ENVIRONMENTAL TOLERANCES OF THREE SPECIES OF FRESHWATER CRAYFISH

by

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A thesis submitted to the
University of Nottingham
for the degree of
Doctor of Philosophy.

Department of Life Science
1993

To Mum and Dad

"They were dwarfish natures capable of growing into monsters if ill chance fostered the process...and were highly susceptible to the encroachments of evil. They were...like crayfish who always retreat into shadow, going backwards rather than forwards in life, gaining in deformity with experience, going from bad to worse and sinking into deeper darkness....."

(Hugo, Les Miserables)

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ABSTRACT

The only species of crayfish native to the British Isles, i.e. *Austropotamobius pallipes*, is presently widely distributed throughout much of England, Wales and Ireland. The main controlling factor behind this distribution is the predominantly "bicarbonate" freshwater system of the British Isles. In geological terms, native crayfish are generally found in areas of chalk, carboniferous or magnesium limestone, or drift deposits of boulder clay. More recently, native crayfish have been affected by a general decline in the quality of Britain's freshwater system largely due to sewage effluent and agricultural. By far the majority of native crayfish records are associated with water bodies of very good or good quality, with the result that many populations of *A. pallipes* are isolated in small, good quality headwaters, due to the degree of pollution in the main rivers acting as "chemical barriers".

The last two decades have seen a number of alien species of crayfish imported into England and Wales for aquaculture and culinary purposes. Deliberate and accidental introductions have resulted in the establishment of populations of these species in the wild, the most widespread being the North American signal crayfish *Pacifastacus leniusculus* and the European slender-clawed crayfish *Astacus leptodactylus*. An important and devastating consequence of the introduction of alien species of crayfish has been the spread of the crayfish plague fungus *Aphanomyces astaci*, possibly initiated by infected *P. leniusculus*. Both *A. pallipes* and *A. leptodactylus* are susceptible to the disease, with the

result that populations of *A. pallipes* have been eliminated from whole lengths of river, such as the River Kennet and the Hampshire River Avon. The nature of the disease and the ease with which it is spread means that crafish is an ongoing problem in England and Wales, with new populations of *A. pallipes* regularly becoming affected.

The distribution of alien species in the British Isles is an artificial one, initially determined by man, although consideration of water quality may have determined the choice of aquaculture sites. However, established populations of alien species in the wild will be subject to the same constraints as *A. pallipes*, which will affect their subsequent spread and distribution in the freshwater system of England and Wales. A greater tolerance of environmental factors may allow alien species to inhabit waters not currently occupied by the native species, such as polluted or estuarial waters, and possibly threaten populations of *A. pallipes* protected by "chemical barriers".

This study investigated and compared some of the environmental tolerances of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* and consisted of two parts:

1.) Comparison of the tolerance of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* to four common pollutants; chloride, copper, ammonia and lindane, using lethal and sublethal toxicity tests.

No species was found to have a greater overall pollution tolerance on the basis of the toxicants used in this study. Median lethal concentrations (LC_{50}) obtained from lethal studies

with stage II juveniles indicated that *A. leptodactylus* juveniles were most tolerant of chloride, but were very sensitive to lindane. *P. leniusculus* stage II juveniles were least tolerant of chloride, but of equal or greater tolerance when tested in larger juvenile stages, and were most tolerant of copper. *A. pallipes* juveniles were very sensitive to copper, and all three species showed a similar sensitivity to ammonia. However, episodic experiments with ammonia and copper indicated that differential short-term tolerance to toxicants, i.e. less than 24 hours, may be important in determining the outcome of episodic pollution events on crayfish populations.

Salinity studies indicated high resistance to chloride, with all three species able to tolerate salinities up to 50% seawater, implying freshwater crayfish may be able to tolerate estuarine conditions. Accumulation studies with *P. leniusculus* inferred resistance to high levels of copper in the environment, with a substantial storage capacity for the metal and a regulatory mechanism for excretion of excess amounts. However, establishment of populations under high saline or high copper conditions would be limited by the lower tolerance of early life stages. Similarly, sublethal studies implied that sublethal toxicant effects on early life stages may be important in determining presence or absence of crayfish from a waterbody, through effects on recruitment.

2.) Comparison of the thermal relations of *A. pallipes*, *A. leptodactylus* and *P. leniusculus*, using tolerance, growth and respiration experiments.

Results from tolerance experiments showed that *P. leniusculus* had a greater overall thermal tolerance, so is more resistant to changes in environmental temperature, such as in waters affected by thermal discharges or in waters affected by drought and reduced flow. In growth experiments *P. leniusculus* grew faster at all temperatures tested and was predicted to grow at temperatures unsuitable for the other species. Therefore, *P. leniusculus* would not only be able to survive and grow in conditions unsuitable for *A. pallipes* and *A. leptodactylus*, but will also grow faster where favourable conditions exist for all three species. Field experiments showed that, although *P. leniusculus* juveniles were smaller on release from the female, they were released earlier and their faster growth rate allowed them to maintain a distinct size advantage over *A. pallipes* juveniles, which was very marked by the end of the growing season. Large size is a key element in the attributes leading to competetive success in other crayfish species. Field observations on mixed populations of plague-free *P. leniusculus* and *A. pallipes* indeed show that signal crayfish are superior competitors, eventually eliminating *A. pallipes*. Therefore temperature effects may be important in determining the outcome of competition in mixed crayfish populations..

ACKNOWLEDGEMENTS

Many thanks are owed to my supervisor, Dr David Holdich for his support and encouragement during the course of this study. Thanks are also due to Professor D. Wakelin and Professor P.N.R. Usherwood, for the provision of equipment and laboratory facilities, and to SERC for providing the financial support.

I am also very grateful to the following people: Jim Reader and Martin Sayer for their technical and statistical expertise and, at times, rather strange sense of humour; Tim Smith, Pete Smithurst and Bob Liddle for scrounging, stealing, making or mending equipment for me; Chris Stanley and Ian Reeve for their friendship and making those dull days in the laboratory so much more tolerable; Martin Frayling for his culinary expertise with crayfish and other things. Finally, I could not have completed this thesis without the support, encouragement and patience of my wife, "Shrimp".

CHAPTER 1

INTRODUCTION TO THE STUDY

1.1 Species of crayfish endemic to Europe

There are approximately 500 species of crayfish known worldwide, occupying a wide range of habitats, with species divided between the Astacidae in the Northern Hemisphere and the Parastacidae. They are the largest and longest-lived of the freshwater Crustacea and are often the dominating benthic invertebrate in a water system with respect to biomass. They are highly opportunistic in terms of feeding habits, performing roles of detritivores, carnivores and herbivores (Hogger, 1988), thus acting as key energy transformers between various trophic levels within an aquatic ecosystem (see Figure 1.1) and may indirectly shape community organization in streams (Hart, 1992). Their high biomass compared to other consumers which cannot readily utilise detritus and living vegetation means they transfer energy from the producer level directly to higher trophic organisms. As a consequence, removal of crayfish may result in rapid ecological change, such as the increased eutrophication and growth of aquatic macrophytes in Swedish lakes and ponds after elimination of the noble crayfish *Astacus astacus* by disease (Abrahamsson, 1973). On an economic basis, crayfish may be the most important inhabitant of many waterbodies. Crayfish are a valuable crop and fetch a high price, particularly in N. W. Europe. The aquaculture potential of crayfish and the methods developed for increasing

production have been reviewed (Holdich and Lowery, 1988; Skurdal *et al.*, 1989; Holdich, 1993).

Europe has a relatively impoverished crayfish fauna with only five native species, all belonging to the family Astacidae. The life histories of these species are adapted to suit the cold water habitats where they naturally occur. Typically, moulting is restricted to the warmer summer months when feeding takes place. Mating and egg-laying generally take place in the autumn in response to changes in day-length and water temperature. The eggs are then carried over the winter by the female until hatching occurs early the following summer. Amongst the astacine crayfish some crayfish are able to adapt to a wider range of environmental conditions than others. Although all members of the Astacidae have similar habitat requirements the most adaptable species are clearly the more successful in terms of distribution and abundance (Hobbs, 1988; Hogger, 1988).

Austropotamobius torrentium Schrank, the stone crayfish, is the least adaptable and is mainly confined to central and south-eastern Western Europe (Laurent, 1988), where it shows a preference for cold, fast-flowing, un-polluted upland streams. *Austropotamobius pallipes* (Lereboullet), the white-clawed crayfish, occurs in Western Europe, including the British Isles (Laurent, 1988) where it is at the northern limit of its range. *A. pallipes* is able to inhabit a much wider range of habitats, including both lentic and lotic conditions. *Astacus astacus* (Linnaeus), the noble crayfish, is widespread in eastern, central and northern parts of Europe (Cukerzis, 1988). Where its distribution overlaps that of *A. pallipes*, such as in north-east

France, it favours larger water courses whereas the latter species inhabits the smaller brooks and streams. *Astacus leptodactylus* Eschscholtz, the narrow-clawed or Turkish crayfish, is endemic to the areas surrounding the Black Sea and the Caspian Sea, but has spread widely within Russia and eastern Finland (Koskal, 1988). It has also been introduced into western Europe (Qvenild *et al.*, 1989) and is now found in Poland, Germany, France, Switzerland and Yugoslavia. It inhabits lakes, ponds, rivers and swamps. In the Ukraine, impoundment of major rivers has created lakes which now contain dense populations of this species. Four sub-species of *A. leptodactylus* are recognised, differentiated by their general appearance and form of the carapace and the chelae. Finally, *Astacus pachypus* Rathke is confined to areas of the Caspian and Asov Seas. Little is known of the biology of this species.

With respect to the commercial and culinary value of native species, *A. astacus* has been regarded as a regional delicacy since the Middle Ages (Spitzy, 1972) and demand for this species is still great in the European market, particularly in Scandanavia and France. However, because of the decline in populations due to crayfish plague *A. leptodactylus* has become more important, with Turkey and Russia becoming the main suppliers to the western European market (Holdich and Lowery, 1988; Holdich, 1993). The small size and slow growth rate of crayfish belonging to the genus *Austropotamobius* makes them relatively unsuitable for commercial exploitation (Rhodes and Holdich, 1979), although the potential for the farming of *A. pallipes* in north-central Ireland has been investigated

(Reynolds, 1989). However, in general *A. pallipes* can only be regarded as being important for small-scale, local consumption (Laurent, 1988).

1.2 Introduction of alien species of crayfish

1.2.1 Europe

In Europe, introductions of alien species of crayfish have largely resulted from attempts to restore crayfish populations and associated markets after crayfish plague has caused a decline in native crayfish stocks, although restocking with European species, especially *A. leptodactylus*, in areas where crayfish plague has "burnt out" has occurred, but with not much success (Qvenild et al., 1989). All the European crayfish species are affected by the disease crayfish plague, caused by the epizootic fungus *Aphanomyces astaci* Schikora. This fungus has been described in detail by Alderman and Polglase (1986, 1988). The emergence of crayfish plague is now generally accepted to have occurred in the Po valley, Italy in the early 1860s, either through deliberate introduction of crayfish harbouring the disease or accidentally, e.g. in the ballast tanks of ships (Furst, 1984). The disease spread rapidly north, east and west and most populations of European species have been severely affected throughout their ranges in mainland Europe.

On entering a watercourse the disease spreads quickly from individual to individual and can result in the total elimination of crayfish from a water body. Mainly as a result of this disease

A. astacus and *A. pallipes* are listed as rare and endangered and rare respectively in the Invertebrate Red Data Book of the IUCN (Wells et al, 1983), although at the time the extent of the distribution of *A. pallipes* in Britain was not fully appreciated (Holdich and Reeve, 1991). In 1988, because of threats to its survival, *A. pallipes* was added to the list of species protected by the Wildlife and Countryside Act, although this was confined to "taking" under Section 9(1) and to "sale" under Section 9(5) (Department of Environment, 1988). Asiatic and Australasian species have been shown to be just as susceptible to crayfish plague as European species (Unestam, 1972) and therefore unsuitable for introduction into European waters. However, all North American species tested are highly resistant to infection by *A. astaci*, with a balanced relationship occurring between host and parasite.

In 1890, *Orconectes limnosus* Rafinesque was introduced from North America into a tributary of the Oder River, Germany, to substitute for losses of *A. astacus*. It is now common in water systems throughout Europe, particularly Germany, Austria, Poland, Russia and France (Momot, 1988). Three reasons for its rapid spread are: 1.) fast reproduction; 2.) resistance to crayfish plague; 3.) resistance to mild pollution. However, it is regarded as a gastronomically inferior species due to its sub-standard taste and has very limited commercial value.

More recently the signal crayfish, *Pacifastacus leniusculus* (Dana), has been introduced into and distributed throughout western Europe (Lowery and Holdich, 1988). This species is native to north-western North America. The range of the species has been

increased by introduction to south-western states, such as California, at the end of the nineteenth century (Goldman, 1972) and more recently into Europe in the 1960s (Abrahamsson, 1973). In the USA the species is capable of populating a wide range of habitats, ranging from sub-alpine streams and lakes to dilute brackish water creeks. Initial imports of signal crayfish into Sweden resulted in outbreaks of crayfish plague (Abrahamsson, 1973). Experimental and field evidence has since shown that both *O. limosus* and *P. leniusculus* harbour the crayfish plague fungus as a latent, chronic infection and can therefore function as vectors for the disease (Unestam, 1972, Persson and Soderhall, 1983). For this reason, a hatchery was developed for signals in 1968 at Simontorp for production of signal juveniles within Swedish borders. Many juveniles have now been exported to other European countries, e.g. Finland, Russia, Greece, Poland, Germany, France and England, resulting in established populations in many cases.

The red swamp crayfish, *Procambarus clarkii* Girard, is another North American species to be introduced in large numbers into western Europe (Holdich, 1993). The species is native to north-eastern Mexico and the southern United States (Huner, 1988), but has been introduced extensively in the USA and other countries, such as Brazil, Belize, Portugal, Spain, France, Kenya, China, Taiwan, Japan and Hawaii. This crayfish is very productive and in Spain the yield presently is approximately 3000 tonnes in natural waters, reservoirs and ricefields and there are now populations in the south-western part of France, in Gard and Brittany (Laurent et al., 1991). However, in many places such

introductions have been damaging, rather than beneficial, with damage to crops and structures such as levees and dams.

1.2.2 The British Isles

Prior to the 1970s *A. pallipes* was the only species of crayfish present in the British Isles, and is presently widespread throughout much of England, Wales and Ireland (Holdich and Reeve, 1991). It should be noted that from here on *A. pallipes* will also be referred to as the "native" crayfish, although there is some debate as to what extent the spread of this species was assisted by man after the last Ice Age. Albrecht (1983) concludes that *A. pallipes* gained access to British waters as a result of human activities. In addition, presence of this species in Ireland may also be of human origin (Reynolds, 1979), introduced by continental monastic orders after the tenth century A.D., together with most other Irish fish species (Reynolds, 1989). However, Laurent (1988) does not hold this opinion, arguing that the small size of *Austropotamobius* lessened the interest for its transplantation. Whatever the origin of the species in the British Isles, it is presently an established and integral component of the freshwater system (Huxley, 1880; Hogger, 1984; Holdich and Reeve, 1991).

Since the 1970s the signal crayfish, *P. leniusculus*, and the Turkish crayfish, *A. leptodactylus* have been imported into Britain (but not Ireland) for culinary and aquaculture purposes (Lowery and Holdich, 1988). As a result of escapes from crayfish farms and deliberate introductions into the wild, these alien

crayfish are now widespread in England and Wales and are forming expending populations in a number of areas, including ones previously occupied by the native species (Holdich and Reeve, 1991). As a consequence, people producing alien crayfish for sale or transfer are required to register with the Ministry of Agriculture, Fisheries and Food (MAFF) under a 1985 Order of the Disease of Fish Act (Alderman and Wickins, 1990). Also, in March 1992 *A. leptodactylus*, *A. astacus* and *P. leniusculus* were placed on Schedule 9 of the Wildlife and Countryside Act, making it illegal without licence to release them, or allow them to escape into the wild,, despite the fact that are now ordinarily resident in the wild (Holdich and Rogers, 1992).

1.2.2(i) *Austropotamobius pallipes* (Fig 1.2)

In England and Wales, populations of native crayfish are presently found in every National Rivers Authority region (Fig 1.3). A recent survey, coupled with records provided by other workers, has revealed over 1000 sites where native crayfish have been found since 1970 (Holdich and Reeve, 1987). Populations have been recorded from both standing and flowing waters, including lakes, reservoirs, inundated quarries, rivers and brooks, but are always associated with base-rich, easily-weathered substrates (Laurent, 1988) (see 2.3.1). Most crayfish records are found below an altitude of 120m, presumably as temperature is a limiting factor at higher altitudes. However, the highest population can be found at 400m in Malham Tarn, Yorkshire.

Native crayfish are distributed throughout most of central

Eire, extending also to the east and west coasts. This includes the catchments of the Boyne, Liffey, Barrow, Corrib and Shannon, the latter draining one sixth of Ireland. There are no known populations or attempted implants of alien crayfish anywhere, principally due to an operating import policy that excludes all non-native species of crayfish.

In Scotland, native crayfish are largely absent. However, a population can be found in north-west Sutherland in a coastal loch, Loch Croispol. This population was introduced in the 1940s. The area is affected by the North Atlantic Drift, which has a warming effect in the winter and spring. This population extends the northerly range of this species by approximately 300 km from Northumberland (Holdich and Reeve, 1991).

1.2.2(ii) *Pacifastacus leniusculus* (Fig 1.2)

There has been one controlling factor dictating the initial distribution of signal crayfish within the British Isles, and that is man. The distribution of signals (Fig. 1.4) can be considered as totally artificial, occurring in a haphazard manner, and not yet influenced by any of the constraints affecting the distribution of the native crayfish. The picture is complicated by the uncertainty concerning the success or failure of many of the 250 or so recorded implants (Reeve, 1990). An unknown proportion of successful implants have failed to develop into viable breeding populations, either because of poor husbandry, or ecological resistance in the form of poor water quality, or unsuitable water chemistry.

An initial import of 1000 signals was made in 1976 from the Simontorp hatchery. After successful trials in the south of England by an entrepeneur a franchise was obtained, resulting in the import of some 150 000 juveniles during 1977 and 1978, and the reported stocking of 245 lakes and ponds. With the increase in the number of crayfish farmers the British Crayfish Marketing Association (BCMA) was established, with encouragement from the Government, in order to establish new growers, to ensure high prices, and to discourage poor quality, dumping and price-cutting. A number of individuals imported signals directly from North America and from suppliers other than Simontorp in Europe.

Crayfish farmers were warned not to introduce signals into the wild, but a certain number have done so, or have not taken sufficient precautions to prevent escapes. Breeding populations account for 17% of known implants, of which 8% represent established populations in natural waters (Reeve, 1990). Signals have become established in both closed (water-filled gravel pits) and open (streams) natural waters. In at least three of these waters, the Rivers Lodden, Whitewater and Kennet, the signals have occupied a vacant niche that was created when native populations were eliminated, presumably by crayfish plague. This is largely due to a similar biology shared between native and signal crayfish, the two species being close ecological homologues.

In some river systems, wild populations of signal crayfish are sufficiently dense to represent a significant, but largely untapped, resource. A single commercial crayfish trapper is operating in the Ouse system (Wiles, pers. comm.) and it is

likely that there is smaller scale exploitation of signal populations in the River Kennet and River Thame.

1.2.2(ii) *Astacus leptodactylus* (Fig 1.2)

The main populations of this species are in the Thames Basin (Fig 1.5). At least four lake and pond systems in London, including the Serpentine and Hampstead Heath and Clapham Common ponds, support large populations of Turkish crayfish (Holdich and Reeve, 1991). In addition they occupy approximately 40 km of the Grand Union Canal, from Camden, west through the city to Bullsbridge where the population splits, west towards the Colne Valley and east towards the Thames. The spread of the species is considered to be assisted by the British Waterways Board through their dredging operations. Silt, containing crayfish, may be dredged from one stretch of the canal to be dumped at another site. All of these populations are thought to be derived, both directly and indirectly, from Billingsgate Fish Market, possibly as discarded stock (Reeve, 1990). Two, largely unmanaged, populations are also known to exist in Suffolk in two eutrophic farm ponds. Recently, records of Turkish crayfish along the length of the River Stour have been reported (Holdich, pers. comm.), presumably originating from the pond introductions.

1.2.2(iv) Other species

There are only two records for *Astacus astacus* in the

British Isles. A breeding population is known to exist in an enclosed pond in the Mendips, although its exact location has not been disclosed (Reeve, 1990). Also, an implant was made into Coldingham Loch, Berwickshire. However, no further details are available.

Procambarus clarkii is imported from the Far East and sold at aquarists, pet shops and garden centres under the name Red, Imperial or Emperor "Lobster". Up until March 1992, there were no confirmed wild populations in the British Isles and there was some debate as to whether the species could survive the British climate. However, a population was present in a bathing pond on Hampstead Heath for an unknown period prior to draining, when the animals were removed (Rogers, pers. comm.).

1.3 Factors affecting the distribution of native crayfish

The distribution of the native crayfish in the British Isles has been affected by the following factors;

1.3.1 Geology

The effect of geology upon the distribution of native crayfish operates through the calcium chemistry of waters associated with particular rock types.

In crayfish, as in all other arthropods, size is increased by moulting. Over 90% of total body calcium is lost at the moult in the shed exoskeleton (10% being retained mainly in the gastroliths). Although some calcium may be recovered by eating

the exuvium, the majority is obtained from the external medium by an efficient uptake mechanism in the gills. Therefore, crayfish require a continual supply of calcium, either in ionic form or as a salt, usually bicarbonate (Greenaway, 1974a). The latter contributes to carbonate formation during calcification of the new exoskeleton. Although some magnesium is also incorporated into the exoskeleton (Greenaway, 1974b), a crayfish population cannot survive if there is insufficient calcium in the environment. As a consequence there is an apparent affinity for calcium-rich strata in the distribution of native crayfish (Reeve, 1990).

In Ireland, crayfish are usually found in catchments underlain by Carboniferous Limestone. This rock type forms much of the Midland Plain and is by far the most common stratum in Ireland. Those crayfish not associated with this stratum are located in areas of Calcareous Till, a glacial drift deposit that covers extensive areas, often to considerable thicknesses (Lucey and McGarrigle, 1987). Crayfish are largely absent from areas surrounding the Midland Plain, where the rock types are generally acid, weather-resistant and deficient in calcareous material (Charlesworth, 1963). Similarly in Scotland, the massive band of old, hard, acid rocks across central Scotland have probably prevented the northerly spread of native crayfish (Jay and Holdich, 1981). The population in Loch Croispol is in an area of Durness Limestone, which would appear to provide ideal conditions for crayfish (Reeve, 1990).

In northern England, a large number of crayfish populations are closely associated with Carboniferous Limestone. Similarly,

in the south and east of England a large proportion of crayfish populations are underlain by Chalk strata, consisting predominantly of friable limestone. The material from both Chalk and Carboniferous Limestone strata can also be found in the form of superficial, drift deposits, formed by glacial erosion and deposition. In particular, Boulder Clay may be of importance to the local distribution of some crayfish populations. This deposit is typically tough, compact and tenacious and is often more permanent than local, solid strata. Calcareous Boulder Clay covers much of eastern England and the Midlands. Such deposits are of particular importance to crayfish where the underlying strata is unsuitable, providing sufficient calcium carbonate for the existence and continuation of a population.

1.3.2 Crayfish Plague

The history of crayfish plague in British waters is debatable, but it is clear that there is no evidence for mass crayfish mortalities from the last half-century and, up until the 1980s, native crayfish were widespread and abundant in many areas of the country (Jay and Holdich, 1981; Holdich and Reeve, 1987). The first confirmed outbreaks of crayfish plague in England (and Greece (Theocharis, 1986)) occurred a few years after the introduction of signal crayfish, so it is difficult to escape the conclusion that the two events may be linked. Initial imports of signals included imports directly from North America and from

Germany, thereby bypassing the recognised route via Simontorp. However, imports from Simontorp may have also been infected by the fungus. Initial imports into Sweden were shown to be infected (Abrahamsson, 1971) and hyphae of *A. astaci* have been found in commercially supplied juveniles that would have been derived from those initial imports (Persson, 1979). Signals imported from Simontorp have been implicated with a plague outbreak in Greece (Theocharis, 1986). Also, crayfish obtained from a commercial supplier were shown to be infected with the fungus and were indirectly responsible for a plague outbreak in native crayfish stocks at Nottingham University (Alderman et al, 1990).

Crayfish plague has been confirmed as responsible for the following mortalities of native crayfish. In the Thames Basin, between 1981 and 1988, populations were eliminated in the catchments of the Lea, Colne, Wey and Darent (Reeve, 1990). The initial outbreak in 1981 in the Lee system is detailed in Hogger (1984). Also in 1981, a mass mortality occurred in the Sherston branch of the Bristol Avon. Further mortalities followed in the Tetbury branch of the river in 1982 and 1983 (Alderman et al., 1984). In 1984, native crayfish were eliminated from a 65 km stretch of the Hampshire Avon in a matter of weeks and the cause was rapidly diagnosed as an *A. astaci* infection (Alderman et al., 1984). Additional outbreaks have occurred in Ireland, in a number of loughs in the Shannon system, in 1987 (Reynolds, 1988) and in the Dowles Brook catchment of the Wyre Forest in 1988 (Reeve, 1990). Crayfish plague has also been implicated, but not confirmed as the cause, in the loss of native crayfish from the River Lodden and its tributary the Whitewater, and from the

entire length of the River Kennet.

In some cases, evidence would strongly indicate that signal crayfish implants were responsible for the plague outbreak. However, a number of workers have shown that crayfish plague can be transmitted on vectors other than crayfish. Human vectors have figured prominently, both through the initial import of infected signal crayfish and through other activities. There is a suggestion that *A. astaci* was introduced into the River Lee on equipment used by biologists studying another crayfish population (Lowery and Hogger, 1986). Similarly, the outbreak at Nottingham University was probably due to students collecting macrophytes with nets from tanks containing infected signal crayfish, then using the same nets in tanks holding natives (Alderman et al., 1990). Divers and fishermen have been implicated in the transfer of *A. astaci* to Ireland (Reynolds, 1988). Other vectors implicated in the transfer of *A. astaci* include salmonids (Alderman et al., 1987; Hall and Unestam, 1980), waterfowl and mammals, such as otters and mink.

According to Reeve (1990), since the initial mortalities in 1981, an estimated 22% of native crayfish populations in England and Wales (post-1970) have either been eliminated, or are at risk due to the presence of crayfish plague in the catchment. This is likely to increase due to the ease of dissemination of *A. astaci* and the enormous reservoir for the disease in wild signal crayfish populations.

1.3.3 Water Quality

The first survey of water quality in rivers in England and Wales was organised by the former Ministry of Housing and Local Government in 1958. This was followed by surveys organised by the Department of the Environment (DoE) in 1970 and 1975, with intermediate updating for the years 1971 and 1972. In 1980, the National Water Council (NWC) was given the task of organising the survey and presenting the results (NWC, 1981). This survey used a revised river classification system (NWC, 1978), which was adopted by water authorities in England and Wales and the DoE. Further surveys were carried out in 1985 (DoE, 1986) and 1990 (National Rivers Authority, 1991).

The water classes specified in the NWC scheme are broadly related to the potential use of the waters, especially in support of fisheries and are as follows:

Class 1A

Waterbodies in this class represent water of high quality suitable for potable extraction, and have high amenity value e.g., as game or other high class fisheries. Average Biochemical Oxygen Demand (BOD - determined from a 5 day carbonaceous test) is probably not greater than 1.5 mg/l and there should be no visible evidence of pollution. The class limiting criteria (95 percentile) are as follows;

i) Dissolved oxygen (DO) saturation greater than 80%.

11) BOD not greater than 3 mg/l.

iii) Ammonia not greater than 0.4 mg/l.

iv) Non-toxic to fish in European Inland Fisheries Advisory

Commission (EIFAC) terms. (Based on "Water Quality Criteria for Freshwater Fish". Alabaster and Lloyd (1980)).

Class 1B

This represents waters of less high quality than Class 1A, perhaps due to a high proportion of high quality effluent present or because of the effects of physical factors, such as canalisation, low gradient or eutrophication, but is usable for substantially the same purpose. Criteria are as follows;

i) DO greater than 60%.

ii) BOD not greater than 5 mg/l (average BOD probably not greater than 2 mg/l).

iii) Ammonia not greater than 0.9 mg/l (average probably not greater than 0.5 mg/l).

Class 2

Such waters are of moderate amenity value, supporting reasonably good coarse fish populations, and are suitable for potable supply after advance treatment. The water should not show physical signs of pollution, other than humic colouration and a little foaming below weirs. Also;

i) DO should be greater than 40% saturation.

ii) BOD should not be greater than 9 mg/l (average BOD probably not greater than 5 mg/l).

Class 3

These waters are polluted to such an extent that fish are absent, or are only sporadically present. The water may be used for low-grade industrial purposes, but has considerable potential for further use if cleaned up. Also;

i) DO should be greater than 10% saturation.

ii) They are not likely to be anaerobic.

iii) BOD is not greater than 17 mg/l.

Class 4

These are grossly polluted waters, which are inferior to Class 3 in terms of oxygen and are likely to be anaerobic at times. In EIFAC terms they are incapable of supporting fish.

Class X

Insignificant watercourses and ditches, not usable, where the DO is greater than 10% saturation.

In most instances, chemical classification given above will be suitable. However, there may be circumstances where BOD, dissolved oxygen levels, or ammonia concentration may be outside the stated level for a class, e.g. extreme weather conditions (flood or drought) or when a water body is dominated by plant growth. Also, as the classification is based on a few chemical parameters, there may be cases where water quality is markedly reduced by chemicals other than those used in the classification. Where this is the case, the classification is made on the basis of the biota actually present.

Reeve (1990) tabulated the distribution of native crayfish populations in England and Wales within the various grades of water quality, according to the river quality survey of 1985 (DoE, 1986). This data is shown in Table 1.1.

It can be seen from the table that the greatest proportion of native crayfish records are allied to Class 1A and 1B waters. In comparison, the number of records allied to waters of class 3 and 4 are negligible. This would strongly suggest that waters of

class 3 and 4 are lethal to crayfish. Those few records that do exist for these waters probably represent unconfirmed, pre-1970 records for water bodies that have since been downgraded, with the probable loss of the crayfish population. Class 1 waters sustain healthy breeding populations, whereas waters of class 2 seem to be intermediate, with some waters being suitable for crayfish and others not. The class 2 chemical characteristics should therefore represent the limit of tolerance of native crayfish to water quality, with respect to oxygen, BOD, etc. (see above). However, these waters potentially receive a wide variety of mild pollutants, which are too great to estimate accurately the level of pollution that would dictate the presence or absence of a crayfish popualtion.

Associated with water quality is river engineering. In a number of European countries it has been observed that various types of engineering operations (clearing, canalizing, dredging, changing of river beds, damming of rivers, regulation of river levels and stream flows and forest ditching) have caused, directly and indirectly, damage to crayfish populations. The crayfish is very sensitive to changes in habitat, particularly as it is a slow moving bottom dweller, confined to a narrow littoral zone. Operations such as dredging and construction work frequently cause long-term turbidity, with an increase in suspended solids and a decrease in dissolved oxygen content downstream. More directly, Lowery and Hogger (1986) observed an operation to remove aquatic grass, *Glyceria maxima*, from a tributary of the River Lea. Up to 137 crayfish were removed at each operation stroke using a 0.73 m^3 dredge. More important are

permanent alterations to the crayfish habitat, such as removal of favourable habitat features, e.g. stones, vegetation and steep banks (Niemi, 1977).

1.4 Introduction to the present study

Certain parallels may be drawn between the spread of alien species of crayfish in England and Wales and the dramatic range expansion of the rusty crayfish, *Orconectes rusticus*, in the mid-western United States. The factors behind this range expansion are not fully understood, although much of the spread has resulted from accidental and intentional introductions by man (Butler and Stein, 1988). This species has displaced endemic species adjacent to its range in Indiana and Ohio, and is having a similar effect on native species in other areas (Capelli, 1982). Once in a new habitat *O. rusticus* has several attributes (e.g. higher metabolic and growth rates, aggressive behaviour, greater resistance to low pH, reproductive interference with other species and a greater fecundity), which may allow it to survive and compete successfully with native species (Berril et al., 1985; Butler and Stein, 1988).

Such attributes may contribute to the expansion of alien species of crayfish in Britain, although the problem is complicated by the fact that some *P. leniusculus* populations carry crayfish plague, to which *A. pallipes* is highly susceptible. However, studies on mixed populations of plague-free *P. leniusculus* and *A. pallipes* (Holdich and Reeve, unpub.; Holdich and Domaniewski, unpub.) have shown that *P. leniusculus*

eventually eliminates *A. pallipes* through competition for resources and through cannibalism. Factors which allow this species to do this may include higher growth rate and larger adult size, higher fecundity, timing of reproduction and moulting, and greater aggression. Similarly, *A. leptodactylus* seems superior to other European species of crayfish in several respects, especially in warmer climates. It is reported to grow and propagate faster and has a wider habitat preference. Its temperature optimum is higher than for other species and it is very tolerant to high temperatures (Fürst, 1989).

With regards to resistance to environmental factors, it has been shown that polluted waters (Classes 2, 3 and 4) can effectively restrict the range of the native species (see 1.3.3). In the Trent Basin, crayfish are absent from many rivers they used to inhabit. Although, crayfish plague has positively been identified in the Wyre Forest area (Reeve, 1990) and implicated in the Wye and Derwent (Holdich, pers. comm.), elsewhere in the region, water pollution has been implicated, particularly sewage, heavy metals and pesticides. Little data exists on the effects of environmental parameters on *A. pallipes*, although there is a great deal of information on general physiology and ecology, and population dynamics (see Holdich and Lowery, 1988, for reviews). Other studies on this species have been limited, and include the effects of abnormal levels of pH (Jay and Holdich, 1976), toxicity studies with lindane and cadmium (Mees, 1983) and the effect of simulated farm waste effluents (Foster and Turner, 1993). Similarly, little data exists to suggest whether the two introduced species are more or less tolerant of environmental

factors, the only study to date being the effect of nitrite on *P. leniusculus* (Harris and Coley, 1991). Such data could be used to predict whether alien species could survive in the wild in waters unsuitable for *A. pallipes*.

At present, populations of native crayfish in flowing waters such as in the Trent Basin are generally restricted to the upper reaches of small tributaries, due to the degree of pollution in the main rivers. These stretches of polluted water effectively act as buffers, or "chemical barriers", between populations of crayfish and could therefore prevent the spread of crayfish plague within the river system. Studies on the environmental tolerances of the three species would indicate whether native populations would be at risk from alien species spreading through these polluted waters, if they were introduced into the Trent Basin and other areas.

In addition, some idea may be gained whether alien species can occupy waters in this country not currently inhabited by crayfish. For example, the spread of Turkish crayfish in the River Stour, mentioned above, could eventually lead to them entering a tidal area. They are known to occupy saline conditions in the Caspian Sea (Cherkasina, 1975), as do signal crayfish in the Sacremento Delta in the United Staes (Rundquist and Goldman, 1978). Also, parts of the Trent Basin are polluted by saline waters originating from coal mining activities. Native crayfish avoid these areas (Mees, 1983) but they could possibly be inhabited by alien species.

The present study is the first in which a comparative study has been made of native and alien crayfish species under the same

experimemtal conditions. In order to assess the environmental tolerances of the three species, two main types of study were undertaken. Firstly, a series of lethal and sublethal toxicity tests were performed, using pollutants found routinely in rivers in many parts of the country, particularly in the Trent Basin, i.e. copper, chloride, ammonia and lindane. Secondly, as temperature is such a key environmental parameter, experiments to determine the thermal tolerance of the three species, and the effects of temperature on growth and respiration were carried out. This has particular relevance to waters which are heated by industrial effluents, e.g. from the electricity generating industry, and direct solar heating of waters reduced in flow and volume by drought conditions. Also, temperature is a major consideration in the aquaculture industry, in determining the optimum conditions for production.

Finally, following stocking of crayfish into natural water bodies for farming purposes and the exploitation of wild populations, the potential for the accumulation of toxicants in crayfish tissues is investigated.

Table 1.1: Numbers of *Austropotamobius pallipes* records allied to different NWC class waterbodies in England and Wales (from Reeve, 1990).

NWC water quality class (see text for details)	Length of non-tidal rivers and canals		Number of native records allied to each class	
	Length (km)	Percentage of total	Number	Percentage of total
1A	13511	33	372	42
1B	13912	34	381	42
1A + 1B	27423	67	753	84
2	9740	23	132	15
3	3607	9	7	1
4	646	1	1	0
Totals	41416		893	

Figure 1.1: Diagrammatic food web showing the trophic position of freshwater crayfish (from Hogger, 1988).

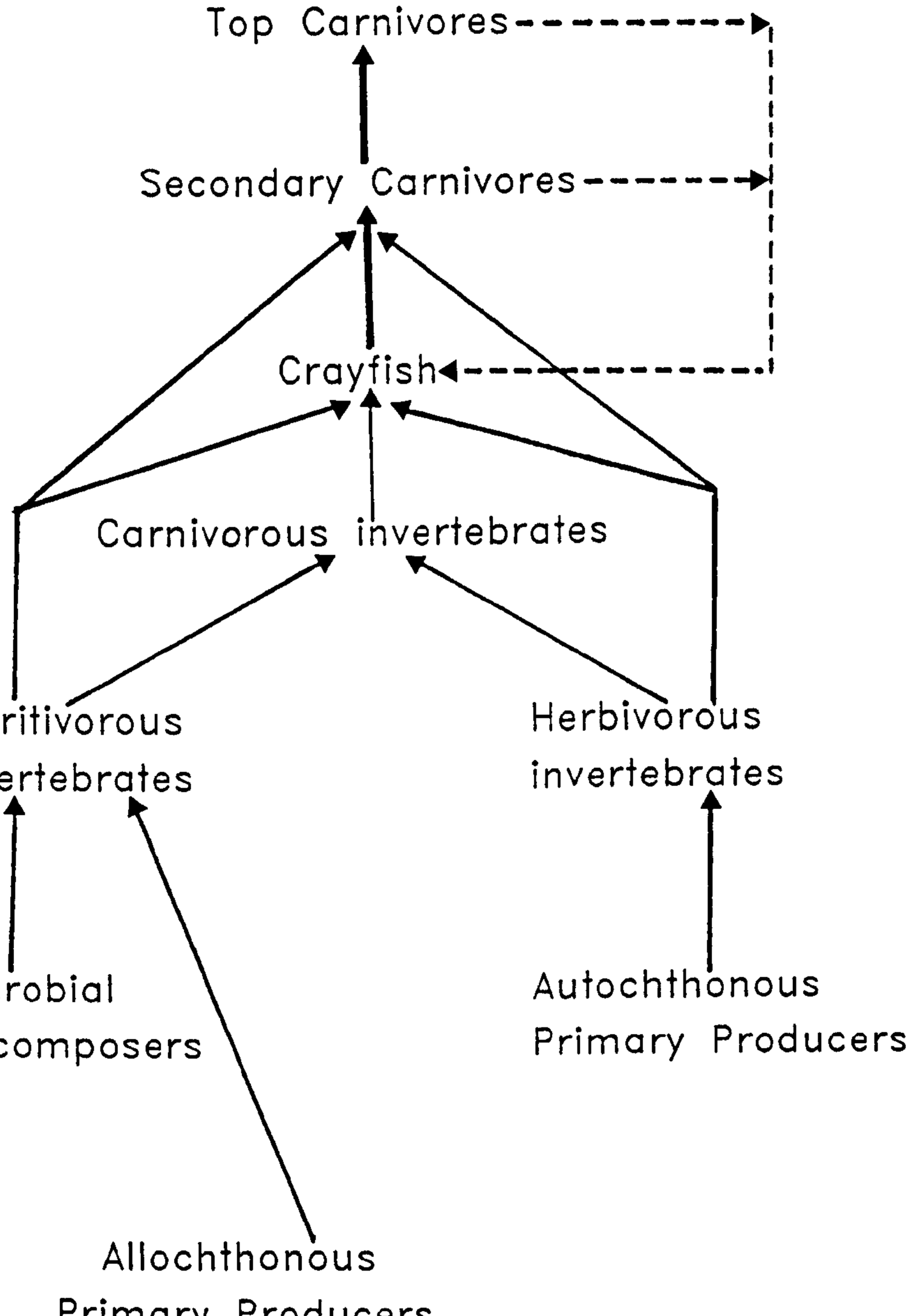
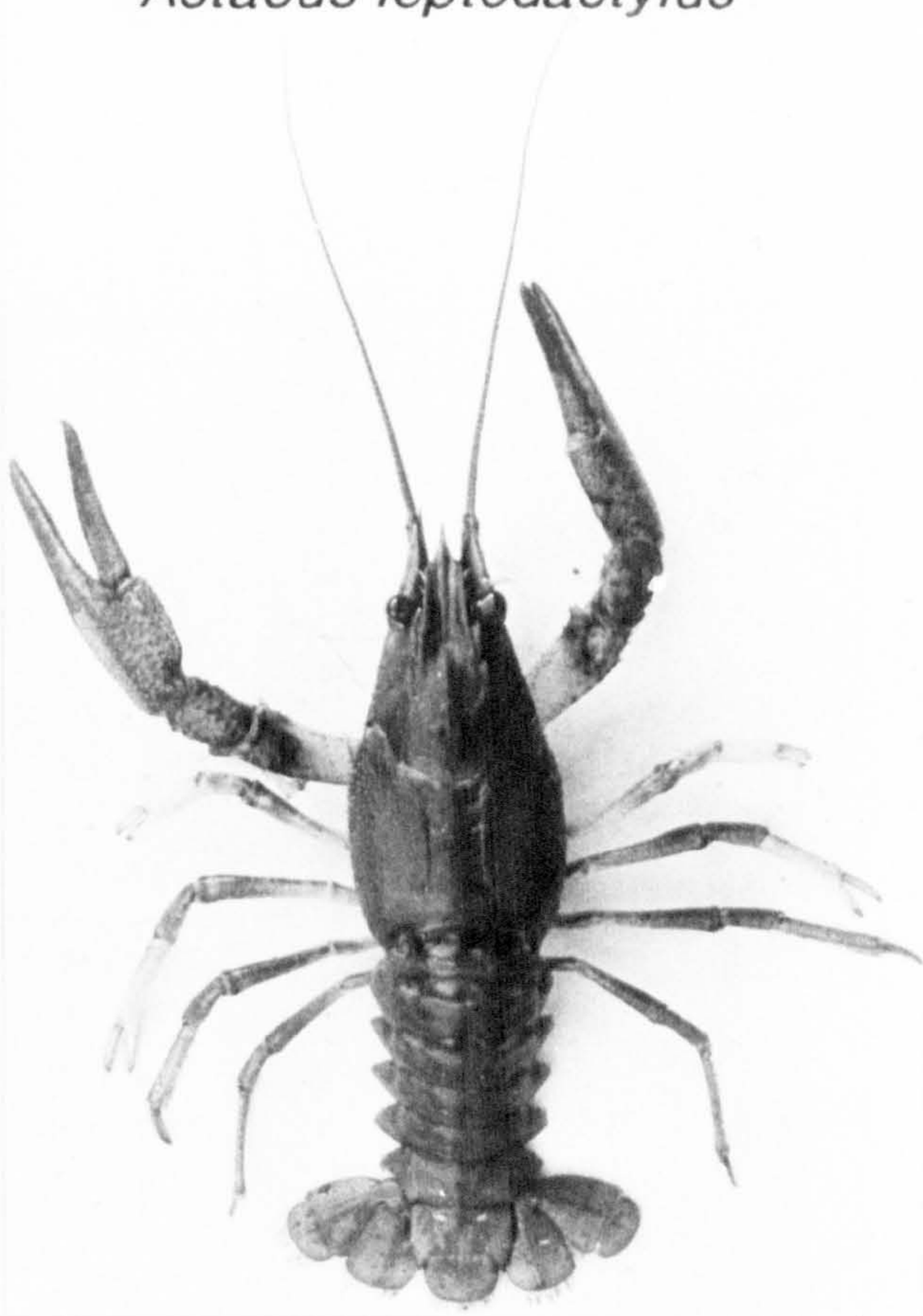


Figure 1.2: Male specimens of *Austropotamobius pallipes* (life size), and *Astacus leptodactylus* and *P. leniusculus* (1/2 life size).

Pacifastacus leniusculus

Astacus leptodactylus



Austropotamobius pallipes

Figure 1.3: The distribution (in 10 km squares) of *Austropotamobius pallipes* in the British Isles based on all records from 1970-1991 inclusive (from Holdich and Reeve, 1991).

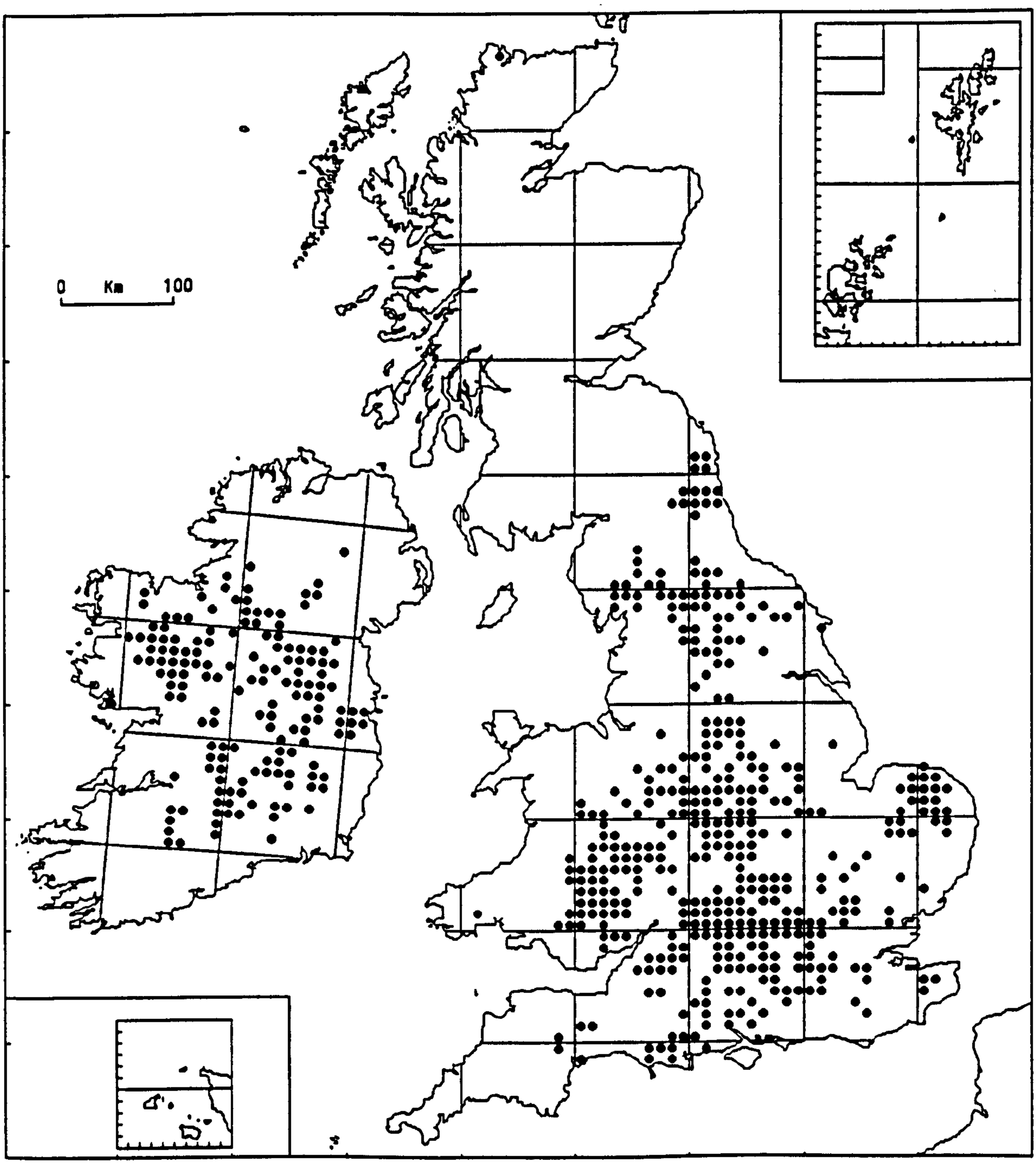


Figure 1.4: The distribution (in 10 km squares) of all known implants of *Pacifastacus leniusculus* in England, Scotland and Wales (from Holdich and Reeve, 1991).

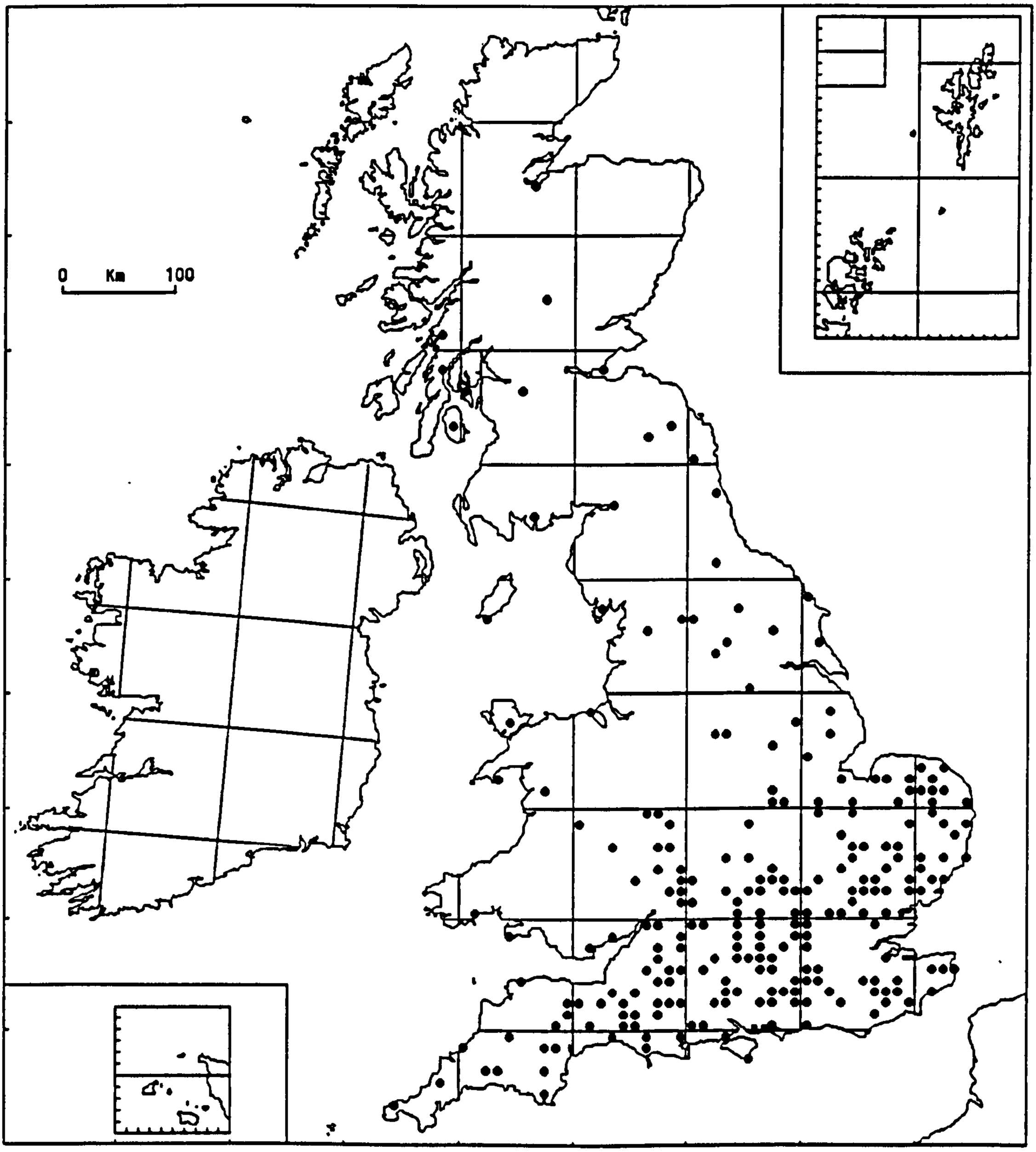
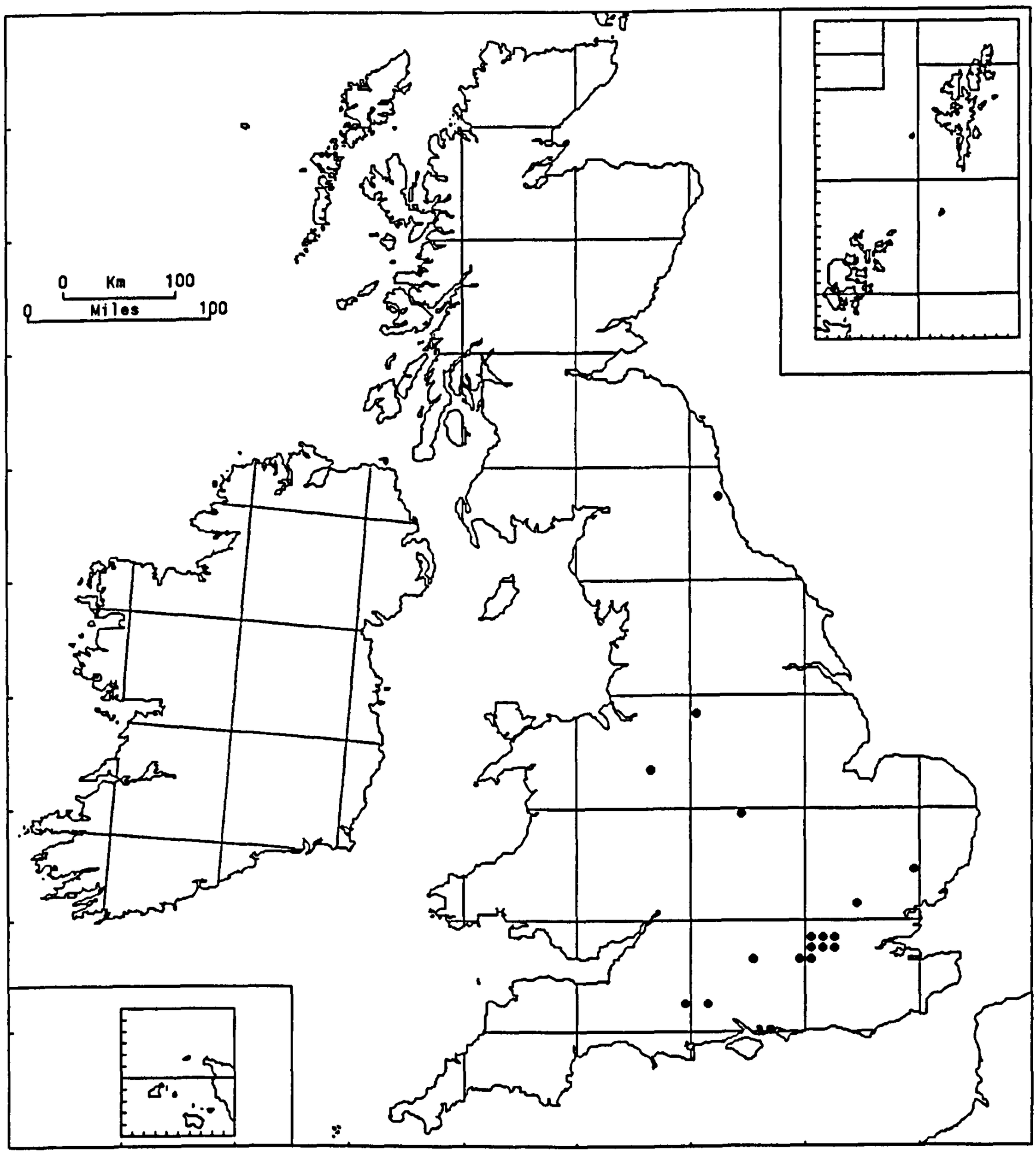


Figure 1.5: The distribution (in 10 km squares) of all known implants of *Astacus leptodactylus*



CHAPTER 2

INTRODUCTION TO POLLUTION STUDIES

Besides geology, one of the major factors influencing the national distribution of *A. pallipes* is water quality (see 1.3.3), with native crayfish associated with grade 1A and 1B waters, suggesting a preference for unpolluted water with dissolved oxygen greater than 60%, and BOD and ammonia less than 5 mg l^{-1} and 0.9 mg l^{-1} respectively. In the Trent Basin, this has resulted in native crayfish populations being confined to relatively small, good quality headwaters, due to the lower water quality of the main watercourses acting as "chemical barriers". Native crayfish would not appear to be able to tolerate the level of pollution in grade 3 and 4 waterbodies, although a number of established populations exist in grade 2 waters. These waters seem to occupy an intermediate position, with some waters being suitable for crayfish and others not. Grade 2 waters receive a variety of mild pollutants, which may include toxic materials or suspended solids, so it is impossible to estimate the exact level of pollution that would dictate presence or absence of a crayfish population. However, these waters appear to represent the limit of tolerance to pollution for a sustainable crayfish population..

The term "pollution" covers a wide variety of definitions. These are reviewed in Warren (1971), but probably the most satisfactory definition of the word is given by Holdgate (1979): *"The introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living*

resources and ecological systems, damage to structure or amenity, or interference with legitimate uses of the environment."

Under this definition, events such as the seasonal de-oxygenation of some waters would not be regarded as pollution, although this has been included under the term "natural pollution" by some authors (Hynes, 1960). However, there are still many pollutants occuring in freshwater that fit the broad definition quoted above. Mason (1981) lists 15 catagories of pollutants found in freshwater, although a more succinct list of pollutant types is given by Jeffries and Mills (1990). These are non-toxic pollutants, toxic pollutants, thermal pollution, pathogens and human/recreational impact. The category of toxic pollutants is by far the broadest, and consequently a large volume of work has been published on the effects of toxins to freshwater organisms. The major types of toxic pollutants have been listed as acids, alkalis, organic compounds, heavy metals, gases and anions (Mason, 1981), and the major sources of these materials are given in Hellawell (1986).

Pollutants can exert a wide variety of toxic effects at different levels of biological organisation and a correspondingly wide range of investigative methods have been used in their study. The main types of toxic effects are given by Sprague (1969) and are defined as:

Lethal toxicity: Toxic action directly causing the death of an organism.

Sublethal toxicity: Toxic action resulting in effects on the organism other than its death.

Acute toxicity: Toxic action (lethal or sublethal) whose effects

manifest themselves quickly (by convention within a few days).

Chronic toxicity: Toxic action (lethal or sublethal) whose effects manifest themselves over a longer period (by convention, within periods of weeks or months, rather than days).

Cumulative: Toxic effect brought about, or increased in strength, by successive doses.

The most commonly used method of quantitatively expressing the effect that a potential pollutant has on an organism is the determination of the "lethal concentration" (LC). This is an acute lethal toxicity test where the lethal concentration is the concentration of chemical required to cause a certain percentage mortality in a given time. Thus a 96 hour LC_{50} is the concentration of a (toxic) material which kills 50% of the test organisms in 96 hours. Acute lethal toxicity tests were initially developed for fish and are reviewed by Sprague (1969) and Buikema *et al.* (1982) and a standard methodology has been adopted to allow direct comparison between tests (HMSO, 1981). Consequently, a wealth of data has been generated on the acute toxicity of pollutants to fish. However, invertebrates are often more sensitive to toxicants than fish (Patrick *et al.*, 1968) and clearly represent a greater majority of the biomass in a natural system. In addition, macroinvertebrates are organisms which fish are often dependent on for food, so that use of macroinvertebrates in toxicity testing is essential for protection of aquatic ecosystems (Maciorowski and Clarke, 1980). As a result, toxicity tests with organisms other than fish have become increasingly common.

The response of crayfish species to pollutants has been

reviewed in Hobbs and Hall (1974). However, much of the information they give on the effects of pollution on crayfish is derived indirectly from investigations of comparative physiology, environmental adaptations, or natural history, rather than from studies directly relating to impacts of particular contaminants. More recently, toxicological research using freshwater crayfish has been reviewed by France (1985). Crayfish more than amply meet the criteria identified by the United States Environmental Protection Agency (Buikema *et al.*, 1982) for the selection of a species for toxicity studies. They:

1.) represent an ecologically important group.

2.) occupy a position in the food chain leading to man or other important species.

3.) are widely available (use of indigenous species is preferable), amenable to laboratory testing and easily maintained in the laboratory. In addition, there is adequate background information on physiology, genetics, taxonomy and their role in the environment.

Leonhard (1979) proposed the use of the crayfish *Orconectes virilis* as "toxicological tools" in the laboratory. However, acute lethal toxicity testing in general, and with crayfish in particular (France, 1985), has been criticised as having little relevance to the determination of the ultimate ecological consequences of pollution. Few experiments have incorporated basic life-cycle information into experimental design. Early life stages may be more sensitive to pollutants than adult stages (Buikema and Benfield, 1979). In addition, few have investigated temporal effects, or have monitored prolonged mortality. More

importantly, toxicity tests are often carried out at unrealistically high toxicant concentrations and have failed to relate toxicity data to field-derived information. In this study, an attempt was made to integrate as many of these factors as possible into the experimental design and subsequent interpretation of the data.

France (1985) also indicated that sublethal experiments formed less than a quarter of all studies on freshwater crayfish. As a result of dilution and dispersion of pollutants from point source inputs in the natural environment, chronic exposure to sublethal concentrations of pollutant is likely to affect a much greater biomass than exposure to acute lethal concentrations (Kleerekoper, 1976). Sublethal effects are those defined as subtle changes in an organism's homeostasis induced by environmental stress, that produces no obvious, or gross short-term damage. Sublethal studies on crayfish have included investigations on the effects of pollutants on repiration in tissues (Hubschmann, 1967) and eggs (Appleberg, 1980), growth (Hubschmann, 1967), reproduction (Brown and Avault, 1974) and bioaccumulation (Zia and Alikhan, 1986).

In this study, the lethal and sublethal effects of four major pollutants on *A. pallipes*, *A. leptodactylus* and *P. leniusculus* were investigated. The pollutants were as follows: chloride, from mining and sewage effluents; copper, from coal mining and industrial effluents; ammonia, from sewage and agricultural effluents; lindane, a pesticide, from agricultural practices. As well as being a unique comparative study of the relative tolerance of the three species to these toxicants, the

data from these experiments may indicate whether *A. leptodactylus* and *P. leniusculus* are more tolerant of pollution in general than the native species. If so, these species could inhabit waters not occupied by *A. pallipes*, or could threaten populations of native crayfish in headwaters protected by so called "chemical barriers".

CHAPTER 3

GENERAL METHODS TO PART 1

3.1 EXPERIMENTAL ANIMALS

The three species of crayfish used in these studies, i.e. *A. pallipes*, *A. leptodactylus* and *P. leniusculus*, were obtained from breeding colonies maintained at Nottingham University. Animals were kept in large outdoor concrete holding tanks, which were subject to natural seasonal variations in ambient conditions. The floor of the tanks were covered with gravel, broken bricks and lengths of drainpipe, to act as hides, and limestone chippings in order to maintain calcium concentrations in the water. Each tank was trickle-fed with Nottingham mains water from the potable supply, which was fed into the tanks via a sprinkler head to aid aeration. Whilst held in the tanks, the crayfish were fed regularly with a proprietry pelleted dog food ("Minced Morsels", Quaker Oats Ltd.). In addition, aquatic weed collected from the field was provided. As a consequence of this a natural flora and fauna developed in the tanks, which consisted of *Cladophora*, and macroinvertebrates such as snails, *Asellus* and *Gammarus*, which may have also contributed to the diet.

There is ample evidence that different stages in the life cycle of aquatic organisms show differing responses to toxicants, with juvenile macroinvertebrates generally being more sensitive than mature animals (Buikema and Benfield, 1979, Pascoe, 1987). Immature animals may be more sensitive for a number of reasons, including; a.) they have a larger surface area:volume ratio and

so have a greater exchange with the environment. b.) they may have a higher initial lipid content, facilitating uptake of lipid-soluble toxicants. c.) they have a greater weight-specific metabolism, thereby facilitating the uptake of toxicants and d.) in arthropods, they moult frequently during the developmental stages. In freshwater crayfish, juveniles hatch and remain attached to the mother for two moults (Holdich, 1992). After the first moult the juveniles (stage II) are semi-independent and can be removed from the female by gentle shaking. For the above reasons this stage was chosen for the initial experiments.

Berried female crayfish of the three species were monitored in the outdoor tanks until their eggs hatched. They were then moved indoors and housed in individual, aerated aquaria at 13 °C and further monitored until the juveniles had moulted to the stage II. The female was then removed and the juveniles held in the tank until required. Larger juvenile stages were used in some experiments and were collected from the outdoor tanks and sorted into size classes. These were also held indoors at 13 °C until required.

3.2 MATERIALS AND METHODS

Efforts were made to standardise experimental procedures, so that tests on the different species would be directly comparable. A number of guidelines have been outlined for toxicity testing by various authors (Sprague, 1969; Buikema *et al.*, 1982) and were incorporated as far as possible. All experiments were carried out in a temperature controlled room (13

+ 1 °C) in a light regime of 12 hours light : 12 hours dark. Animals were fed for at least 48 hours before testing, however no food was provided during the test itself.

3.2.1 ACUTE LETHAL TOXICITY TESTS

Except for the ammonia toxicity test, a static testing regime was used in which animals were individually contained in 5cm diameter petri-dishes. A small square of black nylon mesh was provided for the crayfish to cling to and the dishes placed on a black background, in order to minimize in-test stress. Warlen and Engel (1978) have evaluated flow-through versus static bioassay systems and have concluded that the former should be used where possible as a reduction in toxicity is often observed in static systems, possibly due to a reduction in toxicant concentration due to adsorption, uptake by the test organism and complexation with biologically generated compounds. For this reason copper, chloride and lindane test solutions were replaced every 24 hours and samples of test solutions taken to determine actual toxicant concentrations.

For ammonia it was decided that a continuous flow-through system (Fig 3.1) would be used, due to the difficulty in maintaining a constant concentration of un-ionised ammonia in a static system. Animals were placed on nylon mesh platforms in 400 ml beakers (Fig 3.2). Test solutions were delivered continuously by peristaltic pump through tubing pushed through the base, so that the solutions overflowed into a sump. Replacement of test solutions was 95% in approximately 3 hours. Animals were

prevented from escaping by a petri-dish lid on top of the beaker. Samples of the test solutions were taken every 24 hours.

All materials used in the testing apparatus were acid-washed in 10% nitric acid for 24 hours, then thoroughly rinsed in deionized water. Temperature, pH and hardness were determined daily during the tests. Aerated, dechlorinated Nottingham tapwater was used as diluent in all of the experiments. The chemical characteristics of the diluent water are shown in Table 3.1. No aeration was carried out during short-term toxicity tests in case of volatilisation of the toxicant, especially in the case of lindane.

Preliminary rangefinding experiments were carried out to determine suitable toxicant concentration ranges. Solutions for each test were made up using suitable analytical grade (99.9 %) reagents (B.D.H, AnalaR). Lindane solutions were made up from a 10 % stock solution of gamma - HCH in acetone. Controls in the lindane trials therefore had an amount of acetone added to the water equal to the amount present in the highest concentration of lindane used.

Actual test concentrations were determined as follows. Chloride was measured using a silver nitrate/potassium chromate titration kit (Palintest Ltd., England). Tablets containing pre-determined and fixed amounts of chemical reagent were added to 50 ml aliquots of test solution. Tablets were added and the solution stirred until the end-point of the titration was reached, as indicated by a change in colour of the solution from purple to dark blue. Chloride concentration in mg l^{-1} was derived from the formula (Number of tablets added - 1) x 20.

Copper concentrations were measured by atomic absorption spectrophotometry (AAS) of 50 ml samples acid-fixed (pH 1.0) with 1 ml analytical grade nitric acid. Analysis was carried out using a Pye Unicam SP9 Atomic Absorption Spectrophotometer (Pye Unicam Ltd., Cambridge). A calibration curve was derived from a series of standard solutions made up from deionised water and a 50 mg l^{-1} stock solution of $CuSO_4.5\ H^2O$. Standard solutions were as follows; 0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 mg l^{-1} Cu^{2+} and were acidified as above. Spectrophotometer readings were plotted against standard copper concentration and the calibration curve found to be linear over the range of standards used. Copper concentrations in test solutions in mg l^{-1} were then read directly from the calibration curve using the spectrophotometer reading obtained for each solution.

Determination of total ammonia was carried out using the phenol hypochlorite colormetric method of Harwood and Khun (1970). Analysis of samples was carried out using a Pye Unicam SP600 Spectrophotometer (Pye Unicam Ltd., Cambridge). A calibration curve was derived from a series of standard solutions made up from deionised water and a stock solution of 10 mg l^{-1} $(NH_4)_2SO_4$. Standard solutions were as follows; 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 2.5 mg l^{-1} total ammonia. Percentage absorbance values of the standard solutions were plotted against total ammonia concentration and the resulting calibration curve found to be linear for the range of standards used. Total ammonia concentrations in mg l^{-1} for test solutions were read directly from the calibration curve using the percentage absorbance of each solution. Concentration of un-ionized ammonia (NH_3) was then

calculated from total ammonia concentration and average test temperature and pH using the formulae of Emerson *et al.* (1975):

$$NH_3\ (\%) = 1/(10^{pKa-pH}+1) \times 100$$

Where $$pKa = 0.09018 + 2729.92/T\ (Kelvin)$$

Concentrations of lindane were to be measured by gas liquid chromatography. However, facilities to do so were unavailable at the time of the experiment, so test concentrations are expressed as nominal concentrations.

Ten animals were exposed per concentration and were inspected at 24, 48, 72 and 96 hours, after which any surviving animals were transferred to clean water. During a test death was indicated by a lack of movement of the pleopods and antennae after mechanical stimulation with a blunt probe (Del Ramo *et al.*, 1987). Test mortality data were then analysed by Probit analysis, carried out on a BBC model B microcomputer, running a version of an "Applesoft" BASIC program by Lieberman (1983), modified by Martin (1986). Median lethal concentrations and 95 percentile limits were determined from analysis of percentage mortality at each test concentration with time.

Table 3.1: Chemical characteristics of diluent water (Nottingham Tap Water) used during toxicity experiments.

Parameter	Mean (n=12)	± Standard Error
Temperature (°C)	**13.75**	0.11
Oxygen (mg l^{-1})	**10.03**	0.18
pH	**7.34**	0.23
Conductivity (uS cm^{-1})	**402**	17.1
Hardness (mg l^{-1} $CaCO_3$)	**146**	5.71
Bicarbonate alkalinity (mg l^{-1} $CaCO_3$)	**118**	10.1

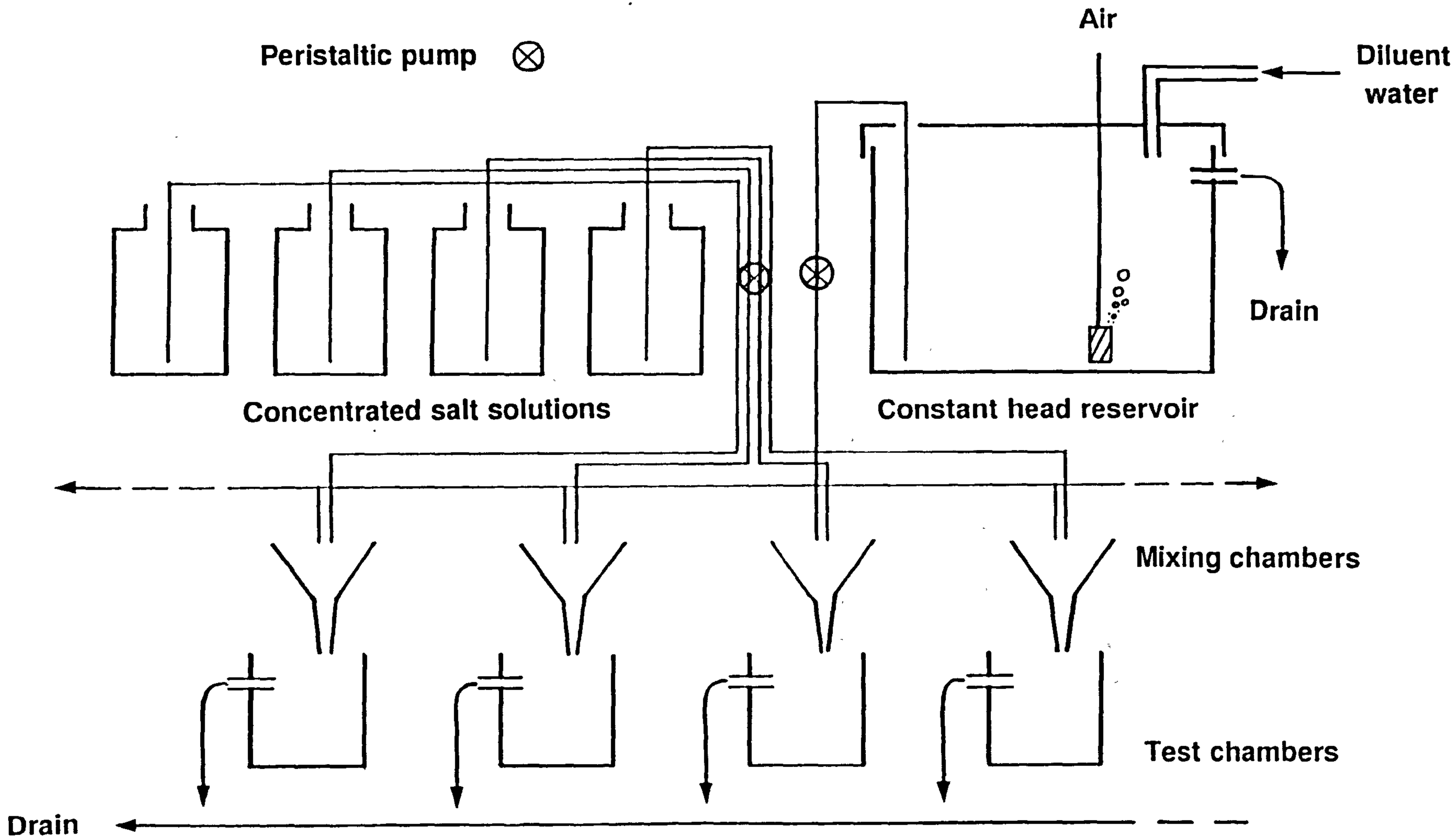


Figure 3.1: Continuous flow apparatus used during ammonia toxicity experiments.

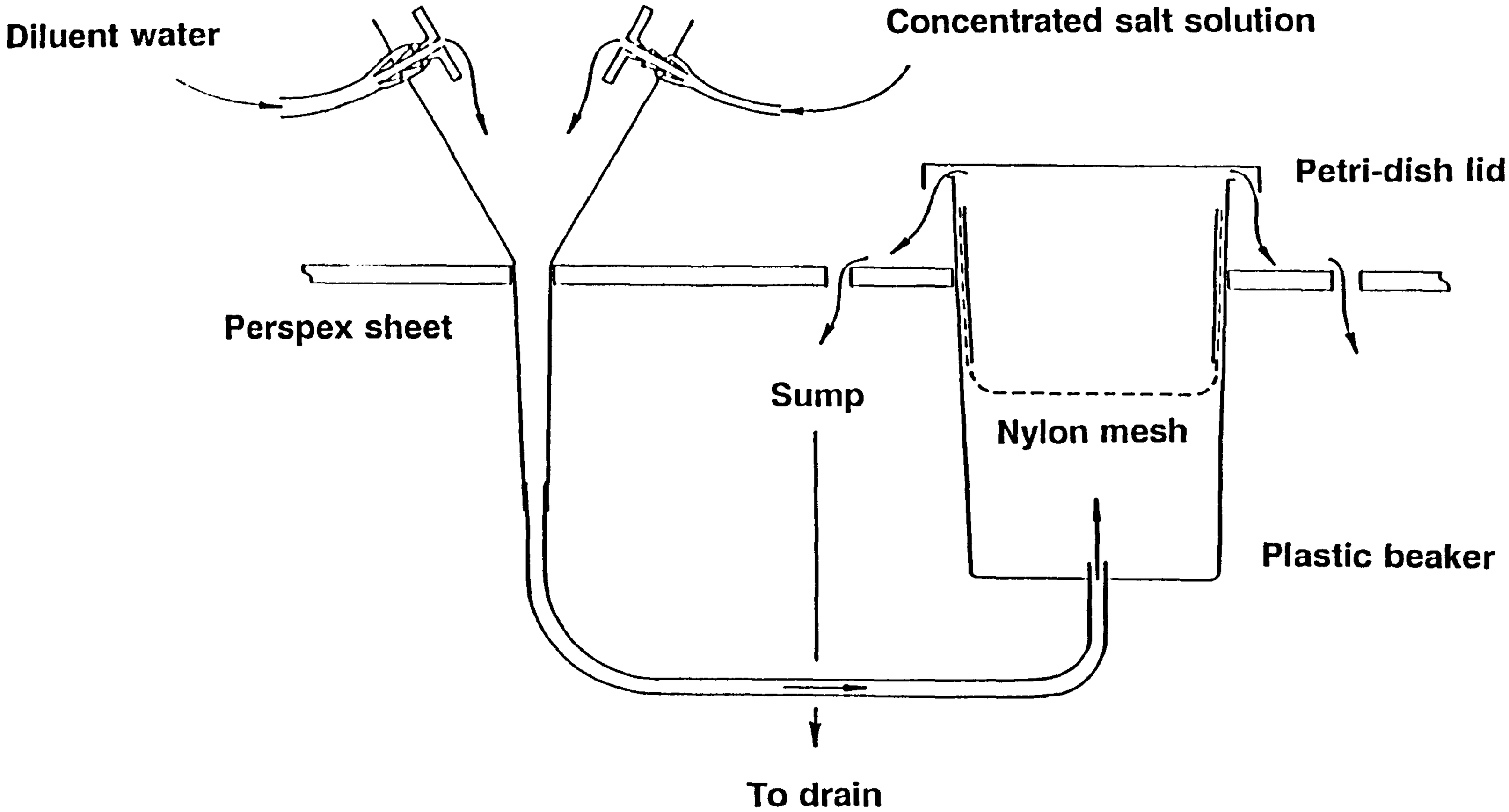


Figure 3.2: Detail of test chamber used for exposure of stage II crayfish juveniles in continuous flow apparatus (from Reader, 1986).

CHAPTER 4

CHLORIDE

4.1 INTRODUCTION

Chlorides may be found in naturally high concentrations in natural brines and in seawater. The amount of inorganic material dissolved in seawater expressed as weight in grammes per kilogram of water (parts per thousand) is termed as salinity (S) and is approximately 35 g kg^{-1} (35 $^{o}/_{oo}$), with chloride the major contributing ion at about 19 g kg^{-1} (19000 mg l^{-1}) (Tait, 1968). Salinity can be determined from chloride ion concentration using an empirical relationship known as Knudsen's Formula:

$$S\ (^{o}/_{oo}) = 0.030 + (1.805 \times Cl\ (^{o}/_{oo}))$$

In the freshwater environment, in waters not associated with saline soils, chloride levels are normally far lower and range between 6 and 130 mg l^{-1} (parts per million) (Jones, 1986), so that chloride concentrations considered normal in a saline environment can be considered as polluting in freshwaters. In this study the effects of chloride on crayfish are therefore investigated under two separate headings: 1.) The lethal and sublethal toxicity of chloride as a pollutant in the freshwater environment ; 2.) The effect of chloride as a component of salinity on osmoregulation and ionoregulation.

4.1.1 TOXICITY STUDIES

In the freshwater environment, elevated levels of chloride may be associated with sewage effluents and industrial wastes. In addition large amounts of chloride salts from salt/grit mixtures, used to de-ice roads in urban areas, can enter water courses, either directly in surface run-off, or by percolation from road-side ditches. In a study of chloride concentrations in run-off from an urban catchment, Kronis (1978) attributed concentrations up to 18 200 mg/l to road salting. Weathering of road-salt stock-piles, which can contain several thousand tonnes of material, may also contribute to the problem. Stockpiles in Yorkshire, containing up to 2000 tonnes of material are known to shrink by 5 % a year (Skelton, pers. comm.), releasing a possible 50 tonnes of chloride salt into the drainage system. This figure is likely to be exceeded in years of high rainfall.

In the United States and Canada, contamination of surface and groundwater by road-salt is a serious problem and strict guidelines exist for its use and storage (Jones, 1986). However, in the United Kingdom, high chloride levels are particularly associated with brine discharges from mines that work deposits of marine coals and associated coal-washing plants. Mine effluents in the Trent Basin may have a chloride content of up to 28 000 mg l^{-1} (Severn Trent Water Authority, 1989) and the streams which receive these discharges may have salt levels of up to 12 000 mg Cl^{-} l^{-1} (Lester, 1975).

Most research into the toxicity of chloride in the freshwater environment has been concerned with fish, although

comparative studies would indicate that invertebrates and micro-organisms are more sensitive to chloride salts (Patrick *et al.*, 1968; Hammer, 1977), especially in a lake environment where seasonal mixing is essential to maintain animal life. In winter, salt-contaminated run-off may enter lakes via feeder streams. The density of the inflowing water may be such that it goes directly to the lake bottom. The resulting stratification prevents mixing in the spring and the anoxic conditions, together with the salt stress, can eliminate all but the most tolerant benthic fauna (Hawkins and Judd, 1972).

Limited data is available regarding the tolerance of elevated chloride concentrations in crayfish. However, adult specimens of *Pacifastacus leniusculus* are reported to be regularly found in brackish water in the United States (Rundquist and Goldman, 1978) and in laboratory studies have been shown to withstand short-term exposure to 75 % seawater (approximately 14 000 mg Cl^- l^{-1}). No laboratory data is available for *Austropotamobius pallipes* or *Astacus leptodactylus*, although the latter species is known to tolerate salinity fluctuations of 5 - 14 $^{o}/_{oo}$ (approximately 2 800 - 7 750 mg Cl^- l^{-1}) in the Caspian Sea (Cherkasina, 1975).

Data on the sublethal effects of elevated chloride levels is similarly lacking. Sharfstein and Chafin (1979) found that in the red swamp crayfish, *Procambarus clarkii*, juveniles could grow at salinities up to 12 % seawater, but that growth rate was inversely correlated with increased salinity. Similar observations were made by Rundquist and Goldman (1978) with *P. leniusculus* juveniles at salinities up to 50 % seawater. No data

exists for similar stages of *A. pallipes* or *A. leptodactylus*.

In this study, the acute toxicity of chloride and the sublethal effect on growth was investigated and compared in the laboratory to determine whether *A. leptodactylus* and *P. leniusculus* are more, or less, tolerant of elevated chloride levels than *A. pallipes*.

In addition, field studies were carried out in the River Leen, a relatively small tributary of the River Trent, rising some six miles to the north of the City of Nottingham.

In its uppermost reaches the river is divided into two branches. The West Branch has its source approximately 3/4 of a mile from the East Branch, near the Newstead Colliery mines. Up until 1983 (STWA, 1988) the West Branch received mine effluent directly from the colliery. This was either from coal-washing activities, for which large lagoons were constructed, or was water pumped directly from underground sources (dewatering water). A feature of this effluent was the high chloride levels (Saleem, 1980). The Severn Trent Water Authority (STWA) report of 1989 states that in 1983 minewater was "removed" from the River Leen, presumably referring to the active discharge of effluent from the colliery. However, high conductivity and chloride content (up to 1400 mg l^{-1}) are still observed in the West Branch, so it is possible that chloride is still entering the river. It may be that surface water percolating through spoil heaps at the source of the West Branch is able to leach large quantities of material into the river.

The East Branch is less affected by pollution and is classed as grade 1B (National Rivers Authority, 1991). Immediately above

the confluence with the West Branch there is a high diversity of macroinvertebrate species, including a population of *Austropotamobius pallipes*, which has previously been used for population studies by Nottingham University (Mees, 1983). The main stream below the confluence occupies an intermediate position. However, crayfish can also be found here. The main purpose of this study was to compare growth and survival of crayfish in cage experiments in relation to environmental levels of chloride salts.

4.1.2 OSMOREGULATION STUDIES

Crayfish are highly successful colonizers of most freshwater systems. However, relatively few species are able to survive exposure to brackish or high saline conditions, such as those found in estuarial waters.

Like other freshwater acclimated animals, crayfish maintain an ionic composition and osmotic concentration of the body fluids substantially above that of the external medium (Wheatly and McMahon, 1982), and are termed osmo- or ionic-regulators. In freshwater, the cuticle provides a good barrier to diffusion. However, the gills have both gas and ion exchange functions (Bergmiler and Bielwalski, 1970; Bielawski, 1971) so cannot be totally impermeable. Therefore, animals have a constant influx of water by osmosis and loss of ions by diffusion down concentration gradients. In crayfish, these problems are resolved by removal of water by the renal organs (antennary or green

glands) and production of copious urine (Lieneman, 1938), active uptake of salts from the gut and by the gills (Shaw, 1960), and some reduction in membrane permeability (Gross, 1957).

An increase in external salinity has several deleterious effects on this regulatory system. The potential osmotic shock, where the animal finds itself in water more concentrated, rather than more dilute, than its blood will result in loss of water by osmosis. The second consequence is salt accumulation in the body. This results from reversed ionic gradients which cause increased diffusion of ions into the body across the gills (and possibly also from drinking of the medium to replace lost body water). Brackish water, or estuarine animals, can be divided into a number of categories on the basis of their physiological responses to changes in environmental salinity. The strategies employed range from conformation, where the ionic and osmotic composition of the body fluids closely follow that of the medium, to regulation, where a constant body fluid composition is maintained independent of the environment, with a number of intermediate strategies between the two (reviewed in Lockwood, 1962). For crayfish to withstand saline exposure, similar compensatory adjustments must occur, and it is this step that limits most crayfish from entry into brackish water (McMahon, 1986).

Relatively few studies have been carried out on the osmoregulation of crayfish in relation to salinity. Herman (1931) kept the crayfish *Potamobius* (*Astacus* ?) *astacus* in 50 % seawater for 6 weeks, but found it could not tolerate higher salinities. Above 50 % the blood was isosmotic to the medium. Lienemann

(1938) immersed *Procambarus clarkii* in different sodium chloride concentrations and found it could not regulate in solutions hyperosmotic to the blood. Bryan (1960), studying sodium regulation in *Astacus fluviatilus* (*astacus* ?), found that after pre-adaption to 200 mM NaCl (roughly isosmotic to the blood) *Astacus* could survive for 2 weeks in 300 mM NaCl. After 5 - 6 days the blood sodium was isosmotic to the medium. More recently, studies have been carried out on *P. leniusculus* which indicate that, in the short term, the species can regulate over a wide range of salinities, and shows hypo-osmotic regulation at high salinities, i.e. maintenance of a blood osmotic concentration below that of the medium (Kerley and Pritchard, 1967; Wheatly and McMahon, 1982). In the United States, *P. leniusculus* adults are regularly reported from brackish waters (Rundquist and Goldman, 1978).

In this study, both the short and long term responses to changing environmental salinity are investigated in *A. pallipes*, *A. leptodactylus* and *P. leniusculus*. Such information could be used to determine whether any of the species are indeed able to adapt to high saline conditions, and are able to extend their range into estuarial waters.

4.2 MATERIALS AND METHODS

4.2.1 ACUTE TOXICITY TESTS

Stage II juveniles of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* were individually exposed to a range of

concentrations of chloride in 5 cm diameter petri-dishes. A small square of black nylon mesh was provided for the animals to cling to, and the dishes placed on a black background to minimise in-test stress.

The source of chloride was analytical grade sodium chloride, minimum purity 99.9 %. Toxicity studies with fish (Al-Daham and Bhatti, 1977) indicated that the nature of the cation associated with the chloride ion can affect the level of mortality, with potassium chloride being more toxic than either calcium or sodium chloride. However, in the majority of other studies attention has been focussed on the toxicity of sodium chloride as this is the predominant chloride salt in the environment.

A preliminary range-finding experiment was carried out to determine a suitable nominal concentration range. This was as follows; 0 (control), 3.125, 4.675, 6.25, 9.375 and 12.5 g Cl^- l^{-1}. Actual test concentrations were determined using a silver nitrate/potassium chromate tiration (Palintest Ltd., England) (see 3.2.1). Conductivity of the test solutions was also checked daily using a pHOX 52E Conductivity Meter (pHOX Systems Ltd., Sheffield). A linear relationship was shown between chloride concentration and conductivity (Fig 4.1), so that measurement of conductivity could be used to rapidly check test solutions.

The experiment was also repeated using two larger juvenile stages (10 mm and 18 mm carapace length).

4.2.2 SUBLETHAL TOXICITY TESTS

Stage II juveniles of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* were chronically exposed to a concentration of 1000 mg Cl^- l^{-1} in a continuous flow system. The concentration used was comparable to the mean chloride concentration measured in the west branch of the River Leen.

Approximately 50 stage II juveniles were placed in polystyrene troughs (100 x 20 x 20 cm) filled with aerated, dechlorinated water from the potable supply. Chloride and control solutions made up with conditioned tapwater were delivered continuously to the troughs through PVC tubing via a peristaltic pump at a rate of approximately 200 ml h^{-1}. Temperature and chloride concentration of test solutions were measured on a weekly basis, with conductivity checked daily.

The experiment was carried out in a constant temperature room (15 $\pm$ 0.1 °C) in an artificial light regime of 12 h light and 12 h dark. Short lengths of plastic hosepipe were provided as shelter and the animals fed to excess with "Minced Morsels" (Quaker Oats Ltd.) and a filamentous alga (*Cladophora sp.*) throughout the experimental period.

At the beginning of the experiment, a sample of ten stage II juveniles of each species was taken to provide a baseline. Due to the small size of the animals, dry weight was used as the measurement of growth, with animals placed in an oven at 50 °C for 48 hours prior to weighing. A further ten animals were then removed after 2 and 4 weeks.

4.2.3 FIELD STUDIES

Holding cages were stocked with crayfish and placed in the East and West branches of the River Leen to determine whether there was any difference in growth and survival of crayfish in the West branch due to elevated chloride levels. Two types of cage were constructed:

1.) Adult Cages - These measured 60 x 45 x 15 cm and consisted of a frame of angle iron covered in plastic garden netting, hole diameter approximately 1.3 cm. Each cage was stocked with equal numbers of male and female crayfish (up to a maximum of ten animals per cage), size range 25.0 - 41.0 mm carapace length, collected from the East Branch. The initial carapace length of each crayfish was measured with a 0.5 mm graduated steel rule and the crayfish marked by pleural clipping, so that individuals could be followed through the moulting period. This simply involved removing a small portion from the end of a different abdominal pleura with nail clippers for each crayfish. The clipped end of the pleura was regenerated in successive moults, but remained sufficiently evident over the moulting period.

Lengths of domestic waste pipe and broken drain pipe were placed in the cage to provide refuges, and the cage filled with aquatic vegetation to provide food. The relatively large mesh size allowed free passage of water through the cage and allowed other food items, such as macroinvertebrates, into the cage. Cages were placed in the East and West Branches in May and were subsequently checked for any moults or mortalities every two

weeks, until the end of the moulting season in September.

2.) Juvenile Cages - These were made from black plastic baskets, designed for planting aquatic plants in ponds, measuring 28 x 20 x 18 cm, with a mesh diameter of approximately 2 mm. A lid was made for the basket from perspex sheet, which was clamped into place with plastic page binders. The bottom of the cage was filled with gravel to provide substrate and weight. Each cage was provided with aquatic vegetation and a length of domestic waste pipe.

A single fully-berried female was placed in each cage. The number of eggs on each female was roughly counted, then calculated from the crayfish carapace length using the equation for River Leen females in Rhodes and Holdich (1982). The cages were then monitored weekly, until hatching of the juveniles, then through moulting of the juveniles to the semi-independent stage II. At this point the female was removed and the number of juveniles counted to determine percentage mortality in the period egg - stage II juvenile. Thirty stage II juveniles were then placed in each cage. These were counted at monthly intervals to determine percentage mortality in the juvenile stages.

Samples of invertebrate fauna were collected for biological assessment of water quality at the cage sites using a long-handled net, by 5x30 second kick-samples. Samples were transported back to the laboratory for identification and calculation of biotic indices (Extended Trent Biotic Index and Biological Monitoring Working Party score).

Water samples were collected in rinsed 500 ml polythene bottles at the cage sites, and also below the confluence of the

two branches. Samples were either analysed promptly or stored at 4 °C for a maximum of 24 hours. Analysis was carried out using a variety of colormetric and titrametric techniques (see Table 4.2). Subsamples of 50 ml for metal analysis by atomic absorption spectrophotometry (AAS) were taken and acidified with 0.5 ml of 5 Molar sulphuric acid to ensure metal ions remained in solution.

Conductivity, oxygen, pH and temperature measurements were made on site with a Horiba Water Quality Checker U-10.

4.2.4 OSMOREGULATION STUDIES

Experiments were carried out on adult *P. leniusculus*, *A. pallipes* and *A. leptodactylus* (size range 37 - 54 mm carapace length, average size 45 mm carapace length) from outdoor breeding tanks at Nottingham University. Prior to experimentation, animals were transferred to a constant temperature room at 15 ± 0.1 °C, in an artificial light regime of 12 hours light and 12 hours dark, for an acclimation period of 48 hours. Animals were housed in clear plastic aquaria containing 4 litres of aerated, dechlorinated water from the potable supply. Each aquarium was split into four sections with Perspex dividers, with one crayfish in each division.

Investigation of physiological responses to changing environmental salinity in the three species was carried out by determination of the osmotic and ionic concentration of haemolymph samples, taken by inserting a hypodermic needle through the second abdominal membrane into the sinus surrounding

the heart. Approximately 0.5 ml of haemolymph was removed from each individual. For a crayfish of 45 mm carapace length, the sample taken represents just 7 % of the total blood volume of 7.7 ml, calculated from the formula in Rhodes (1982). All equipment and samples were kept on ice to prevent clotting of the blood.

Samples were placed in Eppendorf centrifuge tubes and spun for 15 minutes at 16 000 RPM to remove blood cells in an Eppendorf 5415C Centrifuge (Eppendorf, Hamburg), housed in a cold room at 5°C. The serum was pipetted off the blood cells, again to prevent clotting, and measurements of ion and osmotic concentration carried out. A preliminary experiment showed that there was no significant difference in osmotic and ion concentrations between whole blood and serum obtained after centrifugation.

Determination of osmotic and ionic concentration was carried out on both haemolymph and test solutions. Osmotic concentration was measured using a Wescor 5500 Vapour Pressure Osmometer (Wescor Inc., Utah). Chloride ion concentration was measured using a Jenway PCLN3 Chloride Meter (Jenway Ltd., Essex). Analysis of sodium ion concentration was carried out on samples diluted 1/4000 with deionised water, using a Pye Unicam SP9 Atomic Absorption Spectrophotometer (Pye Unicam Ltd., Cambridge). All ion results were expressed as mmoles l^{-1} and the osmotic concentration as mOsm kg^{-1}. The temperature and conductivity of test solutions was also recorded.

Individual experimental protocol are detailed below:

4.2.4(i) Stepwise (Short Term) Acclimation

A group of 24 animals was set up in fresh (tap) water for each species, and allowed to acclimate for 48 hours. At the end of the acclimation period, four animals were randomly selected and sampled as controls. The remainder were then transferred to 20 % seawater, made up from "Instant Ocean" stock at 35 $^{\circ}/_{\circ\circ}$ and tapwater. After 48 hours in this medium a second group of four was randomly selected and sampled, with the remaining animals transferred to 40 % seawater. The procedure was repeated in 20 % increments through to 100 % seawater. The conductivity of each seawater concentration was measured in all cases to ensure the quality of dilution from 100 % seawater. After sampling, all animals were returned to the outdoor tanks.

4.2.4(ii) Long Term Acclimation

Groups of 28 animals were set up in fresh (tap) water. After an acclimation period of 48 hours, four animals were randomly selected and sampled as controls. Half of the remaining animals were then transferred to 20 % seawater and the other half to 60 % seawater. After 48 hours, four animals were randomly selected from each seawater dilution and sampled. A third group of four animals was sampled at 7 days after transfer to the seawater dilutions, and the remaining four sampled at 14 days. Over the experimental period the medium was periodically replaced, and the temperature and conductivity monitored.

4.2.4(iii) Reverse Acclimation

Groups of twenty animals were set up in fresh (tap) water. After 48 hours acclimation, four animals were sampled as controls and the remainder transferred to 60 % seawater for an acclimation period of three weeks. During this period the medium was regularly changed and temperature and conductivity monitored. At the end of the three week acclimation, four animals were sampled and the remainder transferred to fresh (tap) water. After 48 hours in this medium, four animals were sampled, followed by further groups of four individuals after 7 and 14 days post-transfer to fresh water.

4.3 RESULTS

4.3.1 ACUTE TOXICITY TESTS

Throughout all toxicity tests, measured temperature and pH was 13.0 $\pm$ 0.1 ^{o}C and 6.99 $\pm$ 0.17 respectively. The mean hardness of the dilution water was 140 mg l^{-1} as $CaCO_3$. There was an increase in the conductivity of the test solutions with chloride concentration, with mean values ranging from 393 uS cm^{-1} in the control, to 34 583 uS cm^{-1} in the highest test concentration.

The percentage mortalities at 24, 48, 72 and 96 hours for *A. pallipes*, *A. leptodactylus* and *P. leniusculus* are shown in Fig. 4.2. For each species mortality increased with chloride concentration and time of exposure. Mean concentrations of chloride ion during exposure were calculated from the measured

test concentrations. From the above data the 24, 48, 72 and 96 hour LC_{50} (median lethal concentration) values have been calculated in Table 4.1 for each species, and are presented in Fig. 4.3. No control deaths were observed in any of the tests. Also, there was no further mortality in animals transferred to clean water at the end of the test period.

In the toxicity tests with the two larger juvenile stages, percentage mortality was insufficient to allow calculation of an LC_{50} before 96 hours exposure. These values together with 96 hour LC_{50} values for stage II hatchlings are plotted against size (total length) in Fig. 4.4.

4.3.2 SUBLETHAL TOXICITY TESTS

Mean chloride concentration and conductivity during the experiment were 106 $\pm$ 22.3 mg Cl^- l^{-1} and 604 $\pm$ 5.94 uS cm^{-1} respectively in the controls, and 950 $\pm$ 64.5 mg Cl^- l^{-1} and 2840 $\pm$ 55.2 uS cm^{-1} in the chloride treatments. Mean temperature was 15.2 $\pm$ 0.13 °C.

Mean dry weight at 2 and 4 weeks, for control and chloride treated animals, is shown in Fig. 4.5 for all three species. In the control treatments, there were significant increases in dry weight at 2 and 4 weeks, with the largest increase observed in *P. leniusculus* juveniles. There was also an increase in dry weight in the chloride treatments. This appeared to be less than that observed in the controls, although analysis of variance indicated that in *A. pallipes* and *A. leptodactylus*, dry weights of animals from controls and chloride treatments were not

significantly different at the end of the 4 weeks. However, a significant difference ($p < 0.01$) between treatments was noted for *P. leniusculus* juveniles. Expression of the mean dry weight in chloride treatments as a percentage of the mean control dry weight (taken as 100 % growth) gave a 49 % reduction in growth due to chloride exposure in *P. leniusculus* juveniles (Fig. 4.6) compared to 14.7 % and 21.9 % in *A. leptodactylus* and *A. pallipes* juveniles respectively.

4.3.3 FIELD EXPERIMENTS

Cage experiments were conducted in the east and west branches of the River Leen in 1991 and 1992. Experiments were set up at the beginning of June and run through to the beginning of September. In 1991 the experiment was terminated due to siltation of the cages following a period of heavy rain and floodwater, which resulted in the death of many of the experimental animals. In 1992, cages in the east branch were vandalised at the end of July, with the subsequent loss of experimental animals, so no data is available for the remainder of the experiment in this year.

Biological and chemical water quality parameters for cage sites in the east and west branches, and for the confluence, are shown in Tables 4.2 and 4.3. Species lists for the sample sites and scoring schemes for ETBI and BMWP biotic indices are shown in Appendix 1 and Appendix 2. Biological quality in the east branch was very good, with pollution-sensitive stonefly and mayfly species present. In contrast, the west branch was of

moderate biological quality. Stoneflies and sensitive mayfly species were absent, although a cleanwater caddisfly and the tolerant mayfly *Baetis* were recorded. The introduced amphipod, *Gammarus tigrinus*, indicative of elevated salt concentrations, was also present in large numbers. The confluence was found to occupy an intermediate position of good biological quality, with some pollution-sensitive species recorded.

From the chemical data, significantly elevated levels of chloride were noted in the west branch. Concentrations ranged from 738 - 1250 mg Cl^- l^{-1}, compared to 68 - 235 mg Cl^- l^{-1} in the east branch, and were on average 917 mg Cl^- l^{-1} in the west branch. Similarly, conductivity was significantly higher in the west branch, ranging from 3300 - 4650 uS cm^{-1}, compared to 620 - 1150 uS cm^{-1} in the east branch. The confluence again occupied an intermediate position, although chloride concentration and conductivity was still significantly higher than in the east branch.

In the cages stocked with adults, two major periods of moulting were noted in both the east and west branch. There was a short period of approximately one week at the end of May, and a more protracted period of 4 - 5 weeks from the end of July through to the end of August, as previously observed in *A. pallipes* by Brown and Bowler (1978) and Pratten (1980). Most animals moulted twice, once in each moulting period. Only one crayfish (a large male of carapace length 49 mm) failed to moult at all, and only one individual failed to moult properly and subsequently died. Apart from mortalities due to siltation of the cages in 1991, this was the only mortality observed during the

experiment.

Mean growth increments for crayfish from the east and west branches are given for comparison in Table 4.4 for 1991 and 1992. There were no significant differences between the increments in corresponding moult periods in 1991 and 1992 in either the east or west branch. The data for the two years were therefore pooled and are shown in Table 4.4. From pooled data, there was no significant difference between the mean moult increments of crayfish from the east and west branch in either the first or second moulting period, although data is lacking for the second moult period in the east branch in 1992. Similarly for the percentage moult increment (PCMI), i.e the moult increment expressed as a percentage of premoult carapace length, there was no significant difference between the east and west branch for either moulting period.

Only one mortality was noted in the adult cages during the experiment. However, it was observed that crayfish in the west branch accumulated a dense fur-like growth on the carapace in the period between moults (Fig. 4.7). Examination by light microscopy of material scraped from the carapace indicated that it mainly consisted of large colonies of a stalked ciliated protozoa, subclass Peritricha, identified as *Epistylis sp.* (Fig. 4.8), together with small amounts of filamentous algae and detritus.

Results of survival experiments for egg and juvenile stages are shown in Fig. 4.9. The initial numbers of eggs counted on the females stocked in cages in the east and west branch on 13/5/92 were 79 and 72 respectively. Stage I juveniles were present on the females on 4/6/92 and it was noted that there was a reduced

number on the female in the west branch. Independent stage II juveniles were present in the cages on 17/6/92, at which point the females were removed and the number of juveniles counted. A total of 58 juveniles were present in the east branch cage, whereas only 15 were present in the west branch cage, giving survival rates for the period from egg stage to stage II juvenile of 73% and 21% respectively.

The percentage survival of 30 stage II juveniles placed in cages in the east and west branch is shown in Fig. 4.9. After 4 weeks, percentage survival of juveniles in the east and west branches was 100% and 27% respectively. No further data is available for the east branch due to vandalism of the cage and release of the juveniles. However, percentage survival continued to decline in the west branch and was only 13% at the end of the 12 week experimental period.

4.3.4 OSMOREGULATORY STUDIES

4.3.4(i) Stepwise (Short Term) Acclimation

Mean osmotic and ionic concentrations from blood analyses of animals stepwise acclimated to increasing salinity are shown in Table 4.5 for each experimental salinity.

Mean osmotic concentration of the blood in mOsmol kg^{-1} is plotted against the mean osmotic concentration of the medium in Fig. 4.10 for *A. pallipes*, *A. leptodactylus* and *P. leniusculus*. Osmotic concentration of the blood increased significantly as the

salinity of the medium increased in all three species; *P. leniusculus* (R=0.883, df=18, p<0.001), *A. pallipes* (R=0.948, df=18, p<0.001), *A. leptodactylus* (R=0.932, df=18, p<0.001). However, comparison of regression lines obtained from log-y converted data, using a BBC model B microcomputer running a "COMPREG" BASIC program (Wiggans et al., 1983), showed that a single line would fit the data as well as three independent lines ($F_{2,56}$=7.199, p=0.002), so there was no significant difference in the osmotic response between the three species.

The diagonal line in Fig. 4.10 represents the isosmotic line, where the concentration of the haemolymph and the medium are equal. This indicates the boundary between hyper- and hypo-osmotic regulation, i.e. values for blood osmotic concentration above and to the left of the line indicate hyper-regulation, while those below and to the right of the line indicate hypo-regulation. It can be seen that all three species were able to hyper-regulate in salinities lower than the blood concentrations, and were able to carry out some degree of hypo-regulation in salinities higher than the blood concentration. Values for the osmotic concentration of the medium where the transition from hyper to hypo-regulation occurred were calculated from the log-y regression lines for all three species, and are shown in Table 4.6.

A similar trend was observed for haemolymph concentrations of ions. Chloride and sodium concentration of the haemolymph in mmol is plotted against that of the medium in Figs 4.11 and 4.12. Chloride and sodium concentrations increased significantly as the salinity of the medium increased, with no significant difference

observed between the three species. A transition from hyper- to hypo-ionic regulation was observed in all three species, and the transition values calculated from log-y regression are shown in Table 4.6.

4.3.4(ii) Long Term Acclimation

As no significant difference between the response to increased salinity in *A. pallipes*, *P. leniusculus* and *A. leptodactylus* was noted in the initial experiments, it was decided that only *P. leniusculus* would be used in further experiments, and provide a model for the other species of crayfish.

Animals acclimated to freshwater maintained haemolymph hyper-osmotic to the medium by about 300 mOsmol. Fig. 4.13 shows haemolymph osmotic concentration with time for animals transferred to 20 % and 60 % seawater. On transfer to 60 % seawater the blood was initially hypo-osmotic to the medium. However, by 14 days post-transfer the blood had become roughly isosmotic to the medium. The increase in blood osmotic concentration over the 14 day exposure period was highly significant ($F=62.76$, $df=3$, $p<0.001$) in animals transferred to 60 % seawater, and was actually found to be significantly above that of the medium at the end of 14 days ($T=4.881$, $df=5$, $p=0.005$). No significant increase in blood osmotic concentration was observed in the animals transferred to the 20 % seawater control.

The blood chloride concentration displayed similar changes

to those for osmotic concentration in animals transferred to 60 % seawater (Fig. 4.14), with a highly significant increase in chloride concentration over the 14 day period ($F=55.70$, $df=3$, $p<0.001$). The major difference was that the blood chloride concentration was still significantly below that of the medium at the end of the 14 days ($T=3.120$, $df=5$, $p=0.05$).

The blood sodium concentration also increased significantly over the 14 day period in animals transferred to 60 % seawater ($F=43.13$, $df=3$, $p<0.001$). Haemolymph sodium concentration was not significantly different from the medium at the end of this period, indicating iono-conformity to the medium (Fig 4.15).

4.3.4(iii) Reverse Acclimation

Animals acclimated to freshwater maintained the blood hyper-osmotic to the medium by over 300 mOsm Kg^{-1}. After exposure to 60 % seawater for 3 weeks the blood osmotic concentration had significantly increased and had become isosmotic to the medium (Fig 4.16). On return to freshwater, blood osmotic concentration declined significantly ($F=329.05$, $df=3$, $p<0.001$), and had rapidly returned to a concentration similar to that seen in freshwater acclimated animals after just 7 days. However, the decline in osmotic concentration continued, albeit less rapidly, over the remainder of the experimental period, so that at the end of 14 days the blood osmotic concentration had declined significantly below the original freshwater value ($T=3.845$, $df=5$, $p=0.013$).

Haemolymph chloride concentration is shown in Fig. 4.17. After 3 weeks exposure to 60 % seawater, blood chloride

concentration had significantly increased, but had not reached iono-conformity with the medium. On return to freshwater, the chloride concentration declined significantly ($F=88.57$, $df=3$, $p<0.001$), and after just 48 hours had returned to a level not significantly different to the freshwater acclimated animals. No further decrease was seen and the blood chloride concentration remained at a similar level for the remainder of the experimental period.

A similar observation was made for blood sodium concentrations in Fig. 4.18. The sodium concentration reached iono-conformity after exposure to 60 % seawater for 3 weeks and, after return to freshwater, significantly decreased ($F=123.16$, $df=3$, $p<0.001$) to reach a level not significantly different from the freshwater acclimated state after 7 days. Blood sodium concentration continued at a similar level for the remainder of the experimental period.

4.4 DISCUSSION

The data from acute toxicity tests shown in Figs 4.2 and 4.3 show interspecific differences in mortality and LC_{50} values. At 96 hours, *A. leptodactylus* stage II juveniles were least sensitive to chloride, and approximately 1.7 and 2.5 times more tolerant than *A. pallipes* and *P. leniusculus* respectively. Stage II juveniles of *P. leniusculus* appeared to be the least tolerant, although there was no significant difference between *A. pallipes* and *P. leniusculus* at 96 hours. However, it can be seen that the 96 hour LC_{50} for each species increases with the size of juvenile

tested. The largest increase was seen in *P. leniusculus* juveniles, with a fivefold increase in tolerance between stage II and 18 mm carapace length juveniles. Although data is lacking for 18 mm carapace length *A. pallipes* and 10 mm carapace length *A. leptodactylus*, *P. leniusculus* juveniles would appear to have an equal or greater chloride tolerance in the larger sizes. Data for *P. leniusculus* would also seem to indicate an upper limit for the LC_{50}, with LC_{50} values tending towards a threshold concentration, after which there is no further increase in tolerance with size. Comparative LC_{50}s and other toxicity data for other freshwater invertebrates are shown in Table 4.7.

Chloride itself is not very toxic, rather it affects ionic and osmotic balance. In freshwaters, osmotic regulation would be the principal response mechanism by which aquatic fauna could adapt to chloride salt pollution. Juvenile crayfish, like other juvenile macroinvertebrates, would generally be more susceptible to osmotic stress due to their large surface area to volume ratio and higher weight-specific metabolism (Buikema and Benfield, 1979), increasing exchange with the environment. Also, their osmoregulatory system may not be fully functional (Rundquist and Goldman, 1978). This would particularly apply to *P. leniusculus* stage II hatchlings, which are smaller than both *A. pallipes* and *A. leptodactylus* stage II hatchlings upon release from the female (see Fig. 4.5), and would explain the rapid increase in chloride tolerance with increasing size.

Toxicity tests in general, and with crayfish in particular, have been criticised (France, 1985) for not dealing with realistic toxicant levels and not relating results to

environmental concentrations. In chloride acute toxicity tests, the calculated LC_{50}s for *P. leniusculus*, *A. pallipes* and *A. leptodactylus* juveniles are higher than toxicant concentrations measured in the field and would indicate that even stage II hatchlings of all three species could survive in the west branch of the River Leen, where the average chloride concentration is approximately 1000 mg l^{-1}. Clearly, the absence of crayfish from the west branch and the observed mortality of *A. pallipes* juveniles in the cage experiments cannot be explained simply on the basis of acute chloride toxicity.

Laboratory experiments indicated sub-lethal effects with continuous exposure to a concentration of 1000 mg Cl^- l^{-1}, with a significant reduction in growth in stage II *P. leniusculus* juveniles. This concentration represents 35% of the 96 hour LC_{50} for *P. leniusculus* juveniles, compared to 24% for *A. pallipes* and 14% for *A. leptodactylus*, so it is not unexpected that sublethal effects were greatest in *P. leniusculus*. However, no significant reduction in growth was noted for either *A. leptodactylus* or *A. pallipes* stage II juveniles in the laboratory experiments. Also, adult *A. pallipes* exposed to an average concentration of 1047 mg Cl^- l^{-1} in the field, over a period of four months, were observed to moult and grow normally, despite extensive colonisation of the carapace by a epizooic protozoan.

Sessile and motile epicommensal organisms occur all over the crayfish body, especially around the gills and on limb joint setae. Some 49 different organisms have been found on crayfish of the genus *Pacifastacus* (Lahser Jr., 1975), including Fungi, Algae, Protozoa, Rotatoria, Nematoda, Annelida and Arthropoda.

Most common are the protozoans *Epistylis*, *Cothurnia*, *Lagenophrys*, and *Zoothamnium* (Alderman and Polglase, 1988). *Cothurnia variabilis* has been reported to be one of the first epizooites o colonize a newly moulted crayfish.

Usually, normal grooming behaviour and moulting keeps numbers of epizootic organisms to manageable levels. However, numbers may build up rapidly if there is a change in crayfish health, or environmental factors. This may lead to a general increase in numbers, or a massive increase in one organism, e.g. *Cothurnia astaci*. Protozoans thrive in an organically-rich environment, such as those found in closely-confined culture conditions or organically polluted waterbodies, presumably due to high levels of bacteria and particulate matter. Protozoa may accumulate so much under these conditions that crayfish have been observed to have a "furry" or "fuzzy" appearance (Huner and Barr, 1991), similar to that seen in *A. pallipes* in the west branch of the River Leen (Fig. 4.7). Although the invertebrate fauna of the west branch was not typically indicative of organic pollution, large numbers of the net-spinning caddis *Hydropsyche* were recorded, which would indicate presence of suspended particulate matter. Also, turbidity was observed to be higher than in the east branch. This may possibly be due to the influence of a fishing pool and colliery lagoons upstream. Dickman and Gochnauer (1978) observed that addition of 1000 mg l^{-1} NaCl to a small stream resulted in a change in the microbiota from an algal dominated fauna to a bacterial dominated fauna, with high densities of the bacteria *Hyphomicrobium* recorded on artificial substrates in the treated area. A combination of particulate

matter from the pool and lagoons, and an increased bacterial density due to the elevated salt concentration would mimic conditions found in organically polluted waters, and would explain the very high densities of protozoa on crayfish from the west branch.

It is unclear whether these growths of protozoa have any deleterious effect on the crayfish, although it is suggested that growth on the gills results in a sublethal depression of performance (Alderman and Polglase, 1988). Vey (1977) observed behaviour of *A. leptodactylus* in ponds containing water with suspended clay particles. Adults in these ponds moved very slowly and the branchial filaments were found to be completely covered in a conglomerate of mineral particles and diatoms. The gill epithelium was therefore effectively separated from the water by this coating, so respiratory exchange presumably must have been reduced. Results from the cage experiments indicated that animals in the west branch of the River Leen moulted and grew normally, so activity such as feeding would not appear to have been affected. It was noted that despite heavy growths of protozoa on the carapace, the eyes, antennae and mouthparts were kept clean, so there may not be a debilitating effect on the adult stages. However, this may not be the case for the egg stage. Vey (1976) observed that *A. leptodactylus* females in water with a high particulate load did not lay eggs, or carried a reduced number of eggs, despite having full ovaries containing large eggs rich in vitellus. When egg-laying did occur a number of eggs showed abortive development. This, possibly combined with chloride stress, may explain the high mortality between the egg and

juvenile stages observed in the west branch of the River Leen.

Also, the mine effluent discharged into the west branch may contain other pollutants, such as heavy metals (Maltby *et al.*, 1987), which could act synergistically with the chloride. However, levels of copper and zinc measured in the west branch were very low and not significantly greater than those in the east branch (Table 4.2.). Elevated concentrations of sulphate were noted in the west branch, which are usually indicative of high levels of iron. However, it was also noted that hardness (as $CaCO_3$) was also higher in the west branch. This increase is due to an ion exchange process, in which sodium cations from a saline discharge or runoff are absorbed by soil particles, releasing calcium and magnesium ions (Jones, 1986). Water hardness is a very important factor influencing the toxicity of heavy metals. In general, LC_{50}s for heavy metals increase with an increase in water hardness (Pascoe *et al.*, 1986). For zinc there appears to be an inverse linear relationship between the logarithms of the water hardness and the toxicity, i.e. a tenfold increase in hardness is accompanied by a tenfold increase in the LC_{50} (Hellawell, 1986). Mean hardness of water in the west branch over the experimental period was 798 mg $CaCO_3$ l^{-1}, so it is unclear as to what degree heavy metal toxicity may contribute to crayfish distribution in the River Leen.

Therefore, it can be seen that the absence of crayfish from the west branch of the River Leen is due to a number of factors, probably affecting the survival of egg and early juvenile stages, and cannot be explained on the basis of chloride toxicity alone. However, the LC_{50} values calculated for stage II juveniles may

represent the upper limits for recruitment of juveniles in waters specifically affected by chloride. On this basis, viable populations of *A. leptodactylus* could possibly be found in waters with a higher chloride salt content than either *A. pallipes* or *P. leniusculus* juveniles could tolerate, which may include estuarine waters. The LC_{50} for *A. leptodactylus* stage II juveniles of approximately 7 g Cl^- l^{-1} corresponds to a salinity of 12.6 $^{o}/_{oo}$, calculated by Knudsen's Formula (see Intrduction 4.1). This would agree with the observation of populations of *A. leptodactylus* in the Caspian Sea in salinities of 5 - 14 $^{o}/_{oo}$ (Cherkasina, 1975), but would not appear to allow establishment of viable populations in environments where the salinity was greater.

Previous studies seem to indicate an apparent greater tolerance of adult *P. leniusculus* to salinity (Kerley and Pritchard, 1967; Wheatley and McMahon, 1982a), with short-term survival possible in salinities up to 70% seawater, and an ability for hypo-osmotic and hypo-ionic regulation in concentrations greater than the body fluids. This has been further investigated in this study.

In the short-term experiments, involving step-wise acclimation of the three species to increments of 20% seawater, no significant difference in the response to increasing salinity was noted between *P. leniusculus* and either *A. pallipes* or *A. leptodactylus*. In freshwater and in 20% seawater, all three species maintained the osmotic and ionic concentration of the haemolymlph significantly above that of the medium. With further increases in salinity there were significant increases in the

osmotic and ionic concentrations of the haemolymph. However, blood concentrations were still significantly below the medium, indicating some degree of hypo-regulation in all three species, with a transition from hyper-regulation to hypo-regulation at a concentration between 40 and 60% seawater (Table 4.4).

In freshwater, crayfish suffer from water gain and ion loss. These problems are overcome by a.) relative impermeability of the cuticle, b.) production of a copious, but dilute urine by the renal system with conservation of ions, and c.) active uptake of salts from gut and gills. An increase in external salinity has several deleterious effects on the osmoregulatory system. These are considered in McMahon (1986), but can be summarised as potential osmotic shock, through loss of water through the gills, and salt accumulation. Hypo-regulation in high salinity would therefore involve replacement of body water and drinking of the medium would appear to be the only source of this replacement water. Crayfish have not been observed drinking measureable quantities in normal freshwater medium (Maulef, 1940; Bryan 1960) and, although the evidence is not considered extensive, studies have indicated that the crayfish *P. leniusculus* does not drink while in a hyper-osmotic medium. Also, the problem with this strategy is that it causes additional salt gain and therefore cannot occur without a mechanism to remove the extra ions. For monovalent ions, Cl^- and Na^+ the only route is the gill epithelium. Therefore, to be able to hypo-regulate, the crayfish must either possess two sets of ion pumps in the gill epithelium (inwardly and outwardly directed), or must be able to switch the direction of the existing pumps. Cells similar to the "chloride"

cells, responsible for the extrusion of ions in seawater exposed euryhaline fish (Keys and Wilmer, 1932), have been reported in the gill filaments of *P. leniusculus* (Morse *et al.*, 1970). However, their function in *P. leniusculus* has still yet to be demonstrated.

In long-term experiments, crayfish exposed to a hyper-osmotic medium became isosomotic to the medium, despite the appearance of the ability to hypo-regulate in the short term. This sequence has been observed in previous studies over equivalent time periods (Kerley and Pritchard, 1967; Henry and Wheatly, 1988). True freshwater decapods normally display a reduced permeability to water and ions compared to marine species. For example, rates of Cl^- efflux in the crayfish *A. leptodactylus* has been measured as 94.7 u-equivalent $100g^{-1}$ hr^{-1} (Ehrenfield, 1974) compared to 824 u-equivalent $100g^{-1}$ hr^{-1} in the blue crab *C. sapidus*. A reduced permeability would contribute to a longer period of equilibration between the haemolymph and the external medium, and therefore may account for the period of apparent hypo-osmotic regulation observed in previous studies in *P. leniusculus* (Kerley and Pritchard 1967; Wheatly and McMahon, 1982a) and in *A. pallipes* and *A. leptodactylus* in this study. Henry and Wheatly (1988) found in *P. leniusculus* that haemolymph osmotic and ionic values did not fully stabilise until 3 weeks after transfer to a hyper-osmotic medium. In contrast, haemolymph of the blue crab *C. sapidus* reached new equilibrium values within 12 hours of transfer to a new salinity.

Henry and Wheatly (1988) also observed that activity of the two major transport enzymes, the Na^+/K^+ ATPase and carbonic

anhydrase, in gill and antennal gland cells was dependent on salinity. In freshwater, conversion of respiratory CO_2 into carbonic acid by carbonic anhydrase (CA) provides H^+ ions, which are exchanged for Na^+ ions in the medium via a proton pump, and HCO_3^- ions which are exchanged for Cl^- (Rankin and Davenport, 1981). CA activity decreased in animals transferred from freshwater to 750 mOsm kg^{-1} over a three week period, with a significant reduction in activity between 7 and 14 days. This was consistent with the time course for protein turnover and deinduction of the enzyme, rather than a short-term deactivation. A similar pattern was observed for the Na^+/K^+ ATPase, particularly in the antennal gland, where significant reductions in activity corresponded with a virtual shut-down in urine production. Therefore, the transient period following transfer to a hyper-osmotic medium, during which the haemolymph osmotic and ionic concentration is below that of the medium, probably does not represent hypo-regulation as first suggested (Wheatly and McMahon, 1982a). Rather, it most likely indicates a prolonged phase during which the active transport mechanisms of the gill and antennal gland are shut down, and in which permeability changes occur in these tissues. However, reverse acclimation experiments in this study indicated a rapid decrease in the blood osmotic and ionic concentration of 60% seawater animals when transferred to freshwater, with levels returning to those seen in freshwater acclimated animals after only 48 hours (Figs 4.16 - 4.18). Henry and Wheatly (1988) found that CA activity did not significantly increase in 750 mOsm acclimated animals until after 48 post-transfer to freshwater, implying induction of the enzyme

via *de novo* synthesis. Clearly this would not explain the rapid changes observed in this study. However, ATPase activity is known to change very rapidly after salinity transfer (Towle *et al.*, 1976) and short-term changes in the activity of gill and antennal gland ATPase were noted by Henry and Wheatly (1988). This would imply that Na^+/K^+ ATPase activity is regulated by activation/deactivation of existing enzyme, and is responsible for the rapid changes in blood composition following transfer to freshwater from a hyper-osmotic medium. Alternatively, observed differences in the rate of conformation to different media may be explained simply by different diffusion gradients. There was a difference of approximately 120 mmol l^{-1} in chloride ion concentration between medium and blood in the long-term acclimation experiment (see Fig. 4.14), compared to a difference of over 300 mmol l^{-1} in the reverse acclimation experiment (see Fig. 4.17). Obviously the gradient for passive diffusion of chloride ion was much greater in the reverse acclimation experiment, so may explain the rapid initial rate of change observed.

Results would indicate that *A. pallipes*, *A. leptodactylus* and *P. leniusculus* show a similar response to increased salinity. The crayfish are hyperosmotic and hyperionic regulators in freshwater and low salinity, but are conformers in high salinity. A change in body ionic status has serious consequences for metabolism and also on body acid-base status, i.e control of H^+ (pH). Disruption of ion balance has the potential to cause fundamental shifts in pH and again seriously affect metabolism (McMahon, 1986). In addition, changes in blood osmolality may

cause serious changes in cell volume or metabolic (or even physical) damage to cells, if the cells did not also control their osmotic pressure. In crayfish, and in all other euryhaline animals tested, this is accomplished by adjustment of free amino acid (FAA) levels, which does not affect the ionic composition of the cells. Wheatly and McMahon (1982b) noted that haemocyanin, the oxygen transport molecule and the major protein in the blood, is broken down in crayfish acclimated to 75% seawater. At the same time, the level of FAA in the blood increases, leading to speculation that a source of FAA may come from the haemocyanin. An increase in FAA in the blood would explain the observation that in long-term acclimation experiments, osmotic concentration of the haemolymph was greater than that of the medium after 14 days in 60% seawater despite an ionic conformity to the medium (Fig 4.13). Breakdown of haemocyanin results in serious depletion of the animal's oxygen carrying capacity, although this is compensated for in some part by an actual increase in oxygen affinity of the remaining haemocyanin, due to the change in ion levels (Wheatly and McMahon, 1982b). However, exposure to a hypersaline medium must seriously reduce the animal's ability to increase oxygen uptake, as would be necessary for an increase in activity, etc. (McMahon, 1988).

A combination of these stresses probably prevent long-term survival of freshwater crayfish in a hypersaline environment. Gilhooly (unpub.) found that the osmotic concentration required to kill 50% of *P. leniusculus* over a 30 day period was 586 mOsmoles kg^{-1}. This concentration is just above the value for the transition point from hyper-regulation to hypo-regulation

calculated in Table 4.4. The transition points calculated in Table 4.4 may therefore represent the upper osmotic and ionic tolerances of *A. pallipes*, *A. leptodactylus* and *P. leniusculus*, and correspond to a salinity of 45 - 50 % seawater. This would agree with laboratory data for other freshwater crayfish species. Herman (1931) found that *Potamobius astacus* could survive for up to 6 weeks in 50 % seawater, but could not tolerate higher salinities. Similarly, Kentall and Schwarz (1964) recorded a 72 - 96 hour LC_{50} at this salinity for *Orconectes virilis* and *Cambarus bartoni*. The data would also agree with field observations of adult crayfish, including *P. leniusculus* and *A. leptodactylus*, in dilute saline environments, such as estuaries. However, it should be noted that the establishment of viable populations under these conditions would depend on the considerably lower tolerance of early juvenile stages.

SPECIES	TIME (HOURS)			
	24	48	72	96
Pacifastacus leniusculus	13.6 (9.83 - 24.4)	5.70 (3.92 - 7.28)	3.40 (2.24 - 4.67)	2.84 (1.75 - 3.99)
Austropotamobius pallipes	10.7 (8.31 - 19.2)	5.81 (4.12 - 7.71)	4.54 (3.41 - 5.55)	4.24 (3.13 - 5.16)
Astacus leptodactylus	10.5 (8.88 - 13.6)	9.06 (7.72 - 10.5)	7.66 (6.56 - 8.95)	7.12 (6.20 - 8.28)

Table 4.1: Median lethal concentrations (LC_{50}) and 95% confidence limits (g l^{-1} chloride) for stage II crayfish juveniles, calculated by probit analysis at 24, 48, 72 and 96 hours.

Table 4.2: Chemical parameters for cage sites and chemical sampling points in the east branch, west branch and the confluence of the River Leen.

Parameter	River Leen East	River Leen West	River Leen Confluence
Temperature[1] (°C)	**12.7** 1.37 5.0-20.0	**12.5** 1.32 5.0-19.1	**12.2** 1.30 4.0-18.1
Conductivity[1] (µS/cm)	**799** 51.8 620-1150	**3859** 103 3300-4650	**2779** 250 1400-4600
Oxygen[1] (mg/l)	**9.97** 0.45 8.22-12.3	**10.0** 0.33 8.70-12.3	**9.74** 0.59 6.91-13.5
pH[1]	**7.79** 0.13 6.50-8.18	**7.87** 0.17 6.58-8.29	**7.90** 0.10 7.18-8.30
Hardness[2] (mg/l $CaCO_3$)	**337** 19.8 227-460	**798** 25.9 747-900	**591** 45.4 400-900
Alkalinity[3] (mg/l $CaCO_3$)	**190** 12.3 130-265	**288** 18.1 148-395	**263** 13.5 173-320
Nitrite[3] (mg/l NO_2)	**0.22** 0.05 0.03-0.72	**0.20** 0.04 0.05-0.42	**0.15** 0.02 0.07-0.28
Nitrate[3] (mg/l NO_3)	**23.4** 7.33 4.80-83.6	**20.6** 5.42 3.52-51.0	**19.1** 4.34 2.99-51.0

Table 2. continued overleaf.

Parameter	River Leen East	River Leen West	River Leen Confluence
Chloride[4] ($mg/l\ Cl^-$)	**130** 15.2 68-235	**1047** 76.8 738-1250	**680** 103 222-1650
Sulphate[3] ($mg/l\ SO_4$)	**125** 6.11 100-162	**295** 30.9 162-530	**195** 8.53 162-232
Phosphate[3] ($mg/l\ PO_4$)	**0.55** 0.26 0.02-2.13	**0.21** 0.16 0.00-1.46	**0.09** 0.03 0.00-0.28
Copper[5] (mg/l Cu)	**0.001** - -	**0.002** - -	**0.001** - -
Zinc[5] (mg/l Zn)	**0.008** - -	**0.012** - -	**0.008** - -

Values shown for each parameter are mean, standard error and range for n=12 samples.
Method of determination as follows:
[1] Horiba Water Checker U-10 (Horiba Ltd., Kyoto, Japan)
[2] Titrametric determination (HMSO, 1981)
[3] Palintest Photometer 2000 (Wilkinson and Simpson Ltd., Gateshead, England)
[4] Jenway PCLM3 Chloride Meter (Jenway Ltd., Dunmow, England)
[5] Pye Unicam SP9 Atomic Absorption Spectrophotometer (Pye Unicam Ltd., Cambridge, England)

SAMPLE SITE	NO. OF SPECIES (BMWP TAXA)	ETBI	BMWP (ASPT)[1]	BIOLOGICAL QUALITY*
EAST BRANCH	29 (20)	11	106 (5.30)	Very Good
WEST BRANCH	17 (13)	7	63 (4.85)	Moderate
CONFLUENCE	14 (12)	8	69 (5.75)	Good

[1] Average Score Per Taxon, calculated from BMWP and number of scoring taxa.
* Based on National Water Council interpretation of BMWP scores, i.e;

0 - 12 Unsatisfactory
13 - 35 Poor
36 - 70 Moderate
65 - 95 Good
90 - 150 Very Good
> 150 Excellent

Table 4.3: Biotic Indices calculated from invertebrate data for the east branch, west branch and confluence of the River Leen.

a.) 1991	FIRST MOULT PERIOD			SECOND MOULT PERIOD		
	n	MI	PCMI	n	MI	PCMI
EAST	4	3.5 (0.29)	10.5 (0.42)	9	3.00 (0.19)	9.57 (0.86)
WEST	9	3.3 (0.30)	10.5 (0.71)	9	2.88 (0.26)	8.48 (0.73)
b.) 1992						
EAST	6	3.75 (0.21)	10.9 (0.06)	1*	3.00	8.70
WEST	7	3.66 (0.40)	9.19 (0.20)	6	2.92 (0.20)	8.10 (0.68)
c.) POOLED DATA						
EAST	10	3.63	10.7	10	3.13	9.96
WEST	16	3.57	10.9	15	2.81	8.29

* No other available data - cage vandalised.

Table 4.4: Absolute (MI) and percentage (PCMI) moult increments of crayfish held in cages in the east and west branch of the River Leen. Values are means with standard errors in brackets, n is the number of crayfish.

Table 4.5: Blood osmotic and ion concentrations of crayfish stepwise acclimated to FW and 20, 40, 60 and 80% seawater for 48 hours. Values are means with standard errors (n=4).

	FW	20%	40%	60%	80%
a.) Osmotic Concentration (mOsm kg^{-1})					
Pacifastacus leniusculus	**414** (23.0)	**443** (5.70)	**453** (23.0)	**525** (6.00)	**628** (15.0)
Austropotamobius pallipes	**411** (6.10)	**428** (4.30)	**471** (9.60)	**511** (5.90)	**573** (7.40)
Astacus leptodactylus	**395** (1.60)	**415** (5.20)	**434** (10.3)	**479** (13.0)	**565** (9.40)
b.) Chloride Concentration (mMoles l^{-1})					
Pacifastacus leniusculus	**215** (9.40)	**219** (4.20)	**223** (12.0)	**262** (4.90)	**351** (9.30)
Austropotamobius pallipes	**204** (2.60)	**216** (2.50)	**226** (4.30)	**260** (3.40)	**284** (4.90)
Astacus leptodactylus	**212** (4.40)	**217** (3.50)	**225** (4.50)	**259** (6.90)	**281** (5.50)
c.) Sodium Concentration (mMoles l^{-1})					
Pacifastacus leniusculus	**169** (29.7)	**187** (29.0)	**192** (40.7)	**266** (11.4)	**300** (35.2)
Austropotamobius pallipes	**157** (1.70)	**182** (2.00)	**181** (7.80)	**241** (10.2)	**260** (9.20)
Astacus leptodactylus	**172** (2.80)	**170** (3.30)	**181** (6.10)	**224** (7.30)	**264** (4.20)

SPECIES	TRANSITION POINT (HYPER TO HYPO-REGULATION)		
	OSMOTIC CONC. (mOsm kg^{-1})	CHLORIDE CONC. (mMoles l^{-1})	SODIUM CONC. (mMoles l^{-1})
Pacifastacus leniusculus	523	265	242
Austropotamobius pallipes	497	240	235
Astacus leptodactylus	470	241	231

Table 4.6: Values calculated from log-y regressions for the transition point from hyper-regulation to hypo-regulation during step-wise acclimation to increasing salinity.

SPECIES	SIZE/ STAGE	LC_{50} (gl^{-1})	TIME (HOURS)	REFERENCE
OLIGOCHAETA				
Nais variabilis		2.267	48	Hamilton *et al.*, 1975.
INSECTA				
Anabolia nervosa		4.260	72	Sutcliffe, 1961.
Limnephilus stigma		4.260	72	" "
Chironomus attenatus		4.857	48	Thornton and Sauer, 1972.
Cricotopus trifascia		5.380	"	Hamilton *et al.*, 1975.
Hydroptila angusta		6.151	"	" "
CRUSTACEA				
Pacifastacus leniusculus	10 mm 20 mm 36 mm	2.848 11.86 15.74	96 " "	This study " "
Austropotamobius pallipes	10 mm 20 mm	4.241 11.96	96 "	This study "
Astacus leptodactylus	10 mm 36 mm	7.117 13.80	96 "	This study "

Table 4.7: Acute toxicity of chloride to selected macrinvertebrates.

Figure 4.1: Chloride concentration (mgl^{-1}) against Conductivity (μScm^{-1}). Equation for regression line is as follows; Chloride concentration = 0.3176 Conductivity - 147.8 (R^2=0.949, $p<0.05$).

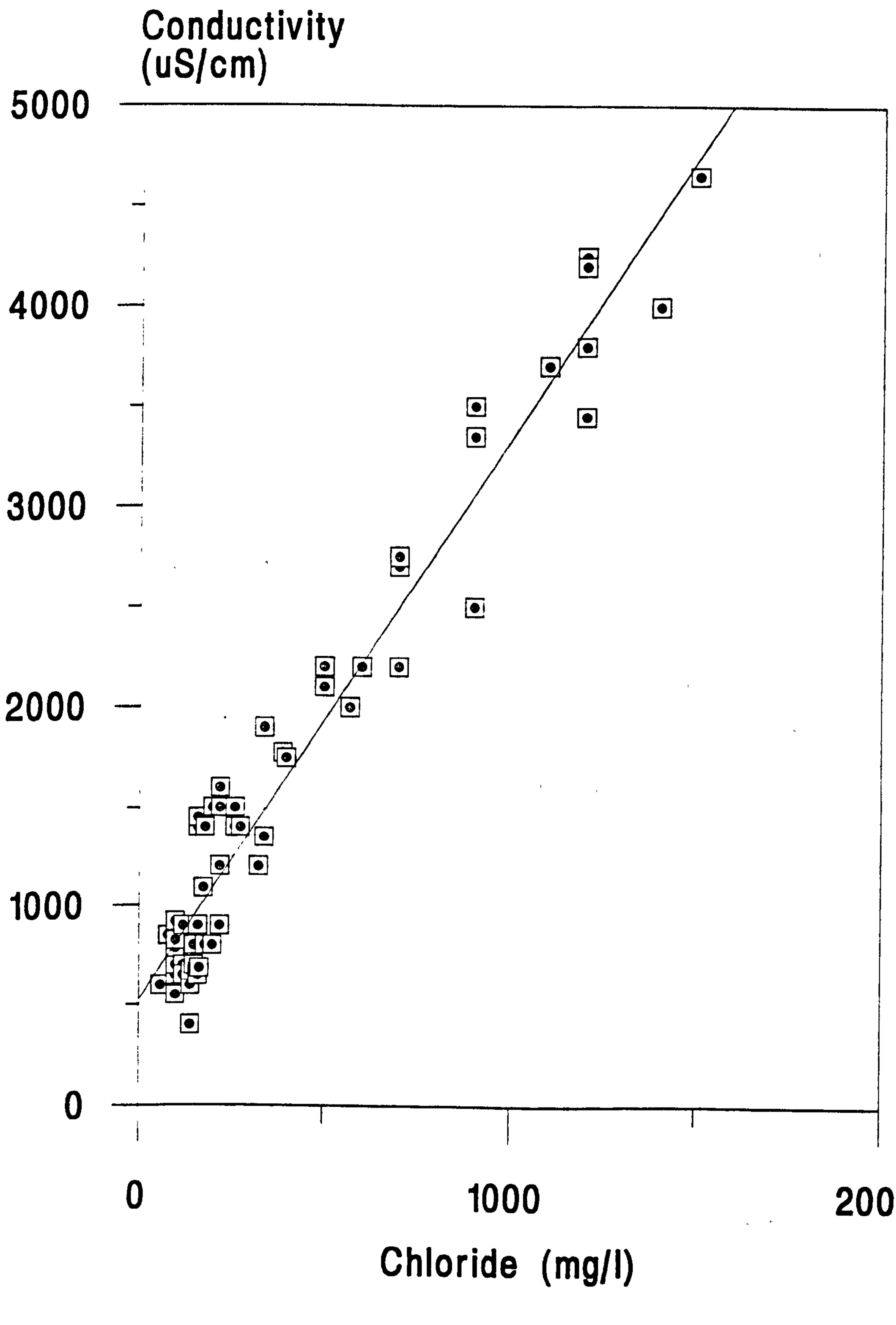
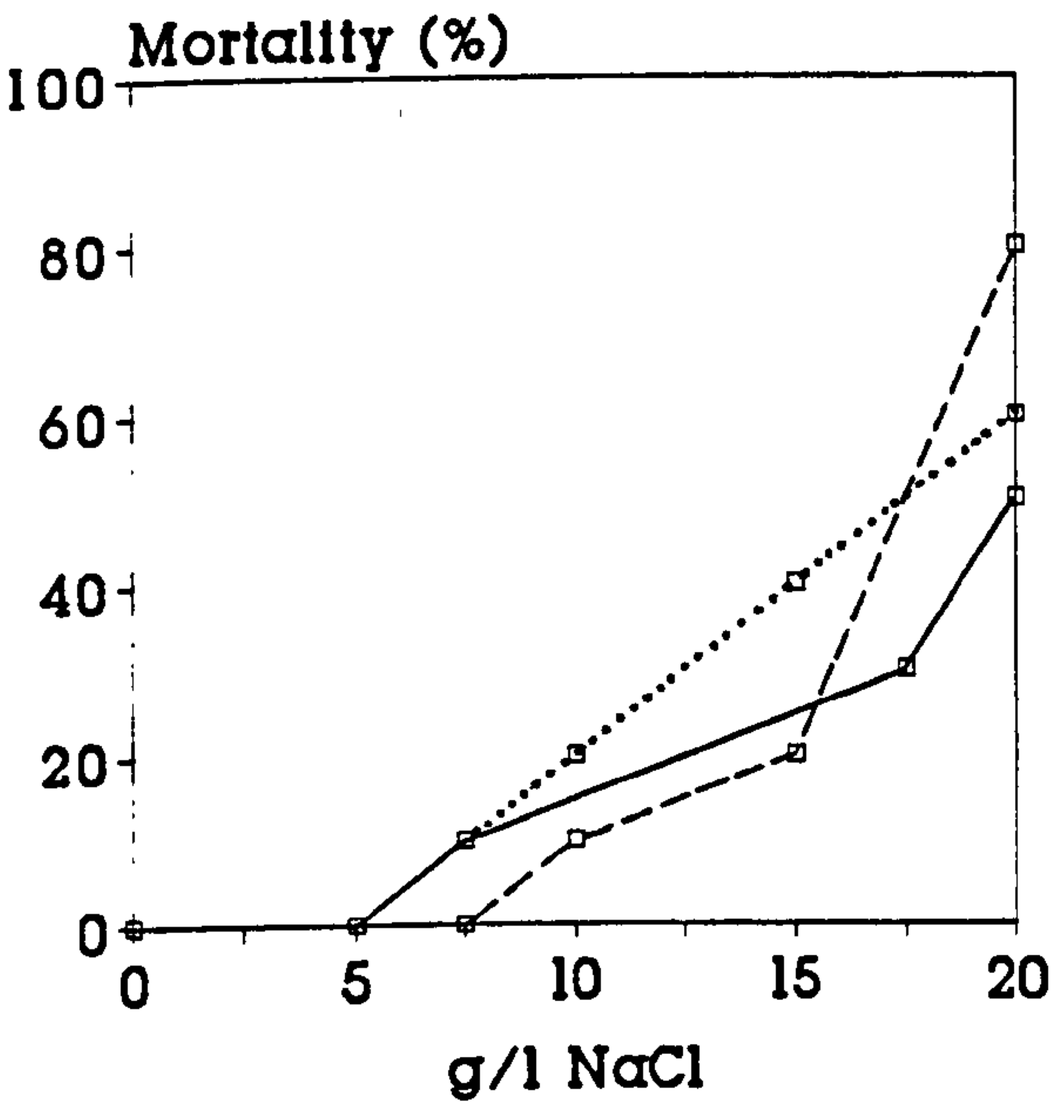
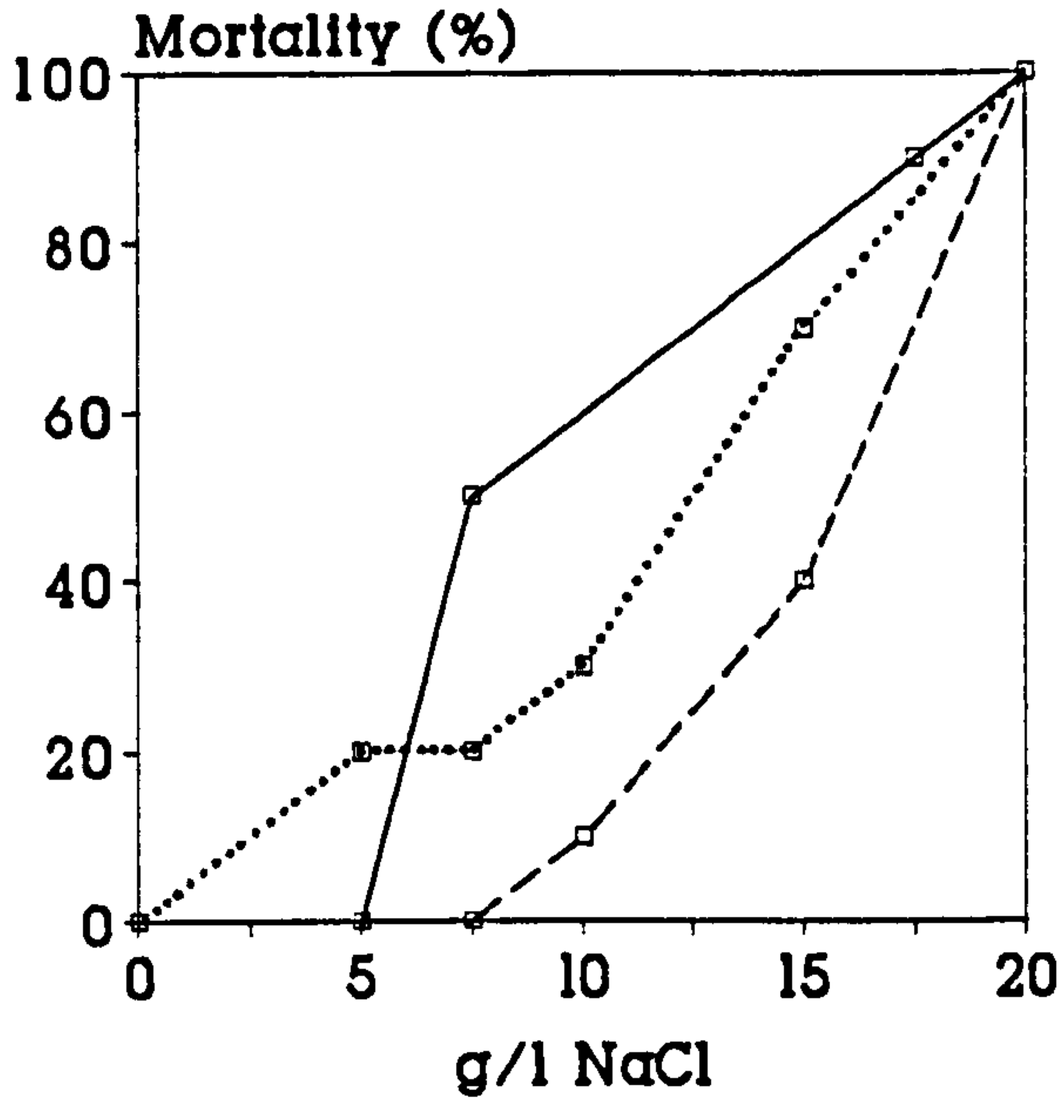


Figure 4.2: Percentage mortality of stage II crayfish juveniles with increasing chloride concentration (——*P. leniusculus*, ……*A. pallipes*,– – –*A. leptodactylus*) at 24, 48, 72 and 96 hours.

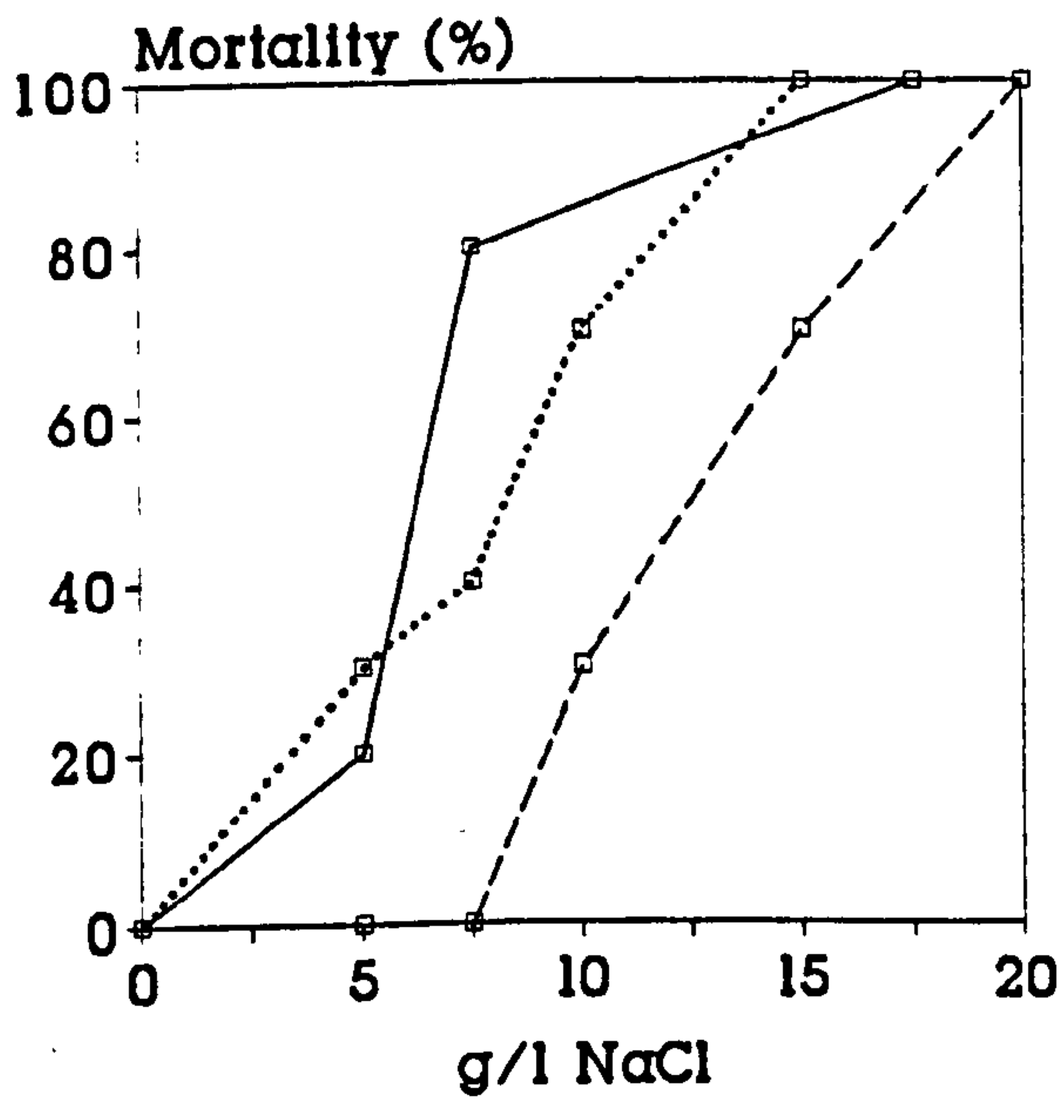
a.) 24 hours



b.) 48 hours



c.) 72 hours



d.) 96 hours

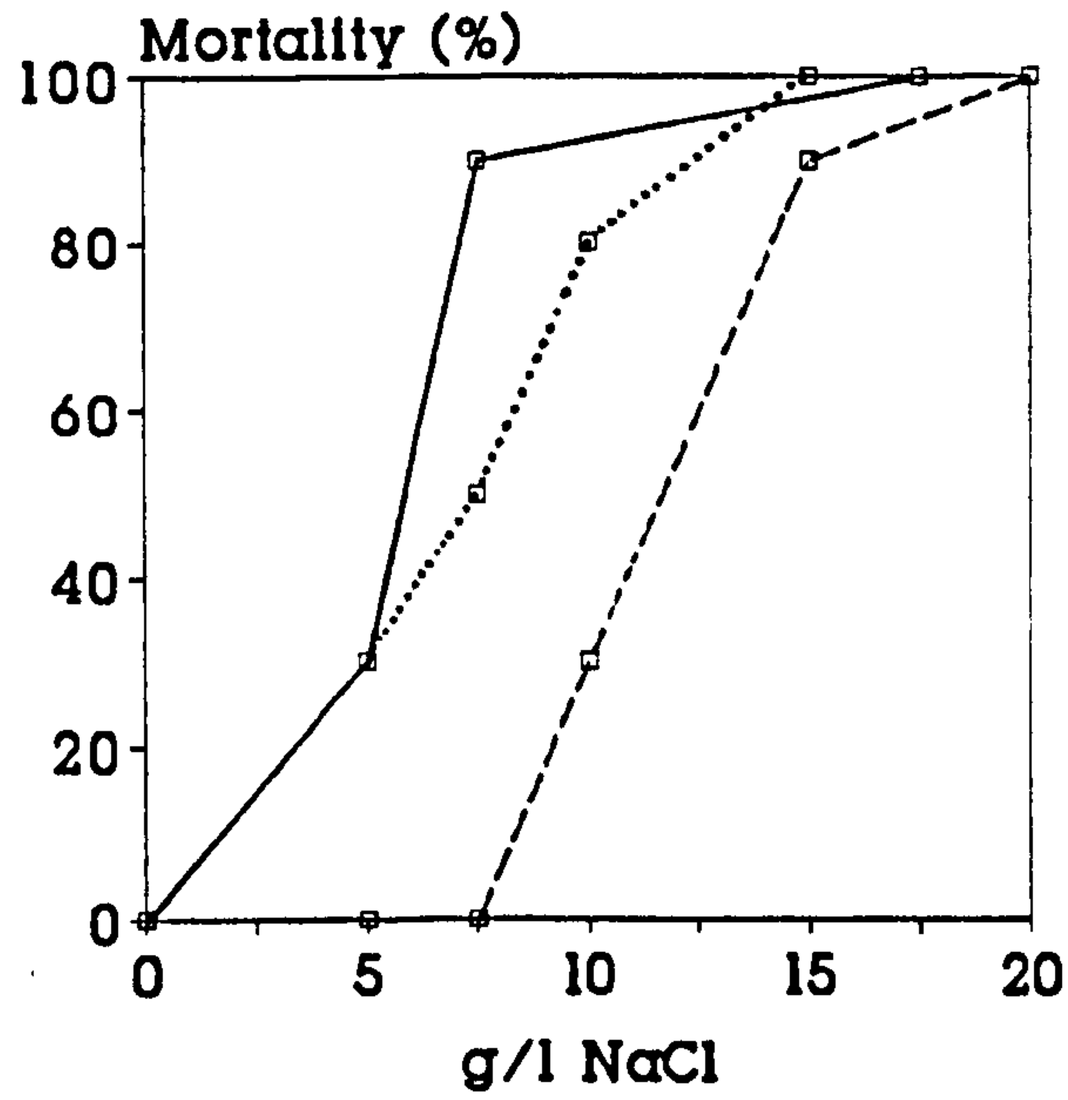


Figure 4.3: LC_{50} (g Cl^- l^{-1}) with time for stage II crayfish juveniles (—◇— *P. leniusculus*, —△— *A. leptodactylus*, ····□···· *A. pallipes*). Error bars indicate 95% confidence limits.

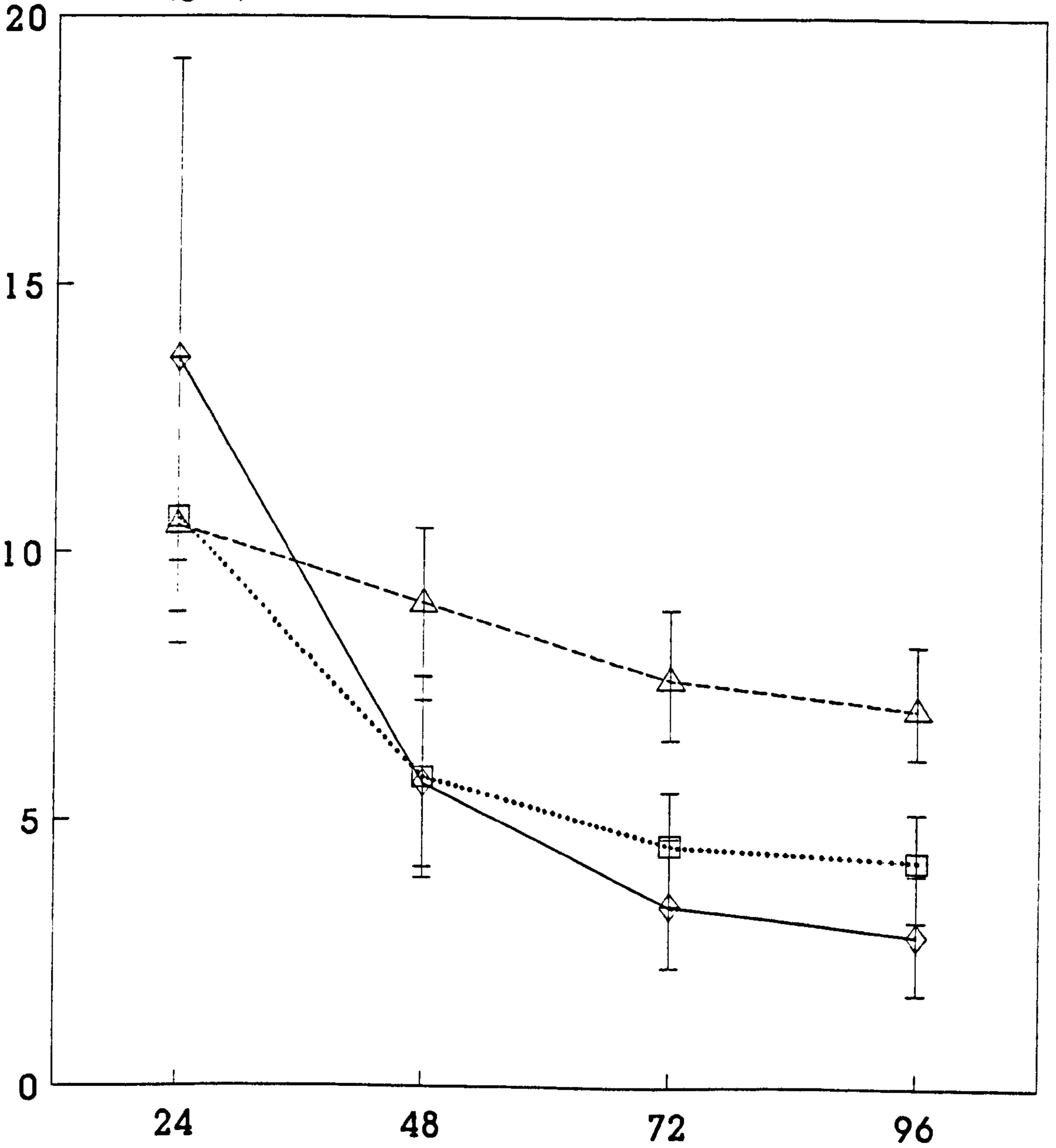
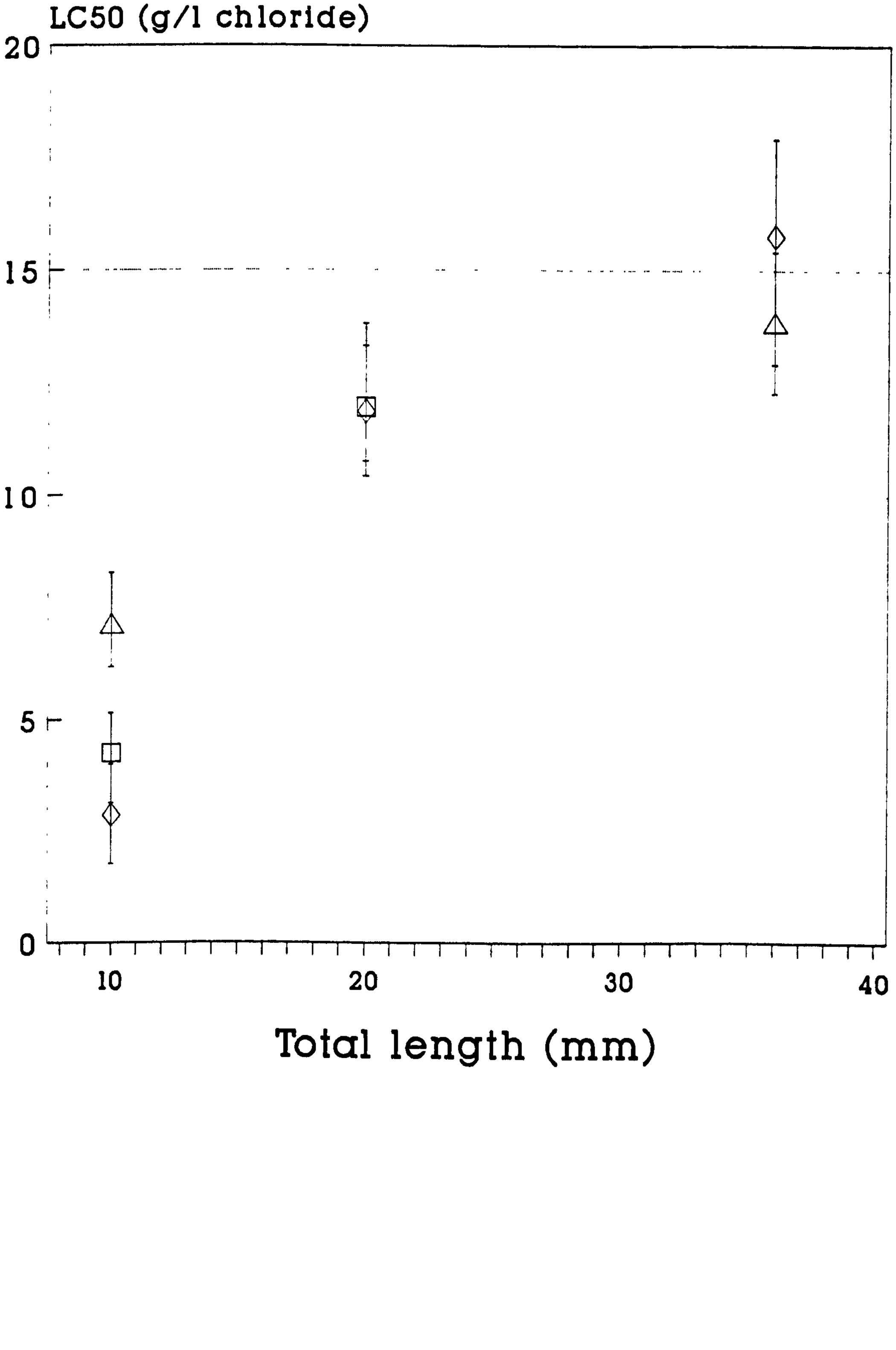


Figure 4.4: 96 hour LC_{50} (g Cl^- l^{-1}) for 10, 20 and 36 mm (total length) crayfish juveniles (◇ *P. leniusculus*, □ *A. pallipes*, △ *A. leptodactylus*). Error bars indicate 95% confidence limits.



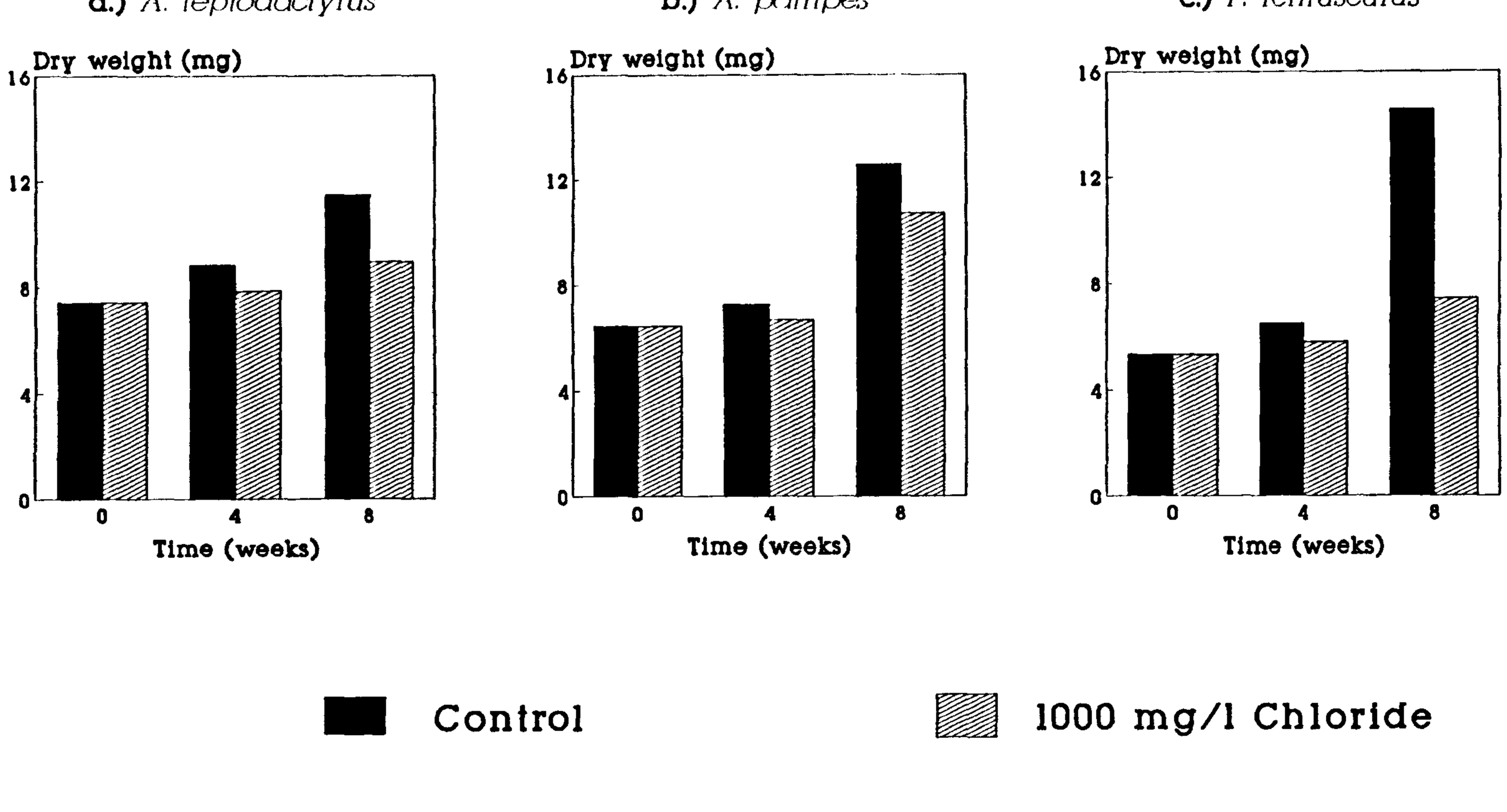


Figure 4.5: Dry weight (mg) of stage II juvenile crayfish exposed to a nominal concentration of 1000 mg l^{-} chloride at 0, 4 and 8 weeks with control (Nottingham tapwater) for comparison; a.) *A. leptodactylus*, b.) *A. pallipes*, c.) *P. leniusculus*. Bars indicate average dry weight (n=10).

Figure 4.6: Percentage reduction in growth for stage II crayfish juveniles exposed to 1000 mg l^{-} chloride. Values indicate significance level compared to controls.

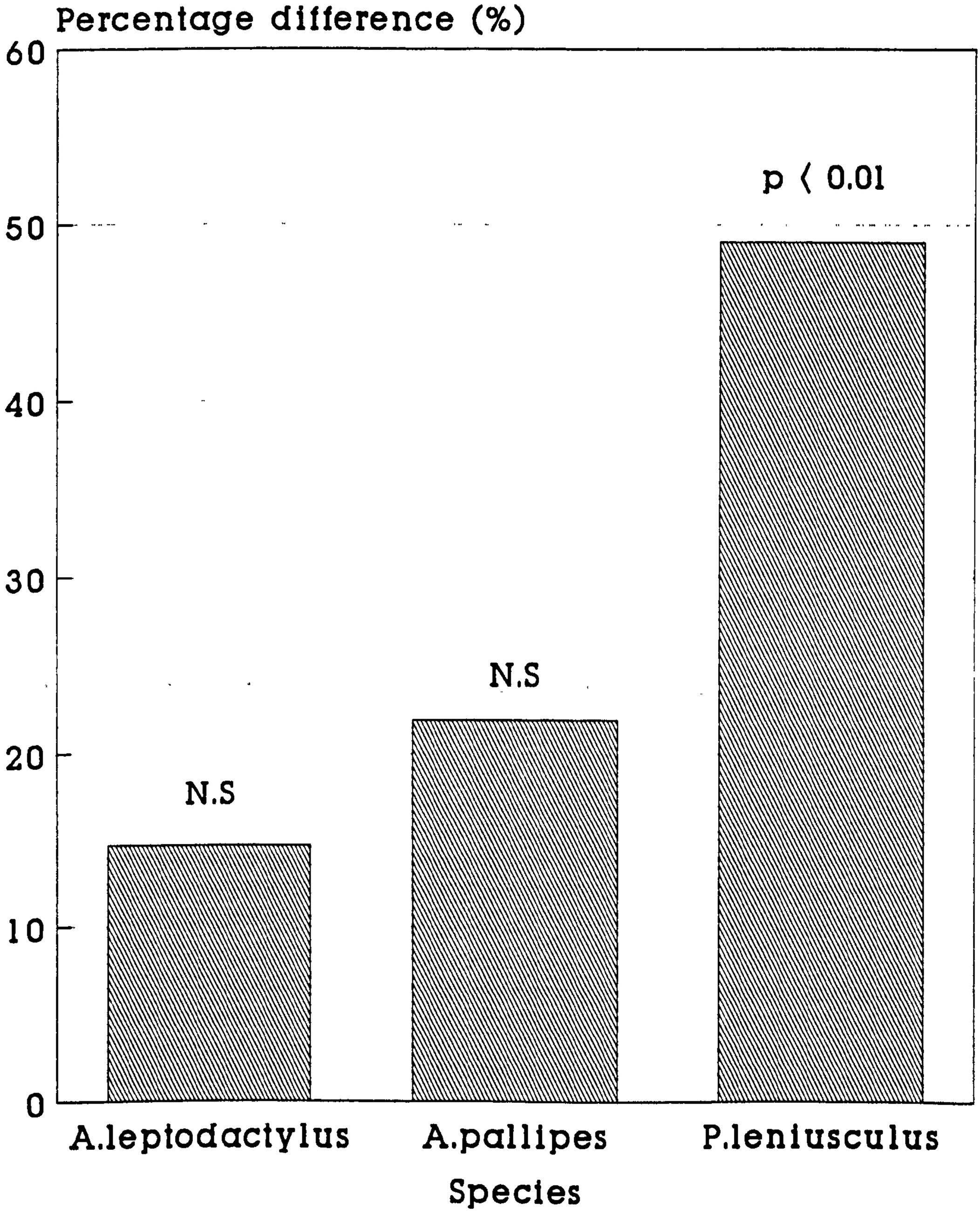


Figure 4.7: Male ***A. pallipes*** from cage experiment in the west branch of the River Leen. Carapace is covered in heavy growth of a epizootic protozoan, ***Epistylis sp***.

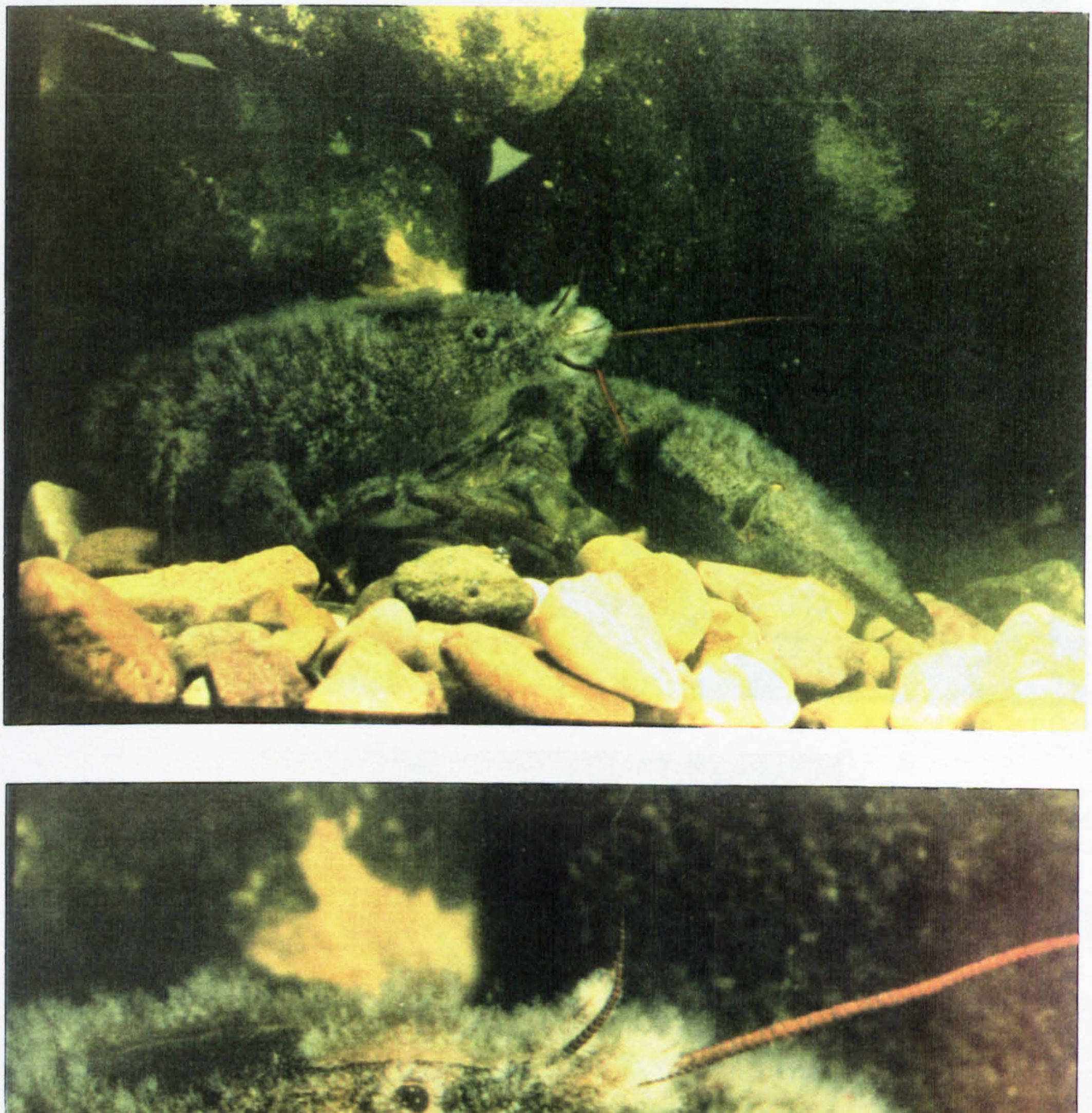
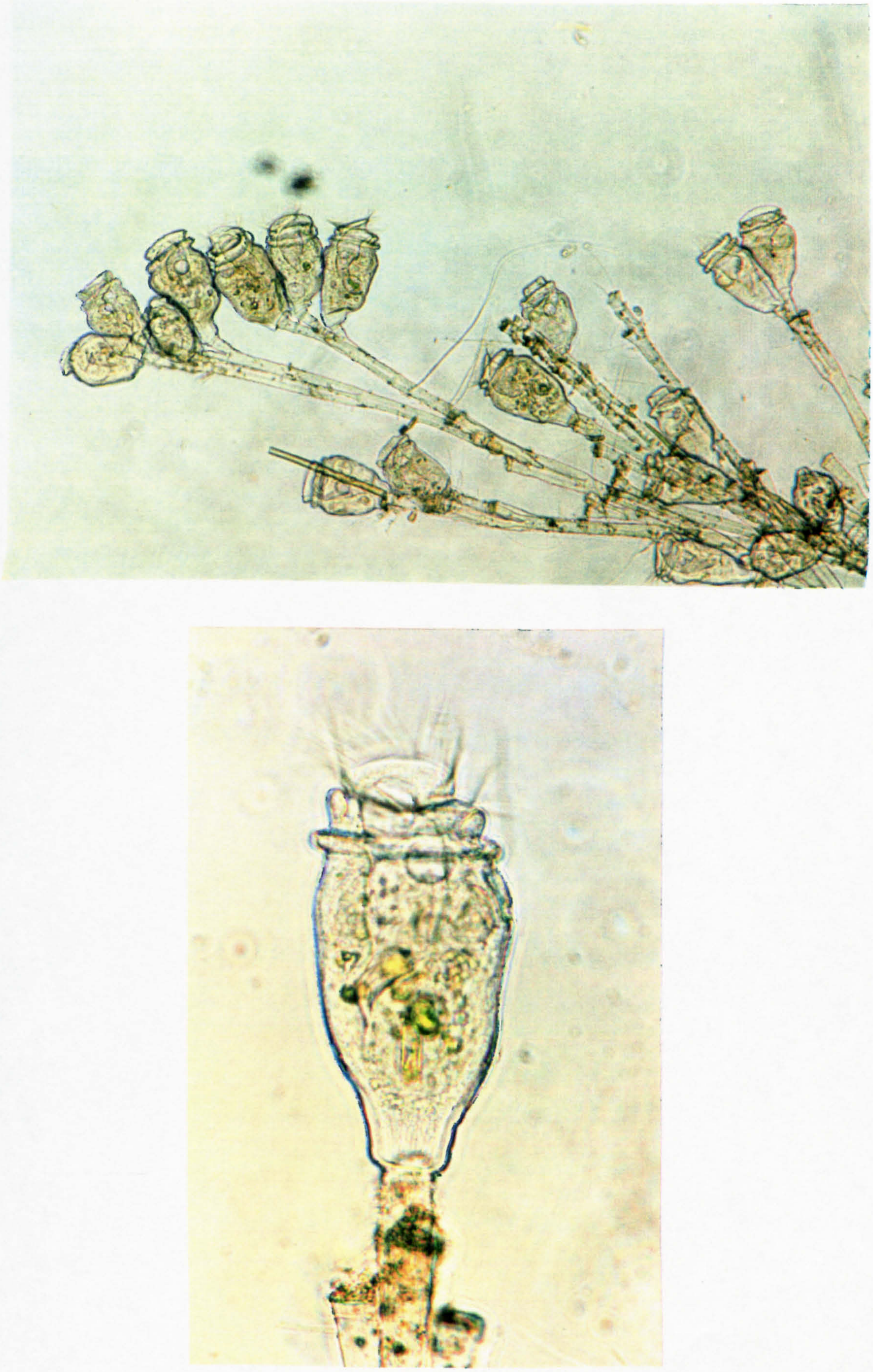


Figure 4.8: Epizootic protozoan ***Epistylis sp.*** taken from the carapace of male ***A. pallipes*** held in a cage in the west branch of the River Leen.



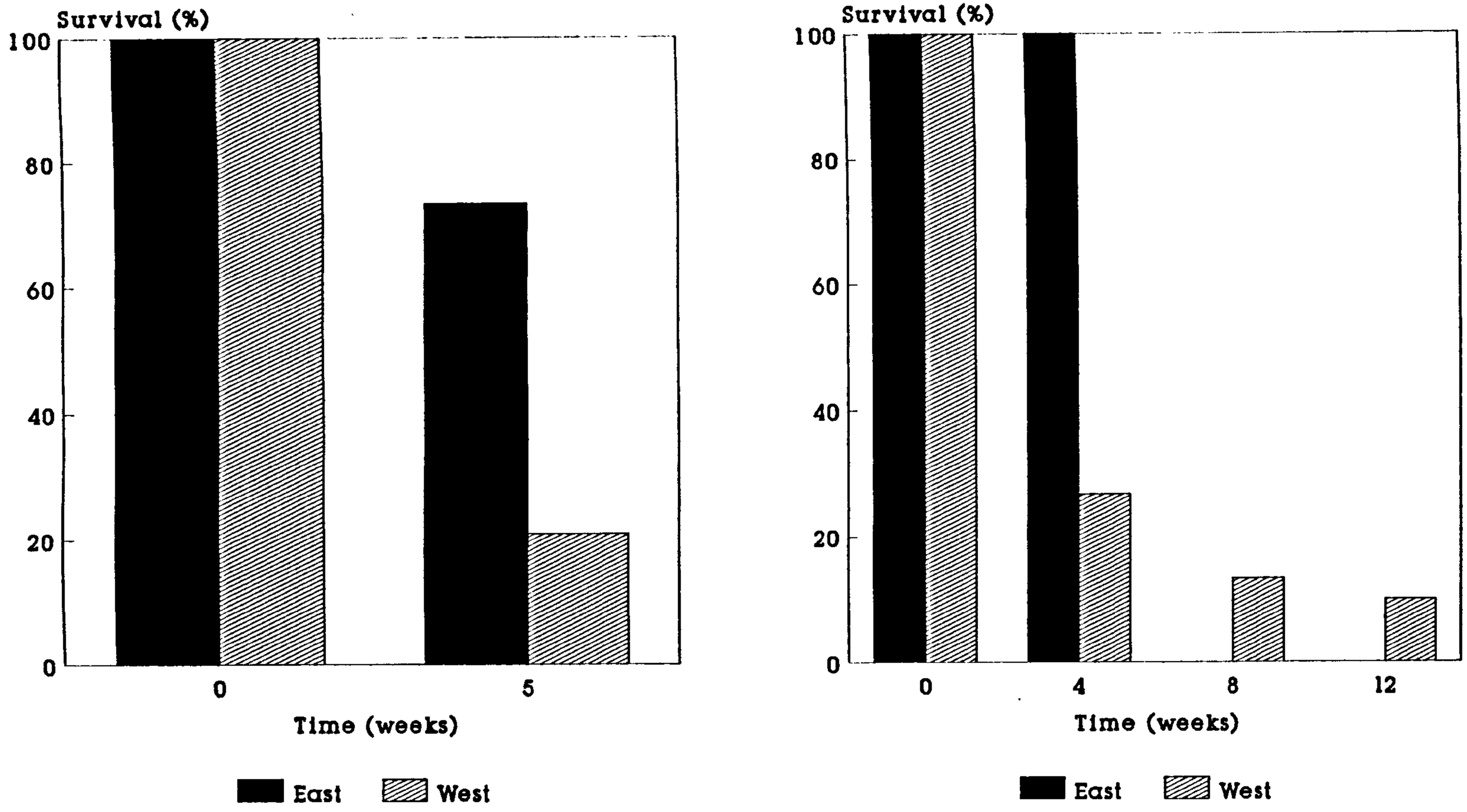


Figure 4.9: Percentage survival of a.) egg and b.) juvenile stages of *A. pallipes* held in cages in the east and west branches of the River Leen (N.B. no data available for stage II juveniles at 8 and 12 weeks in east branch).

Figure 4.10: Blood osmotic concentrations (mOsmol kg^{-1}) for crayfish step-wise acclimated to FW and 20, 40, 60 and 80% seawater for 48 hours (—◇— *P. leniusculus*, ···□··· *A. pallipes*, - - △ - - *A. leptodactylus*). Diagonal line represents points of equal osmotic concentration (iso-osmotic line). Values are means with standard errors (n=4).

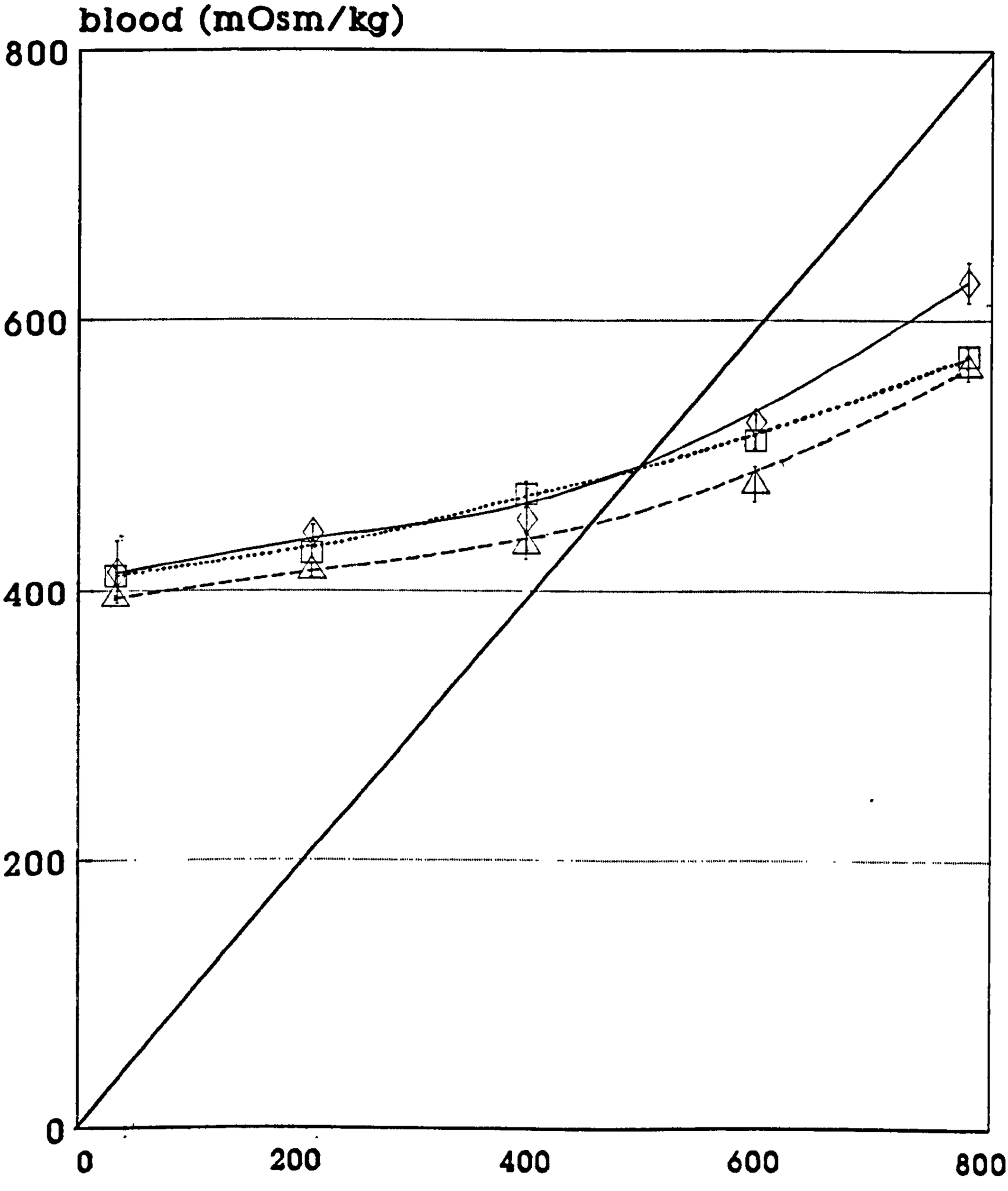


Figure 4.11: Blood chloride concentrations (mmol l^{-1}) for crayfish step-wise acclimated to FW and 20, 40, 60 and 80% seawater for 48 hours (—◇—*P. leniusculus*, ····□····*A. pallipes*, --△--*A. leptodactylus*). Diagonal line represents points of equal chloride concentration (iso-ionic line). Values are means with standard errors (n=4).

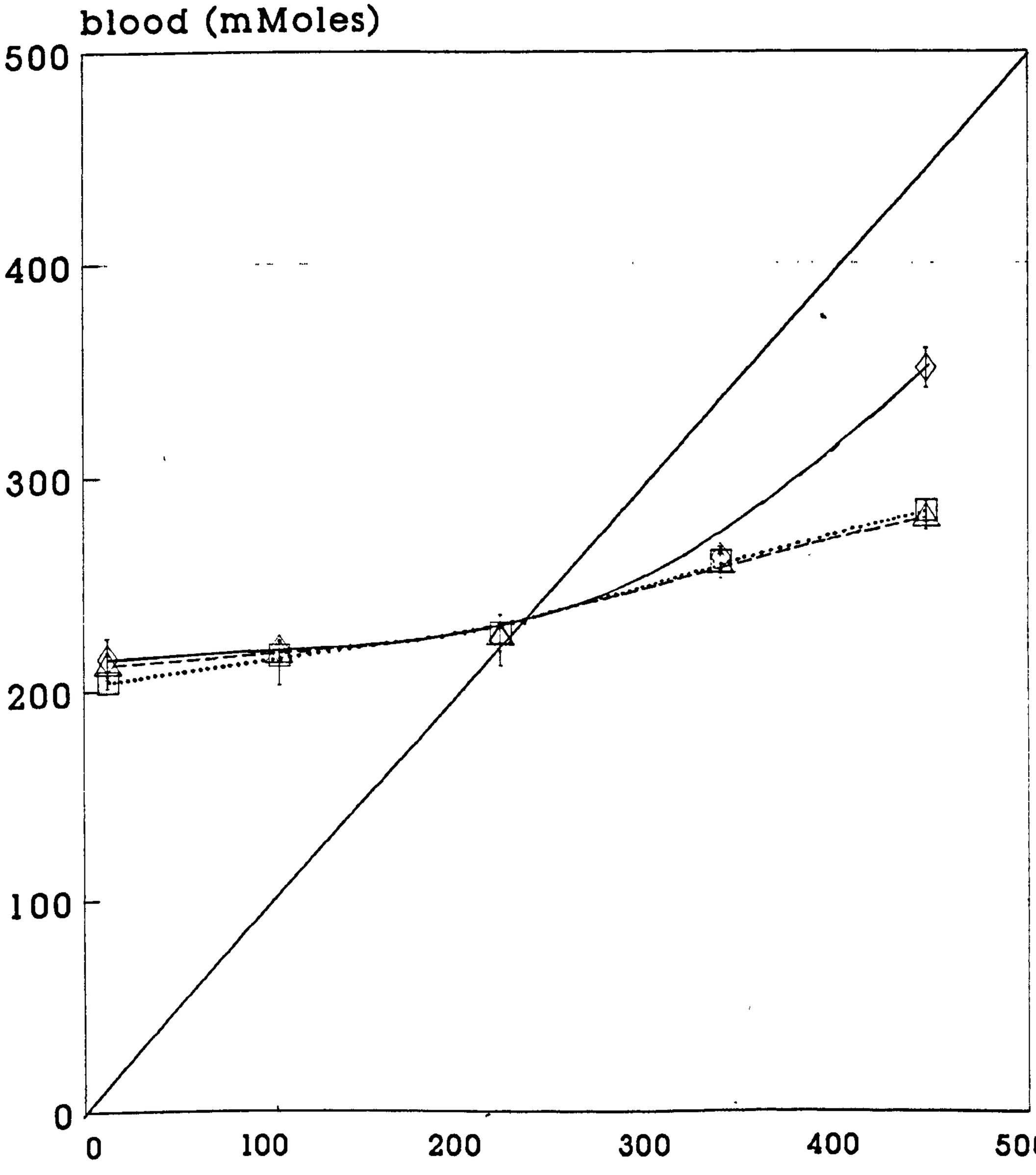


Figure 4.12: Blood sodium concentrations (mmol l^{-1}) for crayfish step-wise acclimated to FW and 20, 40, 60 and 80% seawater for 48 hours (—◇— *P. leniusculus*, ····□··· *A. pallipes*, --△-- *A. leptodactylus*). Diagonal line represents points of equal sodium concentration (iso-ionic line). Values are means with standard errors (n=4).

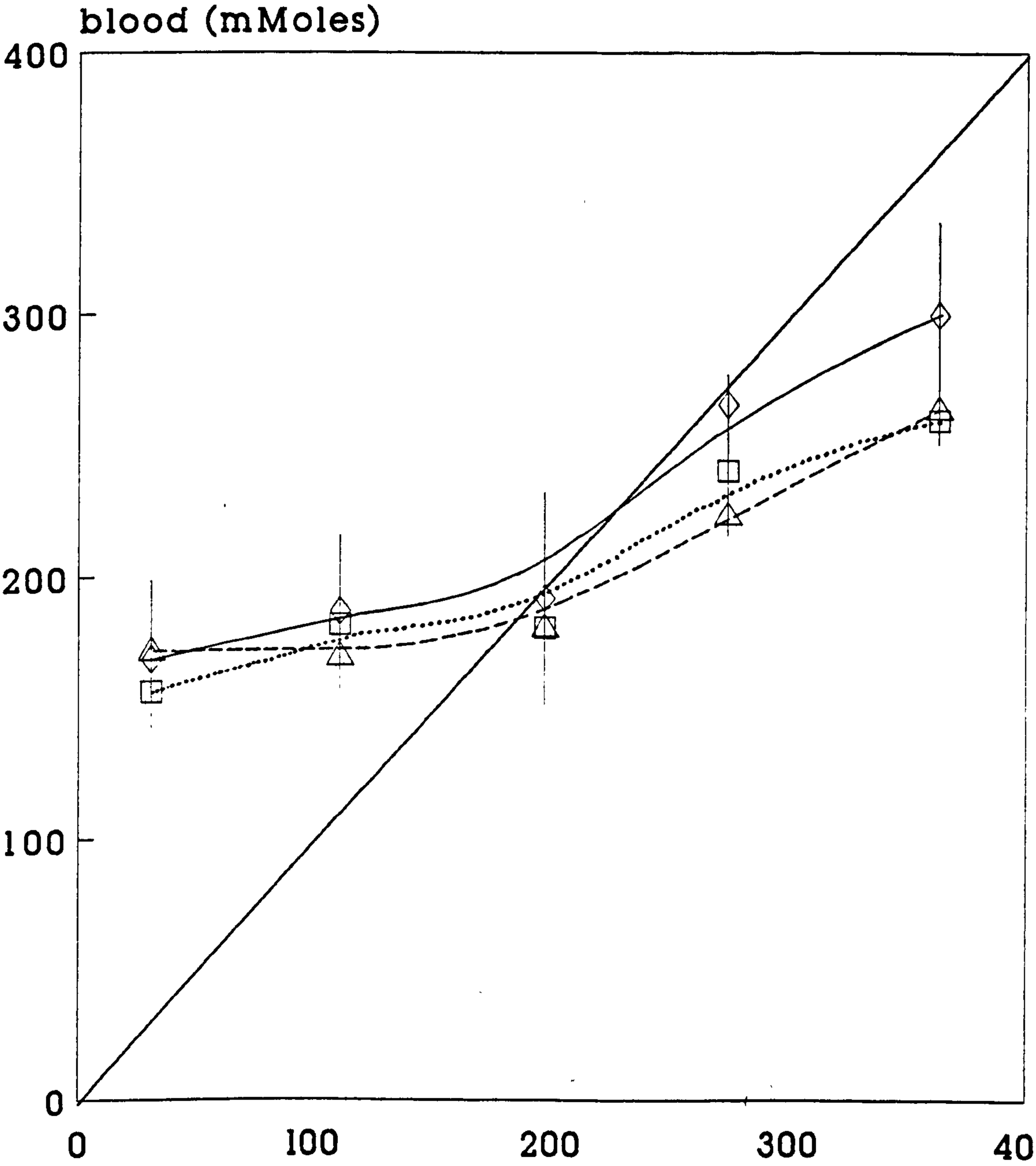


Figure 4.13: Time course changes in blood osmotic concentration in *P. leniusculus* acclimated to freshwater and transferred to 60% seawater. Horizontal lines represent osmotic concentration of the medium at the given experimental salinity. Values are means with standard errors (n=4).

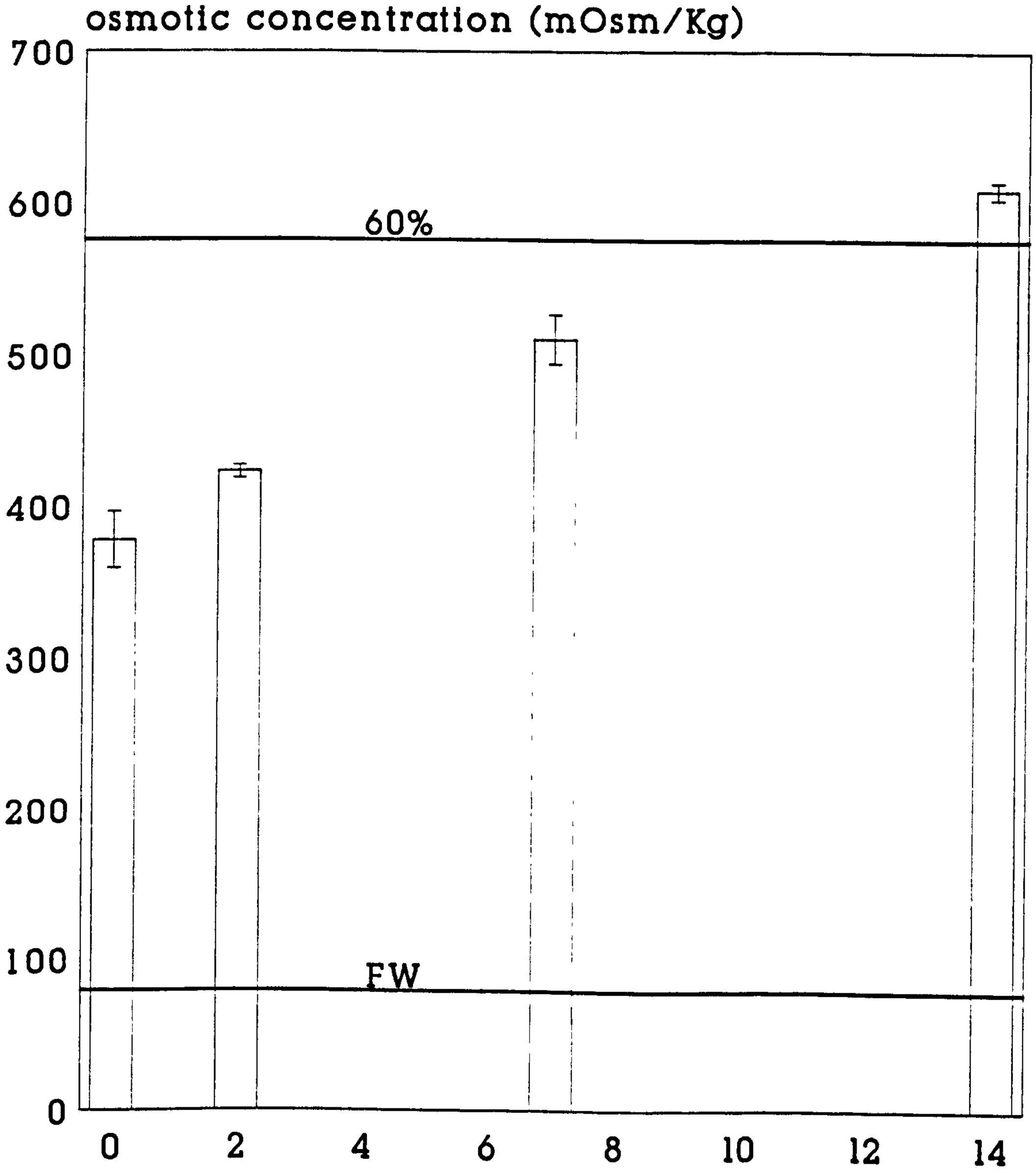


Figure 4.14: Time course changes in blood chloride concentration in *P. leniusculus* acclimated to freshwater and transferred to 60% seawater. Horizontal lines represent chloride concentration of the medium at the given experimental salinity. Values are means with standard errors (n=4).

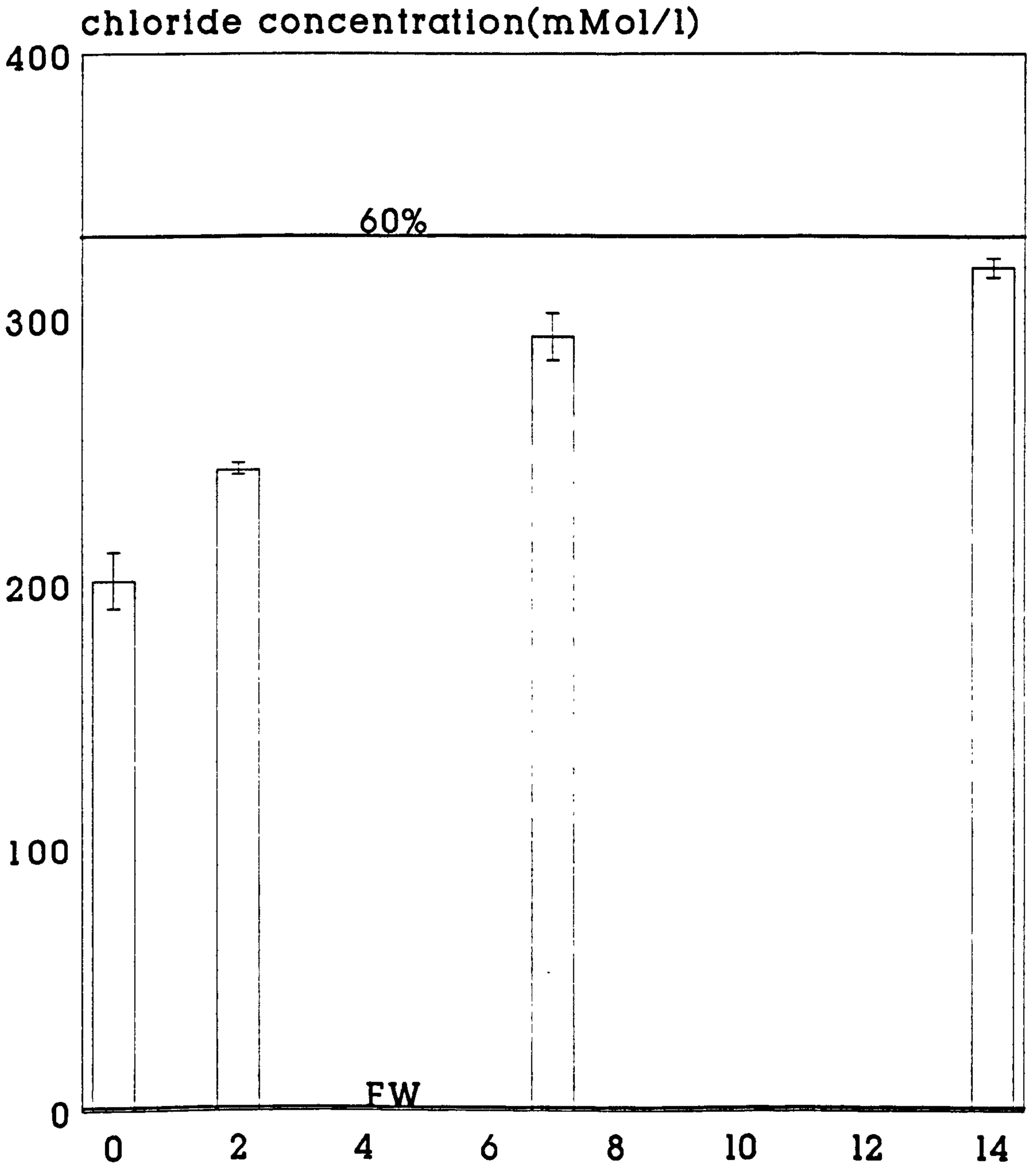


Figure 4.15: Time course changes in blood sodium concentration in *P. leniusculus* acclimated to freshwater and transferred to 60% seawater. Horizontal lines represent sodium concentration at the given experimental salinity. Values are means with standard errors (n=4).

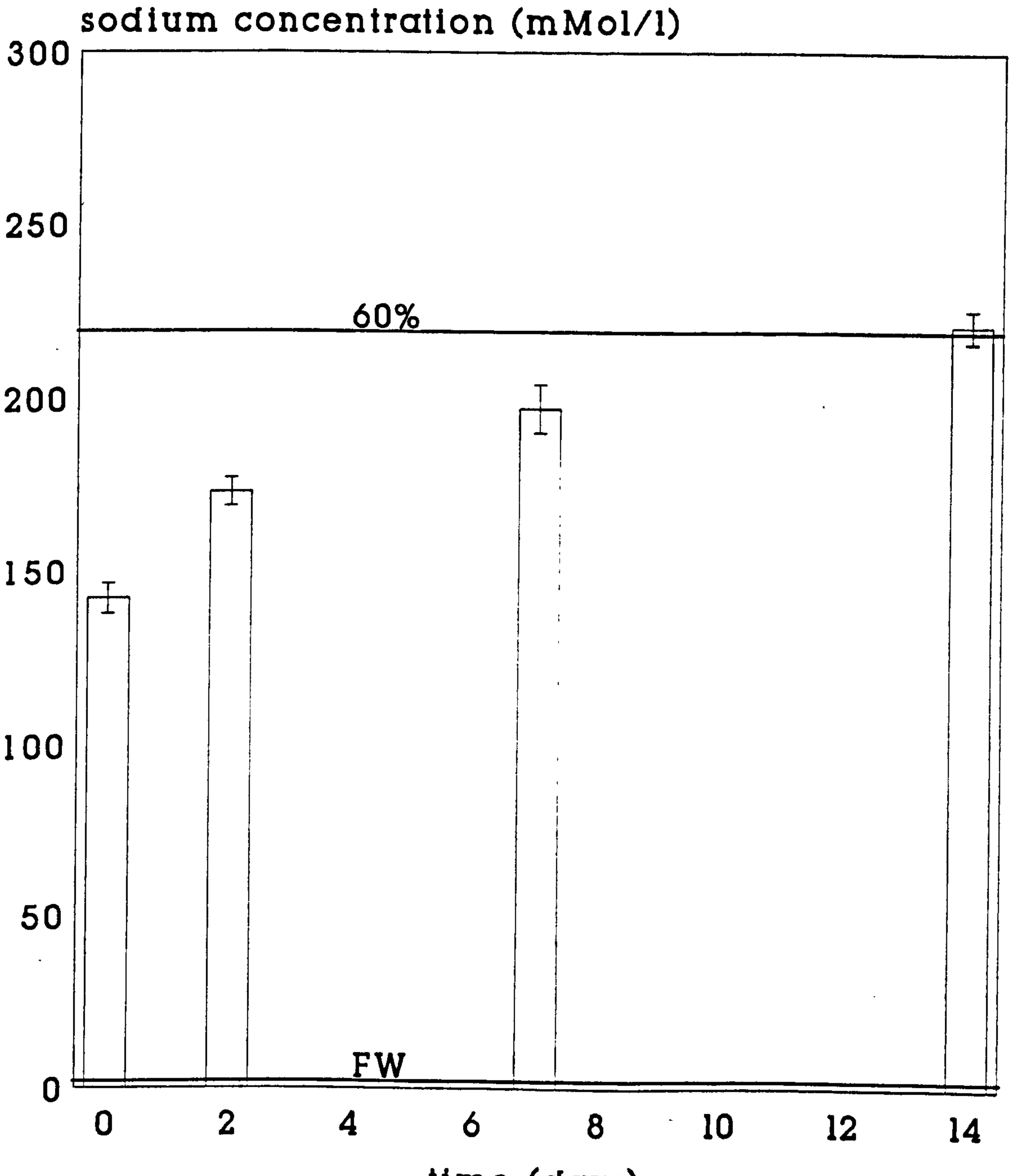


Figure 4.16: Time course changes in blood osmotic concentration of *P. leniusculus* acclimated to 60% seawater and transferred to freshwater. Horizontal lines represent osmotic concentration at the given experimental salinity. Values are means with standard errors (n=4).

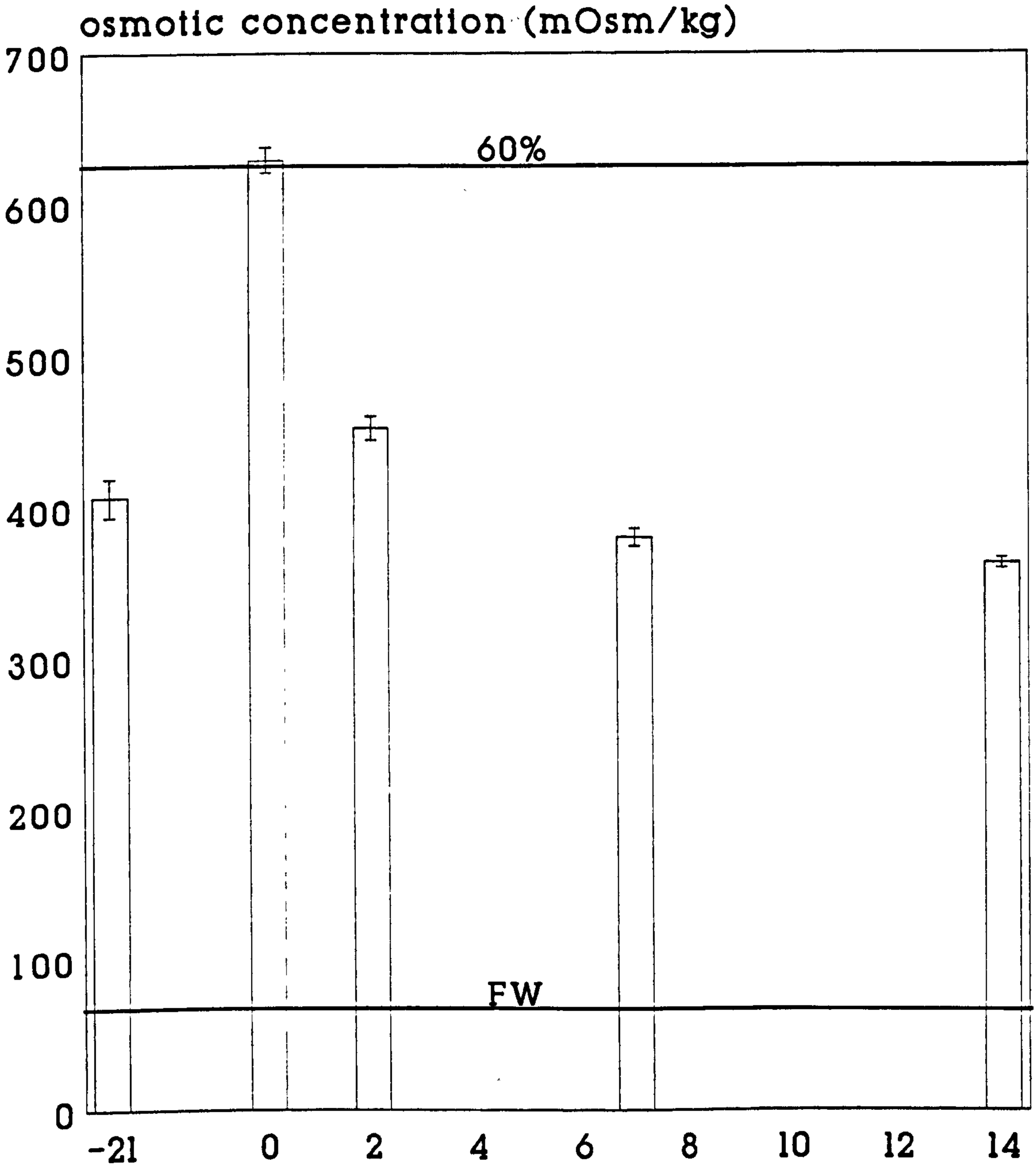


Figure 4.17: Time course changes in blood chloride concentration of *P. leniusculus* acclimated to 60% seawater and transferred to freshwater. Horizontal lines represent chloride concentration at the given experimental salinity. Values are means with standard errors (n=4).

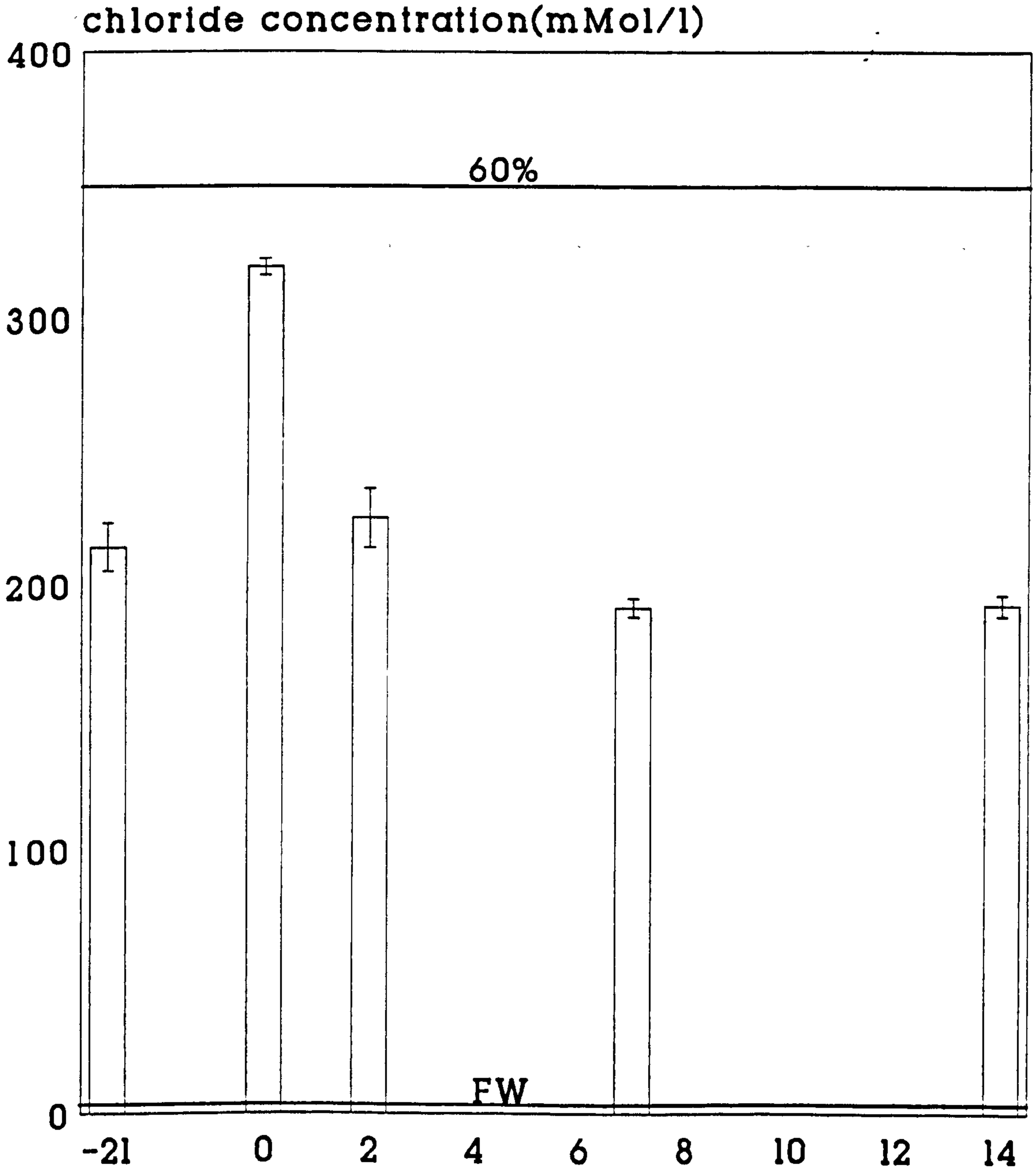
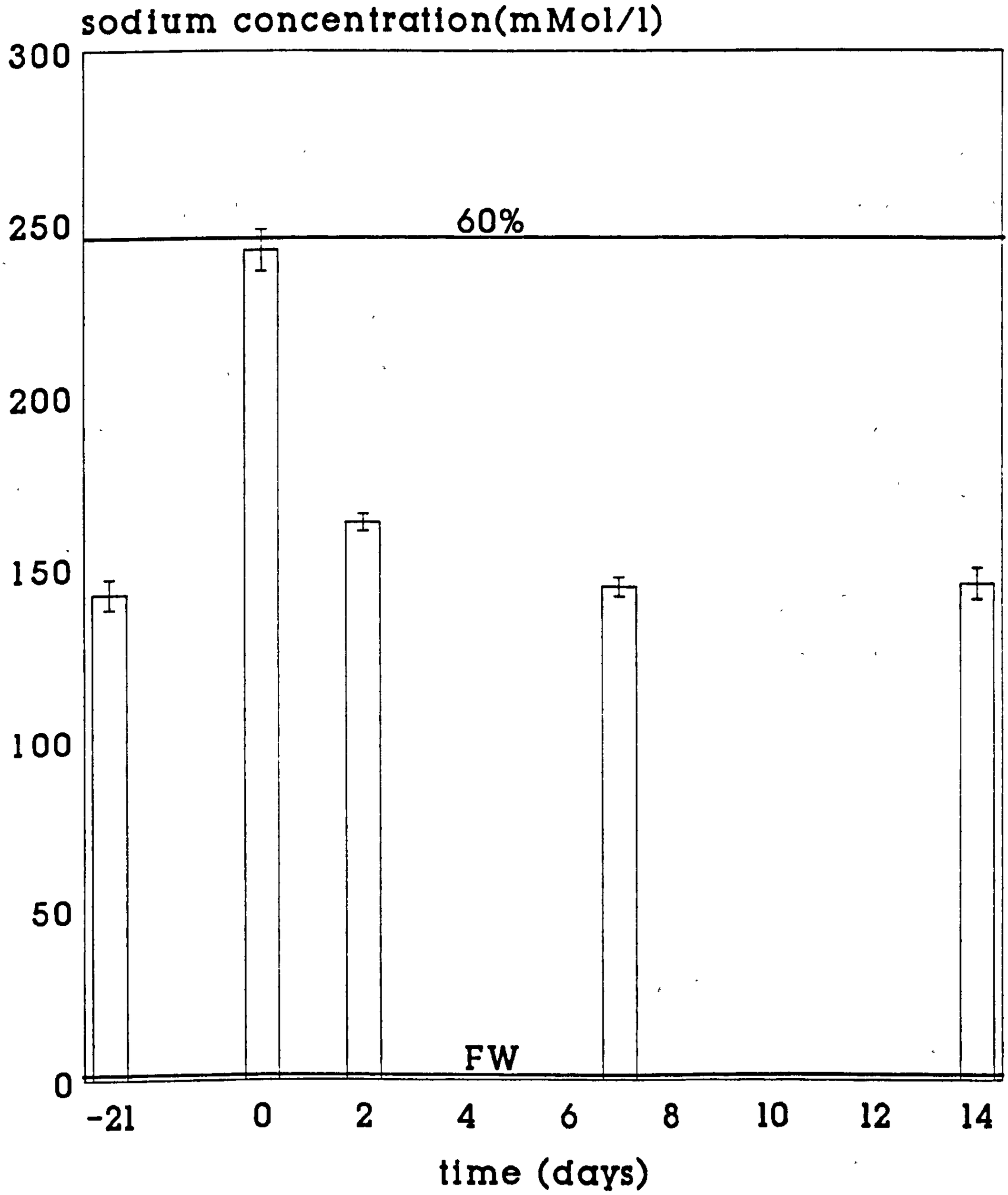


Figure 4.18: Time course changes in blood sodium concentration in *P. leniusculus* acclimated to 60% seawater and transferred to freshwater. Horizontal lines represent sodium concentration at the given experimental salinity (n=4).



CHAPTER 5

COPPER

5.1 INTRODUCTION

Metals are continuously released into air, soil and water from natural and anthropogenic sources. In most waters the concentrations of metals are low, although higher natural concentrations occur in rivers and estuaries which are associated with outcropping metalliferous lodes. As a result the concentrations of metals in natural waters can easily be increased to levels which aquatic organisms have not previously encountered.

Natural fluxes of some metals, such as iron, are similar to those resulting from industry. However, anthropogenic sources of most other metals, such as cadmium, far exceed those from natural processes.

In this study, copper was chosen as a representative of a metal pollutant. Copper enters the freshwater environment primarily in mine drainage and in sewage and industrial effluents, such as those from electroplating and chemical industries. Large quantities may also be associated with storm run-off from urban areas (Tomlinson *et al.*, 1980). In agricultural areas contamination may arise through application of sewage sludge as a soil conditioner which, if from industrial areas may contain copper and other metals. A specific problem of copper contamination of agricultural soils may arise following

application of pig slurry (Hopkin, 1989). The growth rate of pigs is substantially increased by addition of copper to their diet at a concentration of 0.2 mg g^{-1}. The physiological mechanisms behind this are not fully understood, however most of the copper is voided in the faeces which may then be used as a fertilizer. Run-off from contaminated agricultural land into adjacent water courses may constitute a major source of metal contamination. Copper compounds have been directly utilised in the aquatic environment for their molluscicidal and piscicidal properties (Ebele *et al.*, 1990). The toxicity of copper is affected by many environmental factors and therefore high field doses are often required. Copper (II) sulphate is still used extensively as an algicide, specifically to control blooms of blue-green algae, which are very copper sensitive (Luederitz *et al.*, 1989), and also to control growth of macrophytes.

The toxicity of copper is thought to be largely attributable to the Cu^{2+} ion, however it readily forms complexes with a wide range of organic and inorganic substances and is also readily adsorbed onto suspended solids. The toxicity of copper in natural waters, except soft water free from organic matter and suspended solids is therefore likely to be less than that predicted from laboratory tests with clean water, because of the presence of these non-toxic complexes and insoluble precipitates. The proportion of the total dissolved copper present as the free ion has been found to be approximately 1%, but would be smaller in waters with heavy organic loads and those of high pH (>7.5). Other factors which influence toxicity of metals include salinity (Jones, 1973), temperature (Rehwoldt *et al.*, 1972), oxygen

concentration (Lloyd, 1961), hardness (Pascoe *et al.*, 1986) and pH (Bradley and Sprague, 1985).

The toxicity of copper to fish is well documented (Alabaster and Lloyd, 1980) and the European Inland Fisheries Advisory Commission (EIFAC) reccomends that the annual maximum 95 percentile concentration for rainbow trout should not exceed 22.0 ug Cu l^{-1} in soft water (50 mg l^{-1} $CaCO_3$) and 112 ug Cu l^{-1} in hard water (300 mg l^{-1} $CaCO_3$). Invertebrates vary widely in their resistance to copper (Alabaster and Lloyd, 1980), a few organisms being at least ten times and *Daphnia* and *Gammarus* being about five times as sensitive as rainbow trout. However, the majority are either similar to or much more resistant than trout. Acute toxicity studies with crayfish have been limited to a single species, *Orconectes rusticus* (Hubschmann, 1967a). Newly hatched *Orconectes* juveniles were very sensitive, with an "acute toxicity threshold" existing between 0.06 - 0.125 mg Cu l^{-1}. Concentrations as low as 0.015 mg Cu l^{-1} were sufficient to inhibit growth in juveniles, and 0.06 mg Cu l^{-1} was sufficient to prevent growth altogether.

In the same study, a concentration of 3 mg Cu l^{-1} was sufficient to kill 50% of adult crayfish in 96 hours. However, mortality in crayfish transferred to clean water following a 24 hour exposure to 2.5 mg Cu l^{-1} was found to extend over a period of several days, so that at the end of two weeks all animals were dead. This has implications for "episodic" pollution events, where toxicant concentrations may increase to high levels and then decrease over a relatively short period. The ability of crayfish to recover from chemically-induced stress, once toxicant

concentrations begin to decrease, will strongly influence the final percentage of the population that will be affected and a delayed mortality, in the case of copper, may result in an under-estimation of acute toxicity.

Episodic pollution events are likely to increase in importance, as pollution control measures decrease discharges from chronic sources. Further study of short-term exposure of crayfish to copper is therefore required, including the recovery of animals after simulated pollution incidents.

Other studies have been concerned with the uptake and accumulation of sub-lethal concentrations of copper into different tissues (Evans, 1980; Zia and Alikhan, 1989). Organisms that live in and ingest sediment where metals tend to accumulate are known to concentrate trace metals in their body tissues. In a study of two river systems in North Carolina, burrowing mayflies, Ephemeridae and some Chironomidae had the highest concentrations of all metals studied, whilst the carnivores and surface feeding species such as Gyrinidae had the lowest concentrations (Smock, 1983). Similarly, in a study of the River Irwell, Northern England, highest concentrations of metals were found in oligochaetes and chironomids (Dixit and Whitcomb, 1983). In the crayfish *Procambarus clarkii*, and perhaps other species, young crayfish and unmated males tend to bury themselves in mud, remaining inactive for several months (Aiken, 1968). Some authors have suggested use of crayfish as "sentinel" organisms for metal pollution (Alikhan *et al.*, 1990). Crayfish and bivalve molluscs appear to be the most frequently used organisms in metal accumulation studies, possibly as their large size allows easy

collection and provides sufficient tissue biomass for effective analysis (Johnson *et al.*, 1993). Also, crayfish are often a human food source, so some researchers have expressed concern for the role of crayfish in food chain concentration of metals (Anderson *et al.*, 1978). Wild-collected animals may accumulate metals from waters receiving discharges from combined sewer overflows and from storm drains (Stinson and Eaton, 1983). Also, Madigosky *et al.* (1991) found that concentrations of lead, cadmium and aluminium were significantly higher in the tissues of *Procambarus clarkii* collected from roadside ditches, than in those of commercially produced crayfish. Populations of *P. leniusculus* in the River Great Ouse system, Buckinghamshire, are currently commercially exploited (Wiles, pers. comm.) and it is likely that smaller scale exploitation is carried out in the Rivers Kennet and Thame, all of which have large urban centres situated in the catchment.

This study investigates and compares the lethal effects of copper on *A. pallipes*, *P. leniusculus* and *A. leptodactylus* through acute toxicity and episodic toxicity tests. Secondly, the accumulation of copper in the tissues of *P. leniusculus*, and the factors affecting its uptake and loss, are investigated.

5.2 MATERIALS AND METHODS

5.2.1 Acute toxicity tests

Stage II juveniles of *A. pallipes*, *P. leniusculus* and *A. leptodactylus* were individually exposed to a range of copper concentrations in 5 cm diameter petri-dishes. A small square of black nylon mesh was provided for the juvenile crayfish to cling to and the dishes placed on a black background to minimise in-test stress. A preliminary rangefinding experiment was carried out to determine suitable concentration ranges. This was as follows; 0 (control), 0.25, 0.5, 1.0, 2.0, 4.0, and 6.0 mg Cu^{2+} l^{-1}. The source of copper was analytical grade $CuSO_4.5H_2O$, minimum purity 99%. Ten animals were exposed to each concentration in a static testing regime (see 3.2.1).

5.2.2 Episodic toxicity tests

In contrast to the acute toxicity tests, where animals were exposed to a constant concentration of toxicant for up to 96 hours, an exposure period of short duration to a high concentration of toxicant was used to simulate an episodic pollution incident.

Based on the results from acute tests, ten stage II juveniles of each species were initially exposed to a concentration of 5 mg Cu^{2+} l^{-1} for three hours. At the end of this period the copper solution was pipetted off and replaced with uncontaminated water. The animals were then continually observed

for up to 7 days and any mortalities recorded, together with the time of mortality post-exposure.

5.2.3 Accumulation studies

Adult specimens of *P. leniusculus* and (size range 30-45 mm carapace length) taken from breeding colonies at Nottingham University were used in all experiments. Both males and females were used in the study as previous authors found no sex difference in the accumulation of copper (Zia and Alikhan, 1989). Adams *et al.* (1982) found that there were significant changes in the copper content of body tissues in *A. pallipes* at ecdysis. For this reason only intermoult animals were used, and any animlas that moulted during the experiments were excluded from the analyses.

Animals were exposed to test concentrations prepared with a stock solution of $CuSO_4.5H_2O$ in conditioned Nottingham tap water. The experiments were carried out in a constant temperature room at 15.0 ± 0.1 °C, with artificial lighting timed to provide illumination for 12 out of 24 hours. Unless otherwise stated, animals were fed a proprietary pelleted dog food ("Minced Morsels", Quaker Oats Ltd.), copper content 7.2 ± 0.73 µg g^{-1} dry weight.

During exposure periods test concentrations were monitored, together with water hardness and pH. Water samples for metal analysis were acidified 1:50 with 5M H_2SO_4. At the end of each experiment animals were rinsed in deionised water to remove external copper, placed in labelled polythene bottles, and then

killed by freezing. After thawing at ambient temperatures, individual tissues were dissected out for analysis.

Water and tissue copper concentration was measured by atomic absorption spectrophotometry (AAS) using a Pye Unicam SP9 Atomic Absorption Spectrophotometer (Pye Unicam Ltd., Cambridge). Samples of tissue from gill, hepatopancreas, muscle and exoskeleton were dissected out into pre-weighed and numbered 10 ml soda vials and oven-dried at 80 °C, in order to determine the dry weight. Vials had previously been washed in 10% nitric acid and rinsed with deionised water. Samples for analysis were then digested overnight in 5 ml concentrated nitric acid. Tissue that had not dissolved after this stage was completely digested by heating samples in a sand bath (heated to 80 - 100 °C) in a fume cupboard. Samples were allowed to cool, then diluted to 25 ml with deionised water.

In order to establish that the copper present in the tissues was not as a result of analytical procedure, 5 blanks (i.e. vials containing no tissue) were also prepared and analysed. If any copper was detected then the mean value of the 5 blanks was subtracted from each sample value.

Statistical comparisons between and within treatments were made using a non-parametric ANOVA with Conover's multiple range test.

Individual experimental procedures are detailed below.

5.2.3(i) Uptake and distribution of copper in crayfish tissues

Animals were exposed to nominal copper concentrations of 0, 0.125, 0.25 and 0.5 mg l^{-1} Cu^{2+} for 4 weeks at 18 °C. Four animals per concentration were exposed in Perspex aquaria, in 4 litres of experimental medium. The medium was replaced every other day during the exposure period to prevent a decrease in copper concentration due to uptake by the animals and adsorption onto the walls of the aquarium. At the end of the exposure period samples of gill, exoskeleton, hepatopancreas and muscle tissue were analysed in order to determine concentration and distribution of copper in the tissues.

5.2.3(ii) Effect of exposure time

In order to determine the rate of copper uptake in the various tissues, animals were exposed to a copper concentration of 0.5 mg l^{-1} Cu^{2+} for 4 weeks at 18 °C. A group of four animals was removed at the beginning of the experiment (controls) and again at the end of exposure week 1, 2, 3 and 4 for analysis of copper content of gill, exoskeleton, hepatopancreas and muscle tissue. Samples of faeces were also collected for analysis 16 - 18 hours after feeding with minced morsels, to determine whether copper was voided in the faeces during the experiment.

5.2.3(iii) Effect of exposure temperature

Animals were exposed to nominal copper concentrations of 0, 0.125, 0.25 and 0.5 mg l^{-1} Cu^{2+} for 4 weeks at experimental temperatures of 9 °C and 18 °C, to determine the effect of temperature on the uptake of copper in the various tissues. At the end of the exposure period tissue from gill, muscle, exoskeleton and hepatopancreas was analysed for copper content.

5.2.3(iv) Relative uptake from food and water

Copper dosed food pellets (copper content 472±2.5 ug/g) were made by reconstituting dried, powdered dog food ("Minced Morsels", Quaker Oats Ltd.) with a copper solution. Wheat gluten (10% by weight) was added as a binder. Animals were then exposed to copper in food and in solution at 0.5 mg l^{-1} Cu^{2+} in a combination of treatments for 2 weeks at 18 °C;

a.) Control, b.) Copper in food only, c.) Copper in water only, d.) Copper in food and water.

Animals were fed to excess every other day. Food was given in the evening, and any remaining in the morning was removed to prevent leaching of copper into solution. No attempt was made to quantify the amount of food given to each crayfish. At the end of the exposure period, tissue from exoskeleton, muscle, gill and hepatopancreas was analysed for copper content.

5.2.3(v) Depuration from gills and hepatopancreas

Animals were exposed to a nominal concentration of 0.5 mg l^{-1} Cu^{2+} for two weeks, during which period they were starved. Four animals were removed for metal analysis at the beginning of the experiment and at the end of the exposure period to determine initial and final tissue concentrations. Remaining animals were split into two groups and transferred to clean water, which was changed every other day. One group in clean water was fed (minced morsels), while the other group was starved. Four animals were then removed from each group after 2 and 6 weeks in clean water for metal analysis of gill and hepatopancreatic tissue, to determine whether any significant reduction in tissue concentration of copper occurred with increasing time.

5.3 RESULTS

5.3.1 Acute toxicity tests

Throughout all toxicity tests average temperature and pH were 13 °C and 7.45 respectively. The mean hardness of the dilution water was 180 mg l^{-1} as $CaCO_3$.

The percentage mortalities at 24, 48, 72 and 96 hours for each test are shown (Fig. 5.1). For each species, mortality increased with copper concentration and time of exposure. Mean copper concentrations were calculated from the measured concentrations. From these data the 24, 48, 72 and 96 hour LC_{50} (median lethal concentration) values for copper ion (Cu^{2+}) have

been calculated for each species in Table 5.1 and are shown in Fig. 5.2.

No control deaths were observed in any of the tests. Also, no animals were seen to moult during the tests. However, mortalities continued to occur after transfer of animals to clean water. After 10 days the mortality rates of animals that survived the 96 hour exposure were 80 - 100% in all treatments.

5.3.2 Episodic toxicity tests

Percentage mortality with time, following an initial three hour exposure to 10 mg Cu_{2+} l^{-1}, is shown for the three species in Fig. 5.3. For all three species, although no animals died during the initial exposure period, mortalities rapidly occurred after the toxicant was replaced with uncontaminated water, so that after just two days the percentage mortalities were 100%, 90% and 60% for *A. pallipes*, *A. leptodactylus* and *P. leniusculus* respectively.

Figure 5.4. shows time to mortality, post exposure to toxicant, for individual animals plotted on a log scale, against percentage mortality plotted on a probability scale. This allows estimation of the median post-exposure lethal time (peLT50) (Pascoe and Shazili, 1986), that is the time calculated from the beginning of the recovery period in clean water taken for 50% of the animals to die. These were as follows; *A. pallipes* - 6.9 hours, *A. leptodactylus* - 8.4 hours, *P. leniusculus* - 10.1 hours.

5.3.3. Accumulation studies

Throughout the accumulation studies the average temperature and pH of the diluent water (Nottingham tap water) were 18±0.09 °C and 7.11±0.04 respectively. Mean hardness of the diluent water was 130±6.55 mg $CaCO_3$ l^{-1}. No mortalities were observed during any of the experiments.

5.3.3(i) Uptake and distribution of copper in crayfish tissues

Average copper concentrations in the medium over the four week exposure period were as follows; Not detectable (control), 0.12±0.003, 0.24±0.022 and 0.48±0.044 mg Cu^{2+} l^{-1}.

Mean tissue copper concentrations, expressed as µg Cu^{2+} g^{-1} dry weight, in the exoskeleton, muscle, gill and hepatopancreas of *P. leniusculus* in different copper treatments are shown in Fig. 5.5. Data was analysed using rank sum analysis (Meddis, 1984).

In general highest mean tissue copper concentrations were observed in the hepatopancreas and the gills, and lowest in the exoskeleton and the muscle. At the end of the 4 week exposure period a significant increase ($p<0.005$) in gill tissue concentration was observed, with a linear relationship between tissue concentration and the copper concentration of the medium. Similarly for the hepatopancreas, a significant increase ($p<0.001$) in tissue concentration was observed in animals exposed to 0.125 and 0.25 mg Cu^{2+} l^{-1}. However, there was no significant difference ($p>0.05$) between animals exposed to 0.5 mg Cu^{2+} l^{-1} and

the controls.

Differences in copper concentration in the exoskeleton and the muscle were non-significant ($p>0.05$) among the various treatments.

5.3.3(ii) Effect of exposure time

The mean copper concentration in the medium over the 4 week exposure period was 0.46 ± 0.02 mg Cu^{2+} l^{-1}. Average copper concentrations in the gill and hepatopancreatic tissue and in the faeces are shown in Fig 5.6 expressed as µg Cu^{2+} g^{-1} dry weight.

There was a significant increase ($p<0.01$) in the copper concentration in gill tissue over the exposure period. Similarly, for the hepatopancreas a significant increase ($p<0.01$) was observed for the first 2 weeks. However, after two weeks the copper concentration in the hepatopancreas declined, so that at the end of the 4 week exposure period mean tissue concentration was not significantly different from that of the control ($p>0.05$).

A decline in copper concentration in the hepatopancreas was accompanied by a highly significant increase ($p<0.001$) in the copper content of the faeces over the last 2 weeks of the exposure period. Concentrations of copper in the faeces in the first 2 weeks of exposure were relatively high and very variable. Copper content of the food given during the experiment was very low (7.2 ± 0.73 µg Cu^{2+} g^{-1}). However, it is possible that significant amounts of copper were absorbed by the faeces from the medium before it was collected.

5.3.3(iii) Effect of exposure temperature

Average copper concentrations in the medium over the four week exposure period were as follows; not detectable (control), 0.15±0.01, 0.26±0.02 and 0.6±0.09 mg Cu^{2+} l^{-1}. Mean temperatures for the two treatments were 9.0±0.1 and 18±0.1 °C.

Average copper concentrations, expressed as µg Cu^{2+} g^{-1}, in gill and hepatopancreatic tissue for animals exposed to the various treatments at 9 and 18 °C are shown in Fig. 5.7. Differences in copper content of exoskeleton and muscle were non-significant ($p>0.05$) between various treatments, so copper concentrations for these tissues are not shown.

At both temperatures, there was a significant increase in gill and hepatopancreatic tissue concentration at the end of the 4 week exposure period, with a linear relationship between tissue concentration and concentration of copper in the external medium. Comparison between exposure temperatures of gill tissue concentrations indicated an overall significant difference ($p<0.05$), with higher tissue concentrations in animals exposed at 18 °C. No overall difference ($p>0.05$) was seen between copper concentrations in the hepatopancreas, although the tissue concentration in the 0.5 mg Cu^{2+} treatment was significantly greater ($p>0.05$) in animals exposed at 18 °C.

5.3.3(iv) Relative uptake from food and water

Copper content of copper-dosed and undosed (control) food pellets given to animals during the exposure period were 472±2.5

and 7.2±0.73 µg Cu^{2+} g^{-1} dry weight respectively. Concentration in the medium in controls and in the copper treatment were not detectable and 0.48±0.22 mg Cu^{2+} l^{-1} respectively.

Tissue concentrations, expressed as µg Cu^{2+} g^{-1} dry weight, are shown in Fig. 5.8 for the various treatments. In animals exposed to copper in food alone in treatment a, there was a significant increase ($p<0.05$) in the copper concentration of the hepatopancreas. No increase in copper concentration was observed in any other tissue in this treatment.

Increased copper concentrations were observed both in the gill and hepatopancreatic tissue of animals exposed to 0.5 mg Cu^{2+} l^{-1} in solution in treatment b. The copper content of the hepatopancreas in this treatment was slightly greater than that in treatment a, although this was non-significant ($p>0.05$). A further increase in the copper concentration in gill and hepatopancreatic tissue concentration was observed in animals exposed to copper in both food and water in treatment c. However, this was found to be non-significant ($p>0.05$).

5.3.3(v) Depuration of copper from gills and hepatopancreas

Copper concentration in gill and hepatopancreatic tissue, expressed as µg Cu^{2+} l^{-1}, for animals in the various treatments are shown in Fig. 5.9.

After 2 weeks exposure to 0.5 mg Cu^{2+} l^{-1} concentrations of copper in both gills and hepatopancreas were significantly ($p<0.05$) elevated above those of controls. When animals were

transferred to clean water there was a significant ($p<0.05$) decrease in the copper concentration of gill tissue of both fed and unfed animals. However, 6 weeks post-transfer to clean water, these were still elevated above the control levels.

In fed animals, the copper concentration of the hepatopancreas decreased dramatically and were non-significantly different ($p>0.05$) from control levels at the end of the experiment. However, no such decrease was seen in unfed animals. Copper concentrations in the hepatopancreas remained elevated and a small, but non-significant, increase was actually observed.

5.4 DISCUSSION

The data shown in Figs. 5.1 and 5.2 show interspecific differences in mortality and LC_{50} values. Percentage mortality in all three species increased as expected with increasing copper concentration. The LC_{50} decreased with exposure time, tending towards the lethal threshold concentration, or incipient LC_{50}.

At 96 hours, *P. leniusculus* stage II juveniles were least sensitive to copper and were significantly more tolerant (approximately 4.5 times) than *A. pallipes* stage II juveniles. Juveniles of *A. leptodactylus* were intermediate in tolerance, approximately 1.8 times less tolerant and 2.5 times more tolerant than *P. leniusculus* and *A. pallipes* respectively.

Hubschmann (1967a) found that newly hatched (stage I?) *Orconectes rusticus* juveniles were considerably more sensitive than any of the species tested in this study, with an acute toxicity threshold of between 0.06-0.125 mg Cu^{2+} l^{-1}, compared to

a 96 hour LC50 of 0.46 mg l^{-1} for stage II juvenile *A. pallipes*, the most sensitive species in this study. Similar variation in sensitivity within a taxonomic group has been demonstrated for cadmium (Williams *et al.*, 1985). In this study, the mayfly *Baetis rhodani* was approximately 25 times more sensitive than another mayfly tested, *Ephemerella ignita*. Table 5.2 shows acute toxicity of copper to selected invertebrates for comparison with LC_{50} values obtained for stage II crayfish juveniles in this study. Despite a wide range of experimental conditions, it can be seen that crayfish juveniles are in the middle to upper part of the range of sensitivity to copper, and are more tolerant than rainbow trout at a similar experimental hardness and temperature.

Early life stages have been demonstrated to be more sensitive to copper. Hubschman (1967a) found that adult crayfish were much more tolerant of copper than juvenile stages. A concentration of 1 mg $Cu^{2+}l^{-1}$ caused 50% mortality in *O. rusticus* hatchlings in less than 24 hours. In adults exposed to the same concentration, 50% mortality did not occur until after 13 days. In addition, ovigerous females exposed to 0.25 and 0.5 mg Cu^{2+} l^{-1} for the last 13 or 14 days of egg development suffered 52% and 100% egg mortality respectively. Therefore, levels of copper insufficient to cause lethal effects in adult crayfish may cause a significant reduction, or total elimination of the juvenile population, thus affecting recruitment. The relatively higher copper tolerance of *P. leniusculus* and *A. leptodactylus* stage II juveniles may allow establishment of populations in waters with free copper concentrations unsuitable for *A. pallipes* juveniles.

However, this does not take into account sublethal, long-

term effects at concentrations below the 96 hour LC_{50}. In fish, the mode of action of copper is not clear, but acutely lethal concentrations damage the gill and may affect cell processes and enzyme activity (Alabaster and Lloyd, 1980). Hubschmann (1967b) suggests at least two modes of toxic action. In the crayfish *Orconectes rusticus*, acute lethal concentrations above 1 mg Cu^{2+} l^{-1} caused a rapid inhibition of respiratory enzymes, presumably as the detoxification mechanism is overwhelmed. However, at sublethal concentrations below 1 mg Cu^{2+} l^{-1} there was a degenerative effect on cells and tissues, e.g the antennal gland, so that metabolic processes are eventually restricted. Also, *O. rusticus* juveniles exposed to sub-lethal concentrations ranging between 0.015 and 0.06 mg Cu^{2+} l^{-1} showed a progressive reduction in growth with increasing copper concentration (Hubschmann, 1967a). After 30 days, for animals exposed to 0.06 mg Cu^{2+} l^{-1} there was only a 1.4% increase in dry weight when compared to controls. Therefore the threshold level of copper for long-term survival and growth of juvenile crayfish may be much lower than that indicated by a simple 96 hour LC_{50}.

Data from the acute toxicity tests indicated that *A. pallipes* was significantly less tolerant of copper than either of the other two species in the short term (24 hours) as well as the longer term (96 hours). The 24 hour LC_{50} value for *A. pallipes* was 2.13 mg Cu^{2+} l^{-1}, compared to 5.24 and 6.41 mg Cu^{2+} l^{-1} for *P. leniusculus* and *A. leptodactylus* respectively. Implications for short-term or episodic exposure to copper were also seen in the continued mortality of animals transferred to clean water at the end of the acute toxicity experiments. Many

pollution incidents involve the release of a single pulse of toxicant, and the consequent exposure of biota to a high concentration for a short period of time. Following this exposure, animals may be able to eliminate the toxicant and recover from the incident. Green *et al.* (1988) demonstrated that following exposure of *Asellus aquaticus* to phenol, animals that had become immobilised during exposure to the toxicant recovered when transferred to clean water, indicating that if there was no further uptake of phenol, detoxification mechanisms were adequate to deal with the toxicant. Alternatively, uptake and localisation of the toxicant may be so rapid that toxic effects, including death, may occur even if animals are rapidly restored to clean water. In studies with rainbow trout, *Salmo gairdneri*, Pascoe and Shazili (1986) showed that even brief exposure of 32 minutes to 1.0 mg Cadmium l^{-1} was sufficient to bring about the eventual death of 50% of the fish 175 hours after transfer to clean water.

In the episodic experiments, stage II crayfish juveniles were exposed to a 3 hour pulse of 5 mg Cu^{2+}. This concentration in the acute toxicity tests was sufficient to cause 100% mortality in *A. pallipes* juveniles and 60% mortality in *P. leniusculus* and *A. leptodactylus* juveniles in 24 hours. No mortalities were observed during the 3 hour exposure. However, animals did die after transfer to clean water, with 100%, 90% and 60% mortality after 48 hours in clean water for *A. pallipes*, *A. leptodactylus* and *P. leniusculus* juveniles respectively. This not only has implications for the underestimation of copper toxicity, with lower concentrations required to cause mortality in the long term than those indicated by acute toxicity tests, but also

indicates that *A. pallipes* juveniles are less tolerant than *P. leniusculus* and *A. leptodactylus* juveniles of episodic, as well as chronic, exposure to copper. In populations subject to episodic pollution, the proportion of juveniles that are able to recover and survive a pollution event will affect recruitment, and ultimately the viability of the population.

Trace metals can enter the body of an aquatic invertebrate through two points: from the water itself, usually via the gills as the remainder of the exoskeleton is relatively impervious to water and ion movement, and in food via the buccal cavity (Bryan, 1968). Both of these pathways were shown to exist for copper in the crayfish *P. leniusculus*.

In the experiments investigating uptake of copper from water it was shown that copper is taken up by crayfish from the surrounding medium and accumulated in various tissues. In addition, the increase in copper concentration is related to a number of factors, namely concentration of copper in the surrounding medium, time of exposure and water temperature during exposure. Highest tissue concentrations amongst various treatments were observed in the gills and the hepatopancreas, and the lowest in the muscle and exoskeleton. Copper has been shown to be a regulated metal in several marine and freshwater decapods, probably due to its essential biochemical role in the formation of the respiratory pigment haemocyanin (Bryan, 1968). This function is regulated by the hepatopancreas, so would explain the high copper concentrations observed in control animals, and the high level of accumulation under various treatments, in this tissue.

Increases in concentration in gill tissue were related to the exposure time and exposure rate. A linear relationship was shown to exist in each case. Gills are primarily respiratory organs, and so receive large volumes of haemolymph. The presence of high copper concentrations in the gills may therefore be due in part to the copper-based respiratory pigment haemocyanin in the haemolymph. However, it is probable that an increase in copper concentration is due to adsorption of copper onto the gill surface, or binding to membrane proteins. Ghate and Mulherker (1979) reported distension of gill plates and vacuolation and necrosis of gill tissue after chronic exposure of two species of freshwater prawns to copper sulphate, and concluded that the gills were major sites for the accumulation of copper salts. The gills also appear to be accumulation sites for the non-essential metals nickel and cadmium (Mees, 1983; Alikhan *et al.*, 1990). In a large number of respiratory epithelia, in the presence of metals such as Cu^{2+}, Zn^{2+} and Cd^{2+} there is a an increase in production of the protein metallothionein (Kito *et al.*, 1982), which is rich in sulphydryl groups and avidly binds metal ions. However, there is some debate as to whether these proteins are functioning as storage molecules, or are detoxification systems (Simkiss *et al.*, 1982). Also, membrane-bound bodies and proteins have been implicated in the uptake (by pinocytosis) and transfer (by carrier-mediated, or facilitated diffusion) of a number of metals, including copper (Fowler et al., 1981). An increase in the rate of such processes accompanied by an increase in metabolism, would explain the higher gill tissue concentrations seen in animals exposed to copper at 18 °C, compared to those

exposed at 9 °C.

Metals may then pass from the gill into the haemolymph and become bound to haemolymph constituents, such as proteins, polysaccharides and amino acids. Bryan (1967) found that, following an injection of zinc, about thirty times the normal blood concentration could be bound by blood proteins in the crayfish *A. pallipes*. Also, zinc is bound so tightly by blood proteins that, even in waters where concentrations of the metal are very low, the concentration gradient for unbound zinc favours diffusion from the water to the haemolymph via the gills. In the depuration experiments, it was found that concentrations of copper in the gills rapidly dropped when animals, were placed in clean water. This may have been due to desorption of copper from the gill surface or transfer of membrane-bound copper to the haemolymph. The latter explanation is supported by the fact that hepatopancreatic copper actually increased in starved animals transferred to clean water, although there is some evidence that starving crayfish are able to utilise protein from the breakdown of the respiratory pigment haemocyanin. Copper released by the breakdown of the molecule is absorbed and stored in the hepatopancreas.

Copper concentrations in hepatopancreatic tissue increased significantly with increasing rate of copper exposure. As all animals received identical diets during these experiments, the increase in copper concentration in the hepatopancreas could only have been due to copper from the medium entering through the gills and being absorbed from the haemolymph by hepatopancreatic cells. Similar findings in crayfish exposed to copper are

reported by Stinson and Eaton (1983) and Bagatto and Alikhan (1987). In the latter study, the authors observed hepatopancreatic tissue concentrations of 1986 µg Cu g^{-1} dry weight in individuals of *Orconectes virilis* collected from a lake 12 km downwind of copper-nickel smelters. The relatively high concentrations of copper observed in this tissue reflects the substantial storage capacity of this organ for copper. Given the lower concentration of the metal in the hepatopancreas of crayfish from low copper waters, this storage capacity probably far exceeds physiological requirements for the metal.

The hepatopancreas is a highly versatile organ and consists of a compact, trilobed complex of ducts and blindly ending tubules occupying most of the cephalo-thoracic volume, and in intimate contact with the haemolymph. Four cell types have been identified in the epithelium of the tubules, which consists of a single layer of cells. Cell nomenclature arises from Hirsch and Jacobs (1930) who called them Embryonalenzellen (E), Resorptionzellen (R), Fibrillenzellen (F) and Blasenzellen (B). The single letter abbreviations are still used to describe these cells. The E cells give rise to R and F cells by post-mitotic division. F cells produce digestive enzymes and are thought to be transformed into B cells, which secrete these enzymes into the lumen and tubules of the gland. R cells absorb nutrients and store glycogen and lipids in the cytoplasm. However, as well as a digestive function, these cells are involved in metal metabolism. Specific metal binding proteins exist in this tissue in *A. pallipes* (Lyon *et al.*, 1983). Analysis of cell contents by X-Ray microanalysis (Lyon and Simkiss, 1984) showed a variety of

metals in the R and F cells. Also, R cells appear to be able to absorb metals from both the luminal and basal surfaces, i.e both from the stomach and the haemolymph.

When animals were fed copper dosed food there was a significant increase in copper concentration in the hepatopancreas, indicating absorption of copper from the stomach fluid. Bryan (1967) showed that ionic zinc was absorbed from the stomach in *A. pallipes* by replacing some of the stomach fluid with labelled Zn^{2+}. No increase in copper concentration was observed in any other tissue in this treatment, so it is unlikely that copper can penetrate directly from the stomach fluid to the blood and then to other tissues. Any copper that does so must be removed so rapidly by R cells in the hepatopancreas that no obvious change in blood concentration occurs.

Under contaminated conditions the requirement for essential metals must be far exceeded. At high concentrations, both essential and non-essential metals are enzyme inhibitors. Some degree of protection from such high concentrations may be provided by the general binding capacity of blood proteins, polysaccharides and amino acids. However, in crayfish this is temporary and the excess amounts of metals absorbed by the hepatopancreas within two days (Bryan, 1967). Ogura (1959) found copper and iron granules in the hepatopancreas of *Procambarus clarkii*, and it is possible that other metals are stored in the same way. Alikhan *et al.* (1990) observed that in control animals, metal granules were located in the basal cytoplasm of the hepatopancreatic cells, but under various copper and nickel treatments these granules were aggregated in the apical portion

of the cells, i.e. adjacent to the lumen. Ogura (1959) found copper granules in the faecal material, indicating that the hepatopancreatic cells were able to discharge vesicular copper into the alimentary canal.

In this study it was found that in experiment 5.3.3(i) hepatopancreatic tissue copper concentrations in *P. leniusculus* exposed to 0.25 and 0.5 mg Cu^{2+} l^{-1} were not significant. Also, copper concentrations in the faeces of animals exposed to 0.5 mg Cu^{2+} l^{-1} significantly increased over a four week exposure period, accompanied by a decrease in the hepatopancreas. In depuration experiments, copper concentration in the hepatopancreas of starved animals remained high even after transfer to clean water. These data would indicate a regulatory mechanism for the removal of excess copper in the faeces. Such a mechanism for the removal of excess zinc has been described in *A. pallipes* by Bryan (1968), with an intermittant loss of zinc, dependent on whether the animal was feeding, although it was unclear how the zinc entered the stomach from the hepatopancreas. In addition, increased copper concentrations were observed in gill and hepatopancreatic tissue of animals exposed to 0.5 mg Cu^{2+} l^{-1}. However, no further significant increase was seen when animals were exposed to copper in both food and water. Increased concentrations in the blood and hepatopancreas due to uptake from water may therefore reduce absorption of copper from the stomach fluid. This is in contrast to cadmium accumulation from food and water, which has been shown to be additive (Giesy *et al.*, 1980). Cadmium is a non-essential trace metal and has been shown to be assimilated by the same route as copper. However, it does not appear to be regulated by

crayfish (Mees, 1983), although cadmium tissue concentrations eventually reach a steady state.

Copper concentrations in exoskeleton remained more or less constant during the experiments, although in some treatments there was a non-significant increase in the exoskeleton with increased copper in the outside medium. This was probably due to surface adsorption. Bryan (1966) found that uptake of labelled ^{65}Zn by the cuticle of *A. pallipes* seemed to be a surface adsorption process as nearly all of the activity could be removed from the cuticle with sandpaper. However, some authors have reported higher copper concentrations in pigmented tissues (Bryan, 1968). Weiser and Klima (1969) suggested that freshwater decapods are similar to terrestrial isopods, in that they accumulate copper in the hepatopancreas under low rates of exposure, but shift the copper burden under high rates of exposure to the exoskeleton. The exoskeleton may then act as a sink for the excretion of excess metals during the moult cycle. This would appear to be the case for lead as Stinson and Eaton (1983) found that lead concentrations in *P. leniusculus* from a lake receiving urban run-off were highest in the exoskeleton. Rincon-Leon *et al.* (1988) suggested use of crayfish exoskeleton in monitoring lead pollution for this reason.

Copper in abdominal muscle also remained relatively constant with various copper treatments. This tissue is not considered to be a specific physiological site for the storage of copper. However, Stinson and Eaton (1983) found that concentrations of the non-essential metal mercury were highest in muscle tissue of *P. leniusculus* in a lake receiving urban run-off.

As crayfish reside in close contact with sediments, are easily collected and are often a human food source, they may serve as useful indicators of trace metal contamination of lakes and streams. This and previous studies have indicated that different metals distribute to different parts of the body in crayfish, so that analysis of a number of metals in a single specimen can be carried out by separation of different tissues. According to Dallinger (1977) in isopods the limits in which copper is regulated are closely adjusted to the average concentration of all copper sources available in a particular habitat. However, Bryan (1968) found that in several species of marine and freshwater decapods tissue concentrations of copper varied within fairly narrow limits, despite wide fluctuations of the concentration of the metal in the medium. This is not unexpected considering copper is regulated in *P. leniusculus*. However, it does mean that their value as indicators of environmental copper concentration is debatable. In addition, wild-collected animals would appear to offer little risk of consumption of copper in muscle tissue. For 51 sites in the Severn-Trent region during 1988-1989 the average copper concentration was 0.0265 mg Cu^{2+} l^{-1} (Severn Trent Water Authority, 1989a), with the range for the ten most contaminated sites of 0.025 - 0.225 mg Cu^{2+} l^{-1}. Even at the highest environmental level, comparable with the concentration of 0.25 mg Cu^{2+} l^{-1} used in these experiments, the muscle tissue did not accumulate a significant concentration of copper. However, as the copper content of the hepatopancreas was generally higher and more variable than the muscle, caution should be exercised in the

use of hepatopancreas as a food source.

SPECIES	TIME (HOURS)			
	24	48	72	96
Pacifastacus leniusculus	5.24 (3.77-12.4)	2.70 (1.92-3.84)	2.21 (1.56-3.11)	2.09 (1.47-2.94)
Austropotamobius pallipes	2.13 (1.47-3.15)	0.84 (0.56-1.21)	0.63 (0.38-0.93)	0.46 (0.18-0.76)
Astacus leptodactylus	6.41 (3.45-48.5)	2.38 (1.57-3.93)	1.45 (0.91-2.33)	1.16 (0.61-2.06)

Table 5.1: Median lethal concentrations (LC_{50}) and 95% confidence limits (mg Cu^{2+} l^{-1}) for stage II crayfish juveniles, calculated by probit analysis at 24, 48, 72 and 96 hours.

Table 5.2. Acute toxicity of copper (Cu^{2+}) to invertebrates

SPECIES	SIZE/ STAGE	TEMP. (^{o}C)	HARDNESS (mg l^{-1})	LC_{50} (mg l^{-1})	TIME	REFERENCE
OLIGOCHAETA						
Nais communis		20	320	0.06	60 day	Learner and Edwards, 1963
Tubifex tubifex		20	261	0.89	48 hours	Brkovic-Popovic and Popovic, 1977
INSECTA						
Ephemerella subvaria		18	44	0.32	48 hours	Warnick and Bell, 1969
Heptagenia lateralis		10	109	0.50	7 day	Liepolt and Weber, 1958
Acroneuria lycorias		18	44	8.30	48 hours	Warnick and Bell, 1969
Hydropsyche bettini		18	44	32.0	48 hours	" "
CRUSTACEA						
Daphnia magna		21	196	0.09	48 hours	Malacea and Gruia, 1965
A. pallipes	stgeII	13	220	0.46	96 hours	This study
A. leptodactylus	stgeII	13	220	1.16	96 hours	This study
P. leniusculus	stgeII	13	220	2.09	96 hours	This study

Figure 5.1: Percentage mortality of stage II crayfish juveniles with increasing copper concentration (——*P. leniusculus*, ----*A. leptodactylus*,.....*A. pallipes*) at 24, 48, 72 and 96 hours.

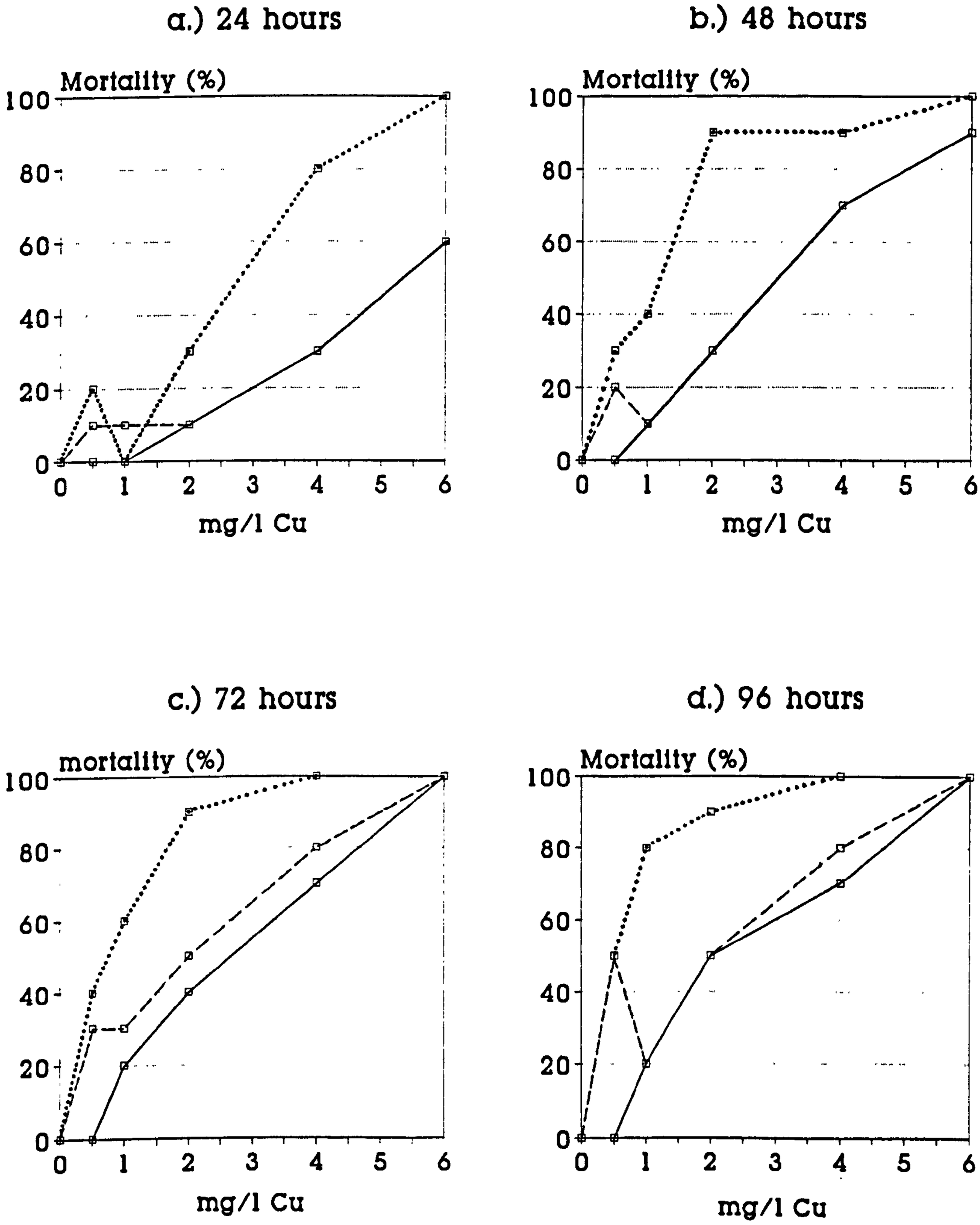
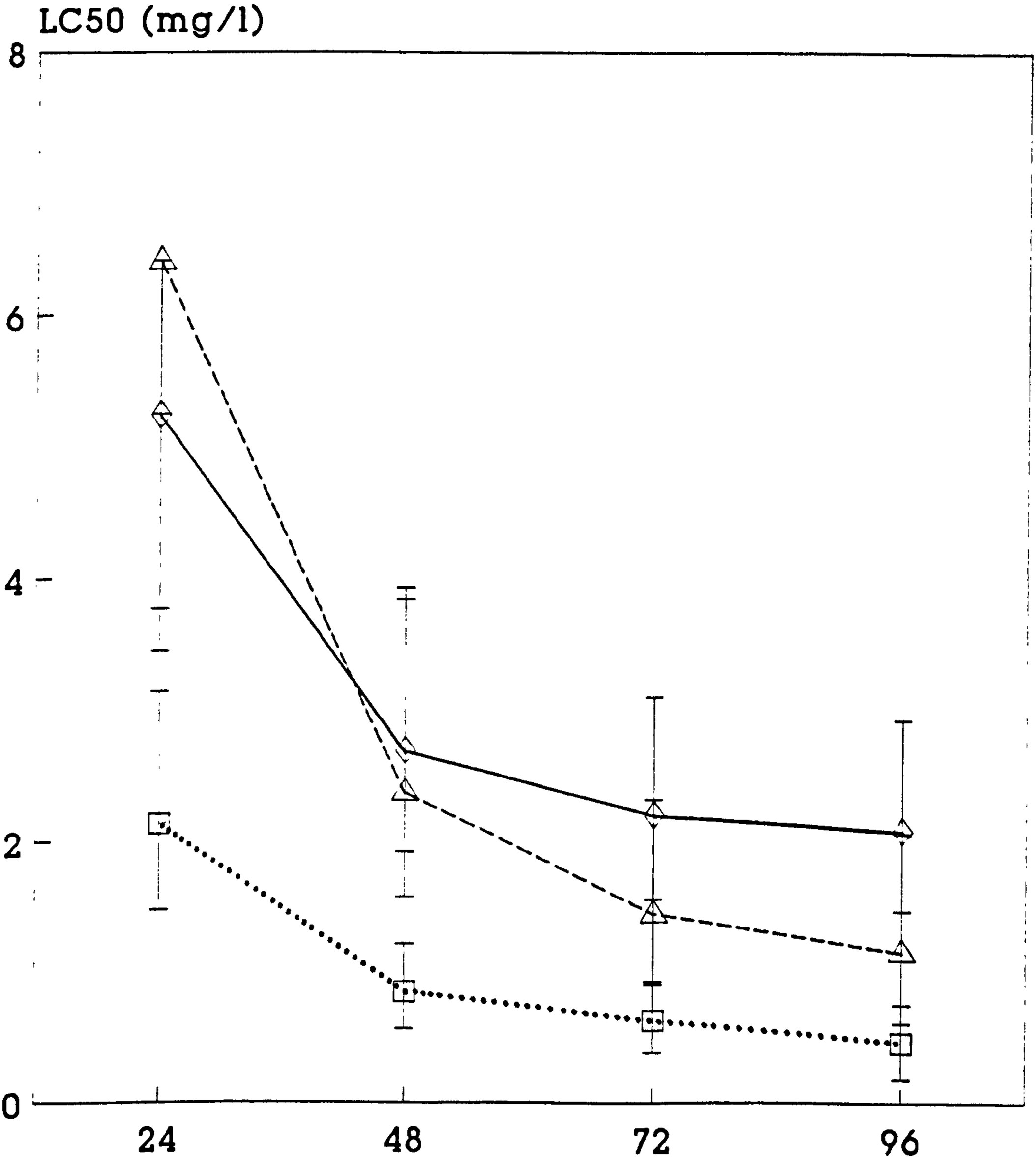


Figure 5.2: LC_{50} (mg Cu^{2+} l^{-1}) with time for stage II crayfish juveniles (—◇—*P. leniusculus*,--△--*A. leptodactylus*,····□··*A. pallipes*). Error bars indicate 95% confidence limits.



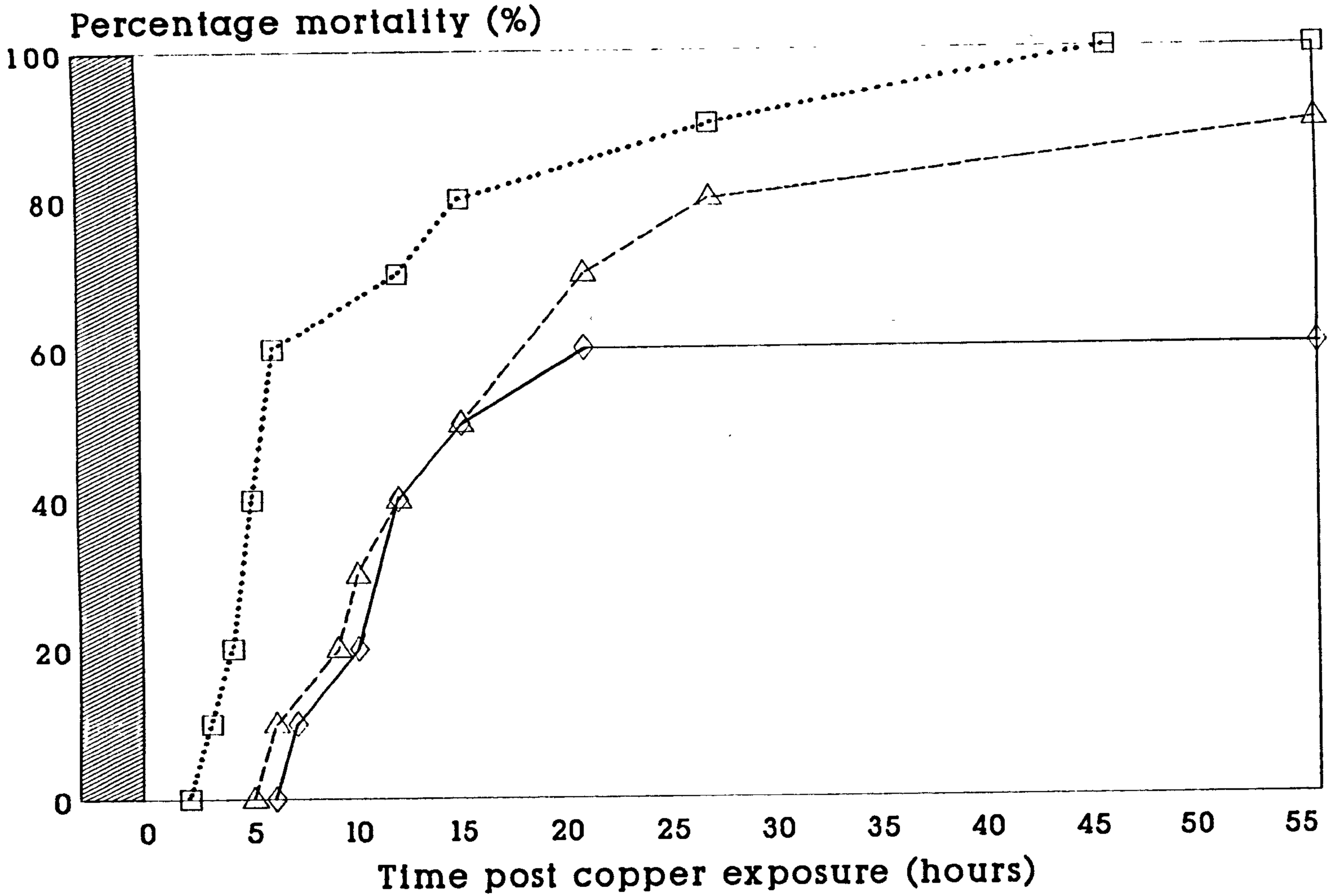


Figure 5.3: Percentage mortality of stage II crayfish juveniles exposed to 5 mg Cu^{2+} l^{-1} for 3 hours (indicated by hatched bar), then transferred to clean water (—◇— *P. leniusculus*, - -△- - *A. leptodactylus*, ···□·· *A. pallipes*).

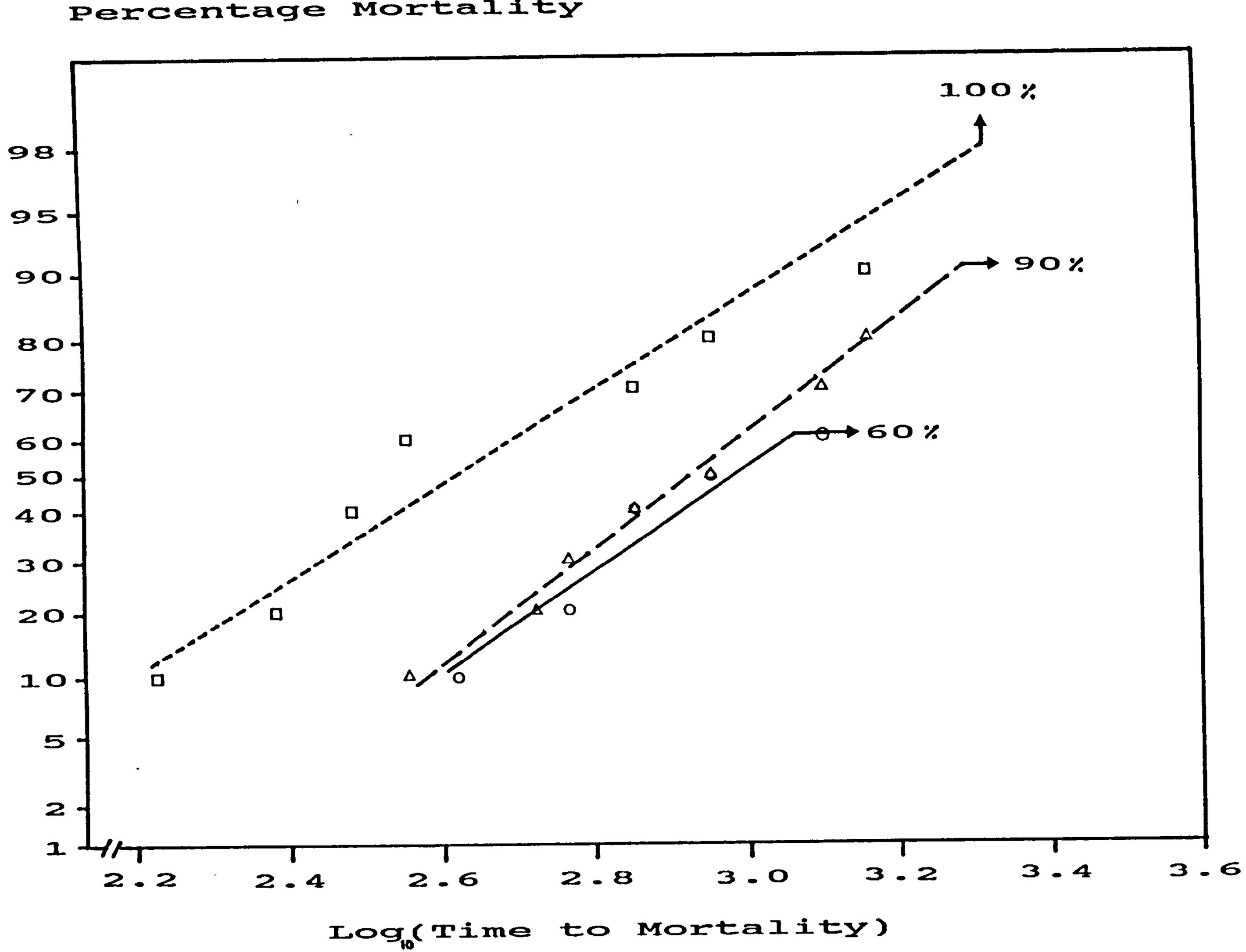


Figure 5.4: Culmative percentage mortality on a probability scale plotted against logarithm of time of death (minutes) post copper exposure for individual stage II crayfish juveniles (———*P. leniusculus*,------*A. pallipes*,– – –*A. leptodactylus*).

Figure 5.5: Tissue copper concentrations (E = exoskeleton, M = muscle, G = gill, H = hepatopancreas) of *P. leniusculus* exposed to 0, 0.125, 0.25 and 0.5 mg Cu^{2+} l^{-1} for 4 weeks at 18 °C. Bars show average tissue concentrations (μg Cu^{2+} g^{-1} dry weight) with standard errors (n=4).

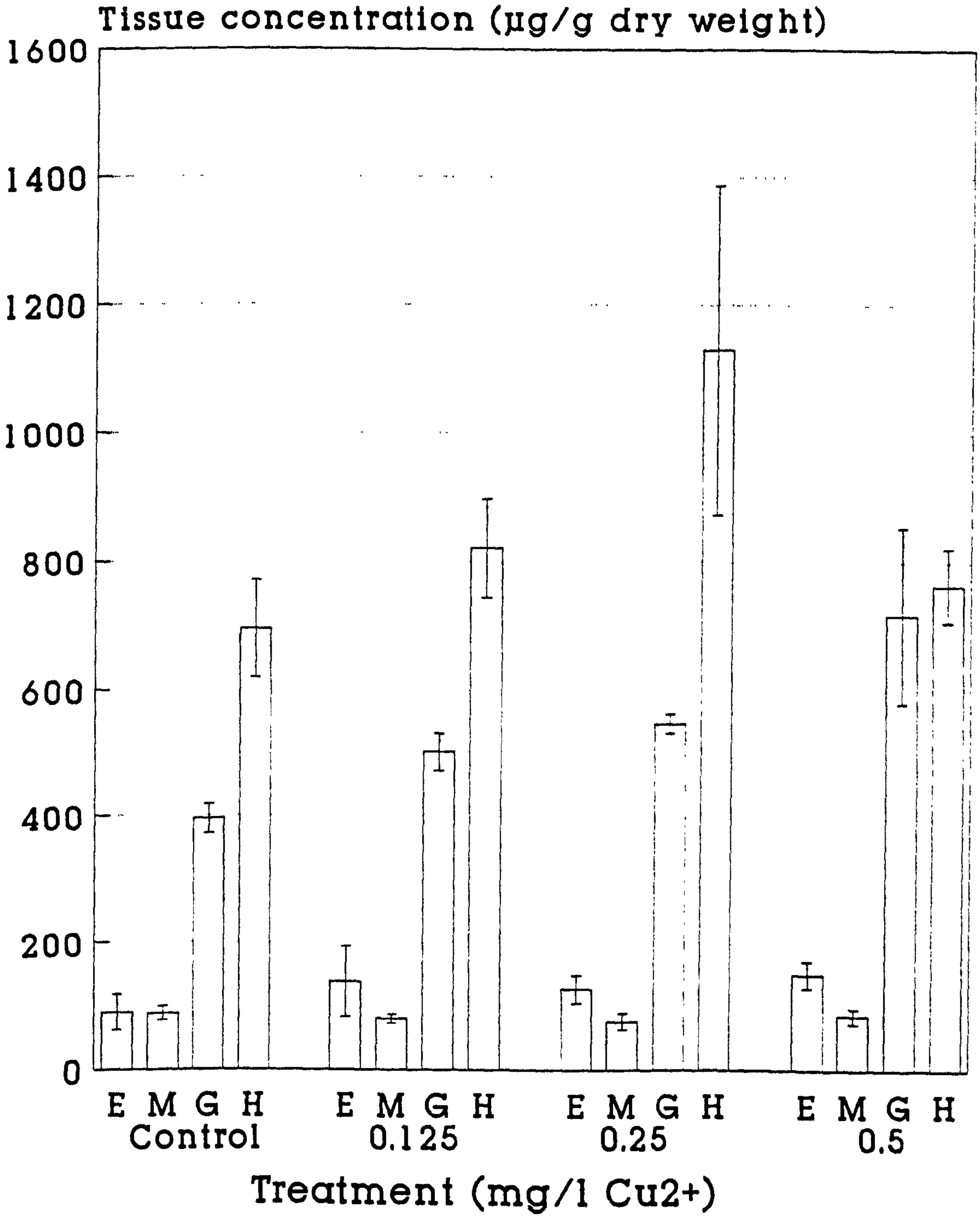


Figure 5.6: Copper concentrations with time in gill (—□—) and hepatopancreatic tissue (--◇--) and in faeces (.....△.....) of *P. leniusculus* exposed to 0.5 mg Cu^{2+} l^{-1} at 18 °C. Values are average tissue concentrations (μg Cu^{2+} g^{-1} dry weight) with standard errors (n=4).

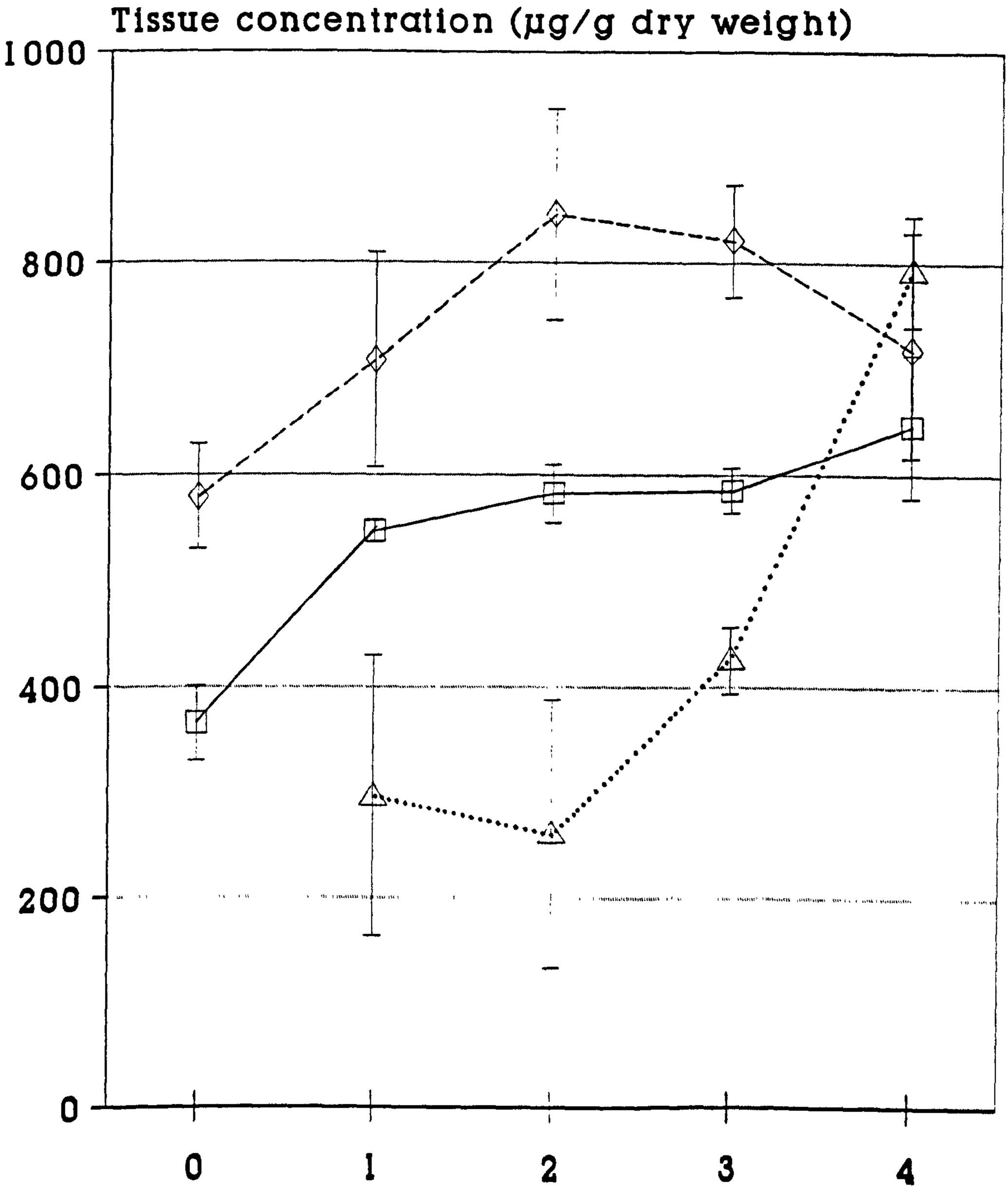


Figure 5.7: Copper concentrations in gill (□) and hepatopancreatic (△) tissue of *P. leniusculus* exposed to 0, 0.125, 0.25 and 0.5 mg Cu^{2+} l^{-1} at 9 (---) and 18 (——) °C for 4 weeks. Values are average tissue concentrations (μg Cu^{2+} g^{-1} dry weight) with standard errors (n=4).

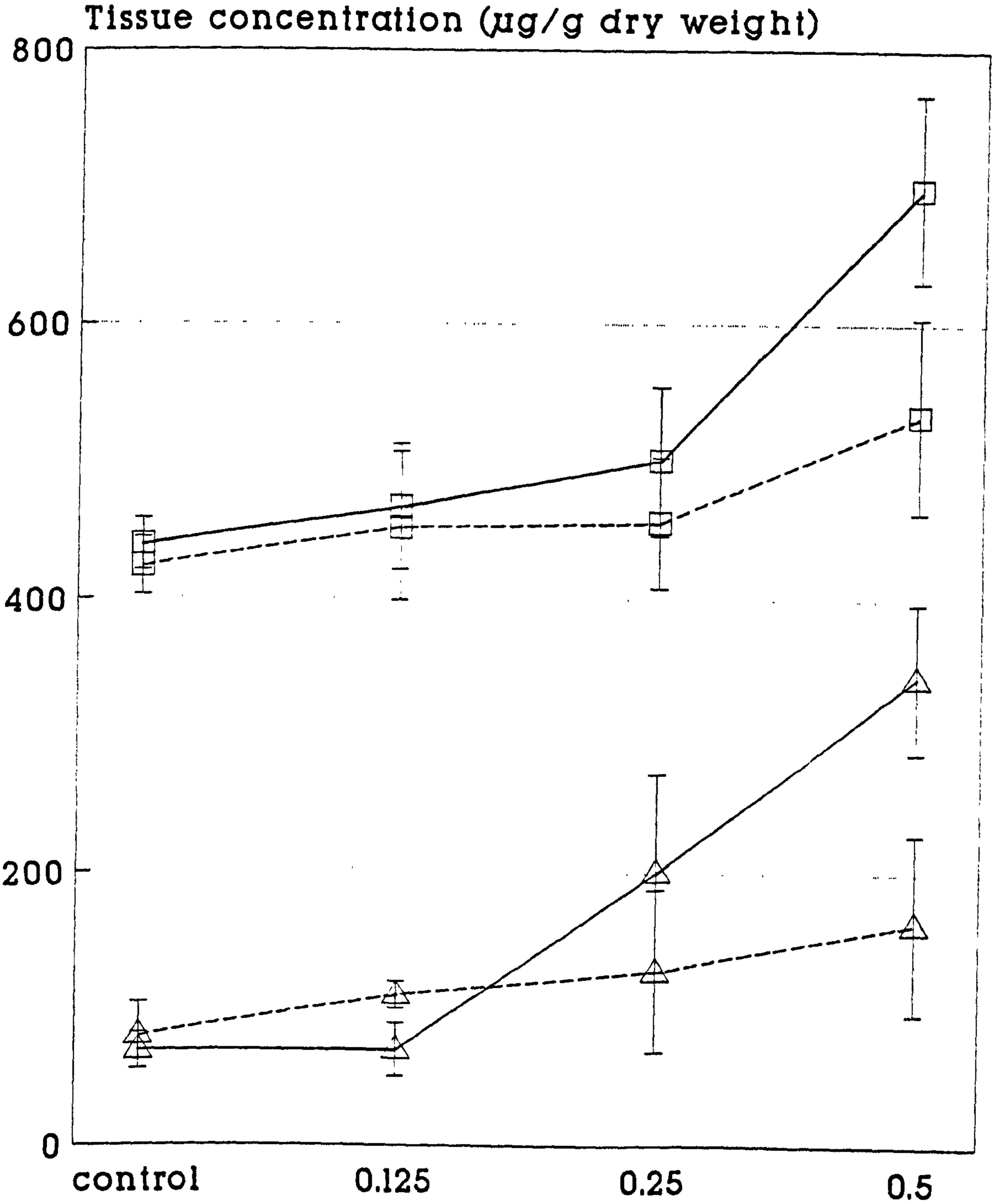
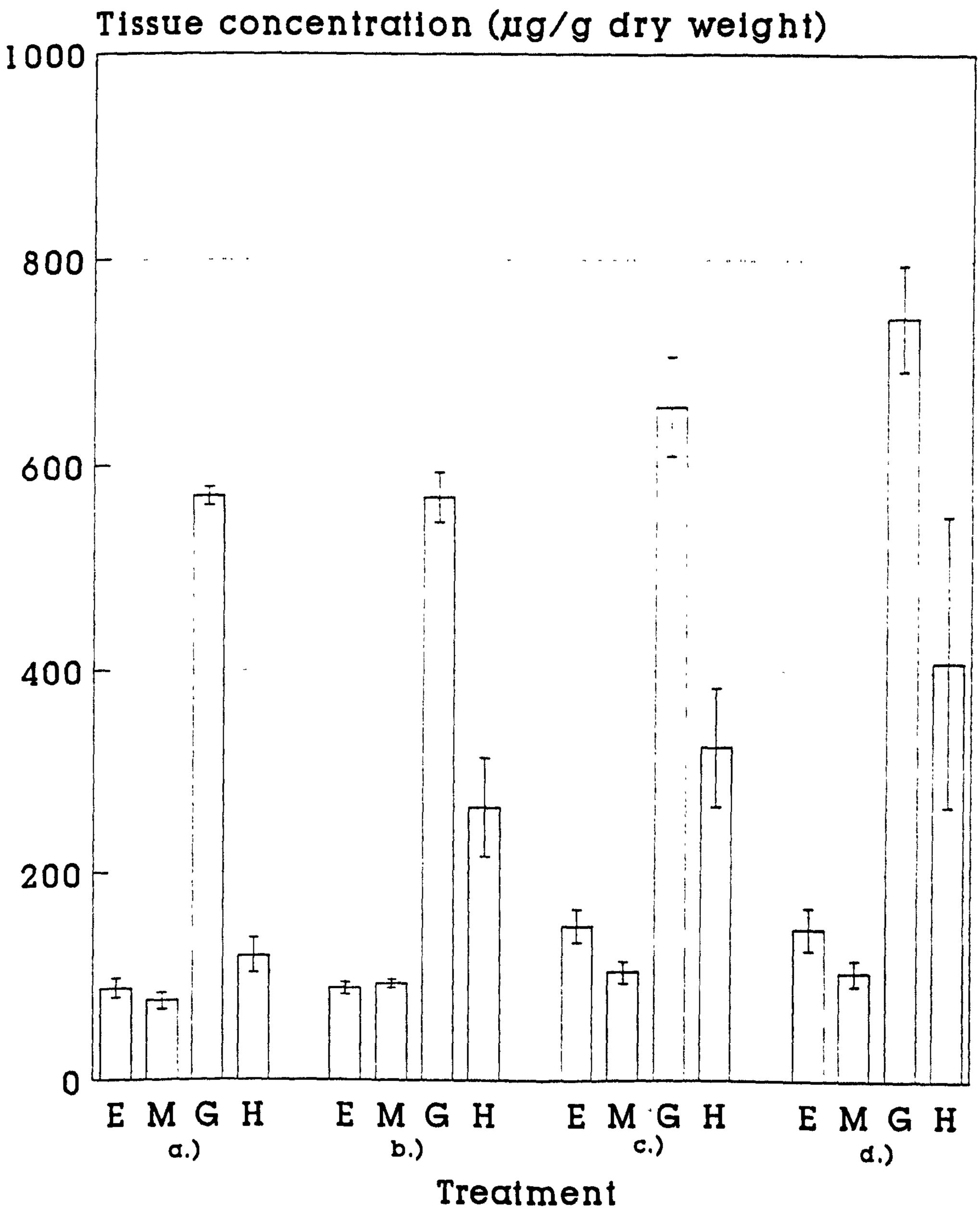


Figure 5.8: Tissue copper concentrations (E = exoskeleton, M = muscle, G = gill, H = hepatopancreas) of *P. leniusculus* exposed to copper in water and food under various treatments as indicated below;

Treatments	Water (0.5 mg Cu^{2+} l^{-1})	Food (472 μg Cu^{2+} g^{-1})
a.)	-	-
b.)	-	+
c.)	+	-
d.)	+	+

Bars show average tissue concentrations (μg Cu^{2+} g^{-1} dry weight) with standard errors (n=4).



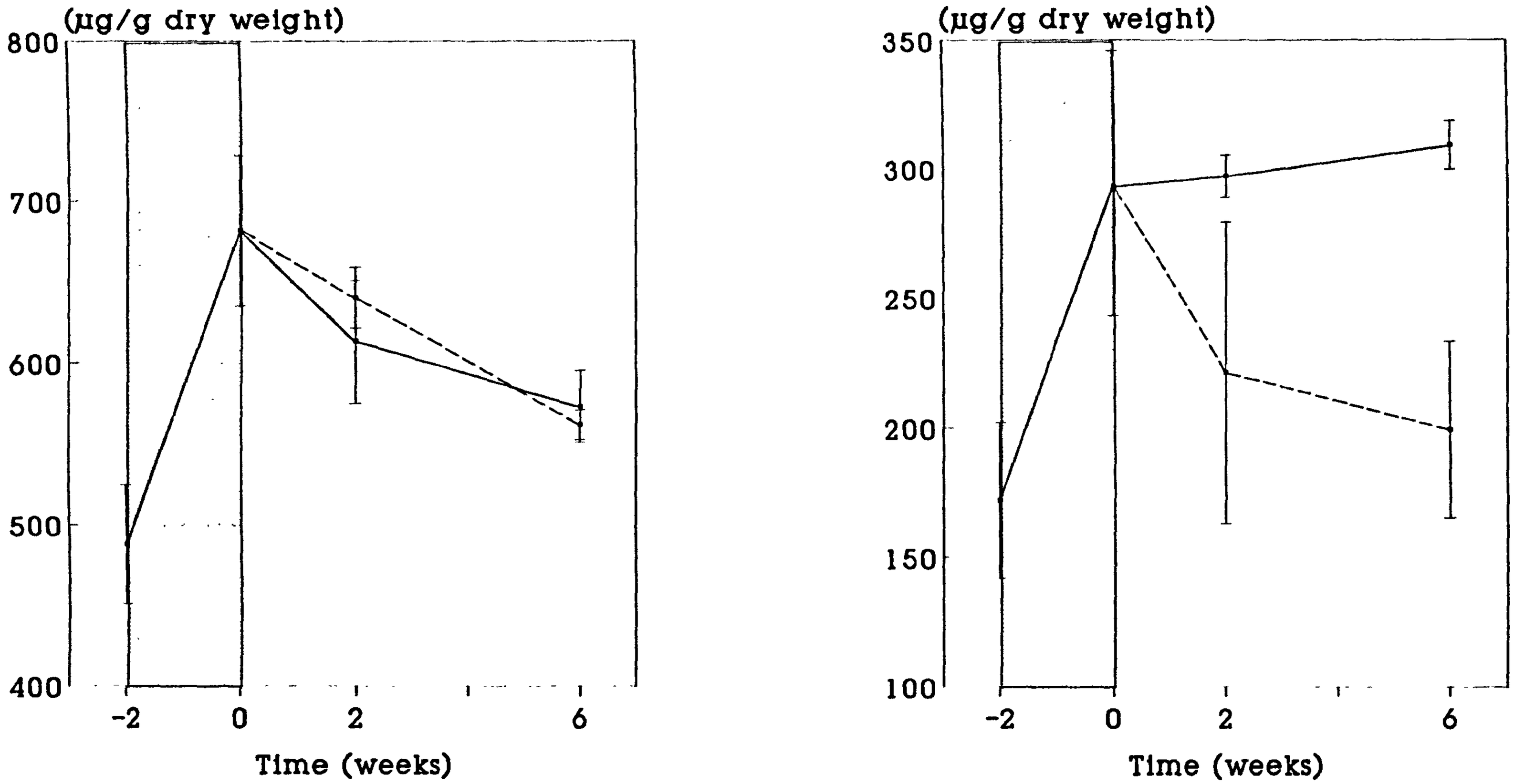


Figure 5.9: Tissue copper concentrations in a.) gill and b.) hepatopancreas of fed (– – –) and unfed (———) *P. leniusculus*, exposed to 0.5 mg Cu^{2+} l^{-1} for 2 weeks (indicated by stippled bar), then transferred to clean water. Values are average tissue concentrations (μg Cu^{2+} l^{-1} dry weight) with standard errors (n=4).

CHAPTER 6

AMMONIA

6.1 INTRODUCTION

Ammonia is readily soluble in water and is present in freshwaters as a normal product of degredation of nitrogenous organic matter, although the concentrations may be very small and subsequent conversion to nitrate may take place (nitrification). The most common pollution source is sewage or sewage effluent, particularly if nitrification of ammonia is inhibited during sewage treatment. Large quantities of ammonia can also be produced by industries, such as those producing coal gas, coke and fertilisers. Another frequent source of pollution is in organic wastes from agriculture, such as silage liquor and cattle slurry. In the period 1985-1989 pollution incidents from agriculture represented 12% of all reported incidents, of which 87% involved organic wastes (National Rivers Authority, 1992). Agriculture-related industries may also be a potential source of pollution. These include creameries, food processing plants and abbatoirs.

In aqueous solution ammonia forms ammonium ions and an equilibrium is reached between the unionised ammonia (NH_3), ionised ammonia (NH_4^+) and hydroxide ions (OH^-):

$$NH_3 + H_2O = NH_4^+ + OH^-$$

The toxicity of ammonia to freshwater fish and invertebrates has been shown to be directly related to the concentration of the

unionised form NH_3, the ammonium ion NH_4^+ having little or no toxicity (Alabaster and Lloyd, 1980, Williams *et al.*, 1986). In a study with *Daphnia pulex* Tabata (1962) concluded that the ionised ammonia fraction was only responsible for 2% of the total ammonia toxicity. The ratio of unionised to total ammonia nitrogen is influenced by pH and temperature, the unionised fraction increasing with rising pH and temperature (Emerson *et al.*, 1975). These factors are therefore of critical importance in determining ammonia toxicity. Low dissolved oxygen concentration can have a marked effect on ammonia toxicity (Lloyd, 1961). Selesi and Vamos (1976) calculated lethal concentrations between 0.2 mg NH_3 l^{-1} at 5 mg O_2 l^{-1} and 1.2 mg NH_3 l^{-1} at 10 mg O_2 l^{-1} for carp. However, in field situations, where low oxygen is likely to be accompanied by high levels of free carbon dioxide, the resulting lowering of pH can more than compensate for increased toxicity caused by low oxygen (Alabaster and Lloyd, 1980). The proportion of unionised ammonia may also be affected to a small extent by high concentrations of dissolved solids (Messer *et al.*, 1984).

An extensive review of ammonia to aquatic life has been compiled by the United States Environmental Protection Agency (U.S. EPA) (1985), with most information consisting of data for fish species. Maximum concentrations for unionised ammonia of 0.02 and 0.025 mg NH_3 l^{-1} have been recommended by the United States Environmental Protection Agency (US EPA) and the European Inland fisheries Advisory Commission (EIFAC) respectively for the protection of fish. Limited work has been carried out to determine the effect of ammonia on invertebrates, with most of

the studies using static test conditions and failing to indicate levels of unionised ammonia. However, the few data which are available show that most invertebrates are more resistant to ammonia than fish, particularly salmonids (Alabaster and Lloyd, 1980; Williams *et al.*, 1986; Arthur *et al.*, 1987) Even sensitive genera such as *Daphnia* are only as sensitive as trout (Malacea, 1966).

In a study of five fish and nine invertebrates (Arthur *et al.*,1987), the least sensitive species was the crayfish *Orconectes immunis* with a mean 96 hour LC_{50} of 18.3 mg NH_3 l^{-1}, compared to a value of 0.53 mg NH_3 l^{-1} for the most sensitive fish species *Salmo gairdneri stonei*. Other studies have also indicated high ammonia tolerance in adult crayfish. Evans (1979) determined a 96 hour LC_{50} of 3.15 mg NH_3 l^{-1} for *Orconectes nais* and in a field experiment, with animals exposed to an artificially raised ammonia concentration, Foster and Turner (1993) estimated a 24 hour LC_{50} of 7.0 mg NH_3 l^{-1} for *A. pallipes*. No acute toxicity data is available for juvenile stages, or for *A. leptodactylus* or *P. leniusculus*. This study therefore investigates the acute toxicity of ammonia to stage II juveniles of *A. pallipes*, *P. leniusculus* and *A. leptodactylus*.

Ammonia pollution may be of a chronic or diffuse nature, but many incidents, particularly those involving organic wastes from agriculture, are of an episodic or intermittent nature, involving high concentrations of toxicant for a short period of time. In flowing waters this passes downstream as a pulse of increasing length and decreasing concentration. Schofield *et al.* (1992) measured definite peaks of ammonia in a stream receiving run-off

from dairy farms, associated with daily cleaning of parlours. In such cases, short-term resistance to high levels of toxicant and the ability to recover when toxicant concentrations begin to decrease will determine the proportion of animals affected in a population. Only one study has investigated episodic ammonia exposure in crayfish. Foster and Turner (1993) simulated the effect of farm waste on *A. pallipes*. The concentration of ammonia in a small stream was artificially raised by addition of ammonium sulphate, to give a square wave pulse of 7-9 mg NH_3 l^{-1} for approximately 24 hours, and the survival of animals in cages above and below the treatment point compared. No data exists for other crayfish species. Therefore a laboratory study of the short-term resistance of *A. pallipes*, *P. leniusculus* and *A. leptodactylus* to ammonia was carried out.

6.2 MATERIALS AND METHODS

6.2.1 ACUTE TOXICITY TESTS

Stage II juveniles of *P. leniusculus*, *A. pallipes* and *A. leptodactylus* were exposed to a range of unionised ammonia concentrations in a continuous-flow system (see 3.2.1) in a temperature-controlled room. Ten animals were placed on mesh platforms in 400 ml beakers. Test solutions were delivered to the beakers via PVC tubing pushed through the base, so that solutions over-flowed into a sump.

The source of ammonia for the tests was ammonium sulphate, minimum purity 98.5%. A preliminary range finding experiment was

carried out to determine a suitable nominal concentration range. This was as follows; 0, 0.75, 0.1, 0.125, 0.15, 0.175, 0.2 and 0.4 g $(NH_4)_2SO_4$ l^{-1}. The pH and temperature of each test solution was measured every 24 hours and samples taken for determination of the actual concentrations of total ammonia (NH_3 + NH_4^+) by the colormetric method of Harwood and Khun (1970) (see 3.2.1). The concentration of unionised ammonia was then calculated using average test pH and temperature from the formulae of Emerson *et al.* (1975).

6.2.2 EPISODIC EXPERIMENTS

Stage II juveniles of *A. pallipes*, *P. leniusculus* and *A. leptodactylus* were exposed to pulses of unionised ammonia in a continuous-flow system. Ten juveniles of each species were exposed to square wave pulses of approximately 10 and 5 mg NH_3 l^{-1} for 6 and 12 hours respectively. The pH and temperature of the water were measured continuously . Samples of test water were taken every two hours and the unionised ammonia concentration determined as above.

Time to mortality for individual crayfish juveniles was noted during the ammonia pulses. Percentage mortality at the end of each pulse and again 24 hours post-exposure was also noted for each species, to determine recovery rates.

6.3 RESULTS

6.3.1 ACUTE TOXICITY TESTS

Throughout all toxicity tests average measured temperature of the test solutions was 14.3±0.09 °C. The mean hardness of the dilution water was 220 mg $CaCO_3$ l^{-1}. The source of ammonia used in the experiment, $(NH_4)_2SO_4$, was found to be a weak acid, so that pH in the test chambers ranged from 7.81 ± 0.09 in the control, down to 7.49 ± 0.03 in the highest concentration (0.4 gl^{-1}). The percentage of unionised ammonia in each chamber was therefore calculated individually using the average pH for different test solutions from the formulae of Emerson *et al.* (1975) (see 3.2.1).

The percentage mortalities of stage II juveniles of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* are shown in Fig. 6.1. For each species mortality increased with ammonia concentration and time of exposure. Mean ammonia concentrations were calculated from measured test concentrations. From the above data the 24, 48, 72 and 96 hour LC_{50} (median lethal concentration) values have been calculated in Table 6.1 for each species, and are presented in Fig. 6.2. No control deaths were observed in any of the tests. Also, there were no further mortalities in animals transferred to clean water at the end of the test period.

6.3.2 EPISODIC EXPERIMENTS

Average temperature and pH of experimental water during episodic experiments were 13.75±0.11 and 8.23±0.03 °C respectively. Mean hardness of experimental water was 266 mg $CaCO_3$ l^{-1}, and oxygen was maintained at saturation levels at 10.08 mg O_2 l^{-1}. Concentrations of ammonia in the 6 and 12 hour pulses, calculated from total ammonia concentrations of 114 and 229 mg NH_4^+ l^{-1}, were 4.58 and 9.16 mg NH_3 l^{-1} respectively at the test pH and temperature.

Cumulative percentage mortality is plotted against logarithm of time to mortality for individual crayfish juveniles in Fig. 6.3. This allowed determination of the median lethal time (LT_{50}), i.e. the time taken for 50% of the animals to die under the ammonia exposure. These were as follows for the 10 mg NH_3 l^{-1} pulse; *A. leptodactylus* LT_{50} = 4.3 hours, *A. pallipes* LT_{50} = 4.4 hours, *P. leniusculus* LT_{50} = 5.2 hours. Mortality in animals exposed to the 5 mg NH_3 l^{-1} was insufficient to determine LT_{50}s directly.

Percentage mortality at the end of the 10 mg NH_3 l^{-1} pulse was 100% for *A. pallipes*, compared to 80% and 60% for *A. leptodactylus* and *P. leniusculus* respectively. No further mortality was observed in the surviving animals after 24 hours in clean water.

6.4 DISCUSSION

The data from toxicity tests shown in Figs 6.1 and 6.2 show interspecific differences in mortality and LC_{50} values. After 96 hours exposure *A. leptodactylus* stage II juveniles exhibit a similar sensitivity to ammonia as *P. leniusculus* juveniles. However, juveniles of the latter species appeared to be more resistant over a short period (24 hours) to relatively higher ammonia concentrations than both *A. leptodactylus* and *A. pallipes*. This was confirmed in episodic experiments. For stage II crayfish juveniles exposed to a 6 hour pulse of approximately 10 mg NH_3 l^{-1}, the calculated LT_{50} values for *A. leptodactylus* and *A. pallipes* juveniles were 4.3 and 4.5 hours respectively, compared to an LT_{50} for *P.leniusculus* juveniles of 5.2 hours. In addition, at the end of the 6 hour exposure period, percentage mortality in *P. leniusculus* was 60%, compared to 80% in *A. leptodactylus* and 100% in *A. pallipes*. Williams *et al.* (1986) also noted differences in short term exposure (<24 hours) to high ammonia concentrations in different species. The mayfly *Baetis rhodani* exhibited a similar sensitivity to ammonia after 96 hours exposure as another mayfly *Ephemerella ignita*, although the latter species was approximately twice as resistant over a short period of time to relatively high ammonia concentrations.

In fish, there is conflicting evidence of the permanence of damage caused by short term exposure to ammonia. Grindley (1946) reported that few over-turned rainbow trout survived on transfer to clean water following ammonia exposure. However, Vamos (1963) found the reverse with carp, and that over-turned fish also had

a greater resistance to subsequent ammonia exposure. In the episodic experiments, no further mortalities were observed 24 hours post-exposure to the 6 hour ammonia pulse, indicating that surviving crayfish juveniles were able to recover from the ammonia exposure. Results would therefore imply that, due to a greater short-term resistance, ammonia episodes sufficient to eliminate *A. pallipes* juveniles would allow a percentage of *P. leniusculus* juveniles to survive. This would ultimately have implications for recruitment of juveniles into a population.

In acute toxicity tests, stage II juveniles of *A. pallipes* were the least sensitive after an exposure period of 96 hours. However, there was no overall significant difference between the three species, with the 96 hour LC_{50}s falling between 2.18 - 4.41 mg NH_3 l^{-1}. In a study of eleven macroinvertebrate species Williams *et al.* (1986) found only a four-fold difference between the most sensitive species, the flatworm *Polycelis tenuis*, and the most tolerant species, *Hydropsyche angustipennis*, the range of 150 hour LC_{50} values falling between 0.5 - 4.0 mg NH_3 l^{-1}. A similar range of sensitivity to prolonged exposure to ammonia has also been reported for fish. In a study of four species of coarse fish, Ball (1967) found the threshold LC_{50} values over periods of 2 - 4 days to be between 0.35 - 0.5 mg NH_3 l^{-1}. This may indicate a similar mode of toxic action in fish and aquatic invertebrates, although mode of action in fish is still unclear. Several authors have demonstrated that exposure of fish to toxic ammonia solutions results in damage to the gill epithelium (Burrows, 1964; Flis, 1968). Also, ammonia may affect water balance by increasing permeability, resulting in damage to the vascular

system (Alabaster and Lloyd, 1980). Studies with *P. leniusculus* showed that ammonia rapidly entered the animal when in the un-ionised state (Harris and Coley, unpub.). Exposure to ammonia caused an decrease in haemolymph sodium and an increase in sodium in the urine, suggesting that ammonia is affecting ionic balance.

LC_{50} data from this and other studies on invertebrates, shown in Table 6.2, would indicate that the crayfish species tested occupy the upper part of the range of tolerance to ammonia, with a very high ammonia tolerance recorded for one species, *O. virilis* (Arthur *et al.*, 1987). The mean concentration of total ammonia determined at 132 sites in the Severn-Trent region in the period 1988 - 1989 was 0.79 mg N l^{-1} (Severn Trent Water Authority, 1989a), with a range for the ten most polluted sites of 2.95 - 5.8 mg N l^{-1}. The 96 hour LC_{50}s for all three species in this study are therefore significantly greater than the average environmental concentration of ammonia recorded in the Severn Trent region, so acute toxicity of ammonia would not appear to be limiting factor in their distribution. However, 79% of populations of *A. pallipes* in the region, and 84% of populations in England and Wales, are found in waters of class 1A or 1B (National Water Council classification). These represent waters of good quality which, according to National Water Council classification, have 95 percentile ammonia concentrations not greater than 0.4 and 0.9 mg NH_3 l^{-1} for 1A and 1B waters respectively. In practice, mean ammonia concentrations are probably not greater than 0.5 mg NH_3 l^{-1}.

Studies on fish have indicated sublethal effects at concentrations as low as 12% of the lethal threshold level (Lloyd

and Orr, 1969). Application of this factor to the 96 hour LC_{50} for *A. pallipes*, *A. leptodactylus* and *P. leniusculus* gives ammonia concentrations of 0.34 - 0.44 mg NH_3 l^{-1}. These concentrations correspond well with the mean concentration of ammonia in grade 1B waters, particularly given the greater resistance of invertebrates to ammonia. It is unclear whether such low levels of ammonia have sub-lethal effects in crayfish, as studies are limited to the effects on ion regulation in one species (Harris and Coley, unpub.). However, it may be that sublethal concentrations have a limiting effect on crayfsih distribution, with short-term resistance to high ammonia concentrations important in determining the extent of mortality during epsodic ammonia pollution.

SPECIES	TIME (HOURS)			
	24	48	72	96
Pacifastacus leniusculus	6.49 (5.42-7.79)	3.57 (3.02-4.22)	3.19 (2.75-3.76)	3.02 (2.47-3.58)
Astacus leptodactylus	4.72 (4.14-5.88)	3.84 (3.51-4.26)	3.21 (2.62-3.74)	2.81 (2.18-3.26)
Austropotamobius pallipes	5.35 (4.53-6.46)	4.28 (3.72-4.97)	4.06 (3.52-4.73)	3.70 (3.23-4.41)

Table 6.1: Median lethal concentrations (LC_{50}) and 95% confidence limits (mg NH_3 l^{-1}) for stage II juvenile crayfish, calculated by probit analysis at 24, 48, 72 and 96 hours.

SPECIES	SIZE/ STAGE	LC_{50} (mg l^{-1})	pH	TEMP (°C)	REFERENCE
OLIGOCHAETA					
L. hoffmeisteri	30-40 mm	1.92	7.8-8.0	11.5	Williams *et al.*, 1986
MOLLUSCA					
L. stagnalis	25-30 mm	1.00	7.8-8.0	11.5	Williams *et al.*, 1986
P. gyrina	adult	1.78	8.0	13.3	Arthur *et al.*, 1987
M. transversum	adult	1.29	8.1	14.6	" "
INSECTA					
B. rhodani	nymph	1.70	7.8-8.0	11.5	Williams *et al.*, 1986
E. ignita	nymph	1.85	7.8-8.0	11.5	" "
H. angustipennis	larva	2.95	7.8-8.0	11.5	" "
CRUSTACEA					
A. aquaticus	8-10 mm	2.30	7.8-8.0	11.5	Williams *et al.*, 1986
G. pulex	8-12 mm	2.05	7.8-8.0	11.5	" "
C. pseudogracilis	adult	3.29	8.0	4.0	Arthur *et al.*, 1987
O. immunis	adult	14.7	7.9	17.1	" "
O. nais		3.15			Evans, 1979
P. leniusculus	stage II	3.02	8.2	14.3	This study
A. leptodactylus	stage II	2.81	8.2	14.3	This study
A. pallipes	stage II	3.70	8.2	14.3	This study

Table 6.2: Acute toxicity of ammonia to invertebrates.

Figure 6.1: Percentage mortality of stage II crayfish juveniles with increasing total ammonia concentration (_____ *P. leniusculus*, ------*A. leptodactylus*,……*A. pallipes*) at 24, 48, 72 and 96 hours.

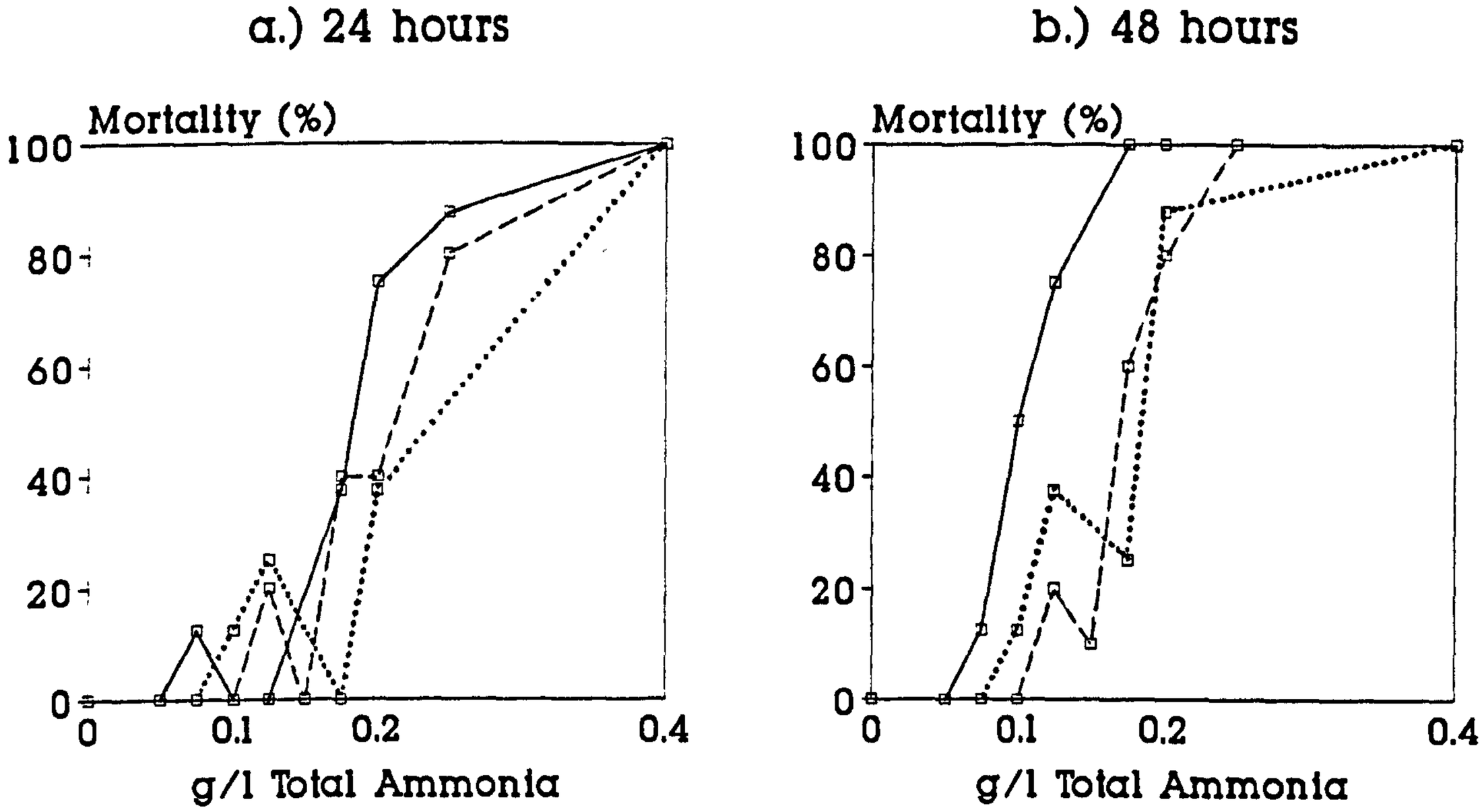
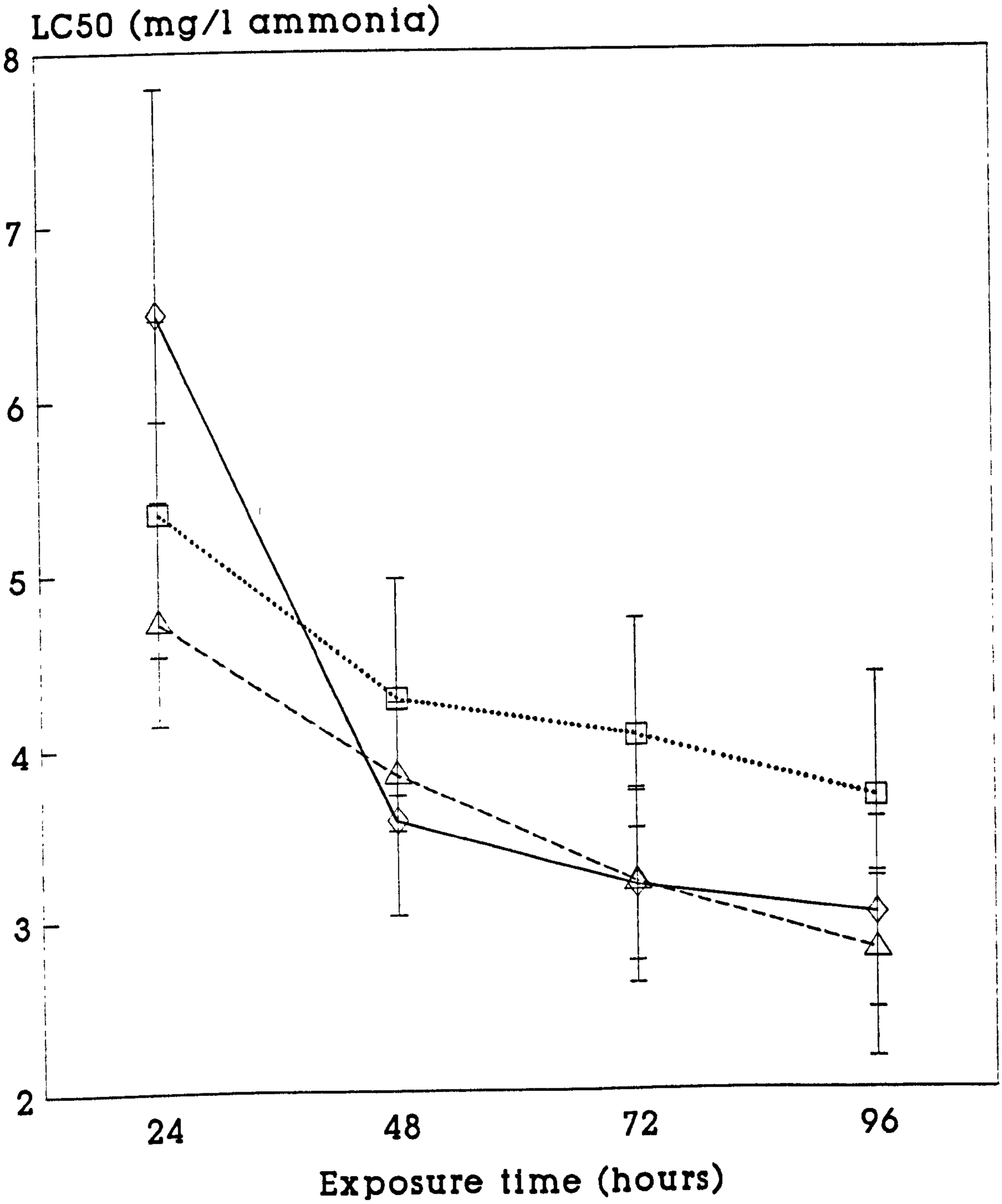


Figure 6.2: LC_{50} (mg NH_3 l^{-1}) with time for stage II juvenile crayfish (—◇— *P. leniusculus*, --△-- *A. leptodactylus*, ···□··· *A. pallipes*). Error bars indicate 955 confidence limits.



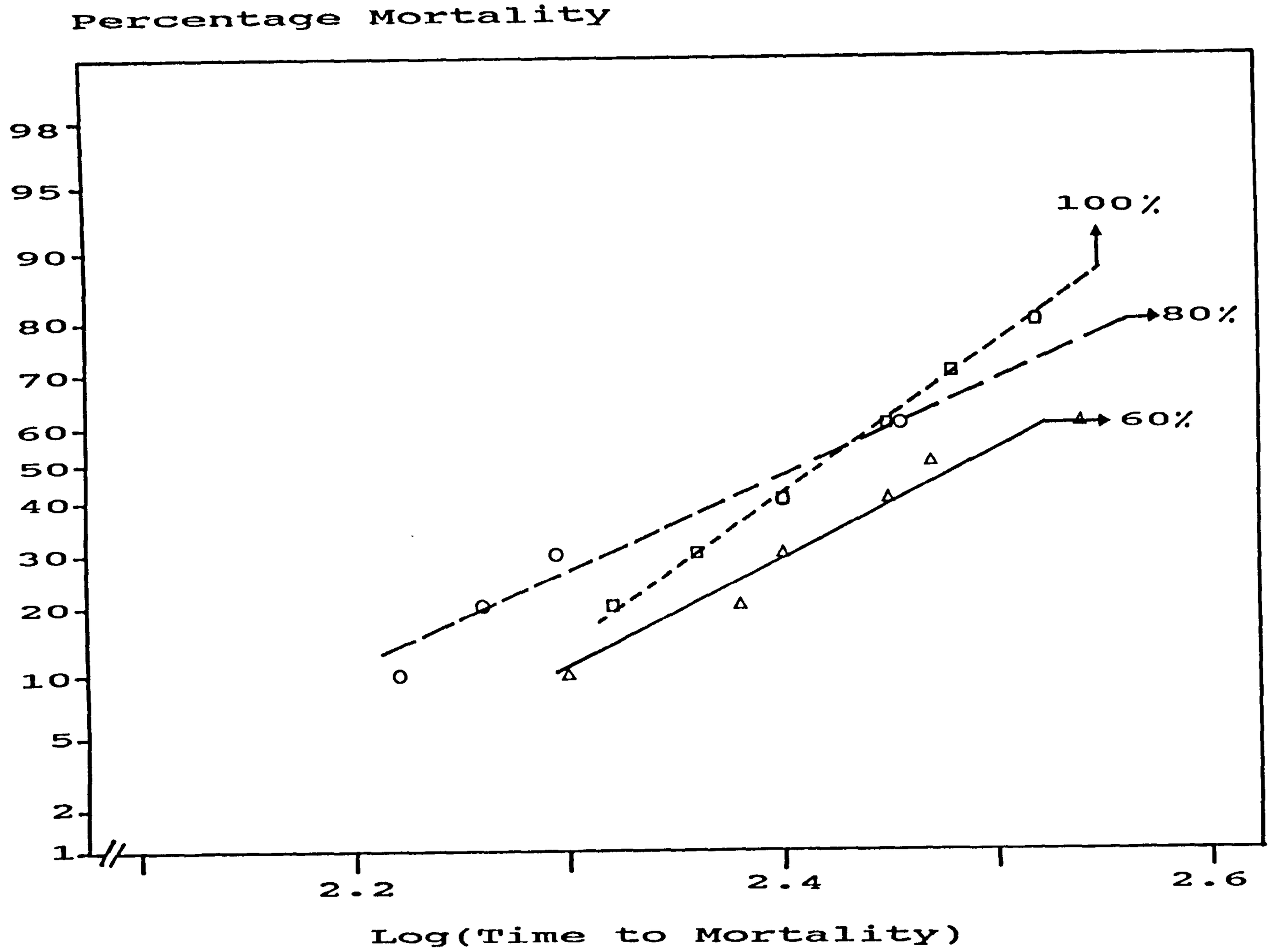


Figure 6.3: Culmative percentage mortality on a probability scale plotted against logarithm of time of death during ammonia exposure for individual stage II juvenile crayfish (——— *P. leniusculus*, -------*A. leptodactylus*, — — —.*A. pallipes*).

CHAPTER 7

LINDANE

7.1 INTRODUCTION

Pesticides include insecticides, herbicides, fungicides and a number of others such as wood preservatives and other chemicals toxic to pest organisms. Few pesticides are "target specific" and therefore affect a range of organisms. At present some 450 active ingredients are available in England and Wales (National Rivers Authority, 1992) and for most the full extent of their toxicity in the aquatic environment is unknown. The sources of potential pesticide pollution in the freshwater environment are from agricultural run-off, which constitutes the major single source of pollution (Edwards, 1970), liquid wastes from manufacturing plants and fall-out from the atmosphere. Disposal of even small amounts of surplus or unused pesticide to foul sewers may result in contamination of rivers as it passes through the sewage treatment process unaltered. Severe pollution incidents may result from accidental or deliberate discharges into streams or rivers. In Swithland Brook, near Nottingham, large numbers of dead native crayfish, including berried females, were found following an accidental spillage of pesticide in April, 1987 (Severn Trent Water Authority (S.T.W.A.), 1989b). No crayfish have been found since, although other sensitive invertebrate species, such as mayflies and stoneflies have since recovered.

In this study, lindane was chosen as a representative of a

pesticide pollutant. Lindane is a popular pesticide used to control arthropod pests on food crops, timber and farm animals and of pesticides screened for in the Severn-Trent region occurs most consistently on a monthly basis, and in the highest concentrations (S.T.W.A., 1989a). It is a broad action organochlorine insecticide made by the direct action of chlorine on benzene in ultraviolet light and does not occur naturally in the environment. Technical grade Hexachlorocyclohexane (HCH) is a mixture of 5 configurational isomers, but it is the gamma isomer that has the insecticidal properties. Lindane is the trade name for the gamma isomer, which is concentrated from HCH at 10 - 18 % to 99 - 100 % as lindane. It is stable to light, heat, air and strong acids, so is relatively stable in the environment and can retain its insecticidal properties for some time. Compared to other organochlorines it is relatively soluble in water, leading to relatively higher residues in the aquatic environment, but has a shorter persistence in soil. Biochemical breakdown by soil organisms, preferentially under anaerobic conditions, occurs at a rate of approximately 10 % in 6 weeks. To aid solubility at the high concentrations necessary for its application lindane may be obtained in the form of wettable powders and dusts, and emulsifiable concentrates.

The United States Environmental Protection Agency (1980) has recommended that levels of lindane in the aquatic environment should not exceed 2 ug l^{-1} and the 24 hour average should only be 0.08 ug l^{-1} in freshwaters. Maximum levels for the Severn-Trent region are below the former value (S.T.W.A., 1989b). However, far greater concentrations may be involved in deliberate or

accidental contamination of streams and rivers, a major source of which is the disposal of used sheep dip containing lindane (Hynes, 1961).

A number of workers have investigated the toxicity (Thurston et al., 1985; Sundaram et al., 1989; Naqvi and Newton, 1990) and bioaccumulation (Naqvi and Newton, 1991) of selected pesticides in crayfish, particularly commercially important species, such as *Procambarus clarkii*, which are collected from the wild for human consumption. Airaksinen et al. (1976) and Mees (1983) carried out toxicity studies with lindane on *A. leptodactylus* adults and *A. pallipes* juveniles and adults respectively. However, no data is available for *P. leniusculus*, and no comparative studies have been carried out under the same test conditions. This study therefore investigates and compares the acute toxicity of lindane to the three species, to determine whether introduced crayfish are more or less tolerant than the native.

7.2 Materials and Methods

Stage II juveniles of each species were individually exposed to a range of concentrations of lindane in 5cm diameter petri-dishes. A small square of black nylon mesh was provided for the juvenile crayfish to cling to, and the dishes placed on a black background to minimise in-test stress. A preliminary range-finding experiment was carried out to determine a suitable nominal concentration range. This was as follows; 0 (control), 0.1, 0.25, 0.5 and 1.0 mgl^{-1} lindane. Test solutions were made up

from a 1 % solution of gamma-HCH in acetone. An amount of acetone equal to the amount found in the highest concentration of lindane was therefore added to the controls. Ten animals were exposed per concentration in a static testing regime (see 2.2.1).

7.3 Results

Throughout all toxicity tests measured temperature and pH were 13.0 ± 0.1 °C and 7.29 ± 0.02 respectively. The mean hardness of the dilution water was 140 mg l^{-1} as $CaCO_3$.

The percentage mortalities at 24, 48, 72 and 96 hours for the three species are shown in Fig. 7.1. For each species, mortality increased with lindane concentration and time of exposure. From these data and the lindane concentrations used during the tests the 24, 48, 72 and 96 hour LC_{50} (median lethal concentration) values have been calculated for each species and are shown in Table 7.1, and are presented in Fig. 7.2. It should be noted that these are nominal values as facilities for measuring the actual test concentrations were unavailable at the time the experiment was carried out. No control deaths were observed in any of the tests and no animals were seen to moult during the tests.

Mortalities continued to occur after the animals were transferred to clean water. After ten days, the mortality rate in animals that had survived the 96 hour exposure was approximately 80 % in all treatments.

7.4 Discussion

As expected, percentage mortality for each species increased with increasing lindane concentration. The LC_{50} for each species decreased with exposure time, tending towards the lethal threshold concentration, or incipient LC_{50} (the concentration at which 50 % survival occurs for an indefinite period). This value could not be determined accurately for the three species since the tests were terminated before the LC_{50} values became asymptotic to the time axis. However, the 96 hour LC_{50} is sufficient for comparative purposes.

Both *A. pallipes* and *P. leniusculus* juveniles exhibit a similar sensitivity to lindane over the 96 hour exposure period (Figs. 5.1 and 5.2). A nominal 96 hour LC_{50} of 0.17 mg l^{-1} for *A. pallipes* closely corresponds to a value of 0.12 mg l^{-1} obtained by Mees (1983). Juveniles of both *A. pallipes* and *P. leniusculus* were approximately twice as tolerant as juveniles of *A. leptodactylus*. Chaisemartin (1974) also noted a high sensitivity of *A. leptodactylus* to DDT and lindane and obtained LC_{50} values for 96 hours of 0.004 and 0.04 ppm, respectively. The latter value is closely comparable to the nominal 96 hour LC_{50} of 0.07 mg l^{-1} lindane obtained in this study. A similar variation within a genus has been reported for two species of *Gammarus* (Sanders, 1969, 1972). The 96 hour LC_{50} for lindane for the species *G. lacustris* was found to be 48 times greater than that of *G. fasciatus*.

After 24 hours exposure, animals in increasing concentrations showed a progressive immobilisation. The limbs

were held stiffly and at different angles from the body in higher concentrations (> 0.1 mg l^{-1}), while at lower concentrations (<0.1 mg l^{-1}) animals were immobile, strongly gripping the mesh square, but still responded to tactile stimulus. Mees (1983) observed that juvenile *A. pallipes* exposed to lindane tended to shed their chelae, possibly a predator escape response, whilst a very characteristic posture adopted by adults was to have the tail thrust straight back and to walk on "outstretched" legs. Accumulation studies using ^{14}C labelled DDT (Airaksinen *et al.*, 1977) and Lindane (Mees, 1983) showed high concentrations of pesticide associated with the gills. Gill function may therefore be directly disrupted with adverse effects on osmoregulatory and respiratory capabilities, as well as the gills being the main route into the animal. In insects it acts as a neurotoxin and a respiratory poison, blocking GABA receptors and causing atoxia and convulsions, which may lead to death by paralysis. This may explain the behavioural responses to lindane observed in these experiments, and it would therefore appear that the mode of pesticide action in crayfish is similar to that in insects. This is to be expected due to their phylogenetic relationship and the specificity of lindane against arthropods.

In a study of the acute toxicity of lindane to ten species of macroinvertebrates, Green *et al.* (1986) found that insect and crustacean species were more sensitive than platyhelminths, molluscs or oligochaetes, but that there was a range of sensitivity in both insects and crustaceans that resulted in an overlap between these groups. A summary of relevant data for crustaceans is shown in Table 7.2.

Generally, concentrations of lindane in freshwaters in the Severn-Trent region are lower than 1 ug l^{-1} (Mees, 1983) and are not likely to result in short-term mortality in crayfish juveniles. However, accidental release of lindane, for example from sheep dips, where levels may rise, albeit briefly, to concentrations sufficient to kill crayfish should be considered. Results from this study would indicate that under such conditions stage II juveniles of *A. leptodactylus* would be least tolerant of lindane pollution, with *A. pallipes* and *P. leniusculus* showing a similar sensitivity. However, in addition to the LD_{50} values obtained it was observed that mortality continued in lindane exposed animals that had been transferred to clean water. For all three species, the mortality rate of previously exposed animals was approximately 80% for all concentrations of lindane. This also has implications for "episodic" pollution, where the ability of crayfish to recover from chemical stress, once the toxicant concentration begins to decrease, will strongly influence the percentage of the population that will be affected. Further investigations may therefore be necessary to determine the short term (< 24 hours) tolerance of the three species to lindane.

Similarly, even at low concentrations insufficient to cause mortalities Lindane has been shown to interfere with the reproduction of molluscs and crustaceans (Bodenstein, 1972; Bluzat and Seuge, 1970). Insecticide induced drift has also been shown to be a behavioural response to sublethal levels of Permethrin (Muirhead-Thompson, 1978) in species of *Gammarus*, while on a cellular level dithiocarbamate fungicides have been shown to cause degenerative changes leading to "black gill"

syndrome in shrimps (Doughtie and Rao, 1983). Therefore, investigation of the sublethal effects lindane on the three species of crayfish is also required.

SPECIES	TIME (HOURS)			
	24	48	72	96
Austropotamobius pallipes	0.35 (0.22 - 0.54)	0.26 (0.16 0.31)	0.22 (0.14 - 0.33)	0.17 (0.10 - 0.28)
Pacifastacus leniusculus	0.44 (0.31 - 0.64)	0.27 (0.20 - 0.39)	0.22 (0.16 - 0.31)	0.17 (0.11 - 0.22)
Astacus leptodactylus	0.45 (0.23 - 1.42)	0.12 (0.06 - 0.22)	0.09 (0.04 - 0.15)	0.07 (0.02 - 0.13)

Table 7.1: Median lethal concentration (LC_{50}) and 95 % confidence limits (mg $^{-1}$ lindane) for stage II crayfish hatchlings, calculated by probit analysis at 24, 48, 72, and 96 hours.

Species	Stage/ size	Temp (°C)	Type of test	96 hour LC_{50} (mg/l)	Reference
Gammarus lacustris	2 months	21	S[1]	0.048	Sanders, 1969
Gammarus faciatus		21	S	0.010	Sanders, 1972
Gammarus pulex		11	CF[2]	0.225	Green et al., 1986
Asellus brevicaudus		21	S	0.010	Sanders, 1972
Asellus aquaticus		11	CF	0.375	Green et al., 1986
Astacus leptodactylus	6-8 g Stage II	 13	S S	0.04 0.07	Chaisemartin, 1974 This study
Austropotamobius pallipes	5 mm CL[3] 28 mm CL Stage II	10 10 13	S S S	0.12 0.48 0.17	Mees, 1983 Mees, 1983 This study
Pacifastacus leniusculus	Stage II	13	S	0.15	This study

[1]S = Static
[2]CF = Continuous Flow
[3]CL = Carapace length

Table 7.2: Acute toxicity of lindane to freshwater Crustacea.

Figure 7.1: Percentage mortality of stage II crayfish hatchlings with increasing lindane concentration
(—— *P. leniusculus*, ---- *A. leptodactylus*, ... *A. pallipes*) at a.) 24 hours, b.) 48 hours, c.) 72 hours, and d.) 96 hours.

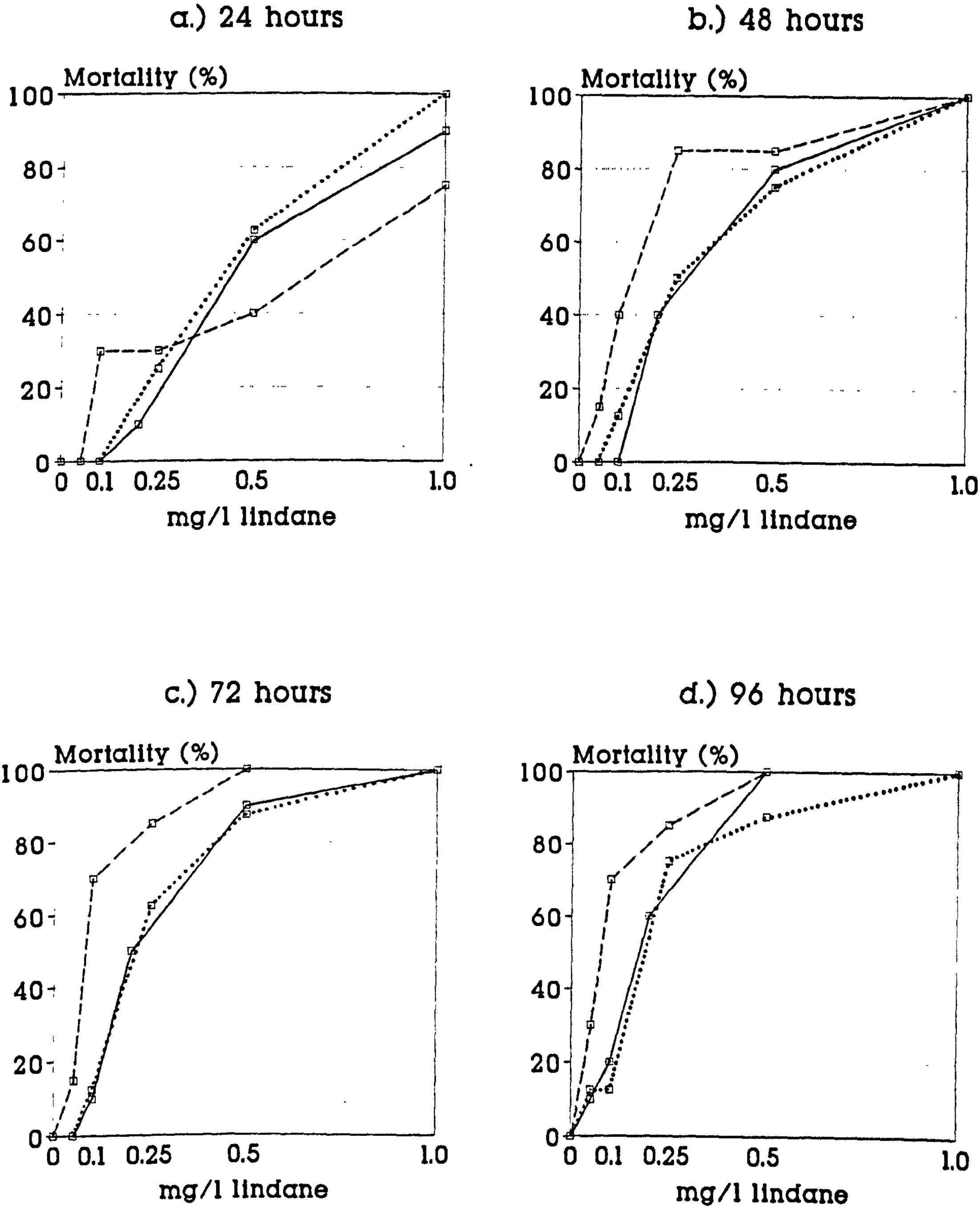
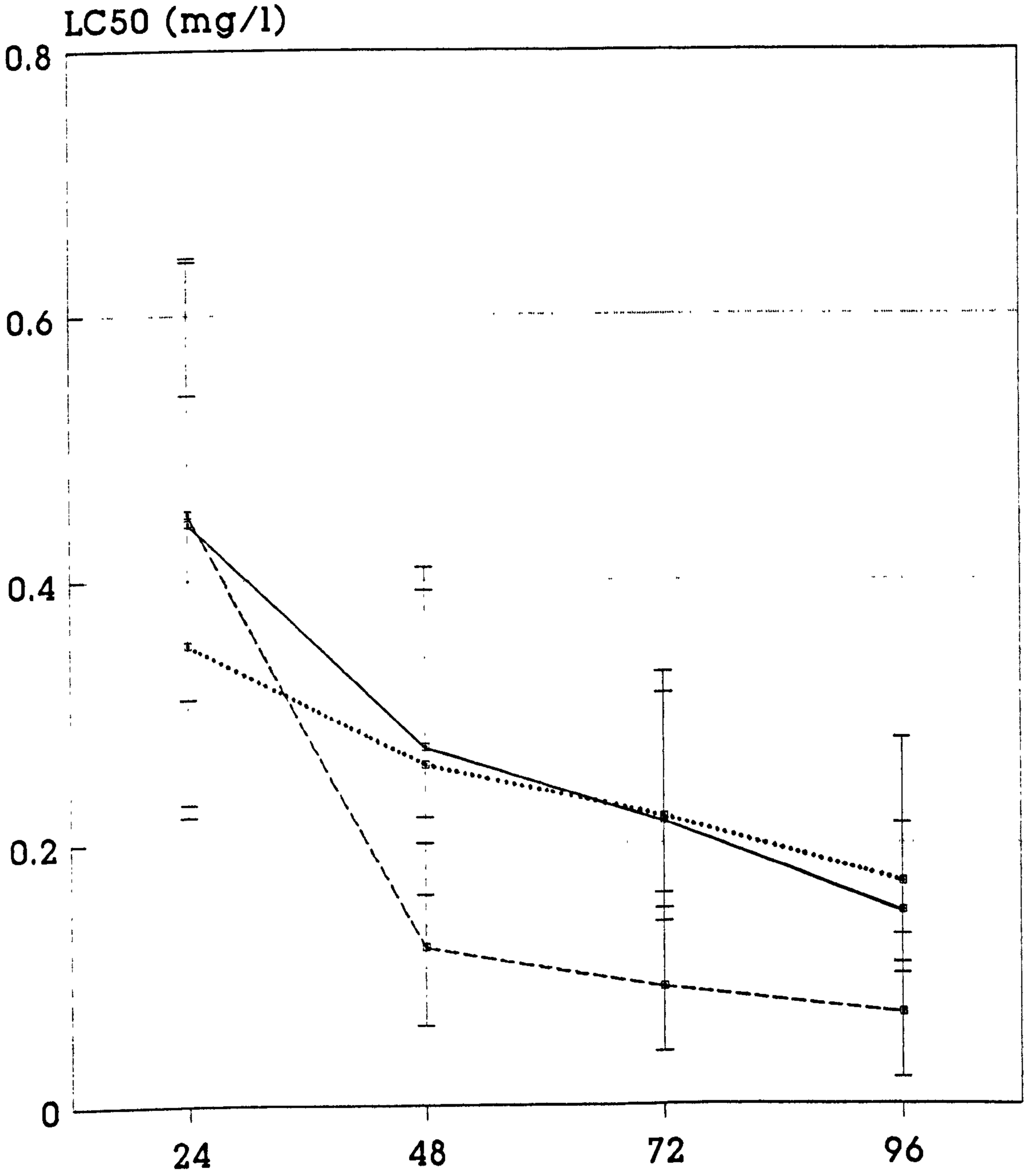


Figure 7.2: LC_{50} (mg l^{-1} lindane) with time for stage II crayfish hatchlings (——— *P. leniusculus*, ----*A. leptodactylus*, ... *A. pallipes*). Error bars indicate 95 % confidence limits.



CHAPTER 8

SUMMARY AND CONCLUSIONS TO POLLUTION STUDIES

The data from this study show interspecific differences in the tolerance of *A. pallipes*, *A. leptodactylus* and *P. leniusculus* to a range of four common pollutants; chloride, copper, ammonia and lindane.

In general, the four toxicants tested can be ranked in order of increasing toxicity to crayfish as follows: chloride (Cl^-) < ammonia (NH_3) < copper (Cu^{2+}) < lindane. Comparison with LC_{50} data for other invertebrate groups would indicate that stage II crayfish juveniles occupy the middle to upper part of the range of sensitivity to all four pollutants and, certainly for chloride, larger juvenile stages are considerably more tolerant than other invertebrates. In addition, 96 hour LC_{50}s indicated that there were interspecific differences in tolerance to chloride, copper and lindane between the three species. However, no species showed a greater or lesser overall pollution tolerance based on the toxicants tested in this study.

P. leniusculus stage II juveniles appeared to be least tolerant of chloride of the three species, but showed equal or greater tolerance when tested in the larger juvenile stages. *A. leptodactylus* stage II juveniles were found to be least sensitive to chloride, but were very sensitive to lindane compared to the other two species. In comparison, *A. pallipes* stage II juveniles had a similar tolerance of lindane as *P. leniusculus* juveniles, but were least tolerant of copper. *P. leniusculus* juveniles were

least sensitive to copper, and all three species showed a similar tolerance of ammonia. Therefore, although providing comparative data for individual toxicants, it is difficult to draw conclusions as to whether the introduced species, *A. leptodactylus* and *P. leniusculus*, have a greater overall pollution tolerance than the native species.

France (1985) criticised toxicity testing for not dealing with realistic toxicant levels and failing to relate results to environmental concentrations. In the acute lethal experiments, for all three species the calculated 96 hour LC_{50}s for each toxicant were generally higher than toxicant levels found in the environment, based on data for freshwaters in the Severn-Trent region (Severn-Trent Water Authority, 1989). This includes those sites most badly affected by a specific pollution, e.g. chloride pollution in the west branch of the River Leen. Therefore, absence of crayfish from these waters cannot be explained simply on the basis of LC_{50} values obtained in this study.

The fact that in field experiments mortality occurred in *A. pallipes* juveniles at chloride concentrations below the 96 hour LC_{50} may indicate an underestimation of chloride toxicity. Although the 96 hour LC_{50} was adequate for comparative purposes in this study, an estimation of the lethal threshold, or incipient LC_{50} (i.e. the concentration at which 50% survival occurs for an indefinite time), could not be made as toxicity experiments were terminated before the threshold was reached. This was true for all of the toxicants tested, so clearly a longer exposure period than the "standard" 96 hours is required to determine the lethal threshold values. Alternatively, although

standardised tests can be used to make comparisons in the laboratory, application of laboratory-derived toxicity data to a field situation may be inappropriate as factors known to modify toxicity, e.g. oxygen, temperature, pH and hardness, vary constantly in the environment. Also, effluents are rarely composed of single pollutants, rather they are often complex mixtures of poisons. An important environmental variable which may influence the toxicity is the presence of another pollutant. If two or more toxicants are present in an effluent then they may interact in their effect on the organisms exposed to the effluent.

Laboratory and field experiments indicated sublethal effects in stage II crayfish juveniles at concentrations below the 96 hour LC_{50}. In chloride experiments, chronic exposure to a sublethal concentration of 1000 mg Cl^{-} l^{-1}, corresponding to average chloride concentrations in the west branch of the River Leen, caused a reduction in growth in juveniles of all three species, but most markedly in *P. leniusculus*. Long-term exposure of *A. pallipes* to a similar concentration in the field caused a reduction in the survival rate of egg and juvenile stages. These data would seem to indicate that sublethal concentrations of pollutants have a limiting effect on crayfish distribution, by affecting early life stages and the subsequent recruitment into a population. Studies with ammonia in fish have shown sublethal effects to occur at concentrations as low as 12% of the lethal threshold level (Lloyd and Orr, 1969). Sublethal studies with crayfish are lacking, so it is unclear whether such low concentrations have any effect. However, calculation of 12% of

the 96 hour LC_{50}s obtained in this study for stage II juveniles gives a range of ammonia concentrations that compares well with mean levels of ammonia seen in grade 1B waters. The fact that crayfish populations are only sporadically present in waters of lesser quality may indicate limiting sublethal ammonia effects, although further investigation would be needed to confirm this.

Values obtained from sublethal toxicity experiments may therefore the threshold level of tolerance to a pollutant. However, the LC_{50} values may be of use in determining the effects of short-term exposure to relatively high toxicant concentrations. As pollution control measures have reduced the number of chronic discharges into watercourses, intermittent or episodic pollution events have increased in importance. These events involve the release of a single pulse of toxicant, which may cause concentrations to rise, albeit briefly, to concentrations sufficient to kill crayfish. Such a pollution episode would be of gretaer importance if there were a marked difference in the relative short-term response of the three species. Comparison of 24 hour LC_{50}s indicated that *A. leptodactylus* and *P. leniusculus* stage II juveniles were more tolerant of copper than *A. pallipes* juveiles in both the short (24 hours) and long-term (96 hours). Also, *P. leniusculus* stage II juveniles showed a greater short-term tolerance to chloride and ammonia than *A. leptodactylus* or *A. pallipes* juveniles, despite having a lesser or equal long-term tolerance. Further experiments with ammonia confirmed the short-term resistance of *P. leniusculus* juveniles. Ammonia episodes sufficient to eliminate *A. pallipes* juveniles would therefore allow a

percentage of *P. leniusculus* juveniles to survive, with full recovery of remaining animals once the episode had passed. This would ultimately have an effect on juvenile recruitment into a population.

Similar results were seen with copper. However, although no mortalities were observed during the copper pulse, stage II juveniles of all three species died on transfer to clean water, with 100%, 90% and 60% mortality after 48 hours in clean water for *A. pallipes*, *A. leptodactylus* and *P. leniusculus* respectively. This has implications for the underestimation of copper, and possibly lindane, toxicity to crayfish, with lower concentrations required to cause mortality in the long-term than indicated by acute lethal toxicity tests. Also, short-term resistance would be important in determining the proportion of crayfish juveniles that are able to recover from an episodic pollution event, which again would ultiumately affect juvenile recruitment.

Salinity and accumulation studies implied a high resistance of adult crayfish to chloride and copper. In salinity studies adults of all three species were found to be relatively tolerant of increased salinity, with the upper limit of tolerance determined by the transition point at which the external medium became more concentrated than the body fluids. This corresponded to a range of salinity of 45 - 50% seawater and would agree with previous laboratory data, and observation of adult crayfish in moderately saline environments. Indeed, data would indicate that all three species could possibly survive in an estuarine environment. Acccumulation studies showed that in *P. leniusculus*

the hepatopancreas acted as a transient store for relatively high levels of copper and that excess amounts could either be stored in a non-toxic granular form in hepatopancreatic cells, or be eliminated from the body in the faeces. Although acute toxicity tests were not carried out with adults, due to problems of size and obtaining sufficient stock, a regulatory mechanism for copper would imply that adults may be tolerant of high levels of copper in the environment. However, establishment of a viable population under conditions of high salinity or high copper would in fact be limited by the considerably lower chloride and copper tolerance of the early juvenile stages.

Therefore, although it is unclear whether *A. leptodactylus* and *P. leniusculus* have a greater overall pollution tolerance than *A. pallipes*, studies would indicate that crayfish distribution may be limited by sublethal concentrations of pollutants, particularly affecting juvenile stages and subsequent recruitment. This would agree with the observation by Reeve (1990) that the majority of crayfish records in grade 2 waters represent non-breeding populations. However, differences in the relative short-term resistance to pollutants, as indicated by acute lethal experiments, may be important in determining the effect of episodic pollution events on crayfish populations.

CHAPTER 9

INTRODUCTION TO THERMAL STUDIES

Temperature is a major limiting factor for aquatic poikilothermic animals. It can influence survival, geographical and local distribution, growth and metabolism, food and feeding habits, reproduction and life histories, movements, migrations and behaviour (Langford, 1983).

These effects can be described under three headings (Fry, 1967): A high or low temperature may kill an organism in a finite time within what would be its normal life span. This is a *lethal* temperature effect. The second effect is on activity and is mediated through the influence of temperature on the rate of biochemical reactions and therefore on the metabolic rate. Most aquatic organisms have little physiological control over their body temperature (i.e. poikilothermic), so their metabolism is greatly affected by the temperature of the water in which they live. This is termed a *controlling* effect. The word activity used here is a very general term to indicate anything from growing to fighting, as controlling effects are very diverse and may have morphometric as well as kinetic implications. Finally, temperature can influence the spontaneous movement of organisms. Mobile organisms exhibit locomotor responses to thermal stimuli. In a temperature gradient animals respond by congregating about a temperature which is characteristic of the species concerned. This is a *directive* effect and the chosen temperature termed the

preferred temperature, or temperature preferendum. These temperature preference or avoidance responses enhance, or optimise, chances of survival and reproduction.

Temperature may also have an indirect effect via other organisms, such as the elimination of a food organism, or the enhancement of a predator or a parasite. More important to aquatic organisms are indirect effects via water chemistry. Nearly every physical property of water is affected by temperature changes, e.g. vapour pressure, viscosity, density, surface tension, oxygen solubility and oxygen diffusity. Very slight differences in density can result in stratification in quiescent bodies of water, which may inhibit vertical mixing and oxygen transfer to lower waters. Evaporation from water bodies increases as rising water temperatures raise the vapour pressure. However, solubility is probably the most important as it is necessary to sustain aquatic life. Low solubilty induced by high temperatures, coupled with an organic load and increased bacterial respiration rates, could lead to severely depleted oxygen concentrations sufficiently low enough to kill fish (Eichholz, 1985). In addition, increased temperature may increase the rates of chemical solubility and biochemical reactions and bring about synergistic effects with pollutants, such as heavy metals.

These biotic effects not only apply to natural systems undergoing warm or cold periods lasting up to several years, but also those systems affected by heat inputs from anthropogenic sources, such as power stations. River and lake water is increasingly used for cooling waste heat from industrial

processes, such as electricity generation. Studies in Britain have shown that thermal effluents from power stations cause a persistant rise in water temperatures downstream of the discharge point. Aston and Brown (1975) measured the downstream influence of Castle Donington power station on the River Trent and found that, on average, temperatures were elevated by approximately 7 °C. Downstream temperatures during the summer ranged between 25-30 °C. Temperatures higher than normal, particularly during the summer, may have detrimental effects for aquatic biota. Organisms may be more susceptible to disease or pollutants, the food supply or the ability to catch food may diminish, and inability to reproduce or compete succesfully with others may indirectly eliminate a species (Eichholz, 1985).

A number of workers have investigated the thermal tolerances and preferenda of mainly North American species of crayfish (Becker et al, 1975; Kivivuori, 1977; Claussen, 1980; Mundahl & Benton, 1990). However, it is difficult to draw conclusions as to to relative thermal tolerances of different species as few comparative studies using the same test procedures appear to have been carried out. In a study of a riverine and a lacustrine population of *P. leniusculus*, Becker et al. (1975) postulated that there may be physiological differences between species and even geographically separated populations of the same species, due to long term adaptation to regional climatic factors.

Bowler et al. (1973) investigated acclimation to temperature and factors leading to death at high temperatures in *A. pallipes*, and there have been a number of studies on growth in this species (Bowler and Brown, 1977; Pratten, 1980). Investigations of the

effects of temperature on this species are in no way comprehensive and data is similarly lacking for *A. leptodactylus*. This study therefore seeks to investigate and compare the effects of temperature on *A. pallipes*, *A. leptodactylus* and *P. leniusculus* through tolerance, growth and respiration experiments.

CHAPTER 10

DETERMINATION OF THERMAL TOLERANCE

10.1 INTRODUCTION

Assessment of the lethal effects of temperature on aquatic organisms, particularly fish, has received considerable attention and there are several reviews detailing methods and terminology (Fry, 1967; Becker and Genoway, 1979). Three basic techniques have been used to determine upper and lower lethal temperatures, although the latter may be indeterminate because of the freezing point of water. These are:

1.) Sudden transfer of animals from a pre-set acclimation temperature to a lower or higher test temperature and exposure until a lethal response occurs. It is conveniant to subdivide this method (Claussen, 1980) into Method 1a (the LT_{50} method) where the time for 50% mortality at a given temperature is recorded, Method 1b (the T_{L50} method) where the temperature at which 50% of the animals survive for a given exposure time is determined, and method 1c (%S method) where the percentage survival at a specified temperature and exposure time is determined.

2.) Acclimation of test animals followed by transfer to test temperatures for a set time. After exposure animals are returned to the acclimation temperature. The temperature after which they do not recover in a set time is regarded as the lethal

temperature (Sprague, 1963).

3.) Heating or cooling of organisms at standard rates, e.g. 1 °C per minute, until death occurs (McFarlane et al., 1976).

Other methods have attempted to judge potentially lethal temperatures by identifying specific criteria prior to the onset of death of the test animals, usually loss of equilibrium, locomotory disorganisation or collapse. Method 3 above provides the basis for the critical thermal maximum or minimum (CTM) method. The term CTM represents both a method and a parameter and is defined by Cox (1974) as:

"the arithmetic mean of the collective thermal points at which locomotory organisation becomes disorganised and the animals loses its ability to escape from conditions that will promptly lead to its death when heated from a previous acclimation level at a constant rate just fast enough to allow deep body temperatures to follow environmental temperatures without a significant time lag".

The CTM approach has been extensively used in studies of poikilothermic vertebrates, i.e. fish, reptiles and amphibians (Fry, 1967), but has rarely been applied to invertebrate groups. However, the methodology can be readily extended to large aquatic invertebrates such as crayfish (Claussen, 1980; Mundhal and Benton, 1990) and is well suited for investigations of thermal tolerance.

Many species are able to acclimate to normal temperature ranges, so that when exposed to increasing or decreasing water temperatures their upper and lower lethal limits change. All of the above methods facilitate investigation of temperature

acclimation if they are repeated over a range of test temperatures. Temperature tolerance polygons can be produced for different species based on acclimation and lethal temperatures (Fry *et al.*, 1946), which clearly illustrate and allow ready comparison of the upper and lower lethal temperature ranges.

10.2 MATERIALS AND METHODS

10.2.1 LETHAL STUDIES

Lethal studies were carried out with stage II juveniles of each species. Prior to experimentation, animals were kept in a constant temperature room at 15 °C.

Animals were exposed to continuously increasing temperatures in 400 ml beakers suspended in a water bath. Water in the bath was continuously aerated to maintain oxygen at saturation level. Water was delivered to the beakers by the water bath pump through PVC tubing pushed through the base of the beaker, so that the water was able to overflow back into the water bath. Ten juveniles of each species were placed on mesh platforms inside the beakers, with two replicates per species. Animals were prevented from escaping by a petri-dish lid on top of the beaker.

The temperature of the water bath was continuously raised from 15 °C and the lethal temperature for each individual crayfish recorded. Animals became moribund at temperatures approaching the lethal temperature and could not maintain an upright position. Actual criteria for death was failure to respond to gentle prodding with a blunt probe.

Two rates of heating were used; 0.5 °C/hr and 2.0 °C/hr. Temperature was measured with a 0.1 °C graduated mercury thermometer and oxygen measured with a pHOX 62TE oxygen meter (pHOX Systems Limited, Shefford, England).

10.2.2 CTM STUDIES

10.2.2(i) Laboratory Experiments

Previous laboratory studies of heat tolerance in crayfish have found no significant interspecific relationship between body size and heat resistance (Spoor, 1955; Bowler, 1963; Claussen, 1980), nor any significant sex influence on heat resistance (Bowler, 1963). Therefore, in this study, eight to ten adults of both sexes (16-33 g wet weight) of each species were acclimated to a range of temperatures in constant temperature rooms (5, 10, 20 and 25 °C) for a minimum of two weeks. During this period animals were kept in darkness.

After the acclimation period, animals were tested using the critical thermal maximum or minimum (CTM) method. For determination of the critical thermal maximum (CTmax) animals were placed in batches of 3 - 4 (depending on size) in an aquarium filled with stirred, aerated water at the acclimation temperature. The aquarium was placed in a water bath, which was heated at a rate of approximately 0.8 °C per minute (Mundahl & Benton, 1990). At this heating rate it was assumed that the internal body temperature of the crayfish increased parallel to the test temperature, without a significant time lag. Critical

thermal minima (CTmin) were determined by placing crayfish in an aquarium placed in a water bath with a cooling coil, which cooled the water bath at a rate of approximately 0.15 °C per minute.

Loss of righting response (LRR) was taken as the endpoint for all experiments, as loss of equilibrium usually precedes death and the righting response is the least resistant motor function to heat (Kivivuori, 1980). Crayfish were turned onto their backs at random intervals until the first individual reached its CTmin or CTmax. The remaining animals were then turned on their backs at 0.5 °C intervals. LRR was judged to have occurred when a crayfish failed to regain an upright posture within 30 seconds of being turned over. After testing, animals were returned for recovery to water at the starting temperature.

Temperature tolerance polygons and tolerance triangles were constructed for each species using procedures outlined by Cocking (1959) and Mirenda & Dimock (1985). Data were analysed using ANOVA and regression procedures.

10.2.2(ii) Field Experiments

In measuring the effects of temperature in the laboratory the animals were held at a constant temperature in darkness for two weeks. This artificial stabilisation of the environment is described as acclimation. However, if the state of the animal is determined entirely by the natural environment prior to testing, it is said to be acclimatized.

To determine the effects of acclimatization on thermal tolerance, adults of each species were removed throughout the

period 28/4/92 - 3/11/92 from tanks containing breeding colonies at Nottingham University and tested for the CTmax, as described above. These tanks were open and, although trickle-fed from the potable supply, were subject to seasonal and diurnal fluctuations in environmental temperature.

Air and water temperatures were measured continuously by temperature probes connected to a Grant 1200 series Squirrel meter/logger (Grant Instruments Limited, Cambridge, England). Initially probes were placed in each of three tanks containing adults of the three species. However, as there was no significant difference in temperature fluctuations or mean tank temperature between tanks over the first two weeks a single probe was used to measure water temperature for the remainder of the period.

Animals were removed periodically from the tanks and tested for the CTmax, as described. All experiments were carried out between 1200 and 1700 hours and the temperature data from the Squirrel meter/logger for the two week period prior to testing was downloaded onto a personal computer using a Grant Instruments analysis program.

10.3 RESULTS

10.3.1 LETHAL STUDIES

The thermal tolerance, as percentage survival, of stage II juveniles of the three species at two rates of temperature increase are shown in Fig. 10.1.

In all three species, survival at temperatures up to 33 °C

was good for both heating rates, with mortality occurring between 33.5-37.5 °C. At temperatures approaching the lethal temperature death was very rapid, with changes of a degree or less causing an increase in mortality from near 0 % to 100 %. The slower heating rate of 0.5 °C did not cause an increase in the lethal temperature for any of the species, as might have been expected. However, there was a difference in the tolerance of the three species with a temperature between 34-34.25 °C sufficent to cause 100 % mortality in *A. pallipes*, compared to 36-37 °C in *P. leniusculus* and 37-37.75 °C in *A. leptodactylus*. The lethal temperature for each species, calculated as an average from values for individual crayfish, is as follows; *A. pallipes* 33.7 °C, *P. leniusculus* 35.3 °C, *A. leptodactylus* 36.4 °C.

10.3.2 CTM STUDIES

10.3.2(i) Laboratory studies

Normal righting response in all three species was accomplished, usually within 5 seconds, with walking legs and chelae and occasionally with a rapid tail flip. As the CTmax was approached limb movements became progressively unco-ordinated and the animals were unable to right themselves. In the CTmin experiment, as the temperature decreased, animals became progressively more sluggish and eventually became moribund.

Both CTmax and CTmin were affected by acclimation temperature. For all three species there is a significant ($p<0.001$) increase in CTmax (Fig. 10.2 - 10.4) with acclimation

temperature. The slopes of the regression lines obtained from the data show the change in temperature tolerance, in degrees Centigrade per degree change in acclimation temperature. The values for each species are as follows: *P. leniusculus* 0.363, *A. pallipes* 0.374, *A. leptodactylus* 0.433. This is equivalent to the acclimation response ratio (ARR) described for amphibians (Claussen, 1977), which assumes a linear relationship between CTM and acclimation temperature. However, comparison of regression lines using a BBC microcomputer running a "COMPREG" BASIC program (Wiggans et al., 1983) showed no significant difference between the acclimation response of the three species.

Comparison by ANOVA at each temperature showed that the upper thermal tolerance of *P. leniusculus* was significantly higher ($p<0.05$) than *A. pallipes* and *A. leptodactylus*. Mean CTmax values for *P. leniusculus* were 1.4-3.0 °C higher than for the other two species. At 5 °C the CTmax for *A. pallipes* was higher than that of *A. leptodactylus* ($p<0.05$). However, there was no further significant difference between these two species over the remainder of the temperature range.

There was also a significant ($p<0.01$) increase in the CTmin with acclimation temperature. However, there was no significant difference between the CTmin values of the three species at any of the acclimation temperatures.

The zones of thermal tolerance of the three species are illustrated by the polygons plotted in Fig. 10.5. The areas of these polygons (expressed as $°C^2$) represent the theoretical sum of thermal variations that each species can tolerate and are shown in Table 10.1. The intersection of a 45 degree diagonal

from the origin to the line defining the upper thermal tolerance indicates the temperature beyond which further acclimation cannot occur and corresponds to the theoretical maximum thermal tolerance of each species (as measured by the CTM method). A perpendicular from this point completes the polygon and allows estimation of the theoretical maximum lower thermal tolerance. These values are shown for each species in Table 10.1.

Similarly, the temperature tolerance triangles in Fig. 10.6. describe the upper thermal tolerance of each species and are quantified by T, the thermal tolerance area, expressed as degrees Centigrade squared ($^{\circ}C^2$). These values are shown in Table 10.1.

10.3.2(ii) Field Studies

Animals were removed from the outside tanks and tested for their CTMax on five occasions over the period 28/4/92 - 3/11/92. During this period the minimum and maximum daily temperatures recorded in the tanks by the datalogger were 5.41 $^{\circ}C$ (22/10/92) and 21.1 $^{\circ}C$ (29/6/92). An example of a graph printout obtained from datalogger readings is shown in Fig. 10.7.

The CTmax obtained for each species is plotted with weekly average temperature, calculated from four datalogger readings for each day, against sampling date in Fig 10.8. There is a correlation between CTmax and average weekly temperature for all three species, with the highest CTmax values recorded in July when the water temperatures were highest. In general, as in the laboratory studies, the CTmax values obtained for *P. leniusculus* were higher than those obtained for the other two species

throughout the sampling period. However, this was not found to be significant. CTmax values obtained from field experiments are also superimposed onto regression lines obtained from laboratory data in Figs 10.2 - 10.4. Field values are plotted against the mean daily water temperature for the two week period prior to experimentation and are closely correlated with laboratory data for all three species. Comparison of regression lines from field and laboratory data sets using a BBC model B microcomputer running a "COMPREG" BASIC program (Wiggans *et al.*, 1983) shows no significant difference between the two data sets, although field data is unavailable for the upper and lower ends of the temperature range used in the laboratory experiments.

10.4 DISCUSSION

The lethal temperatures obtained for stage II juveniles of the three species are shown in Table 10.2, together with a range of values for selected fish and crayfish species. Of the fish species, the Salmonidae have the lowest thermal tolerance found to date, with maximum upper lethal temperatures usually below 25 °C. The Cyprinidae, with the exception of the goldfish *Carassius auratus*, generally occupy an intermediate position with lethal temperatures between 25 and 35 °C achieved through acclimation. The Ameuridae, along with the goldfish, have the highest thermal tolerances of the fish species tested. The available data for crayfish suggests that the majority of species fall into the middle to upper part of this range, with thermal tolerances similar to the Cyprinidae and Ameuridae.

In this study, both *P. leniusculus* and *A. leptodactylus* juveniles were found to be more temperature resistant than *A. pallipes*, so would be less susceptible to a natural, or artificial, increase in water temperature. However, it can be seen from Table 10.2 that the values obtained for *A. pallipes* and *P. leniusculus* stage II juveniles were approximately 3 °C greater than those obtained by Bowler (1963) and Becker *et al.* (1975). Both of these studies investigated the thermal tolerance of adult stages. Mundhal and Benton (1990) found that the thermal tolerance of *Orconectes rusticus* juveniles was significantly higher than adults. Mean CTMax values for juveniles were 0.9 to 2.6 °C higher than those of adults. It is possible that this observed difference between adults and juveniles is the result of ontogenetic differences in heat tolerance. Such differences have been reported in other crustaceans (Sprague, 1963; Craig, 1974). In natural habitats, juvenile crayfish typically occupy shallow areas bordering stream edges where temperatures are highest, whereas adults prefer relatively deeper pool areas where temperatures are lowest. The reason for this segregation is unknown, although it may result from aggressive encounters between adults and juveniles (Butler and Stein, 1985). However, it may also be due to differences in thermal preference.

In all three species a very narrow difference between non-lethal and lethal temperatures was noted, with changes of less than 1 °C causing an increase in mortality from 0 to nearly 100 %. Similar patterns of mortality were observed by Becker *et al.* (1975) in *P. leniusculus*, indicating a high sensitivity to slight temperature changes whenever lethal limits are approached. A

secondary factor in the thermal death of crayfish was noted in this and the above study with the loss of single animals at temperatures several degrees lower than the lethal temperature (see Fig. 10.1). These individuals were stimulated to moult by the increasing temperature and invariably died in the process, indicating that the stress of moulting imposed upon the thermal stress was not tolerated.

Table 10.3 shows thermal tolerance data obtained from CTM experiments for a number of fish and crayfish species. Of the fish species it can be seen that the goldfish *Carassius auratus* again has the greatest overall thermal tolerance. However, according to data obtained by Claussen (1980), at least one species of crayfish, *Orconectes rusticus*, has a substantially greater upper thermal tolerance. Tramer (1977) observed crayfish (species not named) feeding on thermally stressed fish in drought-affected stream-bed pools, indicating the high temperature tolerance of some crayfish species, although in a field situation other factors, such as tolerance of low oxygen, would also be involved.

In this study, in laboratory experiments, all three species showed a similar acclimation response, with changes in CTMax and CTmin with increased, or decreased, acclimation temperature. However, acclimation only had a small effect in extending the zone of thermal tolerance. For example, *P. leniusculus* acclimated to 5°C were able to withstand a temperature increase to 27.6 °C, while animals acclimated to 25 °C were able to withstand 34.75 °C, representing a change of 0.363 °C per degree increase in acclimation temperature. Although response to acclimation

temperature was similar in the three species, and no significant difference was noted between CTmin values, *P. leniusculus* had a higher CTmax at all acclimation temperatures and therefore a greater overall thermal tolerance than both *A. pallipes* and *A. leptodactylus*. The value obtained for the upper thermal tolerance area for *P. leniusculus* in this study is comparable to that obtained by Becker *et al.* (1975) (Table 10.2). In field studies, CTmax values for *P. leniusculus* were also generally higher than the other two species, but not significantly so. In naturally acclimatized animals there are many factors that can influence the response to increased temperature which are eliminated in laboratory conditions. Previous laboratory acclimation studies found no significant sex effect on thermal resistance (Bowler, 1963). However, in a study using naturally acclimatized individuals of *P. leniusculus*, Becker *et al.* (1975) found that thermal tolerance was higher in berried female crayfish during winter than in other individuals, but lower in mature, pre-breeding female crayfish during the autumn. This seasonal variation in thermal tolerance in females may be due to changes in physiological condition in relation to the reproductive cycle (Brett, 1944) and would contribute to a variation in thermal tolerance within a population.

Prolonged exposure to temperatures above the CTMax, or below the CTmin, would result in death, as the animal effectively loses its locomotory organisation and hence its ability to escape from prevailing conditions. Bowler *et al.* (1973) postulated that the progressive loss of coordination during heat death at high temperatures is due primarily to the inactivation of the enzyme

Mg^{2+} - ATPase, which controls membrane permeability to sodium (Na^+) and potassium (K^+) ions. Denaturation of the enzyme causes muscle membrane conductance to increase, resulting in loss of potassium from the muscle to the haemolymph. The high haemolymph potassium concentrations disrupt normal nervous activity, causing the frequency of spontaneous action potentials to increase very rapidly, resulting in progressive loss of co-ordination and death with prolonged exposure. Thermal acclimation would therefore seem to involve a change or changes in the composition of cell membranes, or membrane-bound proteins. The mechanism responsible for eliciting these physiological adjustments is unclear, although it has been shown that the eyestalk neurosecretory apparatus may be involved (Pruitt and Dimock, 1977).

During the CTM experiments in this study, there was a 100 % recovery in experimental animals when returned to their acclimation temperature from the CTM, indicating that they could survive if exposed for a short period to a temperature outside their zone of tolerance as long as temperature returns to normal before death occurs. However, in the natural environment, the period where the animals are unable to function properly due to the loss of limb co-ordination, would make them more susceptible to more temperature-tolerant predators, which in mixed populations would include more resistant species of crayfish, in this case *Pacifastacus leniusculus*.

SPECIES	AREA OF TOLERANCE POLYGON ($°C^2$)	MAXIMUM UPPER THERMAL TOLERANCE (°C)	MAXIMUM LOWER THERMAL TOLERANCE (°C)	AREA OF TOLERANCE TRIANGLE ($°C^2$)
Austropotamobius pallipes	878	34.0	8.0	374
Pacifastacus leniusculus	1061	38.0	7.5	448
Astacus leptodactylus	927	36.0	9.0	383

Table 10.1: Values obtained from temperature tolerance polygons and triangles.

SPECIES	TEMP. (°C)	METHOD	REFERENCE
Oncorhynchus keta	23.8	LT50 (AT 5-20 °C)	Brett, 1952
Hyalella azteca	29.6	24 hour LC50 (AT 10-30 °C)	Sprague, 1963
A. pallipes	30.0	LT50 (AT 8-25 °C)	Bowler, 1963
Cambarus bartoni	32.5	LT50 (AT 15-25 °C)	Cox and Beauchamp, 1982
P. leniusculus	32-33	LT50 (AT 5-30 °C)	Becker et al., 1975
Cambarus acuminatus	33.0	LT50 (AT 4-30 °C)	Mirenda and Dimock, 1985
Rutilus rutilus	33.5	CTM (AT 17-30 °C)	Cocking, 1959
Ameiurus nebulosus	36.5		Brett, 1944
Orconectes rusticus	36.6	12 hour LC50 (AT 4-30 °C)	Spoor, 1955
Carassius auratus	38.6		Fry et al., 1942
Procambarus leonensis	38.9	Constant Heating (1-2 °C per day)	Caine, 1978
A. pallipes	33.7	Constant Heating (0.5-2°C per hour)	This study
P. leniusculus	35.3	"	This study
A. leptodactylus	36.4	"	This study

AT = Acclimation temperature range.

Table 10.2: Lethal temperatures for selected fish and Crustacea.

SPECIES	AREA (°C²)		REFERENCE
	POLYGON	TRIANGLE	
Oncorhynchus keta	468[a]	260[b]	[a] Brett, 1952 [b] McErlean et al., 1969
Salvelinus fontinalis	625	-	Hart, 1947
Rutilus rutilus	770	301[1]	Cocking, 1959
Pacifastacus leniusculus	-	424	Becker et al., 1975
Cambarus acuminatus	1057	495	Mirenda and Dimock, 1985
Carassius auratus	1220[a]	595[b]	[a] Fry et al., 1942 [b] McErlean et al., 1969
Orconectes rusticus	-	702[1]	Claussen, 1980
A. pallipes	878	374	This study
A. leptodactylus	927	383	This study
P. leniusculus	1061	448	This study

[1] Values for triangles calculated from CTM data using procedure outlined in Mirenda and Dimock, 1985.

Table 10.3: Thermal tolerance of selected fish and crayfish species.

Figure 10.1: Percentage survival of stage II crayfish hatchlings (◇ *P. leniusculus*, △ *A. leptodactylus*, □ *A. pallipes*) at two rates of heating (——2 °C hr^{-1}, – – –0.5 °C hr^{-1}).

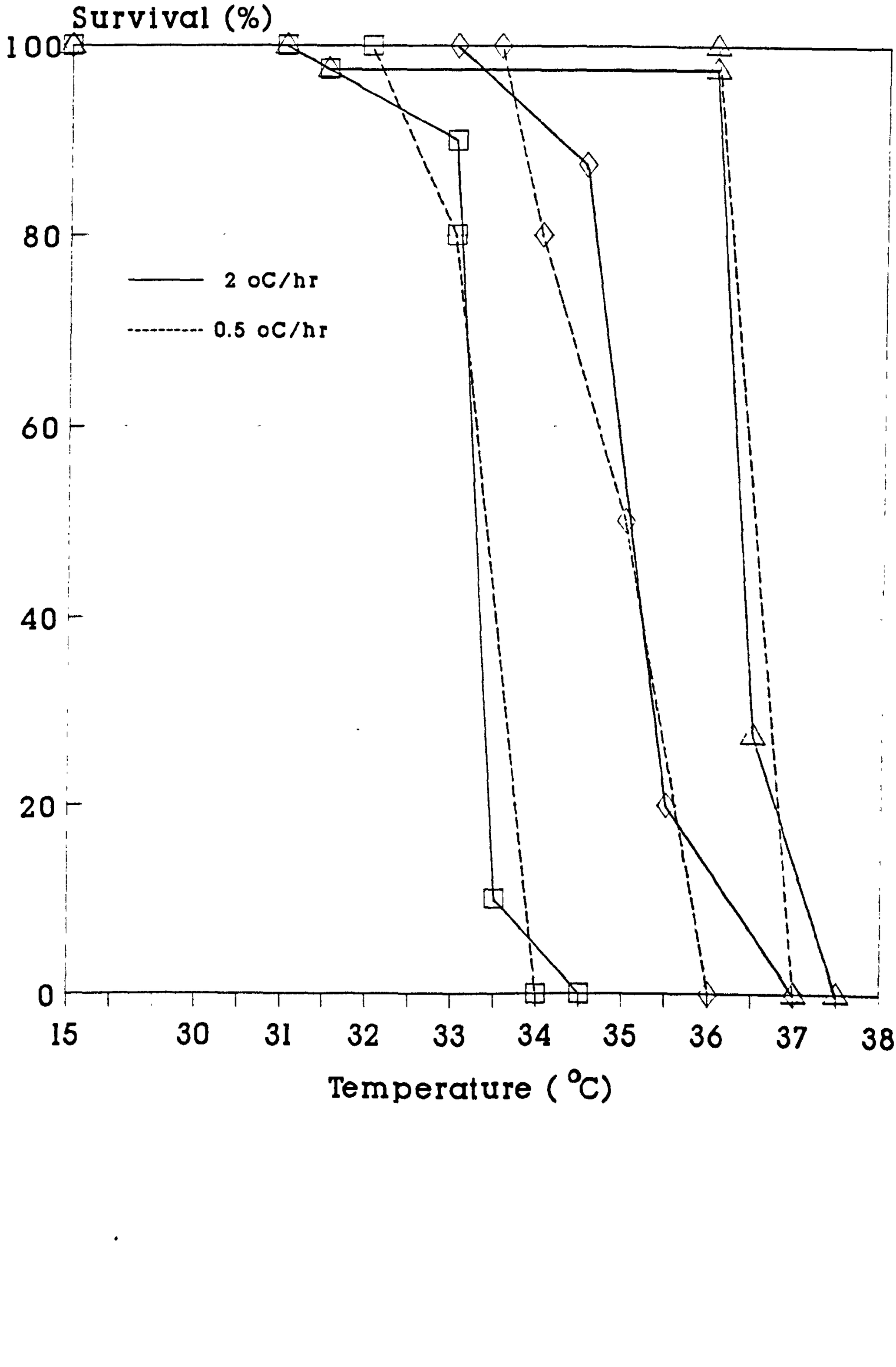


Figure 10.2: Relationship between CTmax and acclimation temperature for *A. pallipes*. Values are means with standard errors (n=4). Equation for the regression line is as follows; CTmax = 0.374 temperature + 23.515, p<0.001. Solid symbols represent values obtained from field experiments with date of sampling.

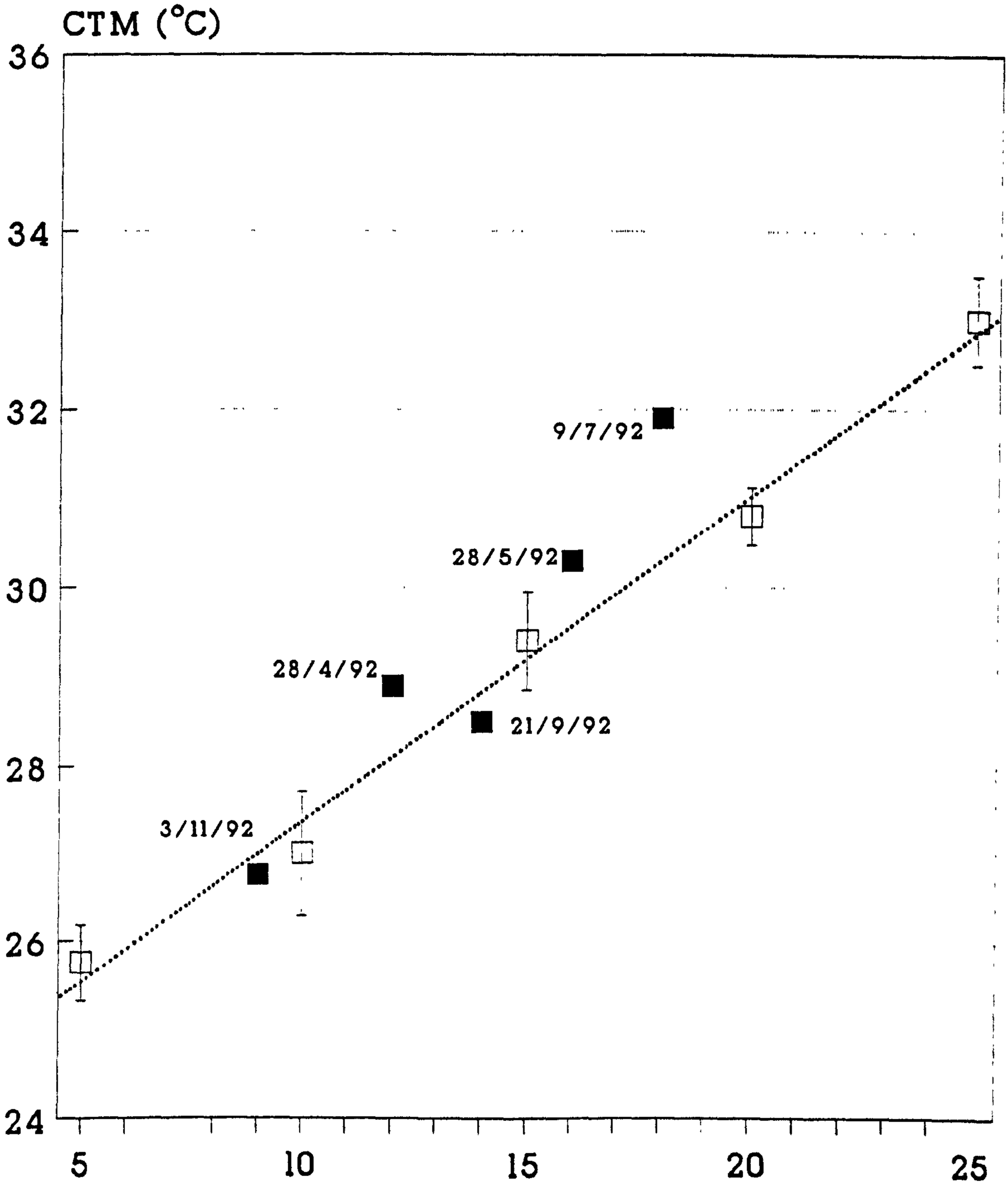


Figure 10.3: Relationship between CTmax and acclimation temperature for *A. leptodactylus*. Values are means with standard errors (n=4). Equation for the regression line is as follows; CTmax = 0.433 temperature + 22.68, p<0.001. Solid symbols represent values obtained from field experiments with date of sampling.

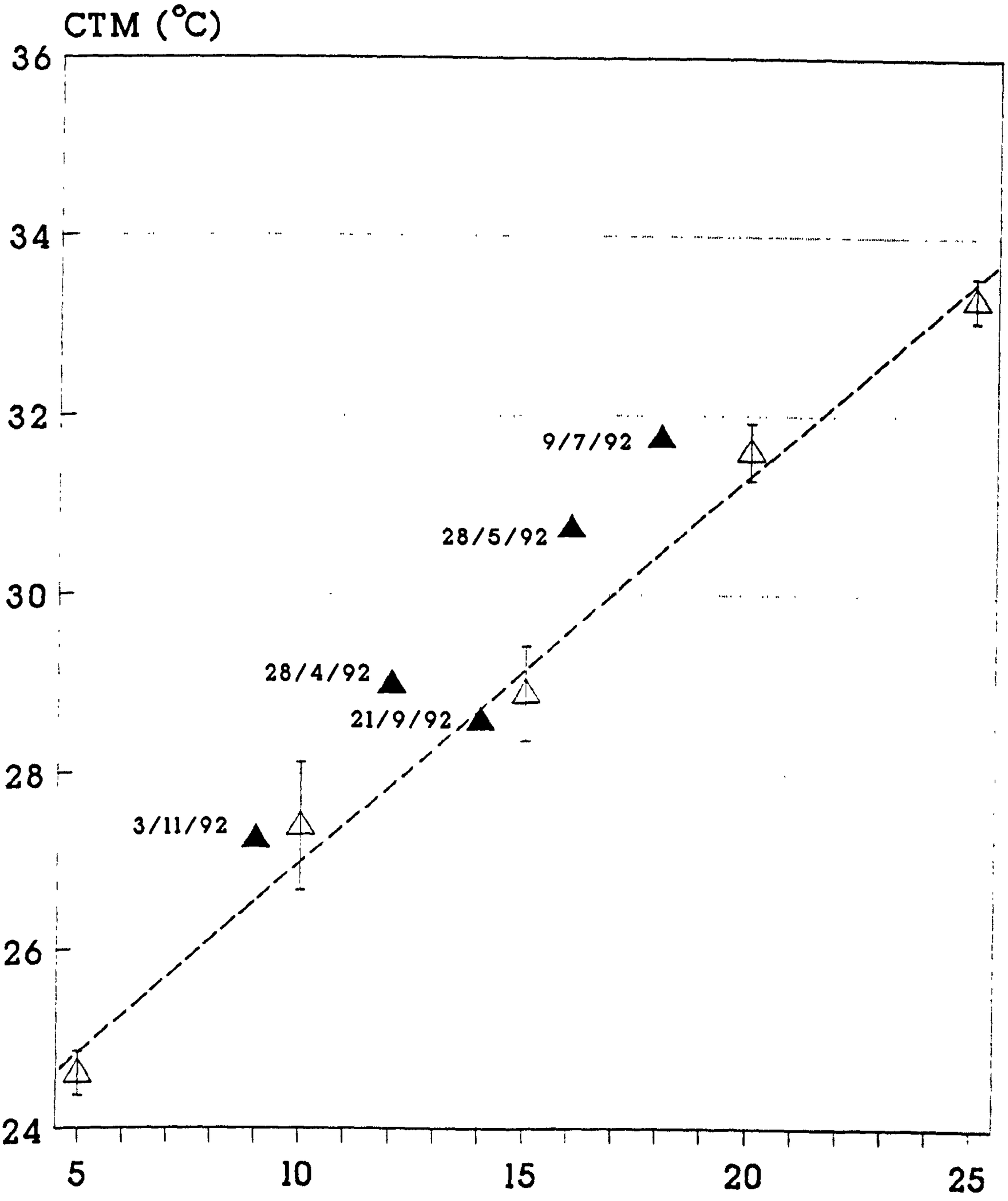


Figure 10.4: Relationship between CTmax and acclimation temperature for *P. leniusculus*. Values are means with standard errors (n=4). Equation for the regression line is as follows; CTmax = 0.363 temperature = 25.55, p<0.001. Solid symbols represent values obtained from field experiments with date of sampling.

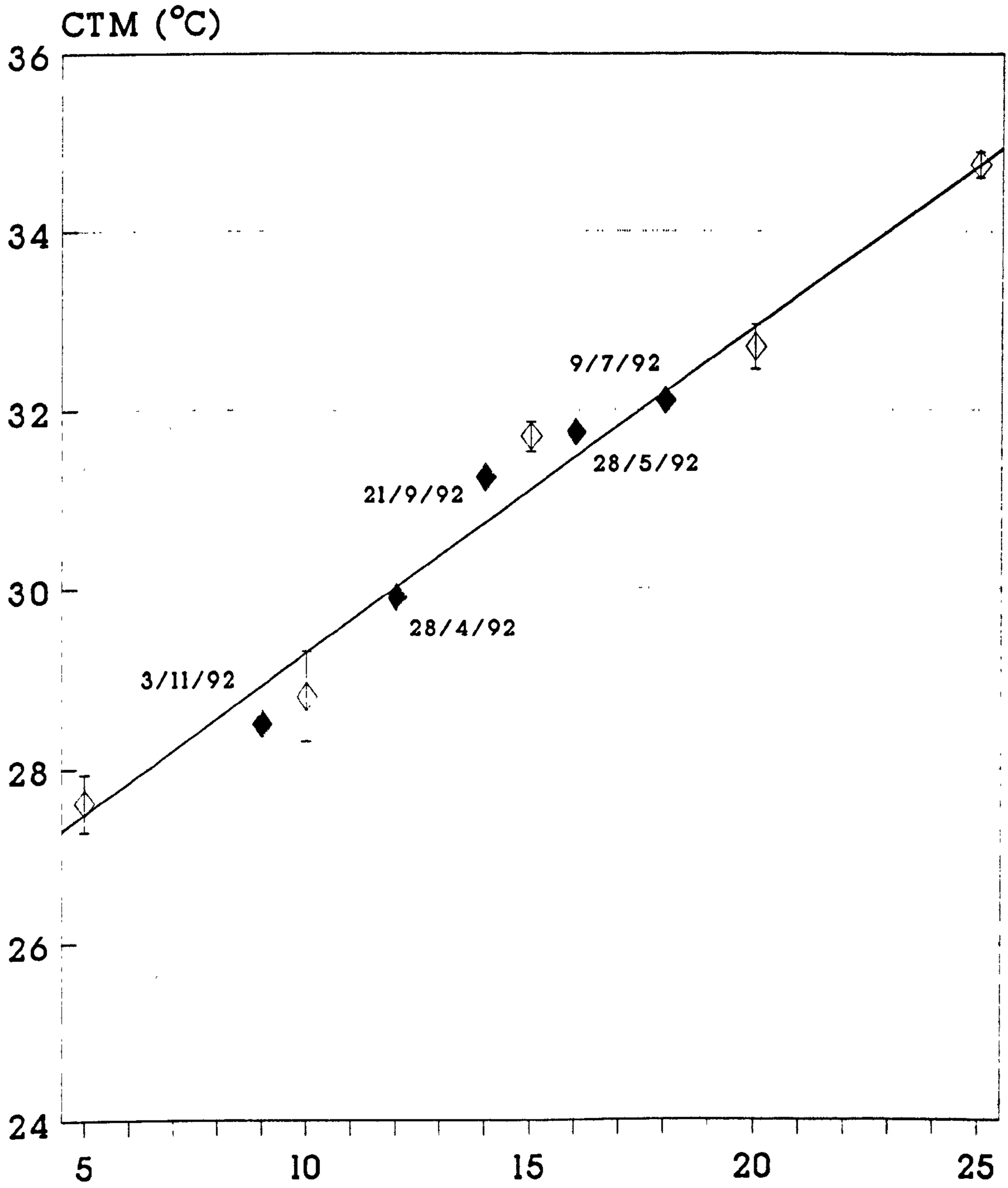


Figure 10.5: Temperature tolerance polygons calculated from CTmax and CTmin data (—●—*P. leniusculus*, – –▲– –*A. pallipes*, — –■— —*A. leptodactylus*).

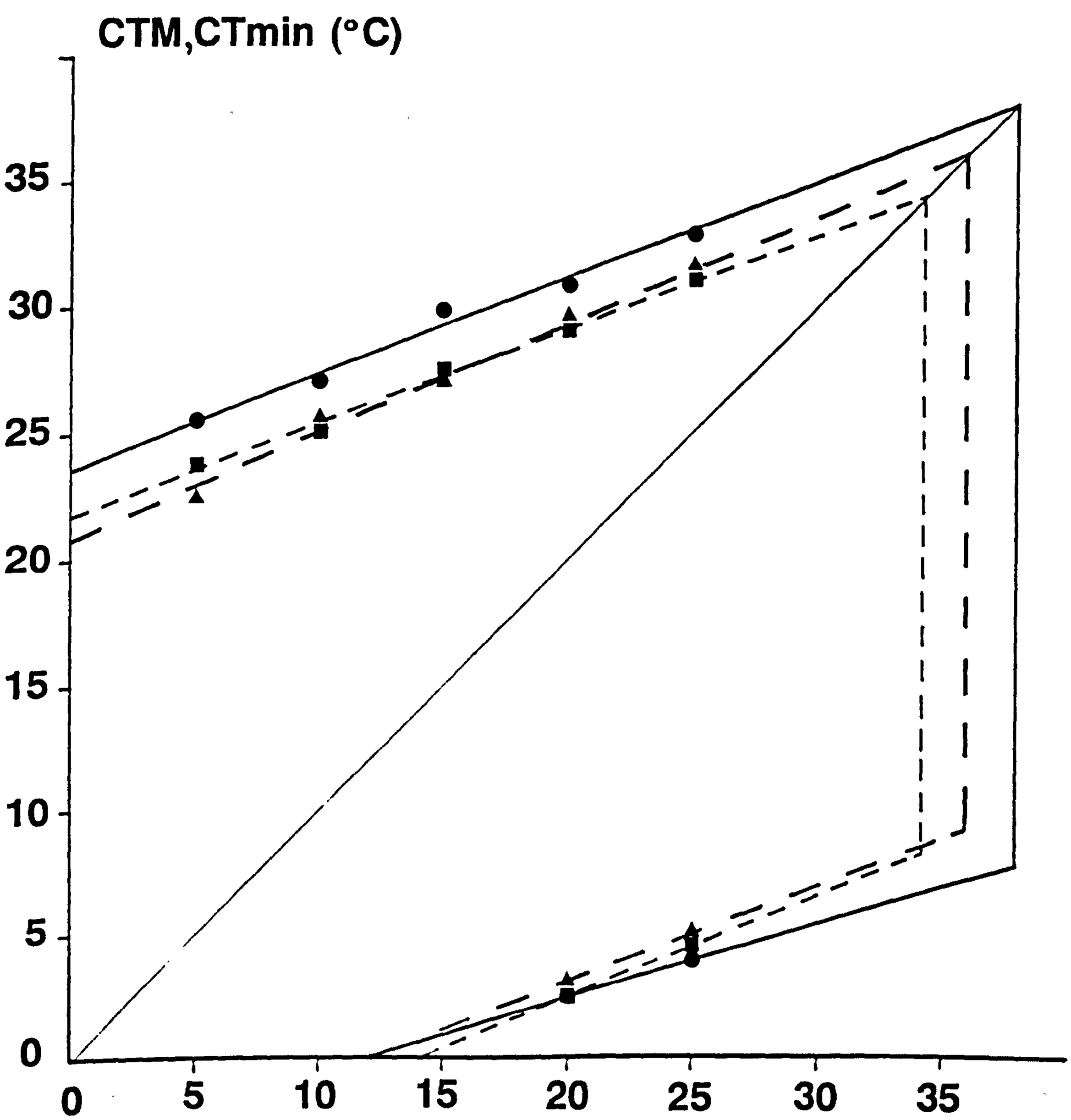
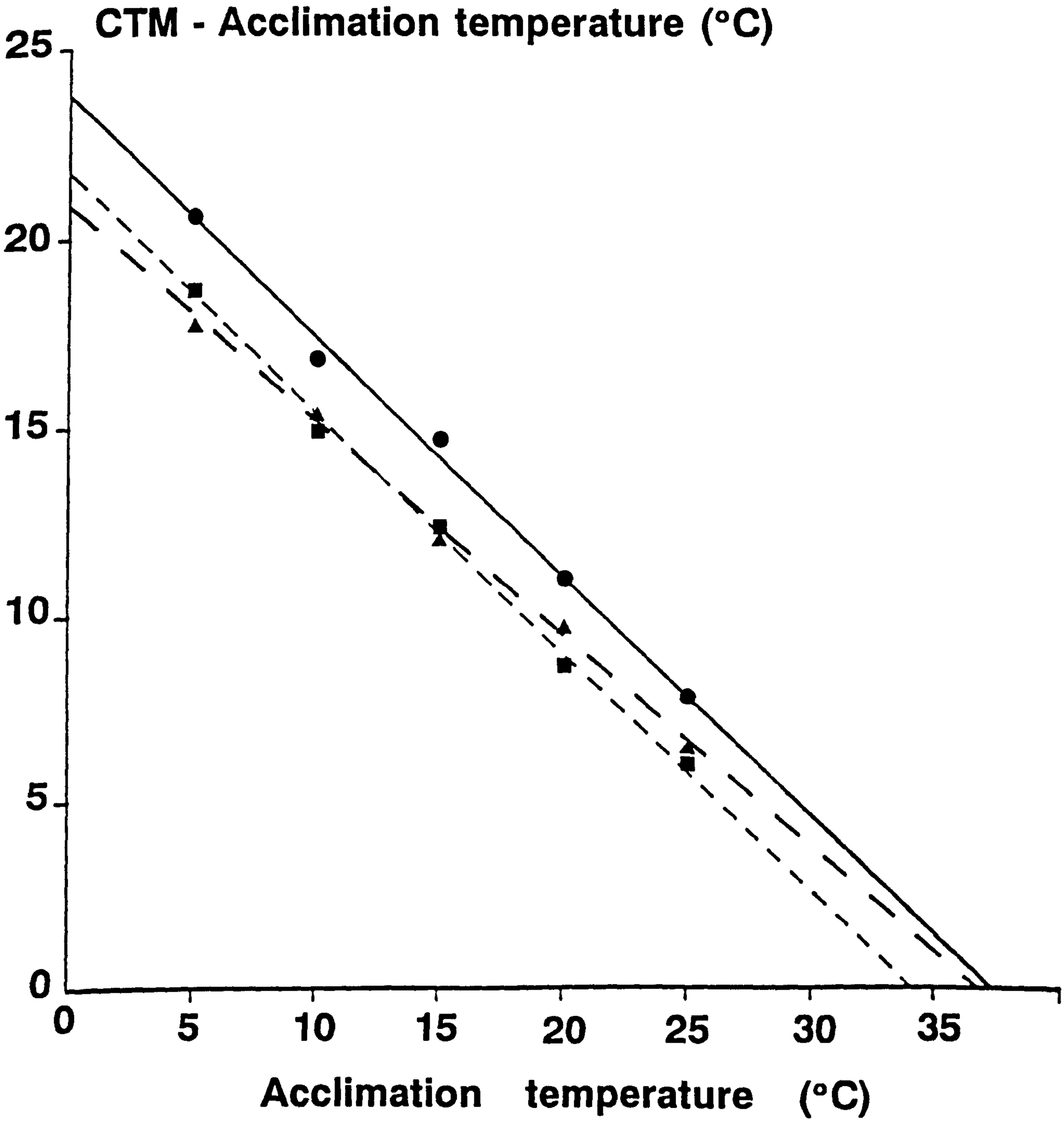


Figure 10.6: Temperature tolerance triangles calculated from CTmax data (—●— *P. leniusculus*, --▲-- *A. pallipes*, —-■-— *A. leptodactylus*).



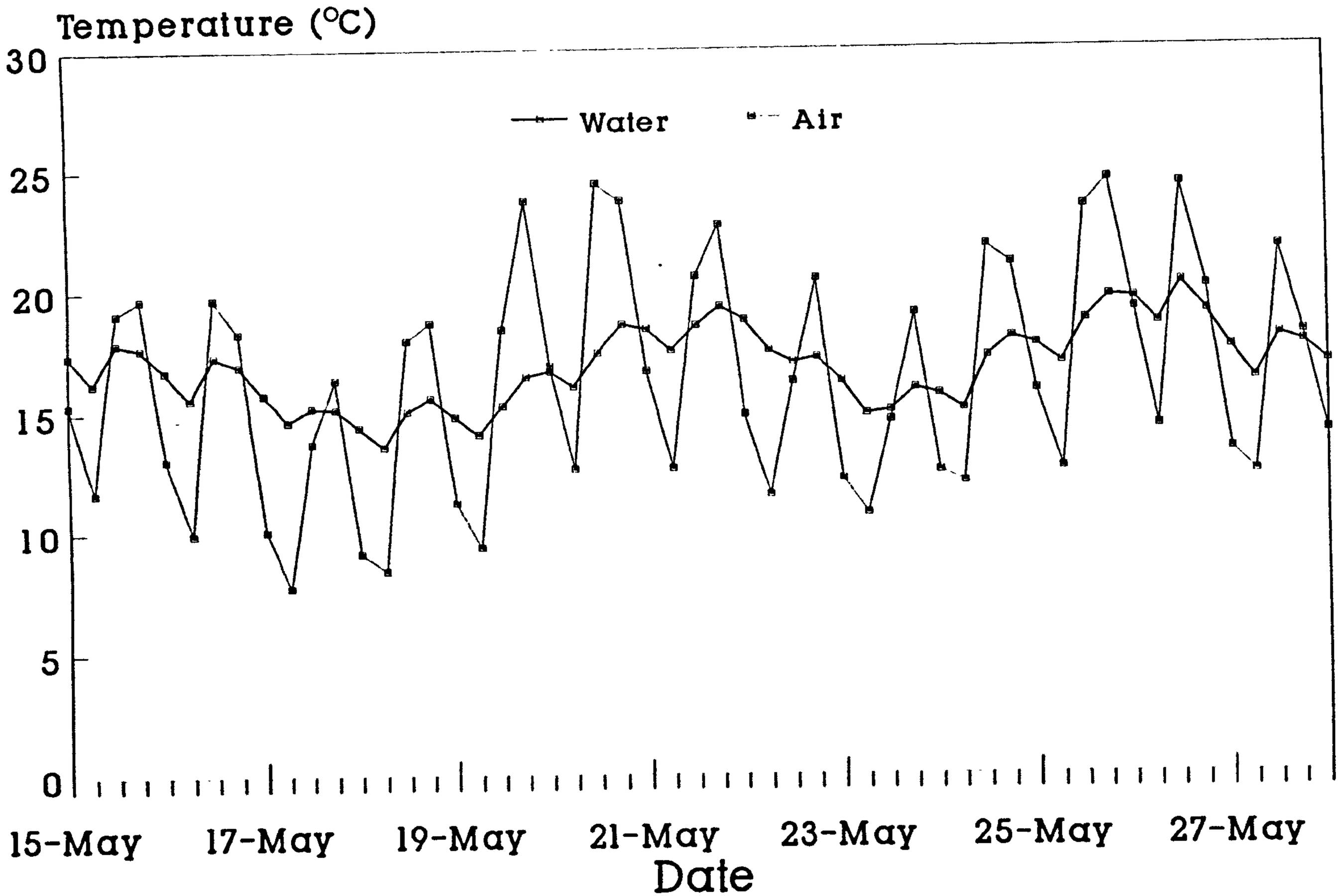
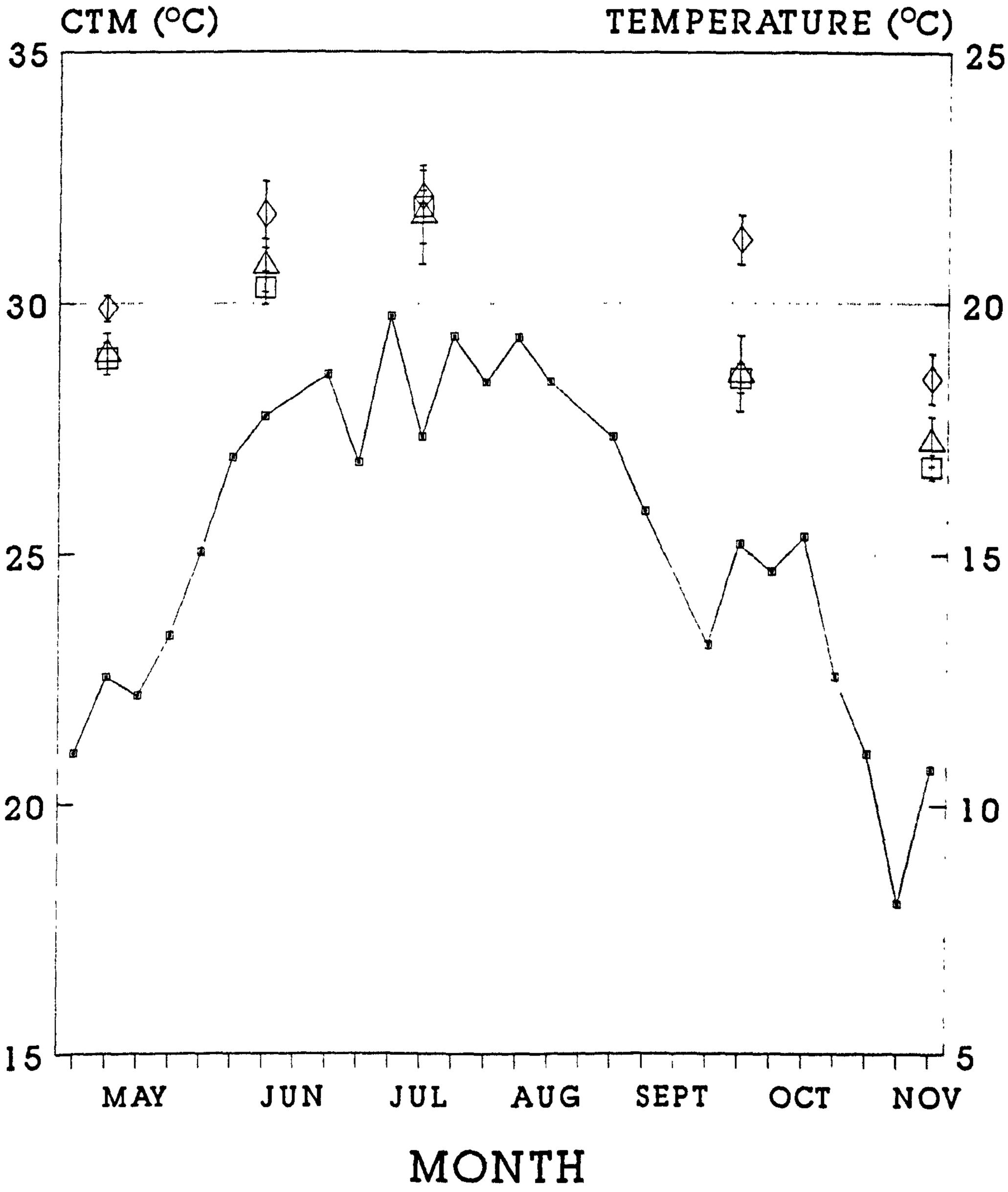


Figure 10.7: Air and water temperatures in outside tanks, measured at 6 hour intervals using a Grant 1200 series Squirrel Meter/Logger, over the period 15/5/92 - 27/5/92.

Figure 10.8: Relationship between CTmax and weekly average temperature in outside tanks, calculated from Squirrel Meter/Logger readings, over the period 28/4/92 - 3/11/92 (◇ *P. leniusculus*, △ *A. leptodactylus*, □ *A. pallipes*). CTmax values are means with standard errors (n=4).



CHAPTER 11

GROWTH STUDIES

11.1 INTRODUCTION

Temperature acts as a controlling factor governing metabolic requirements for food and the rate of processes involved in food processing. If an increase in temperature results in increased metabolism with no increase in feeding rate, then less energy may be diverted into growth. However, work on fish has shown that, given sufficient food, young stages of almost all species show a typical increase in growth rate up to an optimum, after which the relationship is inverse (Brett, 1979).

As might be expected, the optimum temperature for growth of different species generally increases from ecologically cold-adapted to warm-adapted species and appear to be important comparative characteristics of different species (Morrissy *et al.*, 1990). In growth-temperature studies, emphasis is therefore usually given to determination of optimum temperatures (Morrissy, 1990). Also, in aquaculture, nutritional studies are best carried out at the optimum (Morrissy, 1984), and in intensive systems production will be maximised at the optimum temperature, but will be impaired if temperatures deviate from the optimum for any length of time (Lee and Wickins, 1992). However, knowledge of the high and low limits of temperature to growth is equally important in ecological, fishery and aquacultural studies. Such information may help in determining the limits of a natural distribution and

help in making judgements about species translocation and site selection in aquaculture (Morrissy, 1984).

Estimation of the relationship between growth and temperature for a species is usually carried out by rearing animals at many closely spaced and constant temperatures. However, summaries of growth data in the literature are often difficult to compare because of the variety of growth-rate expressions available (Morrissy, 1990). In crayfish, as in all other Arthropoda, an increase in size can only be achieved by periodically shedding the exoskeleton in the process of moulting, or ecdysis. A number of different criteria have therefore been used to describe the size and growth rate of crayfish (Lowery, 1988). The most common are as follows;

1.) Carapace length - the length measured from the tip of the rostrum to the posterior margin at the dorsal midline.

2.) Instantaneous growth rate (Pratten, 1980).

$$G = [\ln(W_t/W_0)]/t$$

where G is the instantaneous growth rate (mg mg^{-1} day^{-1}), W_0 is the initial weight (mg) and W_t is the weight at time t (days).

3.) Moult increment - the increase in carapace length at the moult, measured for individual animals.

4.) Growth Factor - the percentage increase in carapace length at the moult.

The study of growth in crayfish is complicated by the variability of moult frequency and moult increment. In general, when expressed relative to body size (age) the growth increment falls with age. Secondly moult frequency may decline with age. In *Austropotamobius pallipes*, regeneration of lost or damaged

limbs is linked to a smaller moult increment (Bowler and Brown, 1977) and individuals infected with the parasite *Thelohania contejeani* have smaller moult increments than normal animals. Finally, omission of one or more moults by reproductive females leads to their falling behind the growth rates of non-reproductive females of a similar size. Direct estimation of moult frequencies and size of moult increments is usually carried out using mark-recapture methods, and a method was developed for marking individual crayfish with a pattern of spots which corresponded to a number (Abrahamsson, 1965).

Several studies have detailed growth in natural populations of crayfish (Brown and Bowler, 1977; Goldman and Rundquist, 1977; Pratten, 1980). More recently, emphasis has been placed on pond culture (Tcherkashina, 1977; Pursiainen, 1983; Gydemo and Westin, 1988) and development of intensive production methods (Mason, 1979; Morrissy, 1988).

11.2 MATERIALS AND METHODS

11.2.1 Laboratory experiments

Juvenile crayfish were exposed to 15, 20, 25 and 28 °C in constant temperature rooms in an artificial light regime of 12 hours dark : 12 hours light. Two replicates of ten 0+ juveniles of each species were used per exposure temperature.

Confinement of crayfish juveniles in individual compartments has been shown to suppress growth (Goyert & Avault, 1979;

Morrissy, 1990), so animals were housed in 30 litre plastic trays with a floor area of approximately 0.5 m^2. The trays were filled with water from the potable supply, which was continuously aerated and filtered, and air bricks placed in the trays to provide an excess number of shelters. Growth is also known to be inhibited in animals regenerating limbs (Bowler & Brown, 1977), so only fully intact animals were used. A range of sizes of, both sexes allowed identification of individuals at the end of the experiment without the need for marking the animals, which may have affected growth rate. Water characteristics in each tank (temperature, oxygen, pH, conductivity and hardness) were measured every three days.

Animals were weighed (wet weight) at the beginning of the experiment and again after 30 days. The increases in wet weight of individual crayfish were expressed as percentages of initial weight. Data was analysed using ANOVA and curvilinear regression procedures.

11.2.2 Field experiments

To compare the effect of seasonal temperature on the growth of juveniles of the three species, animals were sampled periodically from outdoor tanks at Nottingham University from 13/5/92 to 21/10/92.

Berried females of each species were monitored through egg hatching to the release of stage II juveniles. The date of release of each species was noted and a sample of 15-20 juveniles

taken for measurement. Total length was measured from the tip of the rostrum to the tip of the telson. Animals were then blotted dry and weighed (wet weight). Remaining juveniles were placed in well established concrete tanks, which contained silt, detritus, algae and macroinvertebrates. A sample of 10 animals was subsequently taken from the tanks at approximately four week intervals. Temperature in the tanks was continuously measured over the period using temperature probes and a Grant Squirrel meter/logger (Grant Instruments Limited, Cambridge, England).

11.3 RESULTS

11.3.1 Laboratory studies

Mean tank temperatures for each treatment are shown in Table 11.1 for all three species. There was no significant difference between the tanks at any of the exposure temperatures during the experiment. Over the 30 day experimental period the mean hardness and the pH of the test water were 251 $\pm$ 38 mg l^{-1} $CaCO_3$ and 7.48 $\pm$ 0.15 respectively.

Growth in all three species was temperature dependent (Fig. 11.1). There were significant differences in growth between test temperatures for each species (*A. pallipes* $p < 0.05$, *P. leniusculus* $p < 0.001$, *A. leptodactylus* $p < 0.001$), with maximal growth between 20 - 25 °C for all three species. Comparisons at each temperature with either Bonferroni's inequality (a posteriori t-test) or Kruskal Wallis tests showed that percentage weight increase in *P. leniusculus* was significantly greater (p

< 0.05) than *A. pallipes* or *A. leptodactylus* at all four temperatures. However, there were no significant differences between *A. pallipes* and *A. leptodactylus* at any of the temperatures used.

Curvilinear regressions (Fig 11.2) predicted maximum weight increases for *A. pallipes* at 21.4 °C, *A. leptodactylus* at 21.8 °C and *P. leniusculus* at 22.8 C, although the data would appear slightly skewed towards higher temperatures than the predicted values for the two latter species.

Survival during the experiment was between 80-100 % in all but one treatment. Occasionally an animal failed to moult properly and subsequently died, or was eaten. However, there was a high mortality (95 %) for *A. pallipes* at 28 °C, possibly indicating lethal effects at this temperature.

11.3.2 Field studies

Juveniles were sampled from outside tanks on five occasions over the period 13/5/92 - 21/9/92. It was only possible to sample juveniles of *A. pallipes* and *P. leniusculus* as the entire broodstock of *A. leptodactylus* was lost, possibly due to an outbreak of crayfish plague. During the sampling period the average daily water temperature measured by the datalogger ranged from 11.7 to 21.1 °C.

Measured total length and wet weight of juveniles of the two species sampled are shown in Figs 11.3 and 11.4 respectively, together with sampling date. Independent stage II *P. leniusculus* were found to be significantly smaller ($p<0.05$) than *A. pallipes*

juveniles of the same stage. However, *P. leniusculus* juveniles were also found to be released from berried females approximately three weeks earlier than *A. pallipes* juveniles, by which time they had significantly exceeded ($p<0.001$) *A. pallipes* juveniles in size. In Fig 11.3 it can be seen that on the sampling date 3/6/92 the mean total length of *P. leniusculus* juveniles was approximately 3 mm greater than *A. pallipes*, and this difference had increased to 14 mm by the end of the experiment on 21/9/92.

The relationship between total length and time in days was found to be linear for both species, with the slopes of the regression lines in Fig 11.3 indicating the increase in total length with time. These values are as follows: *A. pallipes*, 0.098 mm total length day^{-1}; *P. leniusculus*, 0.204 mm total length day^{-1}.

Similarly, for wet weight in Fig 11.4, on sampling date 3/6/92 the mean weight of *P. leniusculus* juveniles was approximately 30 mg greater than *A. pallipes*, and by the end of the sampling period this difference had increased to over 800 mg.

11.4 DISCUSSION

In laboratory growth experiments, growth rates of all three species increased from minimum levels at 15 °C to maximum levels between 20 - 25 °C. Westman (1973) reported that growth of *P. leniusculus* increased through the range 7 - 21 °C. Increased growth rates at the higher temperatures probably result from increased calorific consumption (Jones and Momot, 1983). Although crayfish were fed to excess at all temperatures, those at higher

temperatures fed more actively and consumed greater quantities of food. Growth rates were seen to decline at 28 °C. Such a decrease might result from increased metabolic demands approaching the calorific intake, leaving little energy for growth, despite animals being fed to excess. Jones and Momot (1983) observed that *Orconectes virilis* normally expends three times as much energy for maintenance as for growth at 19 °C. Therefore, knowledge of the growth optima is a key factor in determining the aquaculture potential of a species, and for maintainance of maximum production in intensive systems.

The high mortality observed for *A. pallipes* at 28 °C would indicate lethal temperature effects at this temperature for this species. Bowler (1963) estimated that the ultimate upper lethal temperature (UULT) (the highest temperature that can be tolerated for a long period of time, obtained by gradually increasing the acclimation temperature) was approximately 30 °C for *A. pallipes*. This is compared to an estimated UULT of 32 - 33 °C for *P. leniusculus* (Becker *et al.*, 1975). The apparent cause of most deaths in the experiment in all three species was a failure to moult properly. Westman (1973) found that in *P. leniusculus* juveniles post-moulting mortality was lowest at 20 °C, but increased at higher and lower temperatures. Pratten (1980) observed that *A. pallipes* juveniles held at 10 °C failed to undergo a single successful moult. Similarly, Jones (1988) noted that growth of *Cherax quadricarinatus* was negligible at 20 °C (with high survival), but that both growth and survival were poor at 34 °C. This may indicate that the optimum temperature and upper and lower limits for growth actually reflect the optimum

temperature, and limits, for moulting.

Growth optima and upper and lower limits for growth for selected fish and crayfish species are shown in Table 11.2. For the fish species tested, salmonids have the lowest optimum temperatures and the channel catfish the highest, with crayfish species occupying the middle of this range. In this study, the optimum temperature for growth calculated for *P. leniusculus* was higher than those for *A. pallipes* and *A. leptodactylus*. However, despite this, growth of *P. leniusculus* was still greater than the other species at all temperatures tested. Instantaneous growth rates (Pratten, 1980) calculated for the 30 day period (Table 11.3) indicate that the growth rate of *P. leniusculus* is approximately 1.5 - 3 times that of *A. pallipes* and *A. leptodactylus* over the temperature range tested. Curvilinear regressions also predicted that the range of temperatures at which growth will occur is greater in *P. leniusculus* than in *A. pallipes* or *A. leptodactylus* (see Table 11.2), so not only does *P. leniusculus* grow faster at the same temperature as the other species, it will also continue to grow when temperatures are unsuitable for growth in *A. pallipes* and *A. leptodactylus*. In the wild, continued growth at low temperatures may result in a longer growing period in *P. leniusculus* compared to the other species.

In field experiments the growth rate of *P. leniusculus* juveniles was approximately double that of *A. pallipes* over the temperature range 11.7 to 21.1 °C and compares with growth rates obtained for *A. pallipes* and *P. leniusculus* by Hogger (1984). The fact that *P. leniusculus* juveniles were released earlier and grew faster meant that they were able to maintain a distinct size

advantage throughout the experimental period. By the end of the experiment the difference between the two species was very marked, with the largest individual *P. leniusculus* 15.5 mm longer and 1356 mg heavier than the largest *A. pallipes*.

Large size is a key element in the attributes (e.g aggressive dominance, shelter acquisition, reproductive interference) leading to competitive success. The aggressive tendencies of crayfish are well documented (Bovbjerg, 1953; Capelli, 1982), and Capelli and Munjal (1982) recorded interspecific encounters leading to attacks in laboratory experiments. The influence of temperature on growth may therefore be an important factor in determining the outcome of competitive interactions between mixed populations of different crayfish species.

In a field and laboratory study of two species of *Orconectes*, *O. punctimanus* and *O. luteus*, Rabeni (1985) found that larger crayfish were dominant and were able to occupy their preferred environment. Fecundity and eventual recruitment into a population will be enhanced if the size group with the greatest reproductive potential, i.e the largest individuals, are in a preferred habitat, because good habitat implies good food supply, low predation pressure and minimal energy expenditure. In mixed populations of crayfish it is therefore important that the most aggressive species have the most restricted habitat preference. For example, in the above study, Rabeni (1985) found that *O. punctimanus* occupies only a small percentage of the total stream habitat; in slow current and shallow water, and was able to occupy this preferred habitat in the presence of *O. luteus* due

to its larger size. *O. luteus* is more tolerant of a wider range of environmental conditions and is slightly different morphologically, with dorso-ventral flattening of the body allowing it to live in faster current. Thus it is able to occupy much of the remainder of the available habitat, so that the two species are able to coexist in what appears to be a common habitat.

However, where different crayfish species exhibit similar habitat preference, competitive exclusion of one species may occur by the larger, more aggressive species, so that an introduced species may rapidly replace one that occurred naturally (Capelli, 1982). In England, large populations of *P. leniusculus* can be found in waters where *A. pallipes* have been eliminated, presumably by crayfish plague, e.g. the River Kennet (Holdich and Reeve, 1991). Also, a few mixed populations of plague-free *P. leniusculus* and *A. pallipes* exist where the two species occupy the same habitat. The two species have a very similar morphology (Reeve, 1990), so it is not unreasonable to assume that they also have very similar habitat preferences, and may be ecological homologues. In such a mixed population of *A. pallipes* and *P. leniusculus*, studied by Holdich and Domaniewski (unpub.), the trappable population of *P. leniusculus* was always larger on average (length and weight) than that of *A. pallipes*, and over a period of 5 years the population of *A. pallipes* declined and was eventually eliminated.

A parallel may be drawn between *P. leniusculus* and the crayfish *O. rusticus* which is currently undergoing an extensive range expansion in the mid-western United States, probably due

to accidental and intentional introductions, with the displacement of several endemic species. In laboratory experiments, Mundhal and Benton (1990) found that growth of *O. rusticus* was most rapid at water temperatures between 26 and 28 °C, and that populations of *O. rusticus* were most successful (as judged by highest densities, largest average size and absence of any competitors) in larger streams and rivers where temperatures were greater than 20 °C during the summer. However, other species of *Orconectes* are able to maintain population strongholds in cooler, upper reaches of many streams. Butler (1988) reported that the average size of *O. rusticus* females in an Ohio stream decreased in an upstream direction, whereas the size of competing *O. sanbornii* females increased, suggesting a possible differential effect of water temperature on the two species. Cooler water temperatures in headwater areas appear to slow the growth rate of *O. rusticus* and eliminate any size advantage it has over its competitors. Results from laboratory experiments in this study would indicate that *P. leniusculus* is able to continue to grow at lower temperatures than *A. pallipes*, so would not be affected in the same way as *O. rusticus*. Indeed, the results would imply that, given the opportunity, *P. leniusculus* would be able to occupy waters unsuitable for *A. pallipes*, i.e. upland waters and the more geologically suitable areas of Scotland, where the range of the native species is restricted by climatic conditions.

Table 11.1: Tank temperatures during growth experiments. Values are means with standard errors (n=15).

Species	Temperature (°C)			
	15	20	25	28
Austropotamobius pallipes	**14.6** 0.12	**20.2** 0.16	**24.6** 0.15	**28.7** 0.36
Astacus leptodactylus	**14.1** 0.21	**20.7** 0.23	**24.7** 0.16	**28.8** 0.31
Pacifastacus leniusculus	**14.3** 0.16	**19.9** 0.21	**24.7** 0.12	**28.8** 0.26

SPECIES	OPTIMUM (°C)	LIMITS (°C)		REFERENCE
		LOWER	UPPER	
Salmo trutta	12.8	-	-	Elliot, 1975
Oncorhynchus mykiss	17.2	-	-	Hokanson et al., 1977
Cherax tenuimanus	24.1	13.0	30.3	Morrissy, 1990
Orconectes rusticus	26.3	15.1[1]	37.4[1]	Mundhal and Benton, 1990
C. quadricarinatus	27.0[2]	20.0	34.0	Jones, 1988
C. destructor	28.0	15.0	36.0	Mills, 1986
Perca flavescens	28.0	-	-	McCormick, 1976
Ictalurus punctatus	30.0	-	-	Andrews and Stickney, 1972
A. pallipes	21.4	13.5	29.2	This study
A. leptodactylus	21.8	14.3	29.3	This study
P. leniusculus	22.8	12.7	32.5	This study

[1] Calculated from equation for best-fit curvilinear regression for % carapace length increase.
[2] Estimated from data plotted in Morrissy, 1990.

Table 11.2: Growth optima and limits for selected fish and crayfish species.

SPECIES	TEMPERATURE (°C)			
	15	20	25	28
Pacifastacus leniusculus	0.009 (0.001)	0.019 (0.001)	0.022 (0.002)	0.013 (0.002)
Austropotamobius pallipes	0.004 (0.0006)	0.011 (0.001)	0.010 (0.001)	0.004
Astacus leptodactylus	0.004 (0.0005)	0.014 (0.004)	0.014 (0.003)	0.0035 (0.002)

$G^* = \frac{\ln(W_t/W_0)}{t}$ Where W_0 = initial weight
W_t = weight at time t
t = days

Table 11.3: Instantaneous growth rates, G^* (mg mg^{-1} day^{-1}), (Pratten, 1980) calculated for 0+ crayfish juveniles held at 15, 20, 25 and 28 °C for 30 days. Values are means with standard errors (n=10 except for *A. pallipes* at 28 °C where n=2).

Figure 11.1: Percentage weight increase of 0+ juvenile crayfish held at 15, 20, 25, and 28 °C for 30 days.
(■ *A. pallipes*, ▲ *A. leptodactylus*, ● *P. leniusculus*). Values are means with standard errors (n=10 except for *A. pallipes* at 28 ° C where n=2).

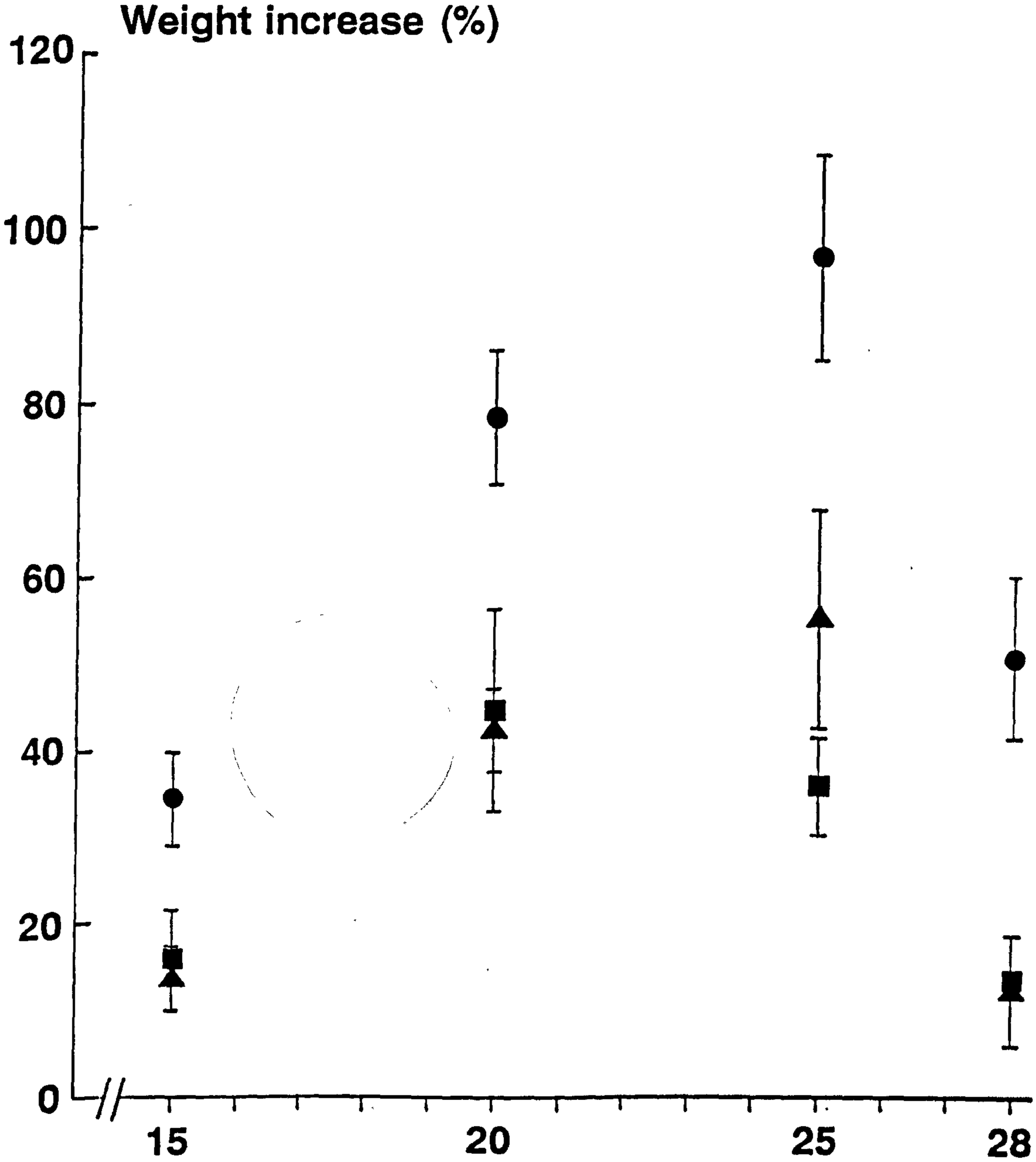


Figure 11.2: Best fit curvilinear regression lines calculated from juvenile growth data (-----*A. pallipes*, — — -*A. leptodactylus*,————*P. leniusculus*). Equations are as follows; *A. pallipes* % weight increase = -295.64 + 31.972 temperature - 0.748 temperature^2, $p<0.05$. *A. leptodactylus* % weight increase = -430.92 + 44.86 temperature - 1.029 temperature^2, $p<0.001$. *P. leniusculus* % weight increase = -371.05 + 40.619 temperature - 0.899 temperature^2, $p<0.05$.

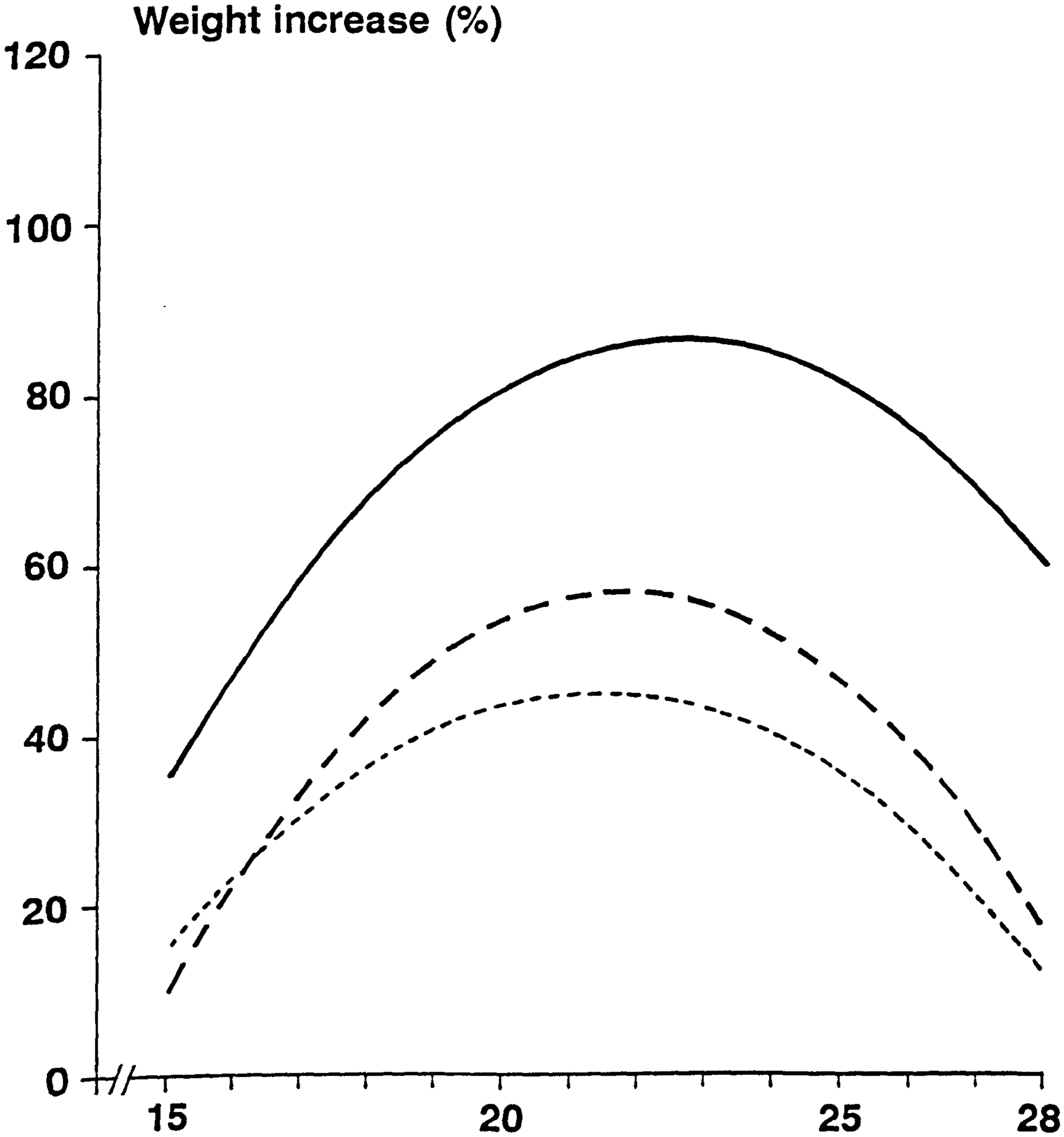


Figure 11.3: Total length of 0+ juvenile crayfish sampled from outside tanks over the period 13/5/92 - 21/9/92 (*A. pallipes*, *P. leniusculus*). Values are means with standard errors.

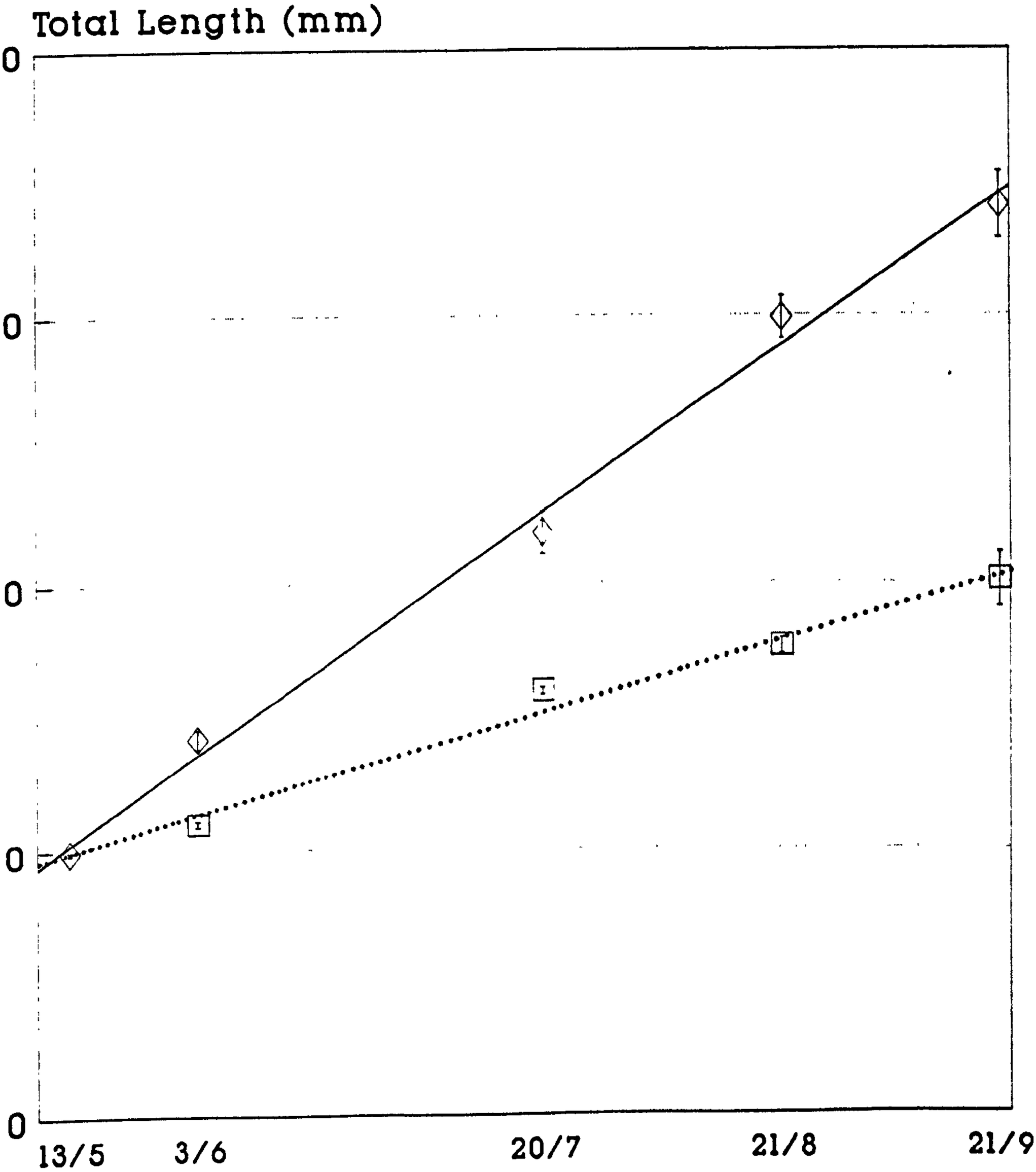
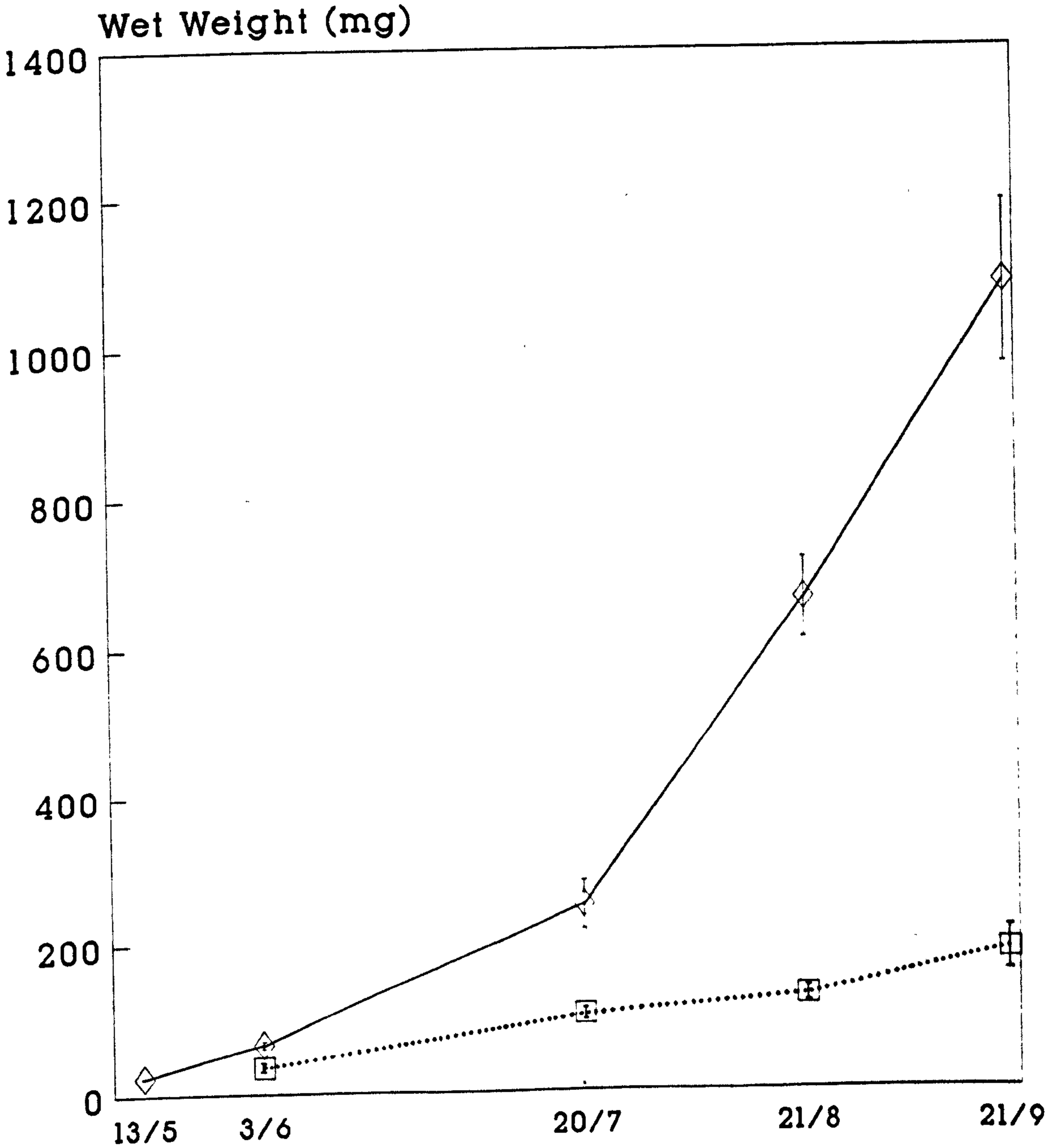


Figure 11.4: Wet weight of 0+ juvenile crayfish sampled from outside tanks over the period 13/5/92 - 21/9/92 (*A. pallipes*, *P. leniusculus*) . Values are means with standard errors (n=10) .



CHAPTER 12

RESPIRATORY STUDIES

12.1 INTRODUCTION

Temperature is a major environmental determinant of metabolic rate in aquatic poikilothermic animals. Most aquatic organisms have no ability to regulate body temperatures so that an increase of 10 °C in environmental temperature increases metabolic rate by approximately 2-3 times unless the organism has an adaptive strategy which counteracts the temperature effects (Armitage and Wall, 1982). A common strategy amongst poikilotherms is metabolic adaptation or compensation (Bullock, 1955) and several types of adaptation response are now recognised (Vernberg and Vernberg, 1970). Such responses are characterised by determination of the rate function of some physiological parameter at one acclimation temperature, followed by redetermination at another. This forms the basis of the Q_{10} relationship for a 10 °C interval (Cossins and Bowler, 1987):

$$Q_{10} = V_{t+10}/V_t$$

where V_{t+10} and V_t are the rates at temperature T+10 °C and T °C. Any two temperatures may be used to calculated the Q_{10} as long as they are far enough apart to allow the difference in rates to be reliably measured. In this case Q_{10} can be determined from

$$\log Q_{10} = 10(\log V_1 - \log V_2)/(T_1 - T_2).$$

For calculation of Q_{10} over an entire temperature range it is more convenient to plot the data according to

$$\log V = \log a + T\log b$$

where the slope b represents the value of Q_{10}. In poikilotherms the majority of values of Q_{10} for biological processes fall between 2-3 (Cossins and Bowler, 1987) and indictate a non-adaptive response to temperature. However, there are cases where the Q_{10} of metabolic response is less than 2 or greater than 3, indicating independence from environmental temperature (Vernberg and Vernberg, 1970).

In fish, the metabolic rate has almost universally been measured by determining oxygen consumption at different acclimation temperatures (Fry, 1971). A review of apparatus and methodology can be found in Fry (1971). Three different levels of metabolism have been identified depending on state of activity. These are termed standard, routine and active levels of metabolism and are defined in Fry (1971). Standard metabolism is an approximation of the minimum rate for the intact organism, determined at zero activity. The routine rate is the mean rate observed during random activity, and the active rate is the maximum sustained rate for a fish swimming steadily. Routine metabolism is the most commonly reported for fish.

Similar methodology and terminology have been applied to Crustacea, including crayfish (Wiens and Armitage, 1961; Wallace, 1972; Taylor *et al.*, 1977; Eggleston and Lustick, 1981; Rutledge and Pritchard, 1981;). Metabolic rate, as measured by oxygen consumption, has been found to be positively correlated with temperature in *P. leniusculus* (Moshiri *et al.*, 1970; Rutledge and Pritchard, 1981). However, few studies, none with crayfish, have been carried out to determine whether metabolic rates vary

between different species over the same temperature range. Momot (1984) hypothesised that a higher metabolic rate would allow a species to incorporate more energy per unit time, grow more rapidly and reach a greater overall size. The importance of body size in competition between crayfish species has been discussed in Chapter 11. Therefore, in this study, comparison of the metabolic rate, as measured by oxygen consumption, in *A. pallipes*, *P. leniusculus* and *A. leptodactylus* was carried out.

12.2 MATERIALS AND METHODS

Oxygen consumption of individual adult intermoult crayfish (28.5 - 46.8 g wet weight), acclimated to temperatures of 10, 15, 20 and 25 °C for a minimum of two weeks, was measured using the apparatus in Fig. 12.1. Crayfish were allowed to acclimate to the apparatus overnight to ensure they were in an unexcited state when oxygen consumption was measured (Taylor, pers. comm.). The apparatus was housed in a constant temperature room with dimmed artificial lighting (12D : 12L) and the respiration chamber partly covered with black plastic, so that animals inside the apparatus could not see the operator. During the acclimation period water was pumped through the chamber via an aerating column to maintain oxygen concentration at saturation levels.

Measurements of oxygen consumption were made over 60 minutes at 10 °C and over 30 minutes for the other temperatures. Percentage oxygen saturation in the experimental chamber was measured using a Yellow Springs Instruments 5331 oxygen probe, connected to a Yellow Springs Instruments Biological Oxygen

Meter, calibrated at 0% and 100% saturation. Water flow through the chamber was shut off at the tap and the chamber outlet clamped off. An initial oxygen percentage saturation reading was then taken, followed by a second reading after the desired time period. Water flow was then restored to bring the oxygen concentration back to 100% saturation. This allowed a number of readings to be made on each crayfish. Finally, animals were removed from the apparatus, blotted dry and weighed.

Oxygen consumption was calculated from initial and final oxygen percentage saturation readings, water temperature, air pressure and animal wet weight using a BASIC program, run on a BBC model B microcomputer, and expressed as µl/g/hr.

12.3 RESULTS

Oxygen consumption increased linearly ($p<0.001$) with temperature over the range 10 - 25 °C for all three species (Fig. 12.2). The temperature coefficient, or Q_{10} was calculated from the slopes of the regression lines in Fig. 12.2 for all three species. This gave the following values; *P. leniusculus* Q_{10} = 2.67, *A. pallipes* Q_{10} = 2.21, *A. leptodactylus* Q_{10} = 2.23. However, comparison of regression lines using "COMPREG" (Wiggans *et al.*, 1983) and comparison at each temperature by ANOVA showed that there was no overall significant difference in metabolic rate with temperature between the three species.

12.4 DISCUSSION

A linear relationship was found between oxygen consumption and temperature for all three species, with Q_{10} values, calculated from the slopes of the regression lines of oxygen consumption against temperature, falling between 2.2 and 2.7. The values obtained for rate of oxygen consumption and Q_{10} are consistent with those obtained in previous studies on Crustacea (Tables 12.1 and 12.2) despite the wide range of temperatures and different methodology used.

The Q_{10} values obtained in this study would seem to indicate a non-adaptive metabolic response to temperature in all three species over the temperature range 10-25 °C, although Figure 12.2 may possibly show some degree of adaptation for *P. leniusculus* and *A. leptodactylus* between 15 and 20 °C. Further measurements of metabolic rate at temperatures within this range would be required to clarify this. Temperature adaptation, or metabolic compensation (Bullock, 1955), is a common strategy amongst poikilotherms, especially inter-tidal animals, which are subject to high and widely varying daily temperatures, and is demonstrated by sigmoidal metabolism : temperature (M:T) curves representing zones of temperature insensitivity, such as in the gastropod *Littorina littorea* (Newell and Roy, 1973). In a study of the temperature response of the crayfish *Orconectes nais*, Wiens and Armitage (1973) could find no temperature-insensitive zone over the range 15 to 35 °C, but the Q_{10} value between 16 and 31 °C was only 1.49, which would indicate some independence from environmental temperature. However, it is still uncertain to what

extent freshwater animals, that experience little daily temperature fluctuation, are able to compensate for the efect of temperature on metabolism. The results from this study would indicate a non-adaptive strategy in the species tested.

No significant difference between the rates of oxygen consumption of the three species was found at any of the experimental temperatures. It was assumed that during the experiments animals were showing settled, or routine, levels of activity. However, oxygen consumption was found to vary greatly, despite animals being left undisturbed overnight before the experiments were carried out. A wide range of parameters besides temperature have been shown to affect oxygen consumption, including oxygen concentration (Wheatly and Taylor, 1981), salinity (Taylor *et al.*, 1977), body size (Wiens and Armitage, 1961) and starvation (Eggleston and Lustick, 1981). Variability under experimental conditions is also associated with animal movement (Dickson and Franz, 1980). This was probably the main source of variation in this study as a relatively narrow size range (28.5 - 46.8 g wet weight) of animals was used and all experiments were carried out at saturated oxygen levels.

An alternative to whole animal respiration studies is to investigate repiration in an excised tissue, such as the gill. Vernberg (1956) found a close correlation between whole animal and gill tissue respiration rates in nine marine decapods. Also, Dickson and Franz (1980) found no significant relationships between gill tissue respiration rates and body size, or dissolved oxygen, which would eliminate some of the variability associated with whole animal experiments. Comparison of gill respiration

rates of four closely related surface and cave species of the genus *Procambarus* (Dickson and Franz, 1980) showed that gill tissue oxygen consumption was lower in the cave species *P. pictus* and *P. pallidus* than in the surface species *P. pictus* and *P. clarkii*, possibly due to physiological adaptations associated with habitat conditions and food availibility. Results from the present study would therefore not necessarily disprove that *P. leniusculus* has a greater basic metabolic rate than *A. pallipes* or *A. leptodactylus*, but could initiate further investigation using tissue respiration techniques.

SPECIES	TEMP. (°C)	O_2 CONSUMPT. (µl/g/hr)	REFERENCE
Astacus astacus	15	30-54	Von Buddenbrock (1948)
Astacus leptodactylus	19-21	70	Bishop (1950)*
Austropotamobius torrentium	19-21	100	Bishop (1950)*
Homarus americanus	12 25	44 88	McLeese (1964)*
Jasus lalandii	8 19	18.6 51.2	Zoutendyk (1989)*
Orconectes immunis	16	68	Wiens and Armitage (1961)
Orconectes nais	16	66	Wiens and Armitage (1961)
Procambarus alleni	25	66	Davison (1956)
Pacifastacus leniusculus	5 30	28.7 77.2	Rutledge and Pritchard (1981)
Austropotamobius pallipes	10 25	14.1 47.7	This study
Astacus leptodactylus	10 25	15.2 50.0	This study
Pacifastacus leniusculus	10 25	14.4 54.3	This study

* Quoted in Precht (1973)

Table 12.1: Oxygen consumption in selected species of Crustacea.

SPECIES	TEMP. RANGE	Q_{10}	REFERENCE
Homarus americanus	12-25	1.7	McLeese (1964)*
Homarus vulgaris	6-16	2.5	Thomas (1953)*
Jasus lalandii	8-10 10-13	2.2 1.7	Zoutendyk (1989)*
Pacifastacus leniusculus	5-25 25-30	2.1 3.4	Rutledge and Pritchard (1981)
Panulirus interruptus	13-20	2.6	Winget (1969)*
Austropotamobius pallipes	10-25	2.2	This study
Astacus leptodactylus	10-25	2.2	This study
Pacifastacus leniusculus	10-25	2.7	This study

* Quoted in Precht (1973)

Table 12.2: Q_{10} values for selected species of Crustacea.

Figure 12.1: Apparatus used to determine oxygen uptake in crayfish, with detail of respiration chamber.

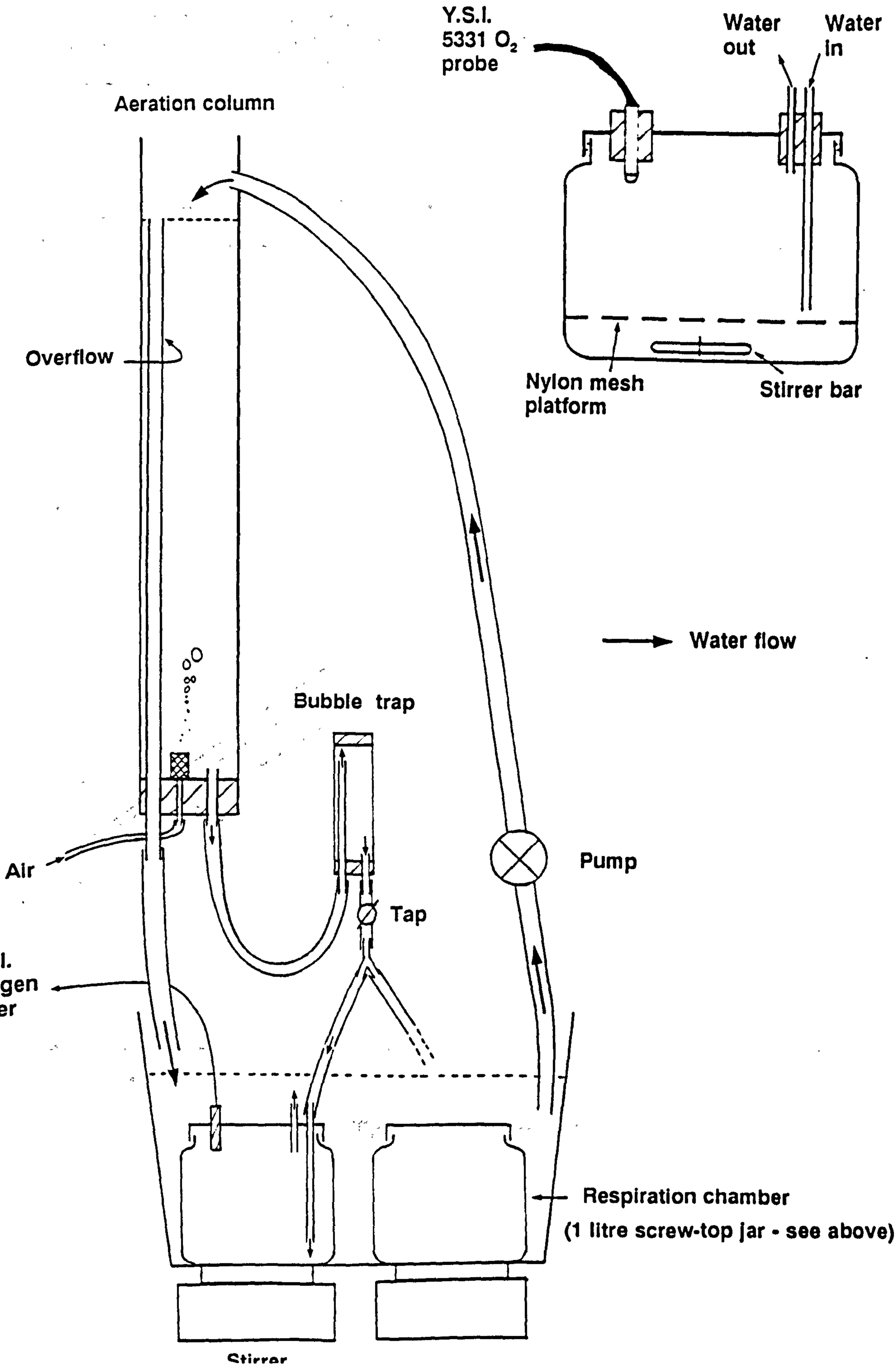
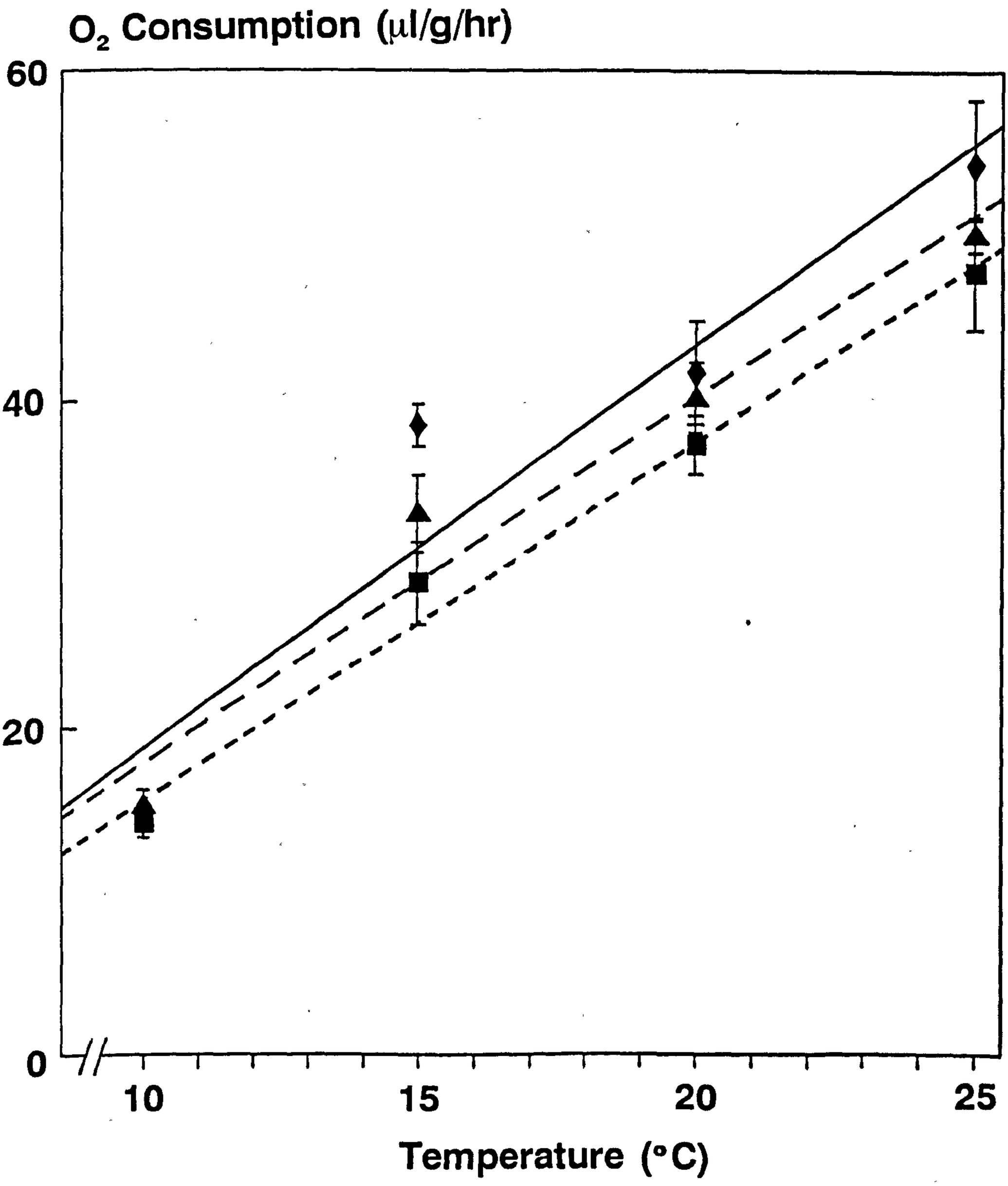


Figure 12.2: Oxygen consumption (μl g^{-1} hr^{-1}) with acclimation temperature (◆ *P. leniusculus*, ▲ *A. leptodactylus*, ■ *A. pallipes*). Values are means with standard errors (n=10). Equations for regression lines are as follows; (———) *P. leniusculus* oxygen consumption = 2.67 temperature - 11.532, $p<0.001$. (-----) *A. pallipes* oxygen consumption = 2.21 temperature - 7.023, $p<0.001$. (– – –) *A. leptodactylus* oxygen consumption = 2.23 temperature - 4.428, $p<0.001$.



CHAPTER 13

SUMMARY AND CONCLUSIONS TO THERMAL STUDIES

All three species of crayfish in this study are widespread in both lentic and lotic habitats in the wild in Britain and may be exposed to seasonal water temperatures ranging from near 0 °C in winter to over 20 °C in summer. In addition to the seasonal changes, aquatic animals are exposed to a daily pattern of temperature fluctuations, the magnitude of which is controlled by season, immediate climatic conditions and, for flowing waters, the state of discharge (Hellawell, 1986). These studies indicate that all three species have the ability to acclimate to a changing temperature regime.

Temperatures may be elevated above the natural range by anthropogenic activities, e.g. thermal effluents from power stations, or natural processes, e.g. direct solar heating of waters reduced in flow and volume by drought. Results from tolerance experiments showed that *P. leniusculus* has a higher CTmax and a greater overall temperature tolerance than *A. pallipes* and *A. leptodactylus* and so is more resistant to increases in environmental temperature. In this study, the CTMax was determined by the progressive loss of co-ordination with increasing temperature, resulting in an inability of the crayfish to right itself. This was shown to be non-fatal and reversible if the animals were returned to their initial acclimation temperature. However, in the natural environment, the period where they are unable to function properly would make them

vunerable to more thermally tolerant predators. In mixed crayfish populations this would include tolerant crayfish species, such as *P. leniusculus*.

In laboratory growth experiments, growth rates of all three species increased from minimum levels at 15 °C to maximum levels between 20-25 °C. However, growth of *P. leniusculus* was higher than the other two species at all the temperatures tested, despite having a higher optimum temperature requirement, and was predicted to occur over a wider temperature range than in either *A. pallipes* or *A. leptodactylus*. In the wild, this may mean a longer growing season in *P. leniusculus* and would also imply that the species would be able to colonize upland regions, where *A. pallipes* is absent due to climatic conditions.

Not only does *P. leniusculus* appear to be able to survive and grow in conditions unsuitable for either *A. pallipes* or *A. leptodactylus*, but it is also able to grow faster where favourable conditions exist for all three species, as seen in "field" growth experiments with *P. leniusculus* and *A. pallipes*. Large size has been shown to be a key element in the attributes (e.g. aggressive dominance, shelter acquisition, reproductive interference) leading to competitive success between different species of crayfish, so that influence of temperature on growth may be an important factor in determining the outcome of competitive interactions between mixed populations of different crayfish species. The higher temperature tolerance and growth rate of *P. leniusculus* may therefore confer a competitive advantage over *A. pallipes* and *A. leptodactylus*, both of which appear to have similar thermal requirements, and facilitate the

spread of *P. leniusculus* to the detriment of the native species. Certainly, cage experiments with *A. pallipes* and *P. leniusculus* that are free from crayfish plague (Holdich and Reeve, unpublished data), and instances where populations of *A. pallipes* and plague-free *P. leniusculus* have come together in the wild (Holdich and Domaniewski, unpublished data) have shown *P. leniusculus* to be a superior competitor, eventually eliminating *A. pallipes*.

CONCLUSIONS TO THE STUDY

This thesis consisted of two parts. The first part investigated the relative pollution of *A. pallipes*, *A. leptodactylus* and *P. leniusculus*, using lethal and sublethal toxicity testing methods with four common pollutants: chloride, copper, ammonia and lindane. The second part investigated the thermal relations of the three species, using tolerance, growth and respiration experiments. The common theme in both parts was to determine whether the introduced species, *A. leptodactylus* and *P. leniusculus* are more or less tolerant of environmental factors than *A. pallipes*.

No species showed a greater overall pollution tolerance, based on the toxicants tested in this study, although interspecific differences in tolerance were noted for chloride, copper and lindane. Median lethal concentrations (LC_{50}) obtained from lethal studies using stage II juveniles indicated that *A. leptodactylus* juveniles were most tolerant of chloride, but were very sensitive to lindane. *P. leniusculus* juveniles were least tolerant of chloride, but were of equal or greater tolerance when tested in larger juvenile stages, and were most tolerant of copper. *A. pallipes* juveniles were very sensitive to copper compared to the other two species, and all three species showed a similar sensitivity to ammonia. In general values obtained in this study agreed with the limited available data for other crayfish species, and indicated crayfish to have an equal or greater tolerance compared to other invertebrate groups of the toxicnats tested.

Although providing a comparison of the relative tolerances of the species, the LC_{50} values obtained in the study may not have much relevance to a field situation, where environmental factors and interaction with other pollutants may substantially modify toxicity. Sublethal studies showed effects at toxicant concentrations below the LC_{50}, so may be more important in determining presence or absence of crayfish from waters, particularly where sublethal concentrations affect early life stages and subsequent recruitment into a population. Studies of the effects of sublethal toxicant concentrations on crayfish are particularly lacking, limited to the study of Harris and Coley (1991), so particularly warrant further investigation. Similarly, the relative short-term resistance of the species requires further investigation. Incidence of episodic pollution events is increasing. Such events involve release of a single pulse of a toxicant, so that aquatic organisms may be subject to a relatively high concentration of toxicant for a short time. This is very important if there are marked differences in the short-term resistance and ability of different species to recover from such an event. Experiments in this study showed *P. leniusculus* to have a greater short-term resistance to ammonia and copper. However, these experiments were very limited. The only other study to investigate episodic effects on crayfish is that of Foster and Turner (1993).

An area not covered in this thesis which also would warrant investigation is tolerance of low oxygen levels. Diurnal depletion of oxygen levels may occur in waters reduced in flow and volume by drought, and in those affected by agricultural

wastes such as cattle slurry. Such wastes have characteristically very high BOD, and may severely deplete oxygen levels during pollution events. Different mechanisms for dealing with low oxygen levels have been proposed for *A. pallipes* and *P. leniusculus*. Wheatly and Taylor (1981) showed that *A. pallipes* are regulators, maintaining a constant rate of oxygen uptake by increasing branchial ventilation and heart rate. However, this could not be maintained below a critical oxygen concentration. Moshiri *et al.* (1970) showed that *P. leniusculus* is a conformer, reducing oxygen demand in low oxygen conditions by reducing ventilation and metabolic rate. However, it is unclear which is the better mechanism for dealing with reduced oxygen concentrations. Bergmiler and Bielawski (1970) observed that the gill surface area of *A. leptodactylus* was almost twice that of *Astacus astacus* and postulated that the difference may be an adaptation of *A. leptodactylus* to live in water with a lower oxygen content. Certainly, populations of *A. leptodactylus* in the British Isles can be found in eutrophic conditions, such as in canals and lakes, and in the Serpentine, London, living in contact with anoxic mud.

Thermal studies showed that *A. pallipes* and *A. leptodactylus* had very a very similar thermal tolerance. However, *P. leniusculus* had a greater overall thermal tolerance than either *A. pallipes* and *A. leptodactylus*, and so is more resistant to changes in environmental temperature. This may reflect the wide range of habitats *P. leniusculus* has been recorded from in the United States, including sub-alpine streams and lakes, shallow streams that warm appreciably in the summer, and dilute brackish

water creeks (Lowery and Holdich, 1988). Growth experiments also indicated that *P. leniusculus* can grow over a wider temperature range and at a faster rate than the other species, so it is not only able to survive and grow at temperatures unsuitable for *A. pallipes* and *A. leptodactylus*, but is also able to outgrow them at optimum temperatures. Field experiments showed that, although *P. leniusculus* stage II juveniles were smaller upon release from the female, they were released earlier and their faster growth rate allowed them to maintain a distinct size advantage over *A. pallipes*, which was very marked by the end of the growing season. Large size is a key element in the competitive ability of other crayfish species, and experimental work and field observation of mixed populations of crayfish have confirmed *P. leniusculus* to be a formidable competitor.

A greater competitive ability through large adult size and other factors, such as a higher fecundity and greater aggression, may therefore contribute to the rapid spread of *P. leniusculus* in England and Wales, rather than a greater tolerance of water quality. The two species *A. pallipes* and *A. leptodactylus* appear to be similar with respect to thermal tolerance and growth, although the apparent habitat preference of *A. leptodactylus* for slow moving or static eutrophic waters and its restricted distribution in south-east England would not appear to threaten populations of *A. pallipes*. In fact, continued westward spread of *A. leptodactylus* in the Grand Union Canal may bring the species into contact with populations of *P. leniusculus* in the River Colne, where crayfish plague has already been implicated in the disappearance of *A. pallipes* from much of the catchment.

Indeed, continued work on the relative environmental tolerances of the three species may be entirely acedemic in the light of the devastating effect of crayfish plague on *A. pallipes* and *A. leptodactylus*.

APPENDICES

APPENDIX I

MACROINVERTEBRATE LISTS

(A adult; L larva; N nymph)

River Leen East Branch

ANNELIDA

OLIGOCHAETA

Lumbricidae

unidentified species

Lumbriculidae

unidentified species

Tubificidae

Tubifex tubifex

MOLLUSCA

GASTROPODA

Hydrobiidae

Potamopyrgus jenkinsi

Ancylidae

Ancylus fluviatilus

BIVALVIA

Sphaeriidae

Sphaerium corneum

Unionidae

Anodonta cygnea

ARTHROPODA

ARACHNIDA

HYDRACARINA

Hygrobatidae

unidentified species

CRUSTACEA

Gammaridae

Gammarus pulex

Astacidae

Austropotamobius pallipes

INSECTA

TRICHOPTERA

Hydropsychidae

Hydropsyche pellucidula (L)

Limnephilidae

unidentified species 1 (L)

unidentified species 2 (L)

EPHEMEROPTERA

Ephemeridae

Ephemera danica (N)

Heptageniidae

Ecdynurus insignis (N)

Baetidae

Baetis rhodani (N)

PLECOPTERA

Perlodidae

Isoperla grammatica (N)

DIPTERA

Chironomidae

Chironomus thummi (L)

Spaniotoma sp. (L)

unidentified species (L)

Tipulidae

Tipula sp. (L)

Dicranota sp. (L)

Simulidae

Simulium sp. (L)

Dixidae

unidentified species (L)

Ceratopogonidae

unidentified species (L)

Tabanidae

Tabanus sp. (L)

COLEOPTERA

Elminthidae

Elmis anea (A,L)

Dytiscidae

unidentified species (L)

Gyrinidae

unidentified species (L)

Other species:

Bullhead	*Cottus gobio*
Brown Trout	*Salmo trutta*

Perch *Perca fluviatilus*

Common Toad *Bufo bufo*

River Leen West Branch

OLIGOCHAETA

Lumbricidae

unidentified species

Lumbriculidae

unidentified species

MOLLUSCA

GASTROPODA

Hydrobiidae

Potamopyrgus jenkinsi

Ancylidae

Ancylus fluviatilus

BIVALVIA

Sphaeridae

Sphaerium corneum

ARTHROPODA

CRUSTACEA

Gammaridae

Gammarus pulex

Gammarus tigrinus

INSECTA

TRICHOPTERA

Hydropsychidae

Hydropsyche sp. (L)

Limnephilidae

unidentified species (L)

Rhyacophilidae

Rhyacophila sp.

Sericostomatidae

Sericostoma sp. (L)

EPHEMEROPTERA

Baetidae

Baetis rhodani (N)

DIPTERA

Chironomidae

Chironomus thummi (L)

Chironomus sp. (L)

Spaniotoma sp. (L)

Tipulidae

Dicranota sp. (L)

Tabanidae

unidentified species (L)

COLEOPTERA

Elminthidae

Limnius volckmari (L)

Helodidae

unidentified species (A)

Other species:

Bullhead	*Cottus gobio*
Perch	*Perca fluviatilus*

River Leen Confluence

OLIGOCHAETA

Lumbriculidae

unidentified species

MOLLUSCA

GASTROPODA

Hydrobiidae

Potamopyrgus jenkinsi

Ancylidae

Ancylus fluviatilus

BIVALVIA

Sphaeridae

Sphaerium corneum

ARTHROPODA

CRUSTACEA

Gammaridae

Gammarus tigrinus

Astacidae

Austropotamobius pallipes

INSECTA

TRICHOPTERA

Limnephilidae

unidentified species (L)

EPHEMEROPTERA

Ephemeridae

Ephemera danica (N)

Heptageniidae

Heptagenia sp. (N)

Baetidae

Baetis rhodani (N)

DIPTERA

Chironomidae

Chironmomus sp. (L)

Tipulidae

Dicranota sp. (L)

Muscidae

unidentified species (L)

COLEOPTERA

Elminthidae

Elmis anea (L)

Other species:

Bullhead	*Cottus gobio*

APPENDIX II.

BIOTIC INDICES USED IN THE STUDY

The Extended Trent Biotic Index

Groups

The term `Group' here denotes the limit of identification which can be reached without resorting to lengthy techniques. Groups are as follows:

(1) Each species of Platyhelminthes (triclads, flatworms)
(2) Oligochaete worms excluding the genus *Nais*.
(3) *Nais* (worms)
(4) Each species of Hirudinea (leeches)
(5) Each species of Mollusca (snails)
(6) Each species of Crustacea (asellids, gammarids, etc.)
(7) Each species of Plecoptera (stonefly nymphs)
(8) Each genus of Ephemeroptera (mayfly nymphs) excluding *Baetis rhodani*
(9) *Baetis rhodani* (mayfly nymphs)
(10) Each family of Trichoptera (caddis fly larvae)
(11) Each species of Neuroptera (alder fly larvae)
(12) Family Chironomidae (midge larvae) except *Chironomus thummi*
(13) *Chironomus thummi* (= *riparius*) (blood worms)
(14) Family Simuliidae (black fly larvae)
(15) Each species of other fly larvae
(16) Each species of Coleoptera (adult and larval beetles)
(17) Each species of Hydracarina (water mites)

EXTENDED TRENT BIOTIC INDEX

Total number of groups present

		0-1	2-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45
Plecoptera nymphs present	More than one species	-	7	8	9	10	11	12	13	14	15
	One species only	-	6	7	8	9	10	11	12	13	14
Ephemeroptera nymphs present	More than one species *	-	6	7	8	9	10	11	12	13	14
	One species only*	-	5	6	7	8	9	10	11	12	13
Trichoptera larvae present	More than one species #	-	5	6	7	8	9	10	11	12	13
	One species only#	4	4	5	6	7	8	9	10	11	12
Gammarus present	All above species absent	3	4	5	6	7	8	9	10	11	12
Asellus present	All above species absent	2	3	4	5	6	7	8	9	10	11
Oligochaeta or Chironomus	All above species absent	1	2	3	4	5	6	7	8	9	10
All above absent	May be organisms requiring no D.O.	0	1	2	-	-	-	-	-	-	-

• *Baetis rhodani* excluded.
* *Baetis rhodani* (Ephemeroptera) is included in this section for the purposes of classification.

BIOLOGICAL MONITORING WORKING PARTY (BMWP) - SCORE

FAMILIES	Score
(a) Siphlonuridae, Heptageniidae, Leptophlebiidae, Ephemerellidae, Potamanthidae, Ephemeridae (MAYFLIES) (b) Taeniopterygidae, Leuctridae, Capniidae, Perlodidae, Perlidae, Chloroperlidae (STONEFLIES) (c) Aphelocheiridae (BUGS) (d) Phryganeidae, Molannidae, Beraeidae, Odontoceridae, Leptoceridae, Goeridae, Leptostomatidae, Brachycentridae, Sericostomatidae (CADDIS-FLIES)	10
(a) Astacidae (CRAYFISH) (b) Lestidae, Agriidae, Gomphidae, Cordulegasteridae, Aeshnidae, Corduliidae, Libellulidae (DRAGONFLIES) (c) Psychomyiidae, Philopotamidae (NET-SPINNING CADDIS-FLIES)	8
(a) Caenidae (MAYFLIES) (b) Nemouridae (STONEFLIES) (c) Rhyacophilidae, Polycentropodidae, Limnephilidae (NET-SPINNING CADDIS-FLIES)	7
(a) Neritidae, Viviparidae, Ancylidae (SNAILS) (b) Hydroptilidae (CADDIS-FLIES) (c) Unionidae (BIVALVE MOLLUSCS) (d) Corophiidae, Gammaridae (AMPHIPODS) (e) Platycnemididae, Coenagriidae (DRAGONFLIES)	6
(a) Mesovelidae, Hydrometridae, Gerridae, Nepidae, Naucoridae, Notonectidae, Pleidae, Corixidae (BUGS) (b) Haliplidae, Hygrobiidae, Dytiscidae, Gyrinidae, Hydrophilidae, Clambidae, Helodidae, Dryopidae, Elminthidae, Chrysomelidae, Curculionidae (BEETLES) (c) Hydropsychidae (CADDIS-FLIES) (d) Tipulidae, Simuliidae (DIPTERAN FLIES) (e) Planariidae, Dendrocoelidae (TRICLADS)	5
(a) Baetidae (MAYFLIES) (b) Sialidae (ALDERFLIES) (c) Piscicolidae (LEECHES)	4
(a) Valvatidae, Hydrobiidae, Lymnaeidae, Physidae, Planorbidae, Sphaeriidae (SNAILS & BIVALVES) (b) Glossiphonidae, Hirudidae, Erpobdellidae (LEECHES) (c) Asellidae (ISOPODS)	3
(a) Chironomidae (DIPTERAN FLIES)	2
(a) Oligochaeta (whole class) (WORMS)	1

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