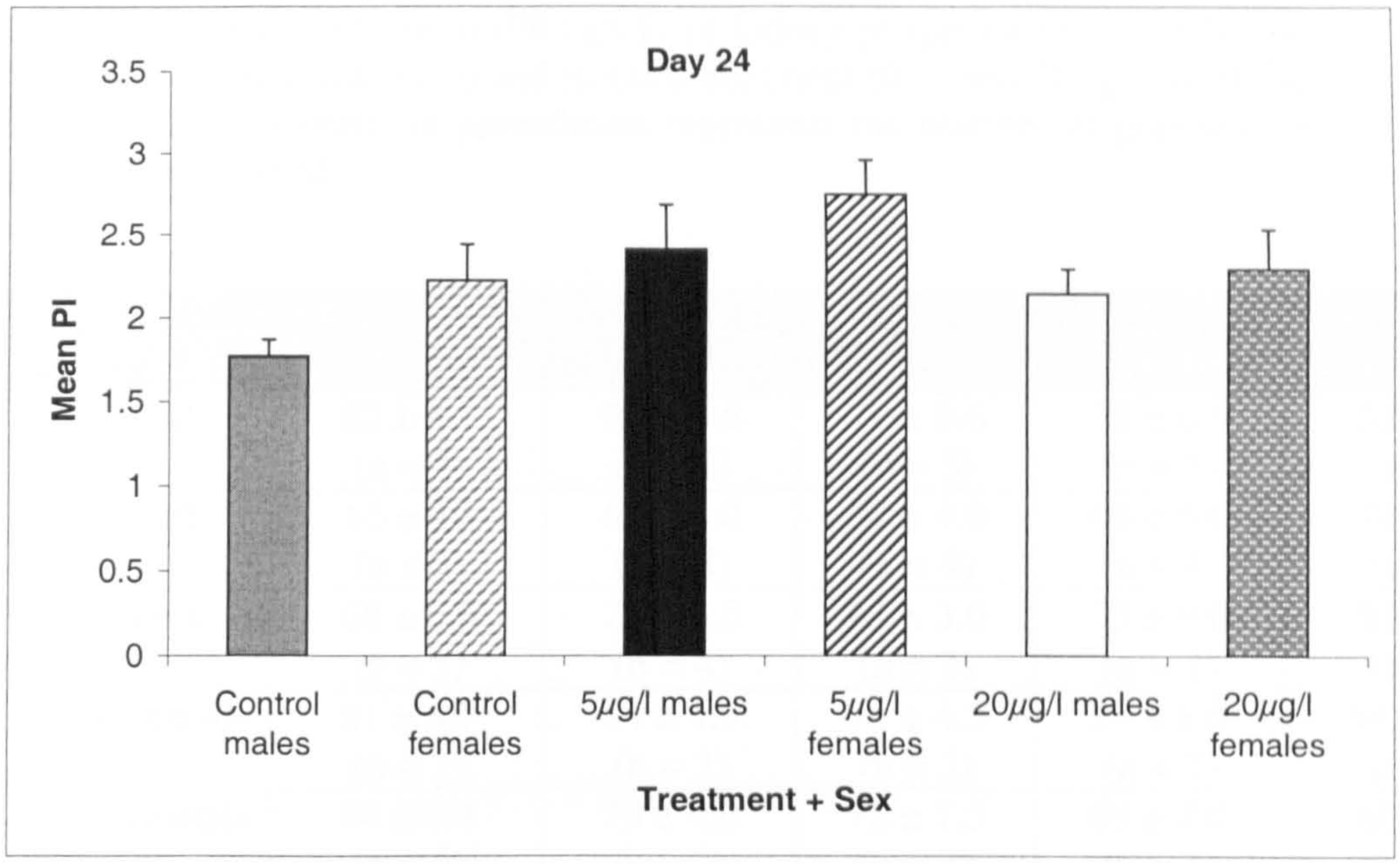


Fig. 7.11a-c. The mean pooled phagocytic index (PI) (± S.E.) of kidney phagocytes from male and female guppies maintained in 0, 5 and 20µg/l cadmium at (a) 6, (b) 12 and (c)18 days post-start of the trial. Pooled values are calculated using data from all 3 tanks in each treatment.

d)



e)

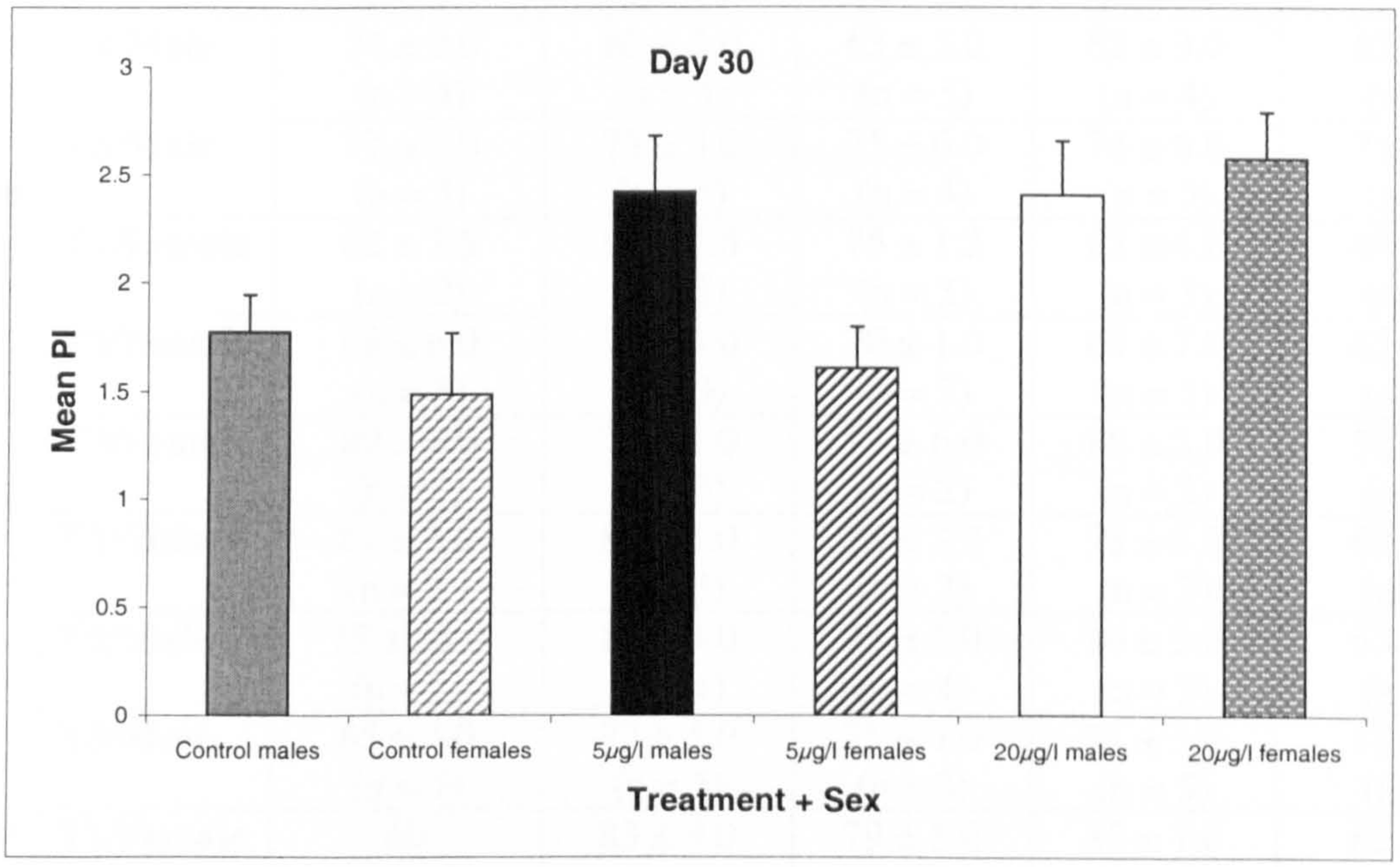
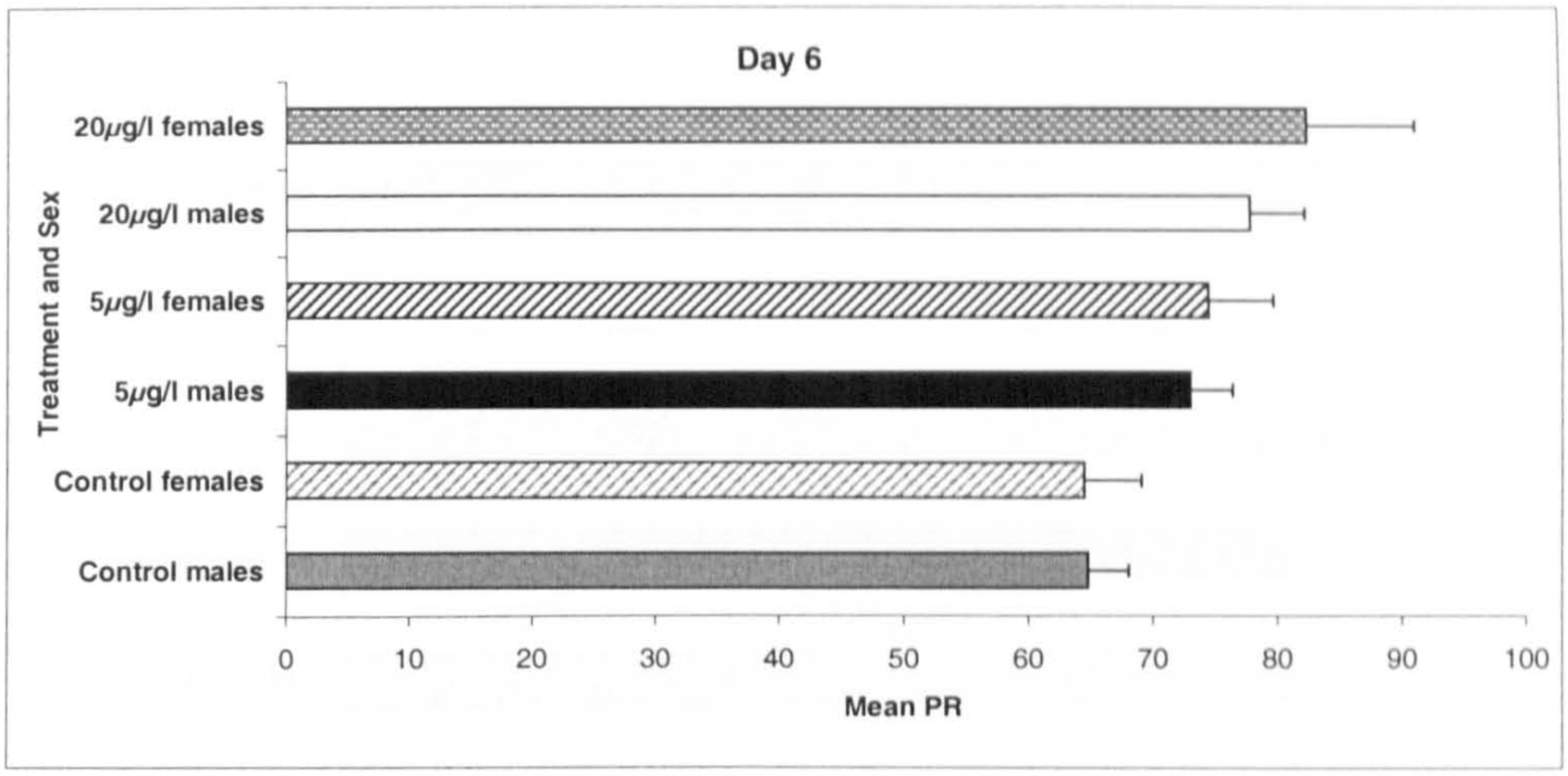


Fig. 7.11 d-e. The mean pooled phagocytic index (PI) (± S.E.) of kidney phagocytes from male and female guppies maintained in 0, 5 and 20μg/l cadmium at (d) 24 and (e) 30 days post-start of the trial. Pooled values are calculated using data from all 3 tanks in each treatment.

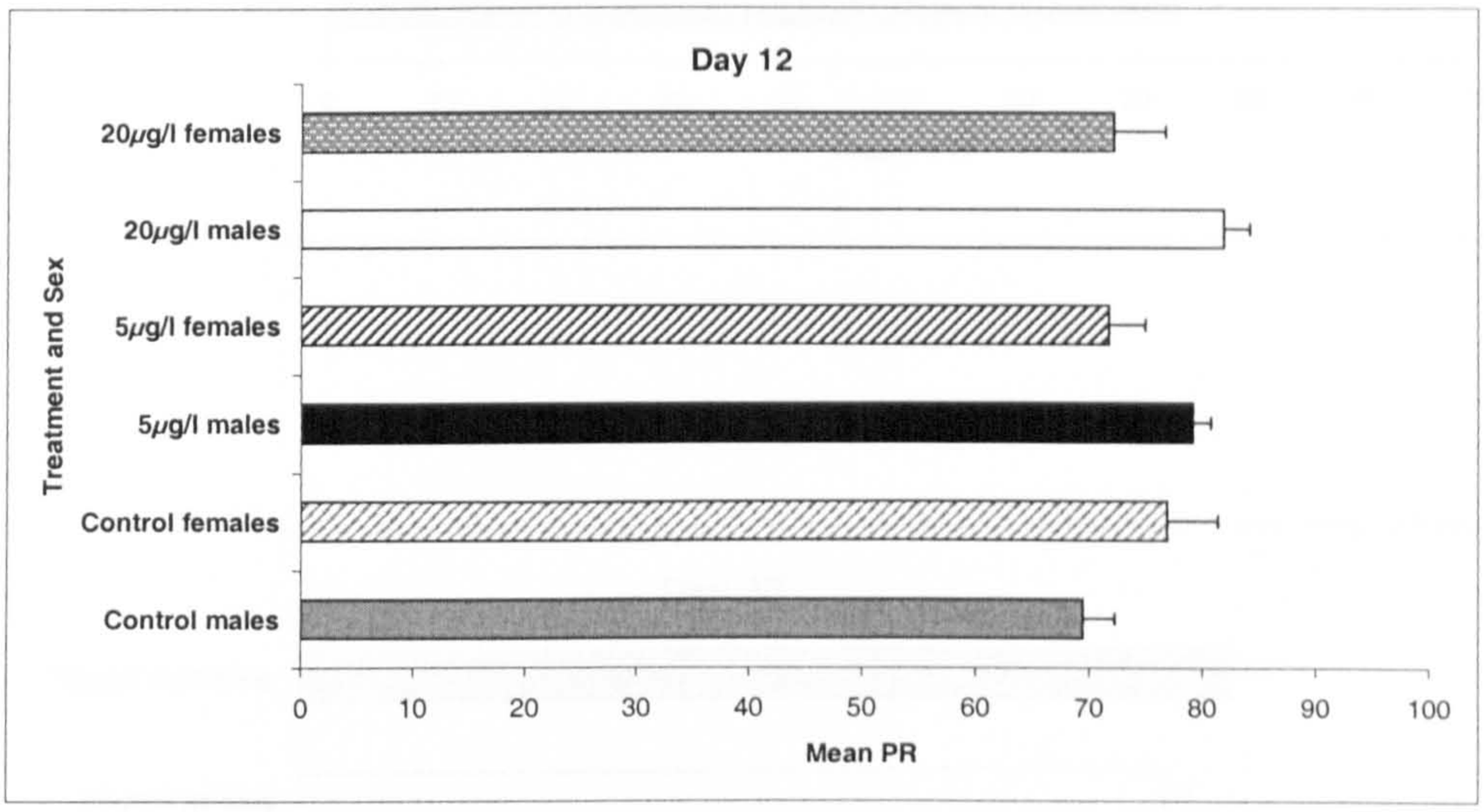
Table 7.4. The mean phagocytic ratio (PR) ±S.E. of kidney phagocytes from male and female guppies in each replicate (1-3) and in each treatment (0, 5 and 20μg/l) over the duration of the trial. (n) number in parentheses represents the number of guppies for which data could be obtained.

Tank/Guppy sex		Mean phagocytic ratio (PR) on days				
		6	12	18	24	30
Control	C1/Male	62 ± 4.1 (n = 5)	69 ± 5.6 (n = 5)	79 ± 8.6 (n = 5)	78 ± 6.6 (n = 5)	52 ± 3.6 (n = 5)
	C2/Male	65 ± 10 (n = 2)	66 ± 1.0 (n = 5)	57 ± 4.0 (n = 4)	68 ± 5.0 (n = 4)	74 ± 7.0 (n = 5)
	C3/Male	68 ± 6.0 (n = 5)	74 ± 6.0 (n = 4)	83 ± 3.0 (n = 3)	75 ± 9.0 (n = 4)	82 ± 6.0 (n = 5)
	C1/Female	51 ± 8.7 (n = 3)	84 ± 4.9 (n = 3)	76 ± 4.5 (n = 2)	85 ± 8.0 (n = 3)	54 ± 11.0 (n = 2)
	C2/Female	66 ± 0.4 (n = 3)	70 ± 4.0 (n = 3)	72 ± 1.5 (n = 3)	69 ± 2.0 (n = 3)	60 ± 4.0 (n = 2)
	C3/Female	77 ± 4.0 (n = 3)	76 ± 17.0 (n = 3)	75 ± 13.0 (n = 3)	78 ± 2.0 (n = 3)	63 ± 23.0 (n = 3)
5μg/l cadmium	T1/Male	66 ± 1.0 (n = 3)	78 ± 2.8 (n = 3)	82 ± 2.9 (n = 3)	73 ± 7.3 (n = 3)	73 ± 11.2 (n = 3)
	T2/Male	74 ± 9.0 (n = 4)	80 ± 3.0 (n = 5)	63 ± 5.0 (n = 5)	82 ± 3.0 (n = 4)	65 ± 5.0 (n = 5)
	T3/Male	77 ± 4.0 (n = 5)	78 ± 3.0 (n = 5)	75 ± 6.0 (n = 4)	76 ± 6.0 (n = 5)	71 ± 4.0 (n = 2)
	T1/Female	62 ± 7.5 (n = 2)	61 ± 2.8 (n = 3)	75 ± 1.2 (n = 3)	85 ± 4.8 (n = 3)	60 ± 7.7 (n = 3)
	T2/Female	68 ± 6.0 (n = 3)	77 ± 5.0 (n = 3)	70 ± 1.0 (n = 3)	68 ± 7.0 (n = 3)	65 ± 4.0 (n = 2)
	T3/Female	89 ± 6.0 (n = 3)	77 ± 1.0 (n = 3)	72 ± 6.0 (n = 2)	88 ± 3.0 (n = 2)	58 ± 2.0 (n = 3)
20μg/l cadmium	T1/Male	62 ± 3.0 (n = 2)	87 ± 3.0 (n = 3)	78 ± 3.0 (n = 3)	78 ± 6.0 (n = 3)	80 ± 2.0 (n = 3)
	T2/Male	75 ± 13.0 (n = 2)	80 ± 4.0 (n = 4)	73 ± 5.0 (n = 4)	86 ± 3.0 (n = 5)	67 ± 4.0 (n = 5)
	T3/Male	85 ± 3.0 (n = 5)	77 ± 5.0 (n = 5)	75 ± 7.0 (n = 3)	71 ± 5.0 (n = 5)	73 ± 3.0 (n = 4)
	T1/Female	60 (n = 1)	83 ± 9.0 (n = 3)	79 ± 5.0 (n = 3)	85 ± 7.0 (n = 3)	82 ± 4.0 (n = 3)
	T2/Female	No data	68 ± 9.0 (n = 3)	84 ± 3.0 (n = 3)	81 ± 5.0 (n = 3)	74 ± 1.0 (n = 3)
	T3/Female	90 ± 6.0 (n = 3)	66 ± 3.0 (n = 3)	82 ± 12.0 (n = 3)	78 (n = 1)	82 ± 8.0 (n = 3)

a)



b)



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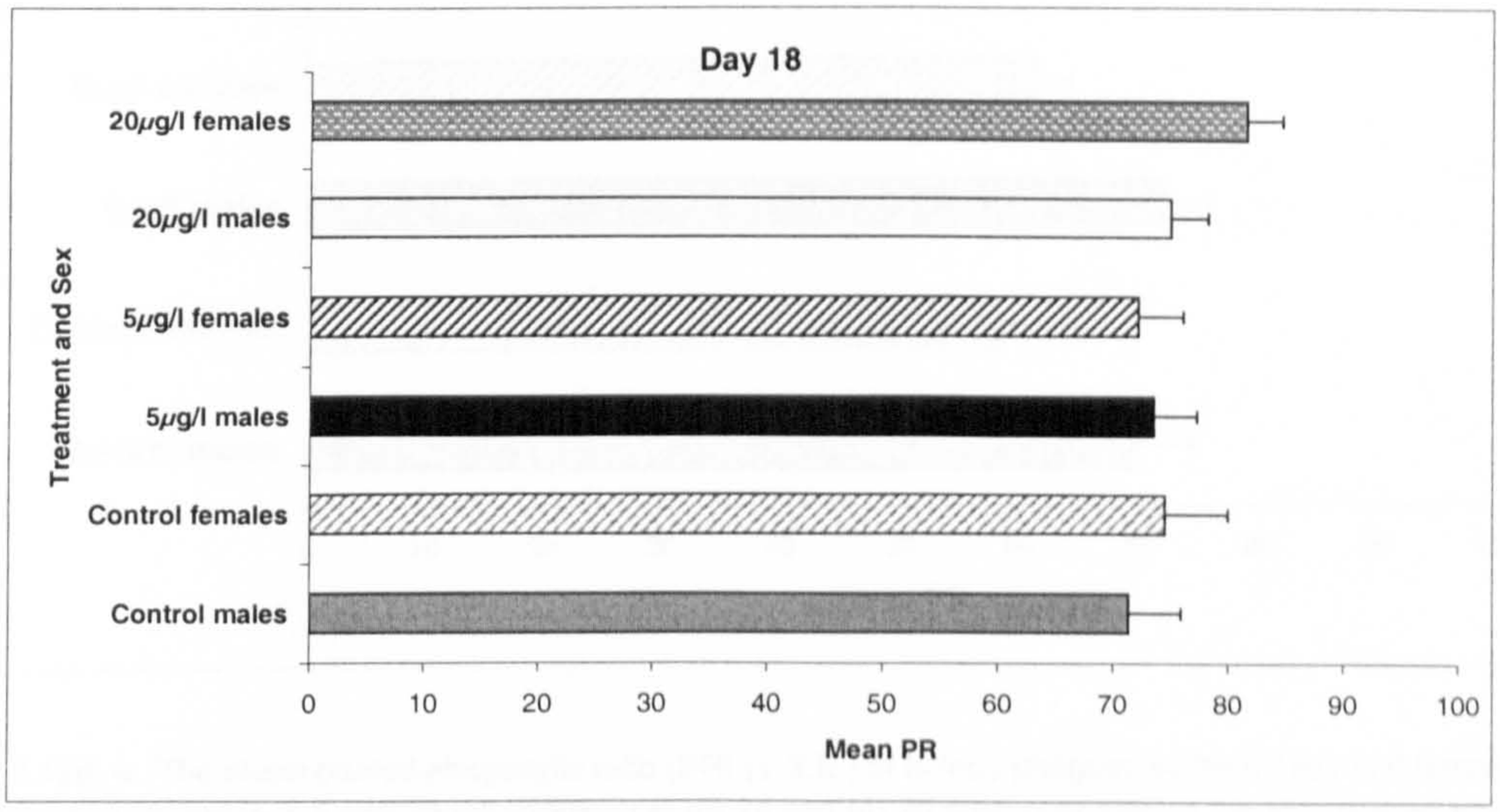
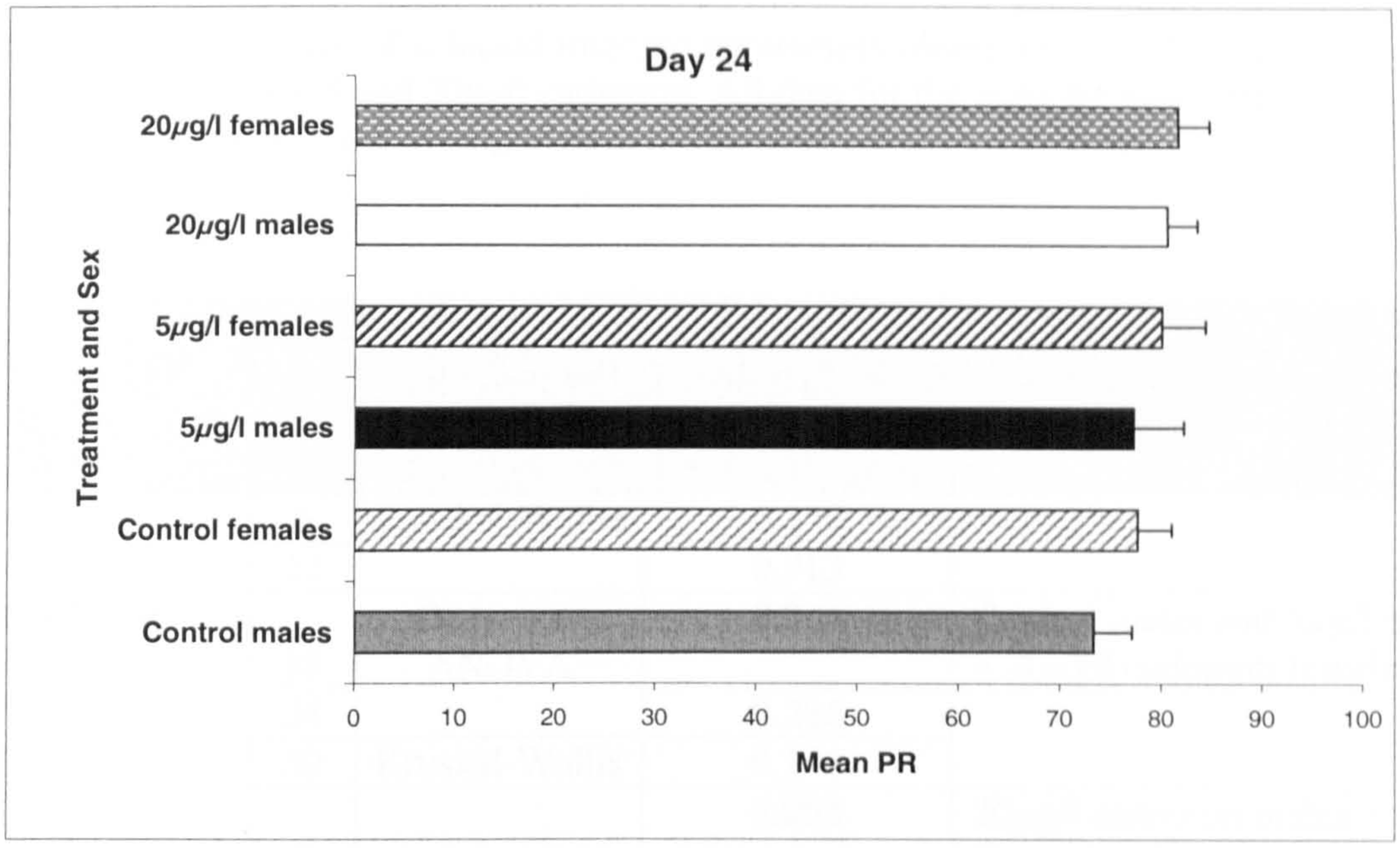


Fig. 7.12 a-c. The mean pooled phagocytic ratio (PR) (± S.E.) of kidney phagocytes from male and female guppies maintained in 0, 5 and 20μg/l cadmium at (a) 6, (b) 12 and (c)18 days post-start of the trial.

d)



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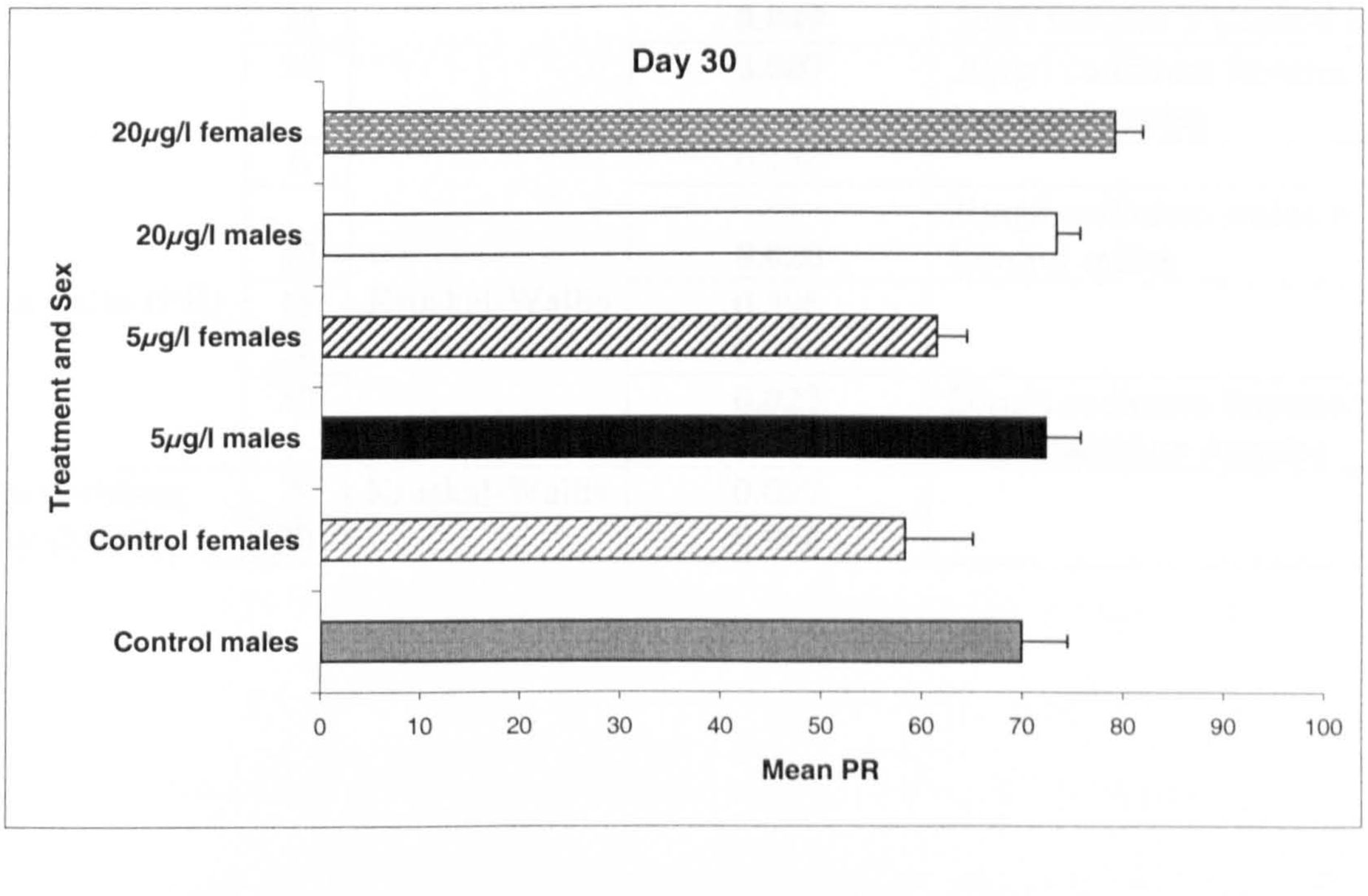


Fig. 7.12d, e. The mean pooled phagocytic ratio (PR) (± S.E.) of kidney phagocytes from male and female guppies maintained in 0, 5 and 20μg/l cadmium at (d) 24 and (e) 30 days post-start of the trial. Pooled values are calculated using data from all three tanks in each treatment.

Table 7.5. Statistical summary of selected immune parameters measured in male and female guppies exposed to 0, 5 and 20μg/l cadmium. All data for the separate sexes are pooled within treatments. Statistically significant differences are shown in bold.

Analysing different treatments and sexes				
Immunological parameter	**Day**	**Statistical test**	**Statistical significance P**	**Treatments between which differences lie**
Respiratory burst	6	Kruskal-Wallis	0.378	
	12		0.913	
	18	One - way ANOVA	**0.044**	Control males and 5μg/l males > 20μg/l cadmium females
	24		0.286	
	30	Kruskal-Wallis	0.141	
Phagocytic index (PI)	6	One - way ANOVA	**0.008**	20μg/l cadmium males > Control males
	12		0.133	
	18		**0.021**	20μg/l cadmium females > Control males and 5μg/l cadmium males
	24		**0.049**	5μg/l females > Control males.
	30		**0.007**	20μg/l cadmium females > Control females
Phagocytic ratio (PR)	6	Kruskal-Wallis	0.140	
	12		**0.020**	20μg/l cadmium males > Control males
	18		0.598	
	24		0.748	
	30		**0.023**	20μg/l cadmium females > 5μg/l cadmium females
Myeloperoxidase activity (MPO)	6	Kruskal-Wallis	0.097	
	30		0.968	

7.3.5. Myeloperoxidase production by neutrophils

The myeloperoxidase activity (MPO) (mean ± S.E.) of guppy neutrophils was determined at days 6 and 30 of the trial and the results are presented in Figures 7.13-7.15. There are no data presented for the individual replicates at day 6 as the sample size was small due to poor cell number. The day 6 data have, however, been pooled and are shown alongside the pooled day 30 data in Figure 7.14.

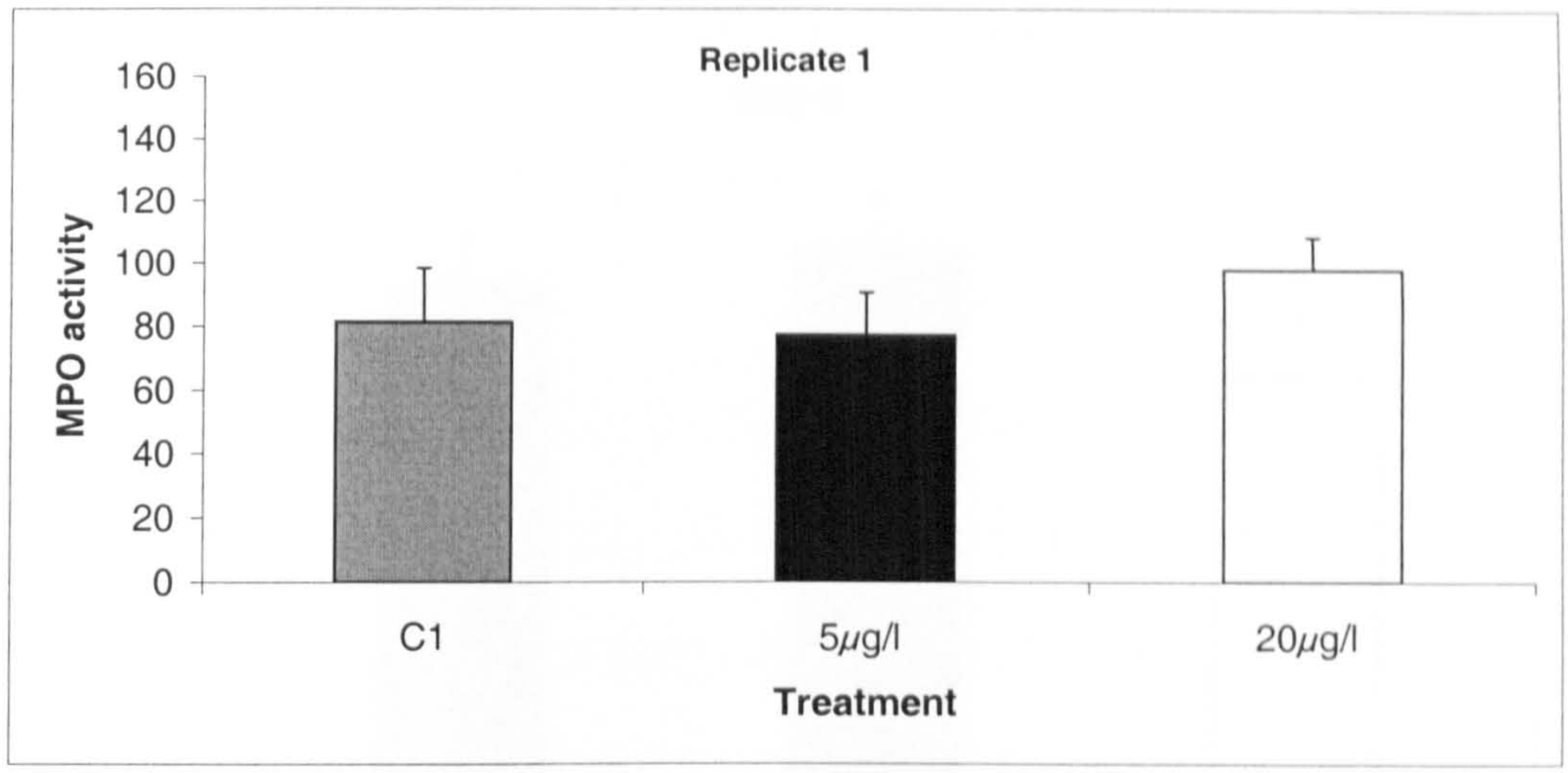
The individual replicates showed similarities in MPO activity at day 30, with control and 5μg/l cadmium-exposed guppies displaying similar levels of activity in all 3 replicates (Fig. 7.13a-c). The MPO of 20μg/l-exposed neutrophils was very slightly elevated in both replicates 1 and 3, compared to the other 2 treatments, and slightly depressed in replicate 2. The pooled data from days 6 and 30 can be seen in Figure 7.14 a, b and, again, the control MPO activity is at a similar level to that from the 5μg/l cadmium-exposed guppies at both time points. At day 6, the MPO activity of 20μg/l cadmium-exposed neutrophils was lower than in the other 2 treatments, while by day 30 this activity was very slightly elevated (Fig. 7.14 a, b).

7.3.5.1. Myeloperoxidase production between sexes

On dividing the guppies from each tank into males and females for analysis, the sample sizes became very small and thus the data for each sex were pooled between replicates and treatments and the data are presented in Figure 7.15 a, b. The standard errors of the MPO activity from each sex in each treatment are relatively high on both sample days. It should be noted that at day 6 the myeloperoxidase activity of male guppy neutrophils was higher than that of the females in all treatments. By day 30, however, the MPO of female guppies was greater in the control and 5μg/l

cadmium groups. The MPO of male and female guppies exposed to 20μg/l cadmium were approximately the same at day 30 (Fig. 7.15b).

a)



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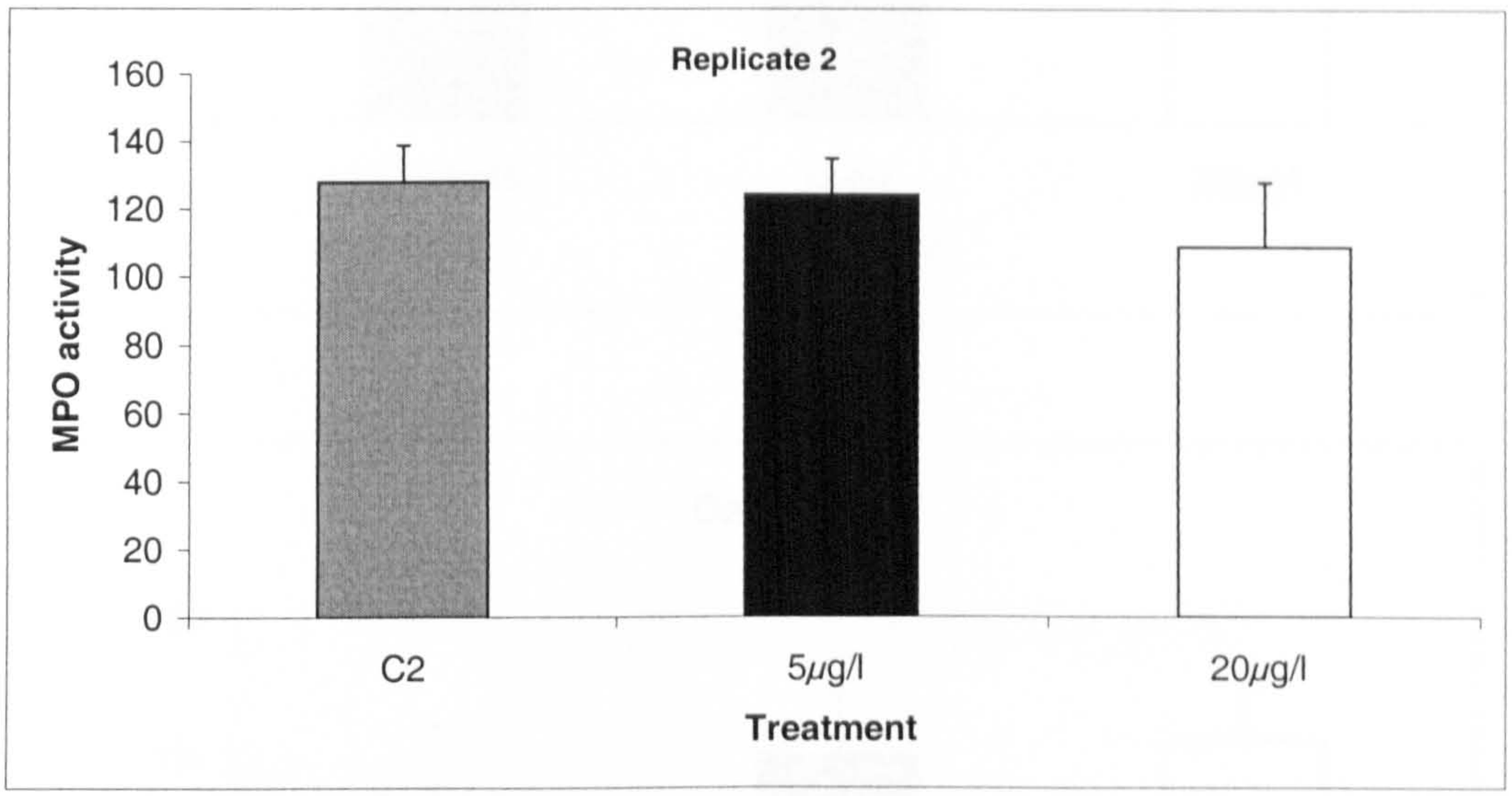

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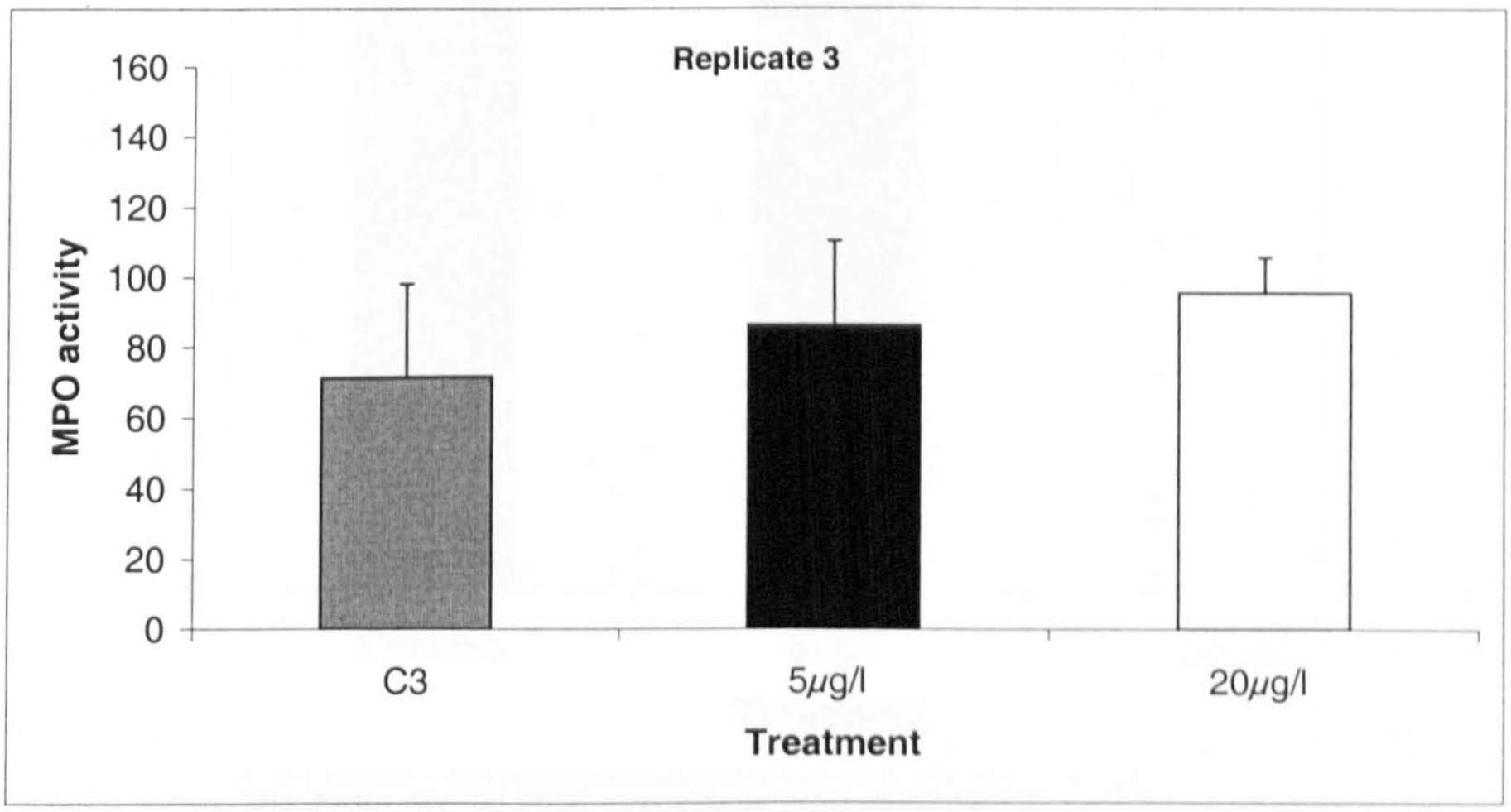
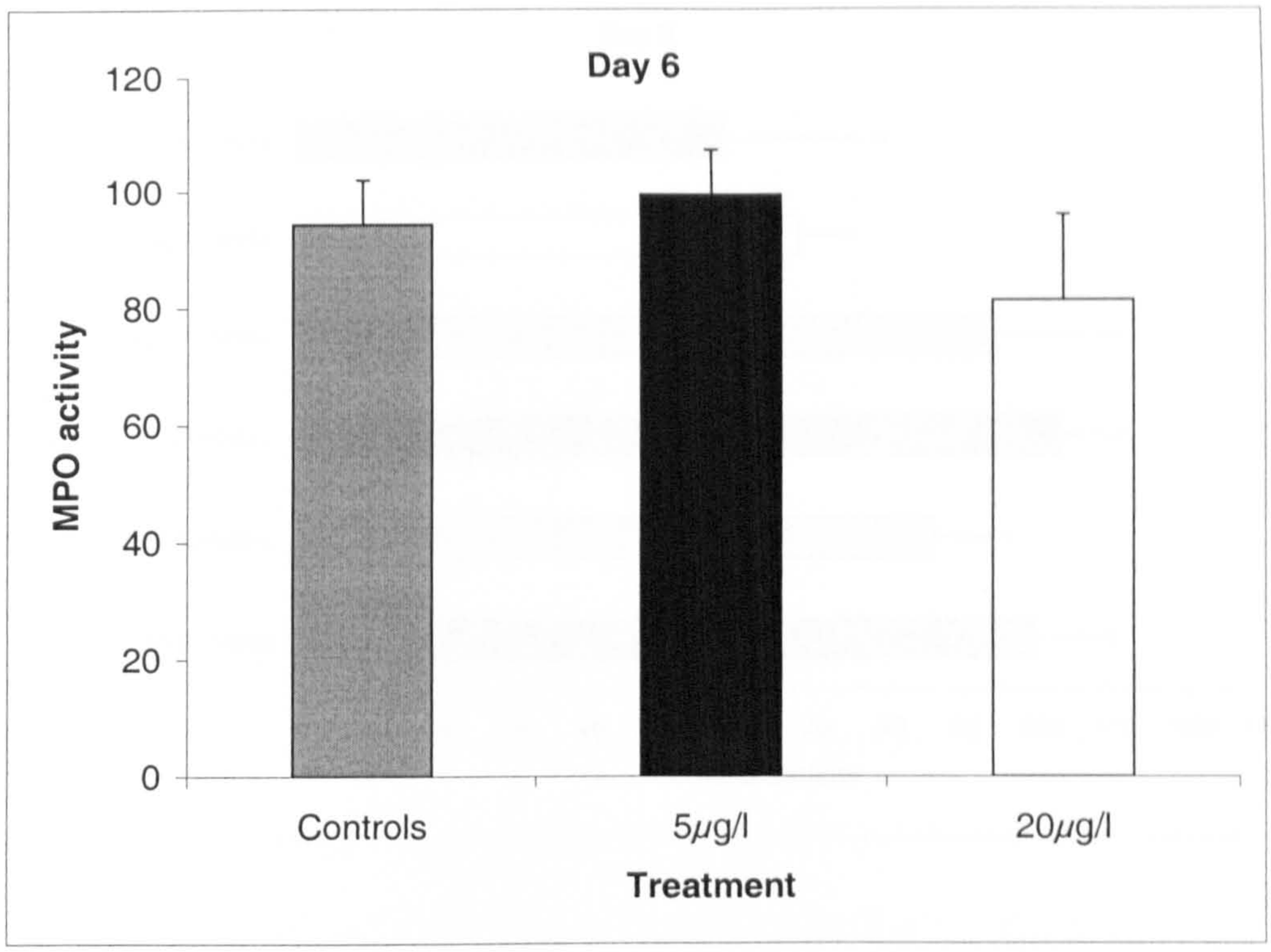


Fig. 7.13a-c. Day 30 - The mean myeloperoxidase activity (MPO) (± S.E.) of guppies maintained in control conditions and exposed to 5 and 20μg/l cadmium in (a) replicate 1, (b) replicate 2 and (c) replicate 3.

a)



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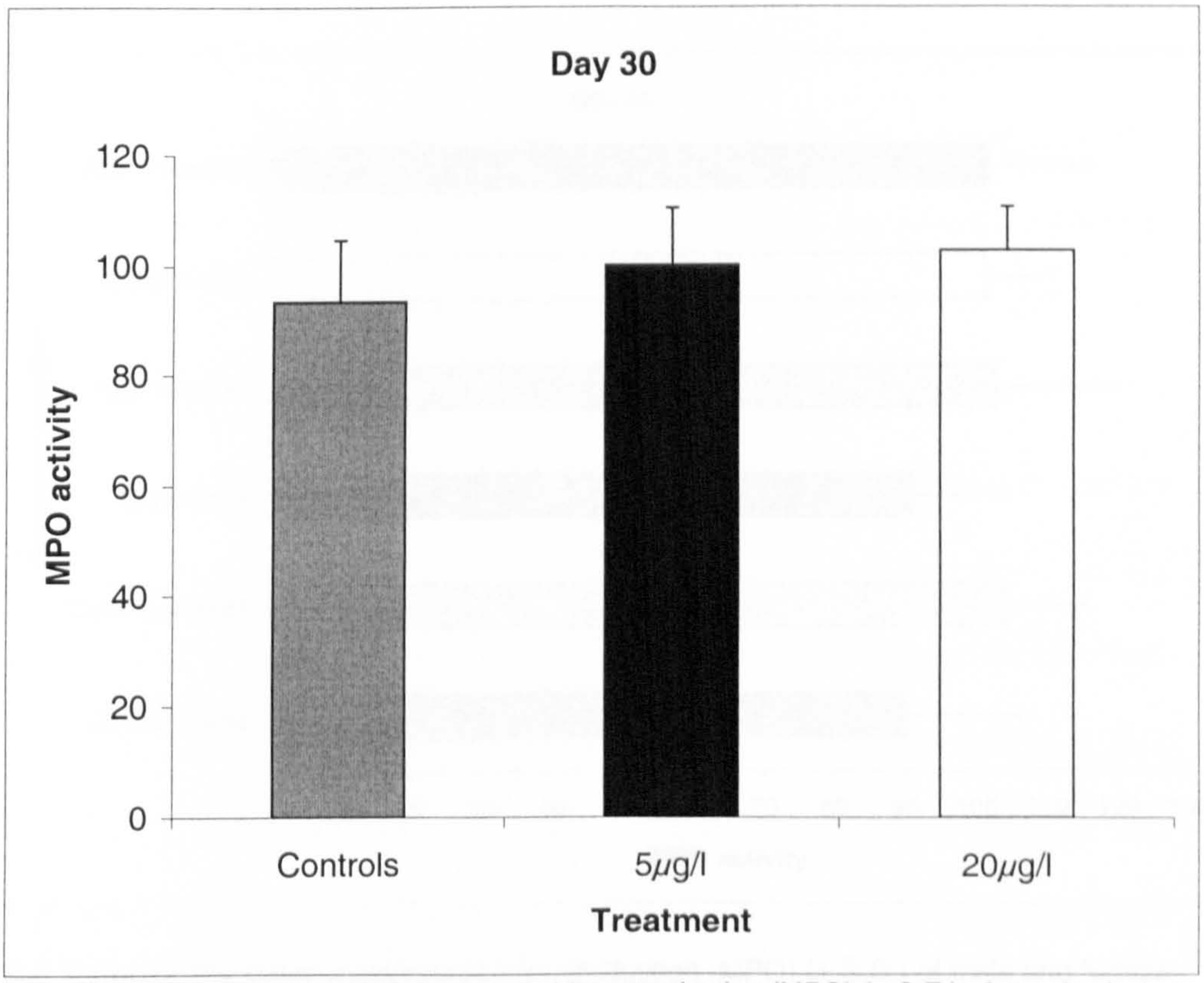
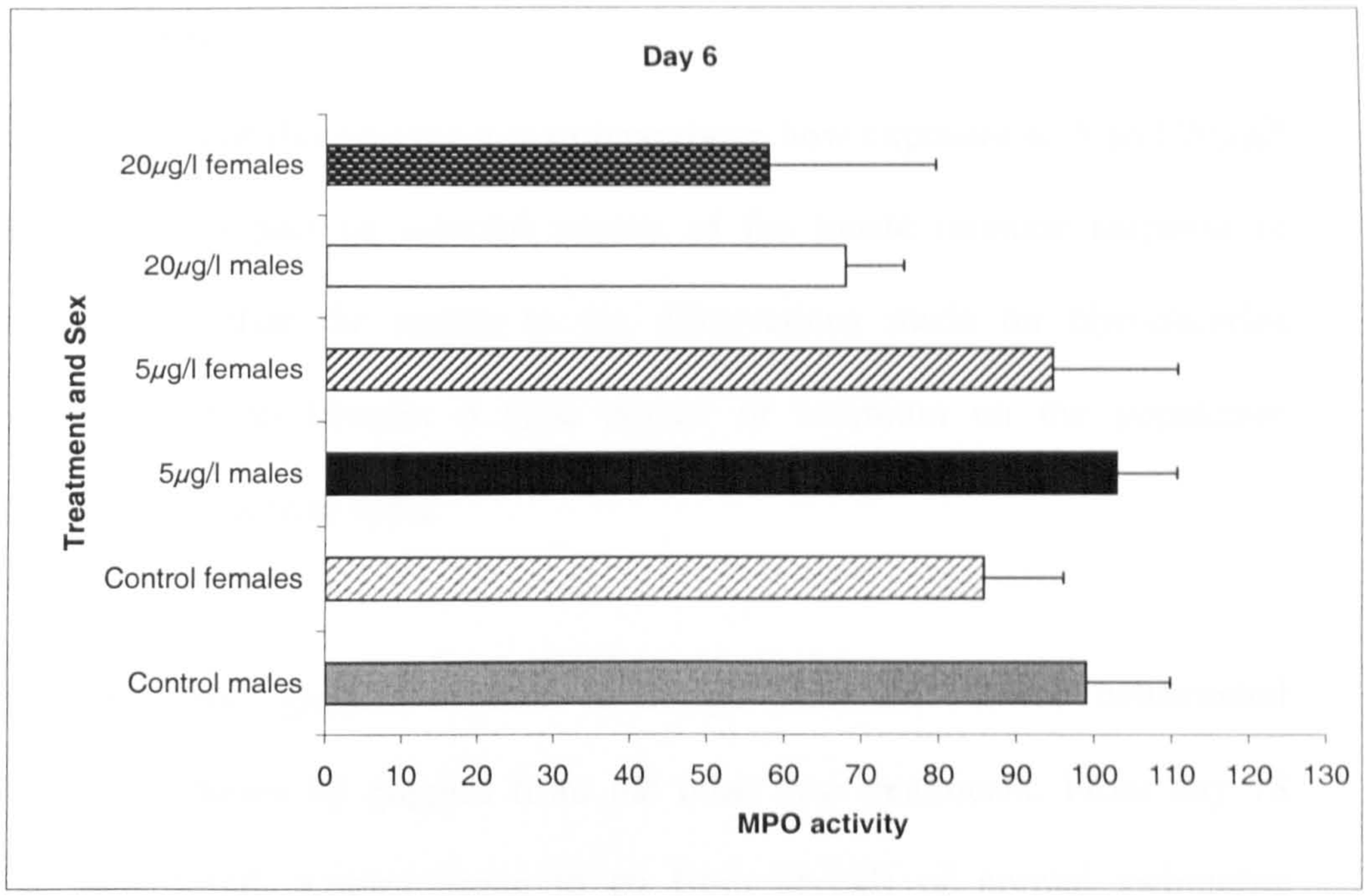


Fig. 7.14a,b. The mean pooled myeloperoxidase production (MPO) (± S.E.) of guppies in 0, 5 and 20μg/l cadmium at (a) day 6 and (b) day 30 of the trial.

a)



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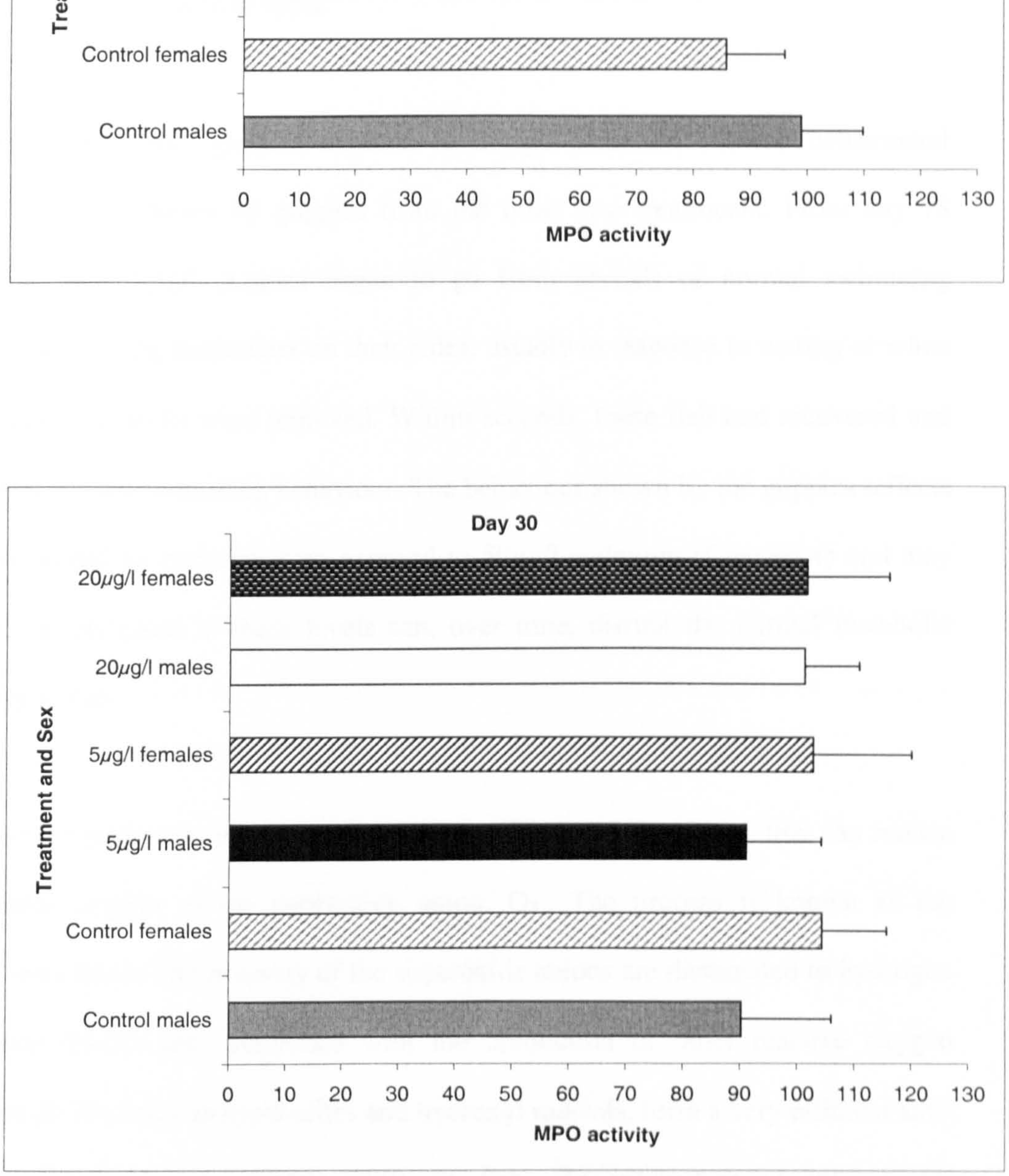

Fig. 7.15a, b. The mean myeloperoxidase production (MPO) (± S.E.) of male and female guppies from each treatment at (a) day 6 and (b) day 30 of the trial.

7.4. Discussion

The principal aim of this chapter was to investigate how exposure to 5 and 20μg/l cadmium would impact on selected aspects of the innate immune response in guppies and to relate the results to the observations made on *Gyrodactylus* population growth in Chapter 6 (The impact of cadmium on the population dynamics of *Gyrodactylus* spp.).

In the current trial, guppies exposed to 20μg/l cadmium showed behavioural differences not shown by guppies from the other two treatments. From day 18 onwards, the 20μg/l guppies began to go from periods of normal swimming behaviour to lying motionless on their sides, usually in response to netting or when the lids of the tanks were removed. Within seconds, these fish had recovered and resumed normal swimming behaviour. The behaviour shown by the guppies reflects that displayed by common carp exposed to 50μg/l cadmium (Chapter 4) and may show that cadmium at these levels can, over time, disrupt the normal metabolic activity of fish.

Phagocytes possess a unique membrane enzyme, NADPH oxidase, that can reduce molecular oxygen to the superoxide anion, $O_2^{\cdot}$. The process is known as the respiratory burst. The majority of the superoxide anions are dismutated to hydrogen peroxide (H_2O_2) and, combined with the production of other reactive oxygen species (ROS), such as hypohalites and hydroxyl radicals, form a very efficient anti-microbial system (Secombes, 1990). In fish, the production of intracellular superoxide anions can be measured by the reduction of the redox dye nitroblue tetrazolium (NBT). The spontaneous respiratory burst of phagocytes and the

stimulated respiratory burst, *i.e.* stimulated with a soluble stimulant such as phorbol myristate acetate (PMA), are measured.

In the current trial, the guppies exposed to 5μg/l cadmium showed little difference in their respiratory burst activity when compared to that of the control fish, implying that, at this concentration, cadmium neither significantly inhibits nor stimulates the killing ability of guppy phagocytes. Zelikoff *et al.* (1995) and Sanchez-Dardon *et al.* (1999) both recorded suppression of the respiratory burst in *Oncorhynchus mykiss* exposed to 2μg/l cadmium and to 1 and 5μg/l cadmium, respectively, an observation also recorded in carp exposed to 5μg/l cadmium in this study (although this was not significant, see Chapter 4 section 4.3.7). Enane *et al.* (1991) found, however, that 17 days exposure of *O. mykiss* to 2μg/l cadmium enhanced the production of the superoxide anion. Zelikoff *et al.* (1996, cited by Zelikoff, 1997) also recorded a significant enhancement of the respiratory burst in medaka *Oryzias latipes* (Temminck & Schlegel) exposed to 6 and 60μg/l cadmium. There are two possible explanations for the similarity in response of control and 5μg/l cadmium-exposed guppies in the current trial. As the 5μg/l level of cadmium used in the current trial was similar to the concentrations used by Zelikoff *et al.* (1995), Zelikoff (1996, cited by Zelikoff, 1997) and Sanchez-Dardon *et al.* (1999), it suggests that the fish species they used are more susceptible to cadmium than guppies. However, as the 20μg/l cadmium-exposed guppies showed an elevated respiratory burst, above that recorded for the controls, it can be seen that higher levels of cadmium can stimulate the respiratory burst response in the guppies in common with the findings of Zelikoff *et al.* (1996, cited by Zelikoff, 1997) and Enane *et al.* (1991). The enhanced respiratory burst of the 20μg/l cadmium-exposed

guppies could be a function of their metallothionien concentration. Youn *et al.* (1995) found that metallothionein levels of greater than 20μM enhanced the respiratory burst of murine macrophages, and it is possible that more metallothionein was produced by the guppies in 20μg/l cadmium and that this was enough to enhance the respiratory burst above both the other treatments. The 5μg/l cadmium concentration used in the current trial may not have been great enough to cause any significant changes to the respiratory burst system directly or via the production of metallothionien proteins.

The values obtained for the respiratory burst of guppy phagocytes from all treatments in this current trial were high in comparison with the values recorded from other warm water fish species. For instance, the respiratory burst of *Oreochromis mossambicus* (Peters) and *O. aureus* (Steindachner) have been recorded as 0.13 ± 0.03 and 0.15 ± 0.10 OD 540nm, respectively (Palti, Tinman, Cnaani, Avidar, Ron & Hulata, 1999) compared to 0.89 ± 0.18 to 2.59 ± 0.97 OD 610nm recorded from control guppies in the current trial. Due to the small size of the guppy kidney, the entire organ had to be used to obtain phagocytes for the respiratory burst assay and the resulting cell suspension was known to contain neutrophils, as determined from the myeloperoxidase production assay. Davis & Gallin (1981) described the various functions of neutrophils, including their involvement in microbial killing via the respiratory burst. Thus, it could be expected that, with two cell types (macrophages and neutrophils) capable of producing superoxide, the recorded respiratory burst values for the guppies might be higher than that from fish where macrophages alone have been isolated from the head kidney and used in the assay. Indeed, the highest respiratory burst value recorded

from control guppies was only slightly higher than that recorded in the control carp (2.59 ± 0.97 and 1.57 ± 0.4 OD 610nm, respectively) in Chapter 4, where the entire kidney was also used to obtain the cell suspension. However, in future experiments it may be advantageous to purify the cell population by using a Percoll gradient to ensure the presence of solely macrophages. Purifying the cell population was not carried out in the guppy immunology trial due to the greater amount of time required to carry out this procedure and the fact that the sampling of guppies occurred on four days out of every seven. Bols *et al.* (2001) also indicated that comparisons of the respiratory burst are difficult due to the contradictory results obtained in different experimental set ups. The major sources of variability that were suggested were exposure periods, the type of assay used, the phenotype of the cells used and the stimulatory agent used in the assay. They considered variability between fish species to be an unlikely cause due to the well-conserved molecular machinery responsible for the respiratory burst over evolutionary time.

Another feature of note from the respiratory burst studies in the current trial is that there was little difference between the activity of the phagocytes stimulated with phorbol myristate acetate (PMA) and those left unstimulated, a phenomenon also observed in the carp in Chapter 4. The phagocytes stimulated with PMA should have resulted in a greater respiratory burst than that generated spontaneously. In the absence of PMA, phagocytes undergo spontaneous respiratory burst but the reaction is not as quick as on addition of PMA (Secombes, 1990). The incubation period of the phagocytes in the presence of NBT and in the presence of NBT and PMA was one hour in the current trial. Secombes (1990) demonstrated that in *O. mykiss* the optimum time for the incubation of macrophages, giving the greatest difference

between PMA stimulated and unstimulated phagocytes, is 20 minutes and that, after one hour, there is little difference between both sets of cells. Thus, in future experiments, it would be of use to assess the superoxide anion production over time to find the optimum incubation period for guppy phagocytes where the difference in respiratory burst of stimulated and unstimulated cells is greatest.

The effect of cadmium on kidney phagocytes, rather than phagocytes from sources such as the peritoneal cavity, was determined because of the small size of the guppies and the kidney being the largest phagocyte containing organ.

The phagocytic ratio (PR) and the phagocytic index (PI) of the kidney phagocytes in guppies in the current trial were found to increase with increasing cadmium concentration, a pattern that was consistent over the duration of the 30-day trial. However, the differences were, in general, not statistically significant. Zelikoff *et al.* (1995) recorded a similar enhancement in phagocytosis above the control values after 8 days exposure to 2μg/l cadmium; the difference between the two treatments was statistically significant at 79% and 40%, respectively. Further samples were taken at 17 and 30 days and, although the phagocytic activity in the cadmium exposed fish fell from the day 8 values, the activity remained greater than in the controls (Zelikoff *et al.*, 1995). These authors attributed the initial significant enhancement of phagocytosis in the cadmium-exposed fish to augmentation caused by exposure to low cadmium levels. The later fall in activity was attributed to increasing cadmium burdens, caused by longer exposure and thus increased levels of intracellular cadmium, which may have exerted an adverse effect on phagocytic function. No suggestions as to how increased phagocytic activity may affect the

overall health of *O. mykiss* exposed to low concentrations of cadmium were, however, proffered by these authors. Enane *et al.* (1991) and Sövenyi & Szakolczai (1993) also recorded stimulation of *in vivo* phagocytosis in cadmium-exposed fish. The latter authors attributed the enhanced phagocytosis to increasing numbers of circulating leucocytes in response to stress.

At around the same time as the decrease in phagocytosis in cadmium-exposed fish was observed by Zelikoff *et al.* (1995), phagocytosis in guppies exposed to 5μg/l cadmium for 18 days in the current trial also fell to a level similar to the controls. It is, however, unlikely that the phagocytic suppression seen at day 18 in the current trial was due to an intracellular cadmium build up, as it would be expected that phagocytosis in the guppies exposed to 20μg/l cadmium would be suppressed to an even greater extent. While the phagocytosis of the 20μg/l guppies did fall at day 18, it still remained higher than that of both the control and the 5μg/l guppies. In order to explain the decrease in phagocyte activity (also observed in the respiratory burst) recorded at day 18 in the guppies in the current trial, it is necessary to consult Figures 7.1a-c, 7.8a-c and 7.9a-c in section 7.3. From these figures, it is possible to see that the respiratory burst and the PI and PR of the guppies in replicate 1 showed the most dramatic reduction at day 18 compared to the other two replicates. During the sampling of replicate 1 tanks, unforeseen problems resulted in these guppies being kept on ice for longer than normal and it is possible that this detrimentally affected the phagocytes either by impairing their ability to phagocytose yeast (as seen in the reduced PI) and/or by reducing the proportion of phagocytes capable of engulfing yeast (as seen in the reduced PR) and thus subsequently affecting the respiratory burst.

The reasons for the phagocytosis of cadmium-treated phagocytes in the guppies being greater than the controls are not clear, but several hypotheses can be suggested. The first hypothesis is centred on the high levels of cadmium that build up in the kidney of fish on exposure to aqueous cadmium (Giles, 1988; Suresh *et al.*, 1993; Brown *et al.*, 1986, 1994; Kraal *et al.* 1995; De Conto *et al.*, 1997,1999; and De Smet & Blust, 2001). The high cadmium levels in the kidney, as well as causing some renal damage, may also impact on the adjacent lymphoid tissue, as suggested by Hutchinson & Manning (1995), thus affecting the production and functioning of kidney phagocytes. Weir (1981) noted that the cell surface of phagocytes contains glyco-proteins. Flick *et al.* (1971) note the affinity of cadmium for binding to proteins, and it is possible that Cd^{2+} ions bind to the cell surface glyco-proteins and stimulate the cell membranes, inducing and increasing phagocytosis. Alternatively, Rosenstreich (1981) reported that activated mammalian macrophages are those with specific effector functions, such as the ability to kill intracellular organisms. Phagocytes capable of engulfing yeast and undergoing respiratory burst can be considered to be activated. As the activation of macrophages is induced most effectively by signals generated from activated lymphocytes (Weir, 1981), it is possible that cadmium affected the lymphocytes of the guppies, resulting in the production/activation of a greater number of these cells and thus greater stimulation of the phagocytes. Considering the work of Sanchez-Dardon *et al.* (1999), where 5μg/l cadmium caused stimulation in the proliferative response of the lymphocytes, this hypothesis could in part explain the enhancement of phagocytosis. The stimulation of the lymphocytes in the cadmium-exposed fish could have been the function of the production of methallothionein and the formation of the metallothionein-cadmium complex (Cd-MT) that Lynes, Garvey &

Lawrence (1990) found could induce splenic lymphocyte proliferation. The Cd-MT theory could also explain the higher macrophage activity of the 20μg/l cadmium-exposed guppies, compared to the 5μg/l cadmium-exposed guppies, due to the production of more metallothionein proteins and thus more lymphocytes. However, there are also several reports of decreased numbers of lymphocytes and decreased lymphocyte viability caused by cadmium that counter this argument (Ghazaly, 1991; Witeska, 1998; Kotsanis *et al.*, 2000). Bols *et al.* (2001) reported how phagocytosis can be regulated by C-reactive protein (CRP) (an acute phase protein), whose levels in fish change in response to tissue damage, inflammation and infection. Paul *et al.* (1998) reported a 3-4-fold increase in the level of CRP in the major carp, *Catla catla* (Hamilton), on exposure to cadmium, and a similar increase in CRP in the cadmium-exposed guppies in the current trial could explain their enhanced phagocytic activity. The work of Paul *et al.* (1998) is discussed in more detail in Chapter 4, section 4.4. A final hypothesis of how cadmium enhances phagocytosis in guppies involves the ability of toxicants, and therefore possibly cadmium, to elicit an inflammatory response, in host tissues (Bols *et al.*, 2001). During the inflammatory response, an increase in blood flow and capillary permeability results in an infiltration of macrophages and granulocytes to the affected area (Secombes, 1996). While this tends to occur only at very high toxicant concentrations, it is still a possible cause of the increased proportion of activated phagocytes in the cadmium-exposed guppies. Of these hypotheses, the activation of phagocytes by CRP and lymphocytes should be elucidated further.

Myeloperoxidase (MPO) uses H_20_2 to oxidize many organic compounds and can oxidize chloride ions to hypochlorous acid (HOCl), which has strong antibacterial

properties (Praveen *et al.*, 2000). No studies investigating the effect of heavy metals on the production of MPO in fish could be found, but Siwicki *et al.* (1998) suggested the use of myeloperoxidase production in immunotoxicological studies and have stated that "normal" fish from unpolluted areas should have a total MPO activity of between 95-110 points, while in polluted aquatic environments the value may be reduced to 50-70 points. In the current trial, the MPO activity in the cadmium-exposed guppies was higher than the values suggested by Siwicki *et al.* (1998) for fish from polluted areas, and this could be due to a variety of factors including species specific differences and differences in the biotic and abiotic conditions to which the fish were exposed. However, as the MPO levels in all three treatments were very similar, it suggests that the levels of cadmium used in the current trial were possibly below the threshold level that would trigger a substantial response from this myeloperoxidase enzyme system or that simply MPO in guppies is unaffected by cadmium exposure.

The results from the effect of cadmium exposure on some aspects of the carp innate immune system showed that, after initial subtle alterations to several immune parameters, the carp exposed to 5μg/l cadmium, and to a lesser extent those exposed to 50μg/l cadmium, showed similar values to the controls by the end of the trial. These findings implied that carp are capable of some form of adaptation with regard to low-level cadmium exposure. The only parameter that appeared to be affected by exposure to 5μg/l cadmium in the guppies was phagocytosis, which showed little adaptation over time, remaining consistently greater than the controls. It is possible that, as in carp, guppies do show adaptation to cadmium but that due to species-specific differences, size differences, varying states of sexual maturity and possible

differences in the capabilities of the two species to detoxify cadmium, this adaptation occurs beyond 30 days exposure and was thus not elucidated in this trial. It is also possible that the respiration rates of the two fish species would have differed due to the different temperatures at which they were maintained. Thus, guppies, maintained at 5°C higher than the carp, may have had a greater rate of respiration and thus a greater rate of cadmium uptake from the water than the carp, possibly delaying the time taken to adapt to the metal exposure.

To attempt to elucidate the interactions between the population growth of *Gyrodactylus* spp. and these selected aspects of the guppy innate immune response, two summary tables (Tables 7.6 and 7.7) have been provided at the end of this discussion. Table 7.6 summarises the effect of 5μg/l cadmium on gyrodactylid population growth and on the immune responses of the guppies, and Table 7.7 summarises the sex-related differences observed in parasite burdens and host immune responses. The possible interactions between 5μg/l cadmium, host responses and gyrodactylid population growth will be discussed with regard to the results obtained in both Chapter 6 and the present chapter (Chapter 7). The results of the population growth of G. *turnbulli* exposed to 5μg/l cadmium only will be discussed in relation to the immunology of the guppy, as the sample times for both experiments were chosen to coincide with one another.

Buchmann & Bresciani (1999) found that peritoneal macrophages are capable of binding *in vitro* to the cephalic ducts, the haptor and, after prolonged exposure, to the body of *G. derjavini*. While the effect of the macrophages on *G. derjavini* has not been determined *in vivo*, the authors have suggested that the enzyme rich

products of macrophages found in the host mucus may be able to adversely affect gyrodactylid performance. Following the attachment of gyrodactylids to the host epithelium, there is a localised inflammatory response in the host epidermis that involves infiltration of the tissues by neutrophils and macrophages. Both macrophages and neutrophils can secrete the cytokine interleukin-1 (IL-1) that stimulates mucus secretion via release of the goblet cell contents (Cohan *et al.*, 1991). However, unlike in the *Dactylogyrus extensus-Cyprinus carpio* system, where the rate of oviposition showed some correlation to the phagocytic activity of the kidney phagocytes, there appear to be fewer consistent correlations between the population growth of *G. turnbulli* and the phagocytic activity of guppies. It is possible that, being more mobile than dactylogyrids, gyrodactylids are able to change their position on the host and move away from the localised tissue responses that are mounted by the host and avoid prolonged interaction with phagocytic cells and their products. This idea has been suggested by Richards & Chubb (1998) as an explanation for low-level gyrodactylid infections persisting on adult guppies. The majority of *G. turnbulli* in the current trial were consistently found on the caudal fin of the guppies. Buchmann & Bresciani (1998) reported that macrophages are known to be less abundant on the fins. Thus, by colonising the tail, the parasites may have avoided strong macrophage activity, resulting in there being less association between these cells and the population growth of these parasites, compared to *D. extensus*.

The lack of statistical difference between the respiratory burst activity of the control and the 5μg/l cadmium-exposed guppies suggests that this parameter also has little role in the observed differences in the population sizes of control and 5μg/l

cadmium-exposed *G. turnbulli*. The production of reactive oxygen intermediates (ROS) via the respiratory burst has been shown to have strong larvicidal properties *in vitro* against the digenean *Diplostomum spathaceum* and the cestode *Diphyllobothrium dendriticum* (see Whyte *et al.*, 1989; Sharp *et al.*, 1991). Indeed, a slight but significant elevation in the respiratory burst has been recorded by Buchmann & Bresciani (1999) when pronephric leucocytes were incubated with extract of *G. derjavini*. However, it is not known how Buchmann's findings might apply *in vivo*. Given that the respiratory burst response of the 20μg/l cadmium-exposed guppies was consistently elevated above the controls, future experiments could assess the impact of this cadmium concentration on the population dynamics of gyrodactylids to determine whether this can be related to the elevated respiratory burst activity seen in these guppies at this same cadmium concentration.

It cannot be determined how the production of myeloperoxidase by guppy neutrophils may have contributed to the observed differences in parasite population size, as there was very little difference in MPO activity between any treatment. However, neutrophils accumulate at the site of tissue injury and it is possible that the release of myeloperoxidase, and subsequent production and release of hypochlorous acid, into the epithelium or mucus may adversely the affect the survival of gyrodactylids.

The immune parameters determined in this current trial were chosen based on their extensive use in assessing the toxic effects of cadmium and other pollutants on fish and on the literature demonstrating the *in vitro* effects of kidney phagocytes on gyrodactylids. From the data presented in this chapter, it would seem that the

aspects of the innate defence system investigated here have little effect *in vivo* on the control of gyrodactylid populations. Given the small size of the guppies, and thus the small amounts of tissue and resulting cell suspension available for various assays, it was not possible to investigate a full suite of immunological parameters. To explain the reasons for the differences in the size of the control and 5µg/l cadmium-exposed *G. turnbulli* populations in terms of host responses, other aspects of the immune system must thus be considered.

The complement system is an integral part of the fish immune system and can be activated via the classical or alternative pathway. Buchmann & Bresciani (1999) and Harris, Soleng & Bakke (1998) have shown *in vitro* how complement factor C3, activated via the alternative complement pathway, can kill *G. derjavini* and *G. salaris* Malmberg, 1957, respectively. Complement is found in the mucus and serum of fish (Yano, 1996) and can be produced by macrophages, although its main source is the liver. The *in vivo* effects of complement on gyrodactylids are unknown, but its presence in mucus and its attachment to the carbohydrate epitopes of the parasite was the probable cause of death of *G. derjavini* incubated *in vitro* in mucus scrapings from *O. mykiss* (Buchmann, 1999). Cadmium exposure is, however, known to inhibit complement production in humans (Galebskaya, Ryumina, Bel'tyulov, Sgchebak, Vlasova & Bogomaz, 1991) and to significantly reduce complement factor C3 in rats (Mochizuki, Kikuchi & Ueki, 1988). The fish complement system is known to be very similar to the mammalian system, and thus, it is possible that complement production in the guppies was inhibited to some degree by exposure to cadmium and that this reduction allowed the parasites exposed to cadmium to proliferate compared to the controls. To determine whether

complement levels were responsible for the observed differences in population growth of *G. turnbulli* between the two treatments, future experiments should measure complement levels in the mucus and serum of control and cadmium-exposed guppies.

Bols *et al.* (2001), reviewing the effects of heavy metals on fish, noted how cadmium exposure has been recorded as causing excessive mucus production in the epidermis. As mucus contains a variety of possible detrimental substances to gyrodactylids (*e.g.* complement), it would be expected that *G. turnbulli* on guppies exposed to 5μg/l cadmium would be more adversely affected than those on control fish. However, as stated previously, *G. turnbulli* from both populations were found in greater numbers on the caudal fin of the guppy, where mucus cells are less abundant. Furthermore, the colonisation of the caudal fin was more rapid in the cadmium-exposed *G. turnbulli*, with 30% more parasites in this area at day 12 compared to the numbers recorded on the control fish. It is expected that all the fins offer less harsh environments to gyrodactylids than the skin of the body, but that those that can be brought into contact with the body surface and mucus are slightly less conducive to parasite survival. The earlier colonisation of the caudal fin by gyrodactylids on the 5μg/l cadmium-exposed guppies may have been in response to the greater mucus production by these fish and may have essentially given them a "head start" in reproduction, which, coupled with some of the other factors already considered, may have maintained their numbers above those of the control population.

Albergoni & Viola (1995) recorded an initial suppression in the amount of circulating immunoglobulin (IgM) in the catfish *Ictalurus melas* after a 1 week exposure to 10, 20 and 30μg/l cadmium. The levels of IgM in *I. melas* did, however, increase after 2 weeks of exposure and were no longer statistically different from the controls (Albergoni & Viola, 1995). The initial impairment in IgM production was attributed to cadmium causing conformational modifications in the immunoglobulins, through binding to their disulphide groups, and interfering with the antigen-antibody bond. Harris *et al.* (1998) have shown that host antibodies can attach to *G. salaris in vitro* and Buchmann (1997) and Scott & Robinson (1984) have clearly demonstrated that there is an acquired immune response in *O. mykiss* to infection with *G. derjavini* and in guppies infected with *G. turnbulli*, respectively. Both fish species showed that on reinfection the fish were able to reduce the infection intensity compared to the intensity recorded during their initial infections. It is thus possible that exposure to 5μg/l cadmium caused a suppression of IgM, resulting in the proliferation of the cadmium-exposed parasite population. At 5μg/l cadmium, this suppression may have taken longer than the 1 week observed for *I. melas* (exposed to higher levels of cadmium), but would have allowed the 5μg/l parasites to increase in number quicker than the controls whose antibody response would have essentially been unaffected. This explanation could account for the differences seen in the parasite populations at the end of the trial (days 48-60), where control populations of *G. turnbulli* were seen to decrease and the cadmium-exposed *G. turnbulli* showed little sign of reduction in population size by comparison (see Fig. 6.8). Furthermore, Yano (1996) stated that antibodies activate the complement system of fish. Thus a reduction in antibody production by the

cadmium-exposed guppies would potentially have resulted in the production of less complement, further enhancing *G. turnbulli* population growth on these fish.

It has been documented by Schreck (1996) and Stoltze & Buchmann (2001) that cortisol can act as an immunosuppressant, with stress depressing the antibody-synthesising capability via its effects on lymphocytes (Schreck, 1996). Cadmium exposure and parasitism by *G. derjavini* have both been linked to increases in cortisol production (Pratap & Wendelaar Bonga, 1990; Tort *et al.*, 1996; Stoltze & Buchmann, 2001). The suppression of the antibody-synthesising system by cortisol could also explain some of the observed differences in *G. turnbulli* population growth on control and 5μg/l cadmium-exposed guppies. Interestingly, Pratap & Wendelaar Bonga (1990), on exposing *Oreochromis mossambicus* to 10μg/l cadmium, found that control and cadmium-exposed cortisol levels deviated statistically at days 2, 6 and 14 before falling to control values by day 35. Considering this, it is possible that the cortisol level of the cadmium-exposed guppies in the current trial could have been raised above the controls resulting in antibody suppression. In contrast to the findings of Pratap & Wendelaar Bonga (1990), the results from Chapter 4 of the current study showed that cortisol levels of the control and 5μg/l cadmium-exposed carp deviated little from one another. Extrapolating cortisol responses from one fish species to another is difficult, as the response is dependent not only on fish species but on various factors, including the experimental conditions, handling procedures and the health status of the fish. Thus to determine the impact of cortisol on the guppy immune response, future experiments should use a similar method to that of Stolze & Buchmann (2001) to analyse cortisol levels. Due to the small size of the *Oncorhynchus mykiss* fry and

the difficulty of extracting enough plasma for the assay, they sonicated and centrifuged the whole fry and determined the cortisol concentration in the whole body fluid. Such a method could also be applied to the analysis of cortisol and serum complement levels in guppies in future experiments.

Despite the obvious possibility that host immune responses control gyrodactylid infections, the possibility that cadmium affects the parasites directly cannot be ignored. Cadmium exposure may directly enhance reproduction by gyrodactylids. However, due to the parasitic nature of gyrodactylids, it is not possible to separate the direct impact of cadmium on the parasites from the indirect impact via the host responses. It can thus be suggested that gyrodactylid population increases on cadmium-exposed guppies are a function of some host responses, together with direct cadmium exposure.

During the experiment looking at the population growth of *G. turnbulli* on control and 5μg/l cadmium-exposed guppies, it became apparent that there was a difference in the parasite infections on male and female guppies (Chapter 6). Thus, a secondary aim of this trial was to see if differences in the immune responses of control and 5μg/l cadmium-exposed male and female guppies could be detected. The sex-related results from the G. *turnbulli* trial (Chapter 6) and the immunology trial are summarised in Table 7.7 at the end of the discussion.

A feature worthy of note is that only 22.2% of the female guppies exposed to 20μg/l cadmium became pregnant during the trial, compared to 68.8% of the control and 68.8% of the 5μg/l cadmium-exposed guppies. Oestradiol is essential for the

regulation of vitellogenic growth of developing oöcytes in fish (Fournier *et al.*, 1998). Le Guevel, Petit, Le Goff, Metivier, Valotaire & Pakdel (2000) stated that cadmium-mediated inhibition of vitellogenesis in *O. mykiss* has been attributed to cadmium acting as an endocrine disrupter and affecting a number of oestrogen signalling pathways. A similar disruption in the 20μg/l cadmium-exposed guppies could account for the reduced number of pregnant fish.

All of the immune parameters investigated in the current trial showed small sex-related differences. However, in light of the apparent lack of correlation between these parameters and the population growth of *G. turnbulli,* as detailed above, it is hard to relate these parameters to the differences in parasite burden that were observed on the male and female guppies in Chapter 6. As stated in the previous chapter (Chapter 6), the increased number of *G. turnbulli* on the female guppies from day 6 to day 24/30 could have been a function of more female guppies being infected initially, compared to the males, allowing the population growth of gyrodactylids to be accelerated above the males during this time. The subsequent acceleration of gyrodactylid infection on male guppies from around day 30 onwards could be the result of competition for female guppies and androgen production. Before both the *G. turnbulli* population dynamics trial and the immunology trial, the male and female guppies had been maintained in separate tanks. During this holding period, the levels of testosterone in the male guppies in both the control and the 5μg/l cadmium treatment could have been considered to have been at "normal" levels. However, on mixing the sexes, the males would have started competing with each other for females to mate with (discussed in Chapter 6), and this could have resulted in a gradual rise in testosterone and cortisol levels and subsequent

suppression of the antibody response. The immunosuppressive effects of testosterone and cortisol could have built up slowly over time, reaching significant levels at day 24 (5μg/l cadmium-exposed guppies) and day 36 (controls), resulting in *G. turnbulli* numbers exceeding those on the females. Interestingly, Kime (1984) demonstrated that *O. mykiss* exposed to 0.05 and 0.5mM cadmium showed significantly elevated testosterone production above the controls and this could account for the parasite burdens on the cadmium-exposed guppies in the current trial peaking earlier and remaining higher than those on the control males. Buchmann (1997) found that testosterone injections caused an increase in *G. derjavini* numbers on *O. mykiss*, but could not elucidate whether it was the immunosuppressive effect of the sex hormone on the host, or the direct effect of the hormone on the parasites, that was causing the population increase. Determining the mechanisms by which differences in the immune responses of male and female guppies affect *G. turnbulli* population growth requires further elucidation. It would be interesting to investigate the possible relationship between the immune response and the reproductive system of guppies, as well as incorporating assays to look at aspects of the specific immune response such as antibody production and lymphocyte activity.

In conclusion, it would appear that the complex nature of host-parasite interactions requires further work to elucidate the exact immune mechanisms that are responsible for controlling the population growth of *G. turnbulli*. In particular, a study of the antibody response is suggested, as changes to this appear to be the most likely explanation for the differences in population growth of *G. turnbulli*. Extrapolating the immune responses recorded in guppies in this current trial to the

population dynamics of *G. turnbulli* is a difficult process, reliant on much speculation in the absence of similar *in vivo* studies. It must also be noted that the immunology trial and the *G. turnbulli* trial were carried out completely independently from one another, as assessing the parasite population over time would not have been possible if guppies were being removed from the tanks every six days to be used for immunological assays, since this would have affected the population growth pattern of the gyrodactylids. The effects of cadmium on the immune responses of parasitised hosts would undoubtedly differ from those of an unparasitised host due to parasite-elicited host responses. Future work should attempt, therefore, to involve a study of the immune responses of guppies infected with *G. turnbulli*, so that the responses of both host and parasite can be monitored simultaneously. A further problem in extrapolating between the two trials is that the *G. turnbulli* trial was run in a static system with complete water changes being made daily, while, due to the larger scale of the immunology trial, the guppies were maintained in a flow-through system. It is possible that the daily netting of the guppies and their being anaesthetised and examined every six days, coupled with their being parasitised, may have compounded the stress that the guppies were subjected to and altered the immunological status of these fish, resulting in a significantly enhanced parasite burden. These factors may also apply to the interpreting the sex-related differences in parasite burdens and immune responses. This study has, however, made some progress into the complexities surrounding host-parasite interactions and has suggested that the respiratory burst and phagocytic activity of kidney phagocytes may have less impact on gyrodactylids *in vivo* than has been demonstrated *in vitro*.

Table 7.6. A summary table comparing data gathered from the population growth study of *G. turnbulli* (Chapter 6) with the data collected on selected aspects of the guppy immune response. Data are presented from parasites and hosts exposed to 0 and 5μg/l cadmium. The treatment listed on each day represents that with the greatest mean number of parasites or the greatest immune response.

Experiment 2		**Immunology results (Treatment listed on each day represents that with the greatest immune response)**			
			Phagocytic -		
Day	**Pattern of *G. turnbulli*. growth.**	**Respiratory burst**	**index (PI)**	**ratio (PR)**	**Myeloperoxidase production (High production only)**
6	Controls	5μg/l cadmium	5μg/l cadmium	5μg/l cadmium	Controls and 5μg/l cadmium equal
12		Controls		Equal	
18	5μg/l cadmium	Controls	Controls	Equal	Not analysed
24		5μg/l cadmium	5μg/l cadmium	5μg/l cadmium	
30		Controls			Controls and 5μg/l cadmium equal
36 - 60	5μg/l cadmium	Immunology trial terminated at 30 days			

Table 7.7. A summary table comparing data on sex-related differences in the population growth of *G. turnbulli* (Chapter 6) with the data collected on selected aspects of the guppy immune response. Data are presented from parasites and hosts exposed to 0 and 5μg/l cadmium. The treatment listed on each day represents that with the greatest mean number of parasites or the greatest immune response.

Day	Treatment	*G. turnbulli* population growth (Sex listed is that with the greatest parasite burden at each time point)	Immunology results (Sex listed on each day represents that with the greatest immune response)			
			Respiratory burst	Phagocytic index (PI)	Phagocytic ratio (PR)	Myeloperoxidase production
6	Control	Female	Male	Female	Male + Female ca. equal	Male
	5μg/l cadmium		Female		Female	Male
12	Control	Female	Male		Female	Not analysed
	5μg/l cadmium		Female		Male	
18	Control	Female	Male	Female	Female	
	5μg/l cadmium			Male + Female ca. equal	Male + Female ca. equal	
24	Control	Female	Male	Female	Female	
	5μg/l cadmium	Male	Female			
30	Control	Male + Female ca. equal	Male	Male	Male	Female
	5μg/l cadmium	Male				
36 - 48	Control	Male	Immunology trial terminated at 30 days			
	5μg/l cadmium					
54 - 60	Control	Male				
	5μg/l cadmium	Female				

Chapter 8

Summary

8.1. Summary

This study has provided the first information on the effects of low concentrations of cadmium on monogenean parasites and their hosts. Cadmium, at its permitted maximum of 5μg/l, was found to cause subtle alterations to the reproduction and survival of *Dactylogyrus extensus* and significant alterations to the population growth of *Gyrodactylus bullatarudis* and *G. turnbulli*. At the same concentration, cadmium caused subtle alterations to the non-specific immune responses of the hosts, *Cyprinus carpio* and *Poecilia reticulata*, with these alterations becoming more marked on their exposure to 50μg/l and 20μg/l cadmium, respectively.

The rate of oviposition of dactylogyrids, as with other oviparous monogeneans, can be altered by various factors, including temperature and oxygen availability (Kearn, 1986). This study provides the first evidence that egg production by *D. extensus* can also be altered by exposure to low concentrations of cadmium. At 5μg/l, cadmium caused a subtle elevation in rate of *in vitro* oviposition at 64% of the sample points in the four experiments, and a statistically significant elevation in the *in vivo* rate of oviposition after 9 days exposure (0.24 eggs/parasite/4 h in 5μg/l cadmium and 0.03 eggs/parasite/4 h in the controls). Exposure of *D. extensus* to 5μg/l and 30μg/l cadmium for ca. 9/10 days resulted in some of the greatest differences in egg production between these two treatments and the controls. The results from these trials contrast with the literature available for the effect of cadmium on free-living invertebrates. For example, a suppression of fecundity was observed in the leech

Nephelopsis obscura (see Davies *et al.*, 1995), the freshwater snail *Lymnea stagnalis* (see Gomot, 1998) and the housefly *Musca domestica* (see Raina *et al.*, 2001). Due to the free-living nature of the organisms noted above, the suppressive effect of cadmium must be due to the direct effect of the metal on these organisms. The opposite effect of cadmium on *D. extensus* suggests that host responses play a part in the rate of oviposition of these parasites. The idea of the involvement of host responses in dactylogyrid egg production was strengthened by the *in vitro* and *in vivo* rate of oviposition increasing over time in all treatments, despite experimental conditions remaining the same throughout each trial. Interestingly, an assessment of the impact of cadmium on selected aspects of the innate immune response of carp showed an apparent association of phagocytic activity, principally the phagocytic ratio (PR), with egg production by *D. extensus*. The treatment with the greatest PR at each time point was, in general, the treatment in which *D. extensus* were observed to produce a greater number of eggs. Macrophages have been shown by Buchmann & Bresciani (1999) to attach to gyrodactylids when incubated with them *in vitro* and, while it is not known if such attachment can occur *in vivo*, the possibility cannot be ruled out. As *D. extensus* browses over the surface of the gills, consuming mucus and epithelial tissue, it is entirely possible that phagocytes can attach to the parasite or are ingested by them and that this can affect the integrity of the parasite and result in a form of "stress" response, culminating in their producing more eggs. This observation was particularly noticeable at the day 9/10 sample points where, in all four *D. extensus* biology trials, the 5μg/l cadmium-exposed parasites produced more eggs than the controls and the carp showed a slightly elevated phagocytic response when compared to the controls. Of particular interest is the observation that the higher level of cadmium (50μg/l) resulted in a statistically greater

phagocytic response from these carp compared to the controls, after 9 days exposure, and that, at the same time point, 30μg/l cadmium-exposed parasites produced more eggs than parasites from both other treatments. This suggests that phagocytes may indeed influence dactylogyrid egg production to some extent. However, further work needs to be carried out to assess the impact of phagocytes on *D. extensus* biology through a range of *in vitro* tests to determine how and where macrophages attach to the parasite, coupled with TEM studies of the parasites attached to the gill to determine if these cells are, indeed, capable of attaching to the parasites *in vivo*. Investigating other immune parameters, such as antibody production, by control and cadmium-exposed carp may help to elucidate the host-parasite interactions further. Nonetheless, the implication that phagocytes may play some part in influencing the egg production of *D. extensus* is of great interest and should be pursued further.

While phagocytic cells appear to exert some influence on the rate of oviposition in *D. extensus*, the possibility that cadmium directly affects the parasites cannot be excluded, given the parasites close association with the surrounding water. Future work is needed to analyse the cadmium content of dactylogyrid eggs to determine whether, as recorded in birds by Burger (1994), the parasites use egg production as a method for the excretion of heavy metals. The current trial demonstrated, using atomic adsorption spectrometry, that *D. extensus* exposed to 5μg/l and 30μg/l cadmium for only 9 days are capable of accumulating cadmium to concentrations of 7.76μg/g and 17.91μg/g, respectively. It is possible that the high levels of cadmium in these parasites, compared to the controls (0.6μg/g), required them to excrete as much of this metal as possible through the increased production of eggs. The

accumulation of such high levels of cadmium in the 30μg/l cadmium-exposed parasites after only a short exposure period may also explain why, with prolonged exposure (from day 14-30), the number of eggs they produced was below control values. At this concentration, cadmium may detrimentally affect the reproductive functioning of these parasites, subsequently resulting in a lower rate of oviposition. Electron microscope studies of the adult parasites need to be undertaken to locate any areas of the body, which may show tissue damage as a result of cadmium exposure, with particular emphasis placed on the reproductive organs.

As well as enhancing egg production, 5μg/l cadmium was also found to enhance survival of adult *D. extensus* and oncomiracidia and to significantly accelerate the hatch rate of the parasite eggs compared to the controls. The enhanced survival of adults was attributed to cadmium inhibiting some aspect of the metabolism of *D. extensus*, resulting in their prolonged survival, and, in the oncomiracidia the slight enhancement of survival was attributed to the metal inhibiting the metabolism of their lipid reserves. Morley *et al.* (2001b,c) recorded a similar enhancement of the survival of *Schistosoma mansoni* and *Diplostomum spathaceum* cercariae on exposure to 10μg/l cadmium and suggested that this was due to cadmium inhibiting the enzymes involved with glycogen utilisation, coupled with reduced cercarial activity. Further work is needed to investigate the metabolic functioning of dactylogyrids in order to determine which aspects could potentially be inhibited by cadmium.

This trial has demonstrated the great tolerance of *D. extensus* to cadmium exposure, with parasites, that had never been exposed to cadmium, surviving off the host for

over 16 hours in concentrations of 13,100μg/l cadmium. Only at this concentration of cadmium was the egg production by *D. extensus* significantly reduced, and this high tolerance may explain why only subtle effects of 5μg/l and 30μg/l cadmium on egg production were observed.

The more rapid hatching of the 5μg/l cadmium produced eggs was attributed to a variety of factors, with the most possible explanation being that cadmium inhibits the enzymatic tanning process that hardens the eggshell. By inhibiting this process, the eggshells may not have hardened completely, making it easier for the oncomiracidia to hatch. Future work should attempt, through the use of electron microscope studies, to determine the thickness and integrity of control and cadmium-exposed dactylogyrid eggshells to ascertain whether the tanning process is being impaired. Although cadmium has been recorded to accelerate hatching of larval *Lithodes santolla* (see Amin *et al.*, 1998) and of acridid grasshoppers (see Devkota & Schmidt, 1999), the majority of studies have recorded cadmium as inhibiting hatching (Rafiee *et al.*, 1986; Gomot, 1998; Rayms-Keller *et al.*, 1998; Hook & Fisher, 2001). These latter findings were reflected in the delayed hatching of *D. extensus* eggs, in 30μg/l cadmium and were attributed to the possible production of a greater number of small, less viable eggs under *in vitro* conditions as a result of the added stress of exposure to the higher cadmium concentration.

Mason (1988) stated that water hardness is known to affect the toxicity of cadmium, with the metal being more toxic in soft waters (ca. 50 mg/l $CaCO_3$). The current study demonstrated that in water of hardness ca. 24-26 mg/l $CaCO_3$, cadmium at 50μg/l caused large-scale deviations from normal health, with many carp displaying

prolonged periods of abnormal behaviour, encompassing periods of hyperactivity. Other trials involving carp have exposed them to much higher cadmium concentrations, ranging from 5-35 mg/l, but, due to the experimental water being harder (138-162 mg/l $CaCO_3$), no abnormalities in the behaviour of the fish were recorded (Sövényi & Szakolczai, 1993; Witeska, 1998). Interestingly, exposing guppies to 20μg/l also resulted in their developing abnormal behavioural traits. Although hyperactivity was not recorded in the guppies, they were often seen lying motionless on the bottom of the tank before resuming normal swimming activity. No abnormal behaviour was recorded in either the carp or guppies exposed to 5μg/l cadmium and the difference in behaviour of these two groups explains the more pronounced immunological alterations of both species exposed to the higher cadmium concentrations.

Carp exposed to 50μg/l cadmium showed more exaggerated alterations to their immune responses, compared to the subtle changes recorded in carp exposed to 5μg/l cadmium, suggesting that the lower concentration was too low to elicit a strong response from the fish. However, with the exception of the respiratory burst activity, in all cases where the 5μg/l carp showed differences from the controls, these differences were in the same direction as those of the 50μg/l carp. Of note is that the carp exposed to 5μg/l showed some adaptation to cadmium exposure, with the majority of their immune parameters, while showing some subtle differences from the controls between days 9 to 14, becoming similar to control values by the end of the trial. The adaptation of these carp was thought to be due to the production of detoxifying metallothionein proteins, which the 50μg/l carp were unable to produce in sufficient quantity, resulting in an overall lack of adaptation to their

condition. However, it should be noted that the 50μg/l population of carp showed heterogeneity in their response to cadmium exposure, with some fish remaining viable over the course of the trial, thus showing adaptation to their conditions. Unsurprisingly, given their behavioural response to cadmium exposure, the 50μg/l cadmium-exposed carp displayed many features typical of a stress response, including a reduction in the number of lymphocytes (Schreck, 1996) and increased numbers of granulocytes (Sövényi & Szakolczai, 1993). The latter two features are often associated with increases in the production of cortisol, levels of which remained consistently higher in the 50μg/l cadmium-exposed carp than in those of carp from both other treatments. Hoole (1997) stated that the effects of pollutants on the immune responses of fish may occur directly, or indirectly via changes in the levels of corticosteroids that are produced as part of a general stress response. This trial has provided evidence that, as well as those parameters noted above, the level of cortisol in carp correlates, in general, to changes in the respiratory burst and phagocytic activity of kidney phagocytes. As cortisol levels fell, the activity of the phagocytes increased, suggesting that as stated by Schreck (1996) cortisol acts as an immunosuppressant. Therefore, the changes to the immune function of carp in this study appear to have been mediated via cortisol in all treatments, with the changes being exaggerated in carp exposed to 50μg/l cadmium as the stress response was increased by exposure to the metal.

The accumulation ability of endoparasitic acanthocephalans and cestodes has been thoroughly investigated by Sures and co-workers, but little information is available on the accumulation capacity of ectoparasitic monogeneans (see Chapter 5, section 5.1). As mentioned above, this study has provided the first evidence that *D. extensus*

can accumulate cadmium and that the concentration of cadmium accumulated by this parasite increases with increasing exposure concentration. In light of this, it is suggested that this monogenean is classed as a net accumulator of cadmium. The uptake of cadmium by *D. extensus* may occur directly from the water and/or from the grazing of gill mucus and epithelial cells of carp. The ability of dactylogyrids to accumulate heavy metals is in its infancy due the problems associated with their small size and the large number required to make one sample (50 individuals). Future work may benefit from using larger monogeneans to over come the problems with sample sizes.

Unsurprisingly, exposing carp to cadmium resulted in the concentration of this metal being greater in the tissues and organs of these fish compared to the controls. However, control carp were able to accumulate cadmium from very low background levels in the water (0.06-0.64μg/l) and/or feed (ca. 0.1μg/g), as demonstrated by cadmium levels in some of their organs being, in general, above the detection limit (0.5μg/l) of the atomic adsorption spectrometer. Exposing carp to the maximum permitted level of cadmium resulted in cadmium burdens in the muscle exceeding the permitted level of 0.05μg/g for food fish muscle. While this finding appears to be a matter for concern, the fish were below the size that would be sold for human consumption, and it is suggested that as they grow less cadmium will be stored in the muscle and more will be accumulated in other organs. The current trial found the greatest concentration of cadmium in the gills of carp. Surprisingly, this level was highest in the carp exposed to the lowest cadmium concentration (5μg/l). It is suggested that the higher cadmium concentration caused kidney damage (as recorded by Mohan, 1990), resulting in large quantities of cadmium being excreted

in urine. At the lower cadmium concentration, damage to the kidneys was less likely and thus most cadmium was eliminated from the body over the permeable gill surface, resulting in its being recorded in high concentrations in this tissue. Interestingly, the gall-bladders of the cadmium-exposed carp in the current study were very swollen in comparison to those of the control fish. It is suggested that some cadmium excretion may also occur via the bile. Future work should include an assessment of the cadmium concentration in the gall-bladders and urine and also a histopathological study of the kidney and liver.

One of the most interesting findings of the current study is how cadmium, at concentrations below the permitted maximum (EQS) of ca. 4.0-4.5μg/l, caused significant increases in the population size of both *G. bullatarudis* and *G. turnbulli*. Due to their greater parasite burdens, the swimming ability of the guppies exposed to cadmium became impaired and these fish were removed from the tanks and killed earlier than the controls. This finding is particular interesting given that the Environment Agency (2003) states "*the dangerous substance is not believed to be detrimental to aquatic life at any concentration below its EQS limit*". The results from this study suggest that exposure to low cadmium concentrations could actually be detrimental to fish by causing significant increases to the size of their ectoparasite populations. This is the first study to directly correlate an increase in size of a *Gyrodactylus* population to the effects of an individual heavy metal. Several field studies have observed numbers of gyrodactylids to increase or decrease in response to pollution (see Chapter 6, section 6.1), but in most instances there have been mixed parasite infections on the fish and a mixture of pollutants in the water body, making it very difficult to elucidate exact cause-effect responses.

The population growth of *G. turnbulli* on individual control and cadmium-exposed guppies was monitored over a 60-day period and showed some very interesting results. Whereas *G. turnbulli* infections on control guppies began to decrease at ca. 42/48 days, after showing a rapid increase in number from day 12 to 36, the infections on cadmium-exposed guppies continued to increase at this time point. This observation suggested that host responses may have been affected by exposure to 5μg/l cadmium and a subsequent trial was undertaken to determine the impact of this metal on the immune responses of guppies. However, unlike in the *D. extensus*-carp system, there was no obvious link between any of the guppy innate immune parameters studied and the population growth of *Gyrodactylus* spp. It is possible that, being more mobile than dactylogyrids, gyrodactylids are able to change their position on the host, move away from the localised tissue responses that are mounted by the host and avoid prolonged interaction with phagocytic cells and their products. Richards & Chubb (1998) suggested that the ability of gyrodactylids to move across the body of the guppy and away from localised host responses, was an explanation for low-level gyrodactylid infections persisting on adult guppies. In the absence of a correlation between the innate immune responses studied and gyrodactylid population growth, it is suggested that the suppression of the antibody production was a possible cause of the increased population growth on guppies. Buchmann (1997) and Scott & Robinson (1984) have shown that *Oncorhynchus mykiss* and *Poecilia reticulata* demonstrate an acquired immune response to *G. derjavini* and *G. turnbulli*, respectively, shown by previously infected fish being able to reduce the intensity of reinfection. It is thus suggested that cadmium directly suppresses the antibody response as demonstrated in *Ictalarus melas* by Albergoni & Viola (1995), or indirectly suppresses antibody production by increasing the

levels of cortisol which have been recorded as depressing the antibody-synthesising capability of lymphocytes (Schreck, 1996). With a lowered antibody response, the cadmium-exposed parasites would have been able to increase in intensity at a greater rate than the controls and show less reduction in the population size overtime. However, future experiments are needed to determine the effect of cadmium on the antibody response of guppies to confirm this hypothesis.

Of note from this trial was the emergence of a sex-related difference in the susceptibility of guppies to *G. turnbulli* infection. At the beginning of the trial female guppies were recorded with the greatest parasite burden, but, from day 24 in the cadmium-exposed population and day 36 in the control population, male guppies harboured the greater number of parasites. The reason for the female guppies being most heavily parasitised at the start of the trial was attributed to those guppies initially infected in each tank being female. Possible hypotheses for the greater infection intensity on males later on in the trial included male-male competition caused by a male-biased operational sex ratio in the tanks, immunosuppression in the males caused by the sex hormone testosterone and the larger surface area provided by the fins of the male guppies. However, measuring the body and tail length of both sexes showed statistically greater tail lengths in male guppies in only two tanks. As this difference was not apparent in all tanks, it suggested that a difference in tail length was not the sole reason for the observed sex-related differences in parasite burden. All of the immune parameters monitored (respiratory burst, phagocytosis, myeloperoxidase production) showed small sex-related differences; however, these differences were only slight and cannot explain the difference in parasite number on the two sexes. It is suggested that sex

hormones may contribute to the observed differences in parasite number and the levels of these hormones should be assessed in future experiments.

Unlike the carp, where cadmium affected an aspect of the guppy innate immune response, there appeared to be no obvious adaptation to cadmium exposure. This was apparent in the phagocytic activity remaining consistently higher than the controls at both 5μg/l and 20μg/l cadmium. The possibility that adaptation does occur in guppies cannot be excluded and a longer trial may help to elucidate this hypothesis. Exposure to 5μg/l cadmium did not cause an alteration in the respiratory burst of the guppies and neither of the cadmium concentrations appeared to affect the production of myeloperoxidase by neutrophils.

In conclusion, this study has been the first to try and elucidate monogenean-host interactions in the presence of cadmium through independent investigations of parasite biology and host immunity. While some links between the innate immune parameters studied and monogenean biology have been observed, it is suggested that further work is needed to elucidate fully the complexities of monogenean-host interactions. While the effects of phagocytic cells and their products have been shown to have a strong impact on helminths *in vitro* (Whyte *et al.*, 1989; Sharp *et al.*, 1991; Buchmann & Bresciani, 1999), the results of this current study suggest that the *in vivo* effects of these cells are less pronounced. The impact of cadmium on the specific immune response, particularly antibody production, should, in particular, be the subject of further investigations to help unravel the impact of cadmium on monogenean biology and population growth.

This study specifically minimised factors that may confuse the cause and effect interpretation of observations made in pollution-exposed parasite systems, such as multispecies infections and mixed pollution. Despite these simplifications, the complexities of the two systems under study were still marked, and this highlights the difficulties faced by scientists in determining the exact cause for observations recorded in the field. It is suggested that fully elucidating monogenean-parasite interactions will take extensive work in the future and will require the standardisation of numerous parameters, such as fish age, size and previous health status, in addition to pollutant type, concentration and exposure duration and various water quality conditions, in order to allow comparisons to be drawn between different systems. Nonetheless, the current study has provided a range of interesting findings and has offered several avenues for further investigations. Given that the maximum permitted level of cadmium caused significant increases in the numbers of *G. turnbulli* and *G. bullatarudis*, to the detriment of the guppies, there is a need to review the water quality guidelines with regards to the permitted concentration of this metal.

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Appendix 1 - Water quality methodologies

1. Chlorine levels

Before each trial, the level of chlorine in the incoming water to the experimental system was checked to ensure that the Eheim was working efficiently. Chlorine levels were measured using DPD Total Chlorine Reagent Pillows and DPD Free Chlorine Reagent Pillows (CAMLAB). Chlorine levels never exceeded 0.1mg/l total chlorine and 0.05 mg/l free chlorine.

2. Total water hardness (ppm $CaCO_3$) - 100 ml of water sample was decanted in to a 250 ml conical flask and placed in a fume cupboard. 2 ml of ammonia buffer solution (BDH Chemicals Ltd) and 1 total hardness indicator tablet (BDH Chemicals Ltd) were added to each sample. Samples were shaken and left until the indicator tablets had completely dissolved (c. 30 min). On dissolving, the tablets turned the water a bright pink colour. Each sample was then titrated with 0.01M EDTA solution (BDH Chemicals Ltd) until the colour of the sample had changed from pink to deep blue. The total hardness of the water was then determined using the following calculation:

$$\frac{\text{Volume of EDTA titrated (ml)} \times 1000}{\text{Volume of water sample (ml)}}$$

3. Alkalinity (meq/l) - 100 ml of water sample was placed in a small, clean, conical flask. Five drops of BDH "4.5" indicator solution (BDH Chemicals Ltd) was added to each sample turning the water blue. The sample was then titrated with 0.01M hydrochloric acid (HCl) until the colour changed from

blue to peach. The alkalinity was then determined using the following calculation:

$$\frac{\text{Volume of HCl titrated} \times 0.01 \times 1000}{\text{Volume of water sample (ml)}}$$

Alkalinity is the concentration of bases dissolved in water. These bases are usually bicarbonates ($HCO3^-$) and carbonates ($CO3^-$), and, in rare instances, hydroxide (OH^-) ions. These ions, called buffers, are important because they slow the rate at which the pH changes. Alkalinity is not the same as hardness. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) are primarily responsible for hardness.

The units used in this trial are milliequivalents per litre (meq/l).

1 meq/l = 50mg/l $CaCO_3$.

4. pH - pH was measured on a Philips PW9409 digital pH meter. The machine was turned on, calibrated and left for 10 min to warm up. Allowing the machine to warm up ensured that readings settled quickly and that variation between readings was minimal. The pH probe was left in each 30 ml water sample for 1 min after which the reading was taken.

5. Dissolved oxygen – Dissolved oxygen was measured using a YSI Model 58 Dissolved Oxygen Meter.

Appendix 2 - Cortisol radioimmunoassay

The following methodology for determining cortisol concentrations was adapted from Pickering *et al.* (1987) by I. Berrill at the Institute of Aquaculture. The methodology below was provided by and undertaken by B. North at the Institute of Aquaculture. The author of this work was unable to carry out the assay due to not being licensed to use radioisotopes.

The following methodology was adapted by Berill (2003).

1. Assay Buffer

The following constituents were dissolved in approximately 100 ml of nanopure water in a volumetric flask with the aid of a magnetic stirrer and a heated plate. Once dissolved, the volume was made up to 500 ml with nanopure water, mixed, and chilled to 4°C. Buffer was generally made up the day before each assay and stored at 4°C, but the addition of sodium azide permitted the buffer to be used within a week of being made. All the chemicals used were of Analar grade and supplied by either Sigma or BDH Chemicals Ltd.

Sodium dihydrogen orthophosphate	0.74g
Disodium hydrogen orthophosphate	2.88g
BSA	1g
Sodium chloride	4g
EDTA	0.15g
Sodium azide	0.05g

2. Charcoal Buffer

On the morning of day 2 of the assay, the following constituents were dissolved in a conical flask in 100 ml of nanopure water with the aid of a heated plate with a magnetic stirrer.

Sodium dihydrogen orthophosphate	0.37g
Disodium hydrogen orthophosphate	1.44g
Gelatine	0.25g

Once the gelatine was in solution the charcoal and dextran was added in the following quantities and the buffer was made up to 250 ml with a further 150 ml of nanopure water. The buffer was then left to stir on ice for at least 1 hour before use.

Activated charcoal	1.25g
Dextran	0.25g

3. Radiolabel

[1,2,6,7-^{3}H] Cortisol radiolabel was supplied by Amersham Pharmacia Biotech UK limited, in quantities of 9.25MBq (250μCi). The radiochemical was supplied in 0.25 ml of a toluene:ethanol (9:1 v/v) solution at an initial purity of 99.8%. An intermediate stock solution was prepared by diluting 20μl of the stock solution in 2 ml of absolute ethanol and from this a working solution of approximately 500dpm/100 ml was made (approximately 50 ml in 25 ml of assay buffer). The radiolabel and intermediate stock were then stored at –20°C until required for use.

4. Antibody

1g freeze-dried sheep anti-cortisol serum was supplied by Diagnostics Scotland and hydrated with 20 ml of fresh assay buffer (1:20 dilution) and frozen in polystyrene tubes in 1 ml aliquots. When required the contents of one tube was diluted with a further 20 ml of assay buffer to achieve a 1:400 dilution (enough for an assay of 90 samples in duplicate).

5. Cortisol Standard

A standard was prepared from 1g hydrocortisone, hydrolysed powder, from Sigma, UK. The following stock standards were made:

Stock 1 (50 µg/ml): 10µg cortisol in 20 ml absolute ethanol

Stock 2 (5µg/ml): 100µl Stock 1 in 10 ml absolute ethanol

Stock 3 (50 ng/ml): 100µl Stock 2 in 10 ml absolute ethanol store at -20°C.

400µl of Stock 3 was diluted in 4.6 ml ethyl acetate to create a working standard of 4 ng/ml.

6. Cortisol Assay Protocol

Standards and samples were always assayed in duplicate within a week of the extraction date.

6a. Sample Extraction

1. Add 200 µl plasma sample to separate polypropylene tubes (LP3P: Thermo Life Science, Hampshire, UK)
2. Add 1 ml ethyl acetate (BDH Chemicals Ltd) to each tube and stopper the tubes
3. Spin tubes on a rotary mixer for 1 hr
4. Centrifuge tubes at 1500 rpm (4°C) for 10 min
5. Store at 4°C if not to be assayed immediately

6b. Assay

1. Prepare a serial dilution of cortisol working standard (4 ng/ml) with 200µl of ethyl acetate (12.5-800pg/tube) in LP3P polypropylene tubes. Add 200µl ethyl acetate to a further 4 tubes that act as the zero standard (BO) and the non-specific binding (NSB).
2. Transfer 200µl of extracted samples to separate LP3P tubes (if high levels of cortisol are expected dilute extract with ethyl acetate and convert data at the end of the assay).
3. Dry down the standards and sample extracts in a vacuum oven at less than 35°C then cover the tubes and cool them to 4°C.
4. Add anti-cortisol to all tubes except the NSBs, to these add 100µl of assay buffer.
5. Add 100µl of tritiated cortisol to all tubes.
6. Vortex all tubes and incubate at 4°C for 18 hours.

7. Make up charcoal buffer and stir on ice for 1 hour before adding 1 ml to each tube. Vortex tubes and incubate at 4°C for 30 min.
8. Centrifuge at 2500 rpm (4°C) for 12 min.
9. Transfer 1000µl supernatant to scintillation vials (Canberra Packard Ltd.) and add 4ml of scintillation fluid (Ultima Gold, Canberra Packard Ltd.).
10. Add 4ml of scintillation fluid to 3 empty vials and to two of these vials add 100µl of tritiated cortisol to act as 'Total Counts'. The remaining vial containing just 4ml of scintillation fluid will act as the blank and will determine the background radioactivity.
11. Cap and vortex tubes until the pellet is mixed.
12. Arrange the vials in the scintillation rack as follows: Blank, Total Counts, NSB, Standards in reverse (i.e. 12.5 – 800), Unknown samples. Count for 5 minutes in a scintillation counter (1900 LSA; Canberra Packard).

6c. Calculations

13. Multiply the mean dpm (disintergrations/min) by 1/1.3 (this corrects for the difference between the volume of reagent and per tube and the volume of supernatant counted).
14. Subtract the mean dpm for the non-specific binding from all of the standards and samples.
15. Calculate the percentage binding of standards and samples relative to the corrected total counts:

$$\% \text{ binding} = (\text{standard or sample dpm/mean total dpm}) \times 100$$

16. Plot the percentage binding of the standards against the cortisol concentration on log-linear graph paper (Fig. 1) and read the concentration of the cortisol for the samples from the standard curve (this gives the concentration of cortisol (pg/ml) in 200µl of extracted sample).
17. To correct the samples for the volume extracted, first multiply by 6 (200µl from a total of 1200µl), then multiply this value by 5 to correct for the volume of serum extracted (200µl x 5 = 1 ml) and finally, to convert to ng/ml (x 1/1000).

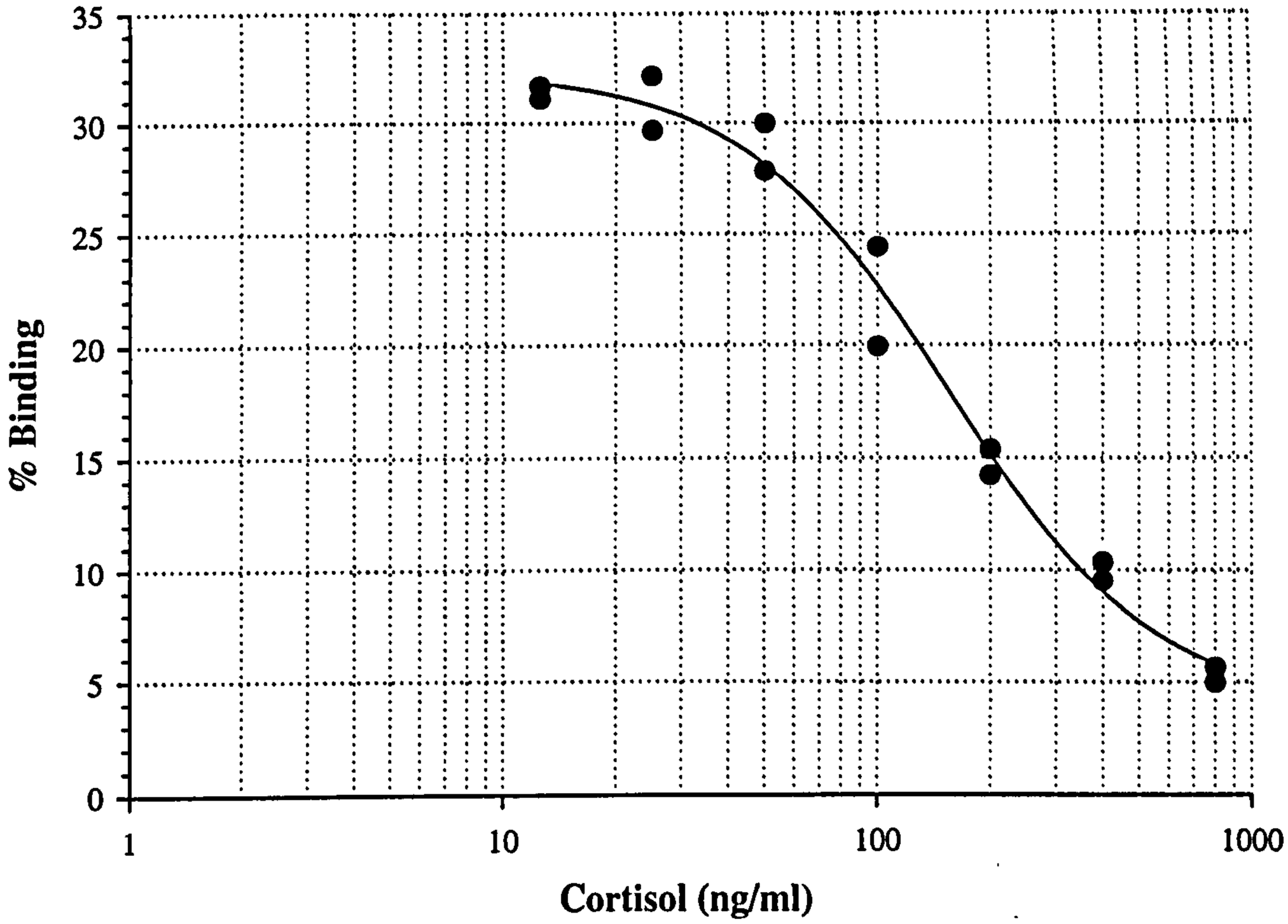

Figure 1. A typical standard curve from a cortisol radioimmunoassay; the concentration of cortisol in a sample is determined by intersecting the standard curve at the point corresponding to the percentage binding in the sample.

Quality Control and Validation

6d. Validation

The sensitivity of the assay (i.e. the minimum amount of cortisol that is statistically distinguishable from zero) was 12.5pg/tube. Two pooled samples of carp plasma were obtained from 'unstressed' fish and from fish, 1-hour after being subjected to standardised handling stress (confined in a net for 1 min) were used as quality controls (QCs) to check the reproducibility of the measurements between each assay. The cortisol concentrations of the 'unstressed' and 'stressed' samples were approximately 5.5 and 40ng/ml respectively. The intra-assay variation was determined by comparing the concentration of an aliquot of the 'stressed' QC at the start and end of each assay and the coefficient of variation was 4.2% (n=5). The inter-assay coefficient variation was 5.7% (n=5). Assays were rejected if the difference between one of the QCs and the average cortisol concentration obtained from previous assays was greater than 2 standard deviations.

To ascertain that the cortisol in the standard was immunologically similar to that in the carp plasma, serial dilutions (1:2) of the extracted carp plasma were used to create an inhibition curve (Fig. 2). When plotted against a serial dilution of cortisol standard there was no statistical significance difference observed between the slope of the lines when the residuals were compared with a T-test using an in-house excel application designed and validated by Iain Berrill.

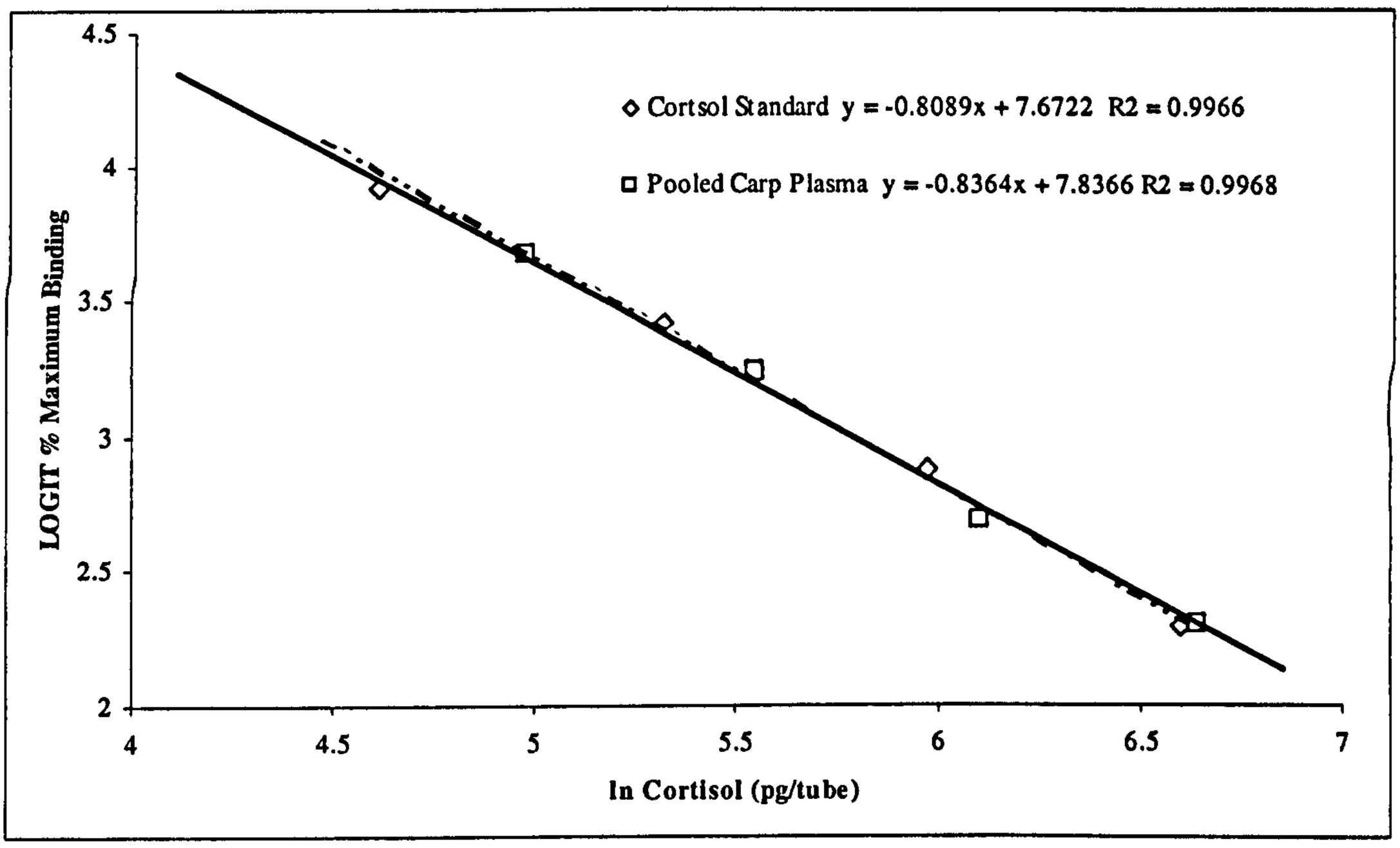

Figure 2. Parallelism of an inhibition curve obtained from a serial dilution (1:2) of 200μl aliquots of extracted carp plasma (extracted from a pooled sample of 200μl plasma in 1ml ethyl acetate) with the cortisol standard curve. Each point represents the mean of duplicate measurements; the X-axis denotes the natural log of the cortisol content in the standards.

Appendix 3a - Pooled respiratory burst results from control carp and carp exposed to 5 and 50μg/l cadmium for 6, 9, 14 and 21 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

		50μg/l cadmium	
Day	**Fish**	**NBT**	**NBT+PMA**
6	1	0.543	0.570
	2	0.349	0.576
	3	0.172	0.275
	4	0.326	0.511
	5	0.200	0.173
	6	0.475	0.606
	7	0.415	0.391
	8	0.291	0.390
	Mean	**0.346**	**0.437**
	SE	**0.045**	**0.055**

		Controls		5μg/l cadmium		50μg/l cadmium	
		NBT	**NBT+PMA**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**
9	1	0.400	0.474	0.126	0.097	0.363	0.468
	2	0.917	0.950	0.124	0.122	0.469	0.571
	3	1.303	1.252	0.667	0.900	0.725	0.705
	4	0.172	0.226	0.237	0.255	0.503	0.450
	5	0.108	0.155	0.243	0.307	0.420	0.197
	6	0.429	0.509	0.410	0.443	0.094	0.0784
	Mean	**0.554**	**0.594**	**0.301**	**0.354**	**0.429**	**0.412**
	SE	**0.190**	**0.174**	**0.085**	**0.121**	**0.084**	**0.095**

		Controls		5μg/l cadmium		50μg/l cadmium	
		NBT	**NBT+PMA**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**
14	1	0.425	0.394	0.133	0.137	1.182	1.188
	2	0.187	0.122	0.212	0.884	0.408	0.424
	3	0.101	0.110	3.017	2.733	0.486	0.472
	4	0.288	0.233	0.759	0.815	0.388	0.467
	5	0.297	0.274	1.059	1.089	0.536	0.483
	6	0.259	0.256	1.381	1.333	1.317	0.759
	Mean	**0.259**	**0.231**	**1.094**	**1.165**	**0.720**	**0.632**
	SE	**0.045**	**0.043**	**0.432**	**0.354**	**0.170**	**0.122**

		Controls		5μg/l cadmium		50μg/l cadmium	
		NBT	**NBT+PMA**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**
21	1	1.369	1.236	0.765	0.702	0.648	0.619
	2	0.556	0.549	0.743	0.726	3.083	2.917
	3	0.960	0.916	0.660	0.642	1.133	1.073
	4	2.378	2.444	0.174	0.197		
	5	1.133	1.082	1.093	1.140		
	6	1.200	1.094	0.552	0.506		
	Mean	**1.266**	**1.220**	**0.665**	**0.652**	**1.621**	**1.536**
	SE	**0.249**	**0.263**	**0.123**	**0.126**	**0.744**	**0.703**

Appendix 3b - Respiratory burst results from control carp and carp exposed to 5μg/l cadmium for 29 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

		Controls		5μg/l cadmium	
	Day	NBT	NBT+PMA	NBT	NBT+PMA
29	1	0.0476	0.073	0.353	0.657
	2	0.033	0.055	0.144	0.305
	3	0.040	0.080	0.048	0.059
	4	0.150	0.259	0.149	0.155
	5	0.988	1.188	0.131	0.133
	6	0.389	0.557	0.06	0.072
	Mean	**0.275**	**0.369**	**0.149**	**0.230**
	SE	**0.153**	**0.181**	**0.044**	**0.093**

Appendix 4 - Surface area of hamuli (μm^3) in control and 5μg/l cadmium-exposed *Gyrodactylus turnbulli*. Both hamuli were measured for each of the 10 specimens in each group.

Control (2 weeks)	Area of hamuli (μm^3)	Test (6 weeks)	Area of hamuli (μm^3)
C1-1	344.23	T1-1	313.85
C1-2	330.11	T1-2	322.79
C2-1	340.61	T2-1	328.67
C2-2	334.47	T2-2	322.35
C3-1	312.54	T3-1	306.01
C3-2	285.08	T3-2	326.05
C4-1	299.69	T4-1	293.37
C4-2	304.31	T4-2	298.03
C5-1	290.58	T5-1	322.18
C5-2	298.20	T5-2	320.04
C6-1	316.20	T6-1	321.04
C6-2	299.51	T6-2	327.75
C7-1	320.13	T7-1	323.00
C7-2	328.41	T7-2	302.21
C8-1	267.91	T8-1	290.75
C8-2	251.26	T8-2	300.69
C9-1	332.24	T9-1	287.70
C9-2	353.43	T9-2	311.76
C10-1	308.53	T10-1	310.41
C10-2	316.51	T10-2	304.26
Mean	**311.6975**	**Mean**	**311.6455**
SE	**5.752353929**	**SE**	**2.913321985**

Appendix 5 - *Gyrodactylus turnbulli* hook morphometrics (μm)

A=Hamulus aperture length; **B**=Hamulus point length; **C**=Hamulus total length; **D**=Hamulus shaft length; **E**=Ventral bar width; **F**=Marginal hook shaft length; **G**=Marginal hook sickle length; **H**=Marginal hook proximal width; **I**=Marginal hook distal width; **J**=Marginal hook sickle aperture length.

Test parasites	A	B	C	D	E	F	G	H	I	J
1	19.78	25.27	56.96	42.33	30.93	26.01	7.85	4.78	7.11	6.99
2	18.95	23.22	54.17	39.12	32.08	26.20	7.57	4.38	4.22	6.92
3	22.27	24.97	57.83	43.16	33.33	23.50	8.34	3.27	2.89	7.75
4	22.53	23.65	57.67	36.78	31.84	25.30	7.26	3.07	4.56	7.33
5	23.62	24.31	59.87	45.81	34.41	23.70	8.70	4.27	4.94	8.24
6	22.07	22.63	56.67	43.85	32.06	22.70	7.58	3.95	4.35	7.49
7	21.56	21.68	55.18	41.70	29.95	23.07	7.65	3.05	3.40	5.78
8	21.23	22.73	57.52	44.11	30.33	25.14	7.86	4.59	4.14	7.60
9	21.51	24.14	55.78	42.80	34.76	23.60	8.69	4.53	6.00	7.07
10	21.01	24.31	56.84	44.43	33.51	23.40	7.82	4.05	4.30	7.66
Mean	**21.45**	**23.69**	**56.85**	**42.41**	**32.32**	**24.27**	**7.93**	**3.99**	**4.59**	**7.28**
SE	**0.42**	**0.36**	**0.50**	**0.85**	**0.52**	**0.40**	**0.15**	**0.20**	**0.38**	**0.21**
Control parasites	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
1	22.28	23.75	58.52	43.52	31.86	26.26	8.01	4.13	3.43	8.02
2	22.26	23.81	57.44	44.45	30.10	23.16	7.23	4.57	4.05	7.27
3	20.88	22.96	57.08	41.05	32.16	25.66	7.71	3.04	3.03	7.18
4	22.44	23.8	56.58	42.3	30.57	26.18	8.01	3.52	3.26	7.92
5	22.63	23.92	58.75	42.64	32.61	24.05	7.35	4.62	3.55	7.18
6	23.51	23.22	58.22	44.00	31.83	25.91	7.87	4.26	4.42	6.91
7	28.02	23.28	56.10	38.10	30.64	24.34	7.41	4.98	4.40	7.20
8	25.96	24.10	61.09	45.63	33.31	22.26	7.56	4.07	3.38	7.55
9	20.00	24.01	53.87	41.91	30.91	23.44	7.34	3.94	3.77	7.17
10	21.76	23.06	57.33	43.64	31.82	22.66	7.53	3.73	3.66	7.31
11	21.05	22.99	56.12	41.56	30.57	24.53	7.93	4.16	3.08	7.47
Mean	**22.85**	**23.52**	**57.26**	**42.53**	**31.45**	**24.22**	**7.59**	**4.09**	**3.66**	**7.32**
SE	**0.77**	**0.14**	**0.60**	**0.67**	**0.33**	**0.43**	**0.09**	**0.18**	**0.16**	**0.09**

Appendix 6a - Pooled respiratory burst results from control guppies and guppies exposed to 5μg/l and 20μg/l cadmium for 6 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

Day	Replicate	Controls		5μg/l cadmium		20μg/l cadmium	
		NBT	NBT+PMA	NBT	NBT+PMA	NBT	NBT+PMA
6	1	0.656	0.394	0.596	0.500	0.514	0.447
		3.725	3.375	0.424	0.341	0.089	0.159
		0.571	0.536	0.609	0.573	0.356	0.456
		2.690	2.900	1.467	1.173	0.940	1.100
		0.409	0.698	0.658	0.535	2.100	2.457
		2.015	1.069	0.726	0.577	1.156	1.422
		1.263	0.683	0.397	0.443	0.191	0.212
		0.111	0.051	2.417	1.517	0.130	0.202
	2	0.419	0.425	1.540	1.690	0.927	1.033
		0.476	0.515	1.586	2.186	5.133	4.467
		1.686	1.943	0.807	1.227	5.800	6.467
		1.006	0.425	1.619	0.897	1.509	1.382
		0.808	0.561	1.521	1.642	2.352	2.286
		0.532	0.376	3.817	3.550	1.086	1.236
		1.844	1.556	0.425	0.450	24.800	16.200
		0.487	0.603	0.4778	0.691	0.225	0.139
	3	0.484	0.742	0.714	1.000	0.036	0.303
		0.579	0.553	0.198	0.208	2.253	6.822
		0.251	0.298			1.644	1.800
		0.256	0.120	0.232	0.388	2.000	0.757
		0.494	0.667	0.415	0.969	2.307	3.375
		0.238	0.163	0.644	2.200	1.550	0.754
		0.545	2.015	0.300	0.505	1.092	0.920
		0.153	0.754	0.310	0.081	2.040	0.940
	Mean	**0.904**	**0.893**	**0.952**	**1.015**	**2.510**	**2.306**
	SE	**0.182**	**0.175**	**0.178**	**0.170**	**1.012**	**0.713**

Appendix 6b - Pooled respiratory burst results from control guppies and guppies exposed to 5μg/l and 20μg/l cadmium for 12 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

Day	Replicate	Controls		5μg/l cadmium		20μg/l cadmium	
		NBT	NBT+PMA	NBT	NBT+PMA	NBT	NBT+PMA
12	1	6.020	3.060	1.465	0.379	5.861	1.515
		7.567	4.667	1.158	0.398	15.050	5.175
		1.600	1.286	8.713	1.963	30.304	6.826
		1.762	1.131	3.761	8.380	1.774	3.954
		1.122	0.561	0.242	0.300	18.850	23.400
		2.000	1.553	13.038	11.600	27.739	24.681
		0.688	0.615	6.923	5.185	27.607	20.675
		1.096	1.151	0.175	0.153	0.701	0.610
	2	0.894	0.867	0.838	0.896	2.375	1.800
		0.512	0.541	0.985	0.845	1.344	0.794
		1.004	0.595	1.900	1.688	1.313	1.638
		0.830	0.725	0.919	1.081	3.050	2.700
		1.023	0.977	2.138	1.438	1.071	1.221
		2.538	1.813	1.070	1.383	0.767	0.807
		7.714	6.393	1.227	0.717	0.438	0.348
		0.694	0.840	0.970	0.799	1.188	1.016
	3	1.311	1.833	1.467	3.578	0.133	0.180
		1.850	3.033	0.918	3.164	1.058	1.467
		1.084	0.879	0.376	0.680	0.483	0.525
		1.090	2.230	1.110	2.940	1.080	1.272
		8.600	23.700	0.756	1.943	0.381	0.525
		2.738	3.025	0.956	1.481	0.781	0.879
		0.318	0.331	0.771	1.195	0.282	0.873
		0.411	0.298	1.028	1.067	2.883	0.550
	Mean	**2.269**	**2.588**	**2.204**	**2.219**	**6.105**	**4.310**
	SE	**0.507**	**0.966**	**0.627**	**0.553**	**2.000**	**1.507**

Appendix 6c - Pooled respiratory burst results from control guppies and guppies exposed to 5μg/l and 20μg/l cadmium for 18 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

Day	Replicate	Controls		5μg/l cadmium		20μg/l cadmium	
		NBT	NBT+PMA	NBT	NBT+PMA	NBT	NBT+PMA
18	1	0.592	0.473	0.125	0.114	0.446	0.508
		3.133	3.733	0.844	0.650	0.292	0.317
		0.650	0.533	1.633	1.575	0.656	0.488
		1.450	0.719	1.100	0.906	0.306	0.311
		0.224	0.182	0.653	0.300	0.352	0.437
		0.957	0.700	1.074	0.563	0.102	0.087
		0.194	0.209	0.844	0.408	0.943	0.657
		0.533	0.368	0.735	0.282	0.045	0.025
	2	0.554	0.746	2.683	1.817	4.840	5.060
		1.614	2.657	0.592	0.492	0.953	0.636
		1.108	1.017	1.433	1.200	3.900	21.333
		0.240	0.249	0.605	0.590	1.733	1.644
		0.507	0.479	0.905	0.576	0.138	0.157
		0.351	0.209	0.711	0.433	0.146	0.533
		0.357	0.390	0.618	0.381	0.287	0.156
		0.256	0.383	0.170	0.180	1.093	0.636
	3	1.670	1.955	0.341	0.394	2.138	1.825
		0.781	0.881	2.443	1.029	1.317	1.310
		4.865	3.581	0.488	0.331	1.775	0.725
		1.307	0.446	0.748	0.457	0.295	0.243
		2.413	0.417	1.608	1.150	0.041	0.056
		0.121	0.187	1.168	0.867	0.385	0.213
		0.128	0.227	0.868	0.472	0.435	0.165
		1.085	1.665	1.573	1.033	0.339	0.176
	Mean	**1.045**	**0.934**	**0.998**	**0.675**	**0.956**	**1.571**
	SE	**0.227**	**0.211**	**0.130**	**0.089**	**0.247**	**0.885**

Appendix 6d - Pooled respiratory burst results from control guppies and guppies exposed to 5μg/l and 20μg/l cadmium for 24 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

		Controls		5μg/l cadmium		20μg/l cadmium	
Day	**Replicate**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**
24	**1**	0.873	3.92666667	2.46	2.2	0.64473684	0.24473684
		0.859	0.61351351	0.857143	0.40714286	2.575	1.4625
		0.879	1.07142857	0.144444	0.10277778	0.64	0.62666667
		2.883	3.492	0.047	0.033	6.900	4.900
		0.897	0.574	0.254	0.180	2.513	3.120
		0.917	0.640	0.066	0.087	0.205	0.168
		0.295	0.246	6.267	7.333	0.3400	0.347
		4.000	0.931	2.005	1.653	0.8667	0.750
				0.645	0.245		
	2	0.612	1.194	1.390	1.443	0.752	0.843
		0.421	0.403	1.023	2.223	0.098	0.110
		0.729	1.962	0.507	0.609	0.263	0.446
		1.200	1.117	3.201	2.227	12.200	5.400
		0.725	1.635	5.043	2.863	0.163	0.186
		0.082	0.067	0.764	0.509	0.119	0.104
		0.258	0.169	1.229	1.577		
		0.180	0.281	2.400	1.438		
	3	0.233	0.232	2.180	1.125	1.210	1.210
		3.440	4.300	0.475	0.742	0.822	2.189
		0.428	0.669	1.973	3.650	2.720	3.240
		0.531	0.485	0.176	0.114	0.367	0.360
		0.309	0.417	1.073	0.873	2.440	3.360
		2.973	2.436	4.033	3.317	0.256	0.281
		0.299	0.360	0.884	0.388	0.122	0.097
		0.676	0.445				
	Mean	**1.036**	**1.032**	**1.593**	**1.441**	**1.779**	**1.460**
	SE	**0.235**	**0.227**	**0.345**	**0.349**	**0.657**	**0.373**

Appendix 6e - Pooled respiratory burst results from control guppies and guppies exposed to 5μg/l and 20μg/l cadmium for 30 days. Respiratory burst response from phagocytes incubated with NBT only and those stimulated by PMA (NBT+PMA).

		Controls		5μg/l cadmium		20μg/l cadmium	
Day	**Replicate**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**	**NBT**	**NBT+PMA**
30	**1**	3.857	2.429	1.105	1.043	1.280	1.350
		2.875	1.688	0.546	0.459	2.680	3.800
		0.792	0.604	1.047	0.857	1.650	1.375
		3.850	1.988	2.100	1.833	0.090	0.120
		0.632	0.632	2.591	2.127	0.474	0.555
		0.486	0.408	1.083	1.089	0.153	0.166
		0.805	0.512	0.664	0.475	0.750	0.558
				1.631	1.217	0.620	0.338
	2	1.260	1.380	3.329	1.821	0.124	0.309
		1.125	1.394	0.677	0.585	0.597	1.106
		2.514	2.071			2.800	4.133
		2.163	2.275	0.575	0.582	2.920	3.520
		0.421	0.828	1.485	0.915	1.071	1.221
		0.146	0.203	2.293	1.781	2.488	3.425
		1.450	1.365	1.351	0.811	4.115	2.400
		0.396	0.378	2.400	1.438	4.430	3.370
	3	0.373	0.365	0.732	0.811	0.869	1.254
		3.867	4.267	0.725	0.919	2.401	2.699
				0.967	1.283	0.126	0.173
		0.477	0.661	0.353	0.470	3.150	6.300
		0.347	0.182	0.813	0.825	0.508	0.700
		0.213	0.132	0.277	0.261	4.467	7.467
		0.276	0.134	0.486	0.323	0.041	0.071
		0.313	0.161	0.114	0.119	0.830	0.665
	Mean	**1.302**	**1.093**	**1.1889**	**0.959**	**1.610**	**1.961**
	SE	**0.274**	**0.221**	**0.175**	**0.115**	**0.297**	**0.410**