

The sublethal salinity tolerance of selected freshwater macroinvertebrate species

A thesis submitted in fulfilment of the requirements for the degree of
Master of Applied Science

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Declaration

I certify that except where due acknowledgment has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third Party is acknowledged; and, ethics procedures and guidelines have been follow.

Tricia A. Paradise

3 February 2009

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Summary

Increasing salinity levels due to agricultural land-use practices pose a threat to freshwater ecosystems in many regions of the world, including Australia. Additionally, climate change may also affect salinity levels in these systems. Despite growing research, our ability to predict the likely severity of the impacts of salinity increases on freshwater ecosystems is still limited. Investigation of the salinity tolerances of macroinvertebrate species occurring in aquatic habitats has shown promise as a means to predict the likely effects of increased salinity. To date, the majority of studies have focussed on the short-term lethal tolerance of these species, despite research indicating likely sublethal salinity effects. While research suggests that early life stages may be more sensitive, most tests have investigated the salinity tolerance of only older aquatic life stage of these species.

This study investigated the sublethal effects of salinity on four macroinvertebrate species commonly occurring in Australian freshwater ecosystems. These were; the introduced worm *Lumbriculus variegatus* Müller (Annelida: Oligochaeta), the introduced snail *Physa acuta* Draparnaud (Gastropoda: Physidae), the shrimp *Paratya australiensis* Kemp (Decapoda: Atyidae) and the midge *Chironomus tepperi* Skuse (Diptera: Chironomidae). The effects of salinity on growth, development and reproduction of these species were investigated through a series of laboratory tests. The tolerances of early life stages of these species were also investigated. The effects of salinity on *C. tepperi* were assessed across a full life-cycle of exposure. The observed sublethal salinity tolerances of the investigated species were compared to their acute salinity tolerances. These relationships were investigated to assess if there is a correlation between the sublethal and acute salinity tolerance of freshwater macroinvertebrate species.

With the exception of *L. variegatus* each of the investigated species displayed life stages with lower salinity tolerances than the eldest aquatic life stage of the species. This was not investigated for *L. variegatus* due to life history traits of the species. Sublethal effects of salinity were observed for all of the investigated species at salinities significantly lower than those affecting their short term survival. In many cases these effects had obvious implications for the survival ability of the species. Examples include zero reproduction and/or growth at salinities displaying minimal impacts on short term survival. Results highlight the importance of consideration of the sublethal effects of salinity.

The pattern of effect with increasing salinity varied between different end points. Many of the investigated effects displayed non-threshold responses with increased salinity. This necessitated consideration of the magnitude of effect. Many end points, particularly growth, displayed optimal levels at slightly elevated salinities.

The results of this study, and published research identified common trends in the relative salinity tolerance of different life stages and sublethal end points. Sublethal responses were generally more salt sensitive than the survival of eggs and juveniles. Growth and reproduction of freshwater macroinvertebrates were found to be highly sensitive to increased salinity. The cumulative effects of salinity on survival, growth, development and reproduction across a full life-cycle of exposure was found to greatly reduce recruitment potential. A 50% reduction in potential recruitment of second generation *C. tepperi* was observed at a salinity more than 80% lower than the 96-hr LC₅₀ (the concentration lethal to 50% of test organisms) for the species.

The sublethal salinity tolerance of the investigated species was closely related to their acute salinity tolerance. This was despite very different acute salinity tolerances of the species. This relationship was also evident in the results of published studies. The ratio between the EC₅₀ (the concentration producing a response in 50% of test organisms) for the most sensitive sublethal end point, recruitment of *C. tepperi*, and the 96-hour LC₅₀ for the species was 0.17. Mean ratios between EC₅₀ values for growth and reproduction end points and LC₅₀ values observed for this and other studies were around 0.45. While further research is required, the proportional relationship between the sublethal and acute tolerance of macroinvertebrate species observed in this study allows the sublethal effects of salinity to be taken into account in aquatic ecosystem management where only acute tolerance data are available.

Common abbreviations

ANOVA	Analysis of variance
C	Centigrade
CI	Confidence Interval
Cl	Chloride
df	degrees of freedom
EC	Electrical Conductivity
EC _x	The concentration effective in producing a response in x% of test organisms
EPA	Environment Protection Authority
hr	Hour(s)
L	Litre
LC _x	The concentration lethal to x % of test organisms
LWA	Land and Water Australia
mg	Milligram
mL	Millilitre
mS cm ⁻¹	milliSiemens per centimetre
n	Sample size
Na	Sodium
NOEC	No observed effect concentration
P	Probability of a type 1 error
r	Pearson's correlation coefficient
SE	Standard error of the mean
SSD	Species sensitivity distribution
TSS	Total soluble salts
TDS	Total dissolved solids
µS cm ⁻¹	microSiemens per centimetre

1.0 Introduction

1.1 Salinity threat to aquatic ecosystems

Salinisation as a result of deforestation and irrigation is a major environmental problem in many regions of the world, particularly Africa, the Middle East, North America and Australia (Williams 1987). An estimated 7% of the world's land area was affected in 1989 (Munns 2002). The problem is widespread in Australia with an estimated 5.7 million hectares considered at risk from dryland salinisation in 2000 and 17 million hectares projected to be at risk by 2050 (ANRA 2003). The most severely affected regions are western and central Victoria, south-west Western Australia, eastern South Australia and central New South Wales (ANRA 2003). In addition to impacts on land, affected areas experience increased salinity of aquatic habitats. Salt makes its way into aquatic habitats through surface run off, direct intrusion of saline ground water and through discharge of saline water from ground water diversion schemes. As salinisation manifests primarily in low lying areas, it is lowland rivers, streams and wetlands that are the most at risk from increased salinity. Salinity increases observed in Victorian rivers to date have ranged from moderate to extreme. For example; salinities twice that of seawater have been recorded in the Wimmera River. In addition to increased salt loads from salinisation processes, alterations to hydrological regimes (for agricultural and management purposes) can contribute to increased salinity of aquatic habitats through the reduced frequency of flushing flows and increasing evaporative concentration of salts (Nielsen *et al.* 2003). Other anthropogenic activities can also lead to increased salt levels in freshwater habitats. Many industrial effluents contain high salt concentrations (Goodfellow *et al.* 2000). Disposal of these effluents to rivers can potentially lead to considerable increases in stream salinity. Saline discharges from mining practices are a particular issue in some regions (Clemens and Jones 1954; Williams 1987; Short *et al.* 1991; Chapman *et al.* 2000). In colder regions of the world, salt applied to roads to prevent ice build up can make its way into surface waters resulting in elevated stream salinities (Williams *et al.* 1999; Blasius and Merritt 2002; Kefford *et al.* 2007c in Lovett *et al.* 2007). Climate change has the potential to lead to increased salinity of freshwater ecosystems in some regions. Changes in rainfall patterns, rates of evaporation and interactions between ground and surface waters could all result in changes in surface water salinities.

Increased salinity of surface waters not only reduces its suitability for agriculture, industry and potable water supply, it can also affect the structure and function of aquatic ecosystems. Despite more than 15 years research into this issue in Australia, there is still limited

understanding of the effects of increased salinity on aquatic ecosystems and limited knowledge with which to predict impacts. Many of the knowledge gaps identified by Hart *et al.* (1991) in an early review of the issue are yet to be addressed in a comprehensive manner. More recent reviews highlight the continuing need for further research in this area and emphasise in particular the need for investigations into effects on sensitive life stages, sublethal and chronic effects (Metzeling *et al.* 1995; Bailey and James 2000; Nielsen and Hillman 2000; Clunie *et al.* 2002; Goss 2003; James *et al.* 2003; Nielsen *et al.* 2003). Natural resource managers continue to face the problem of limited information with which to make salinity related management decisions.

1.2 The use of macroinvertebrates for the evaluation of the effects of salinity on aquatic ecosystems

Macroinvertebrates are fundamental components of most aquatic ecosystems. They represent a large proportion of the abundance and diversity of aquatic ecosystems, are an important food source for fish, birds and other aquatic predators and play a vital role in nutrient cycling. The diversity, abundance and sensitivity of aquatic macroinvertebrates has led to them being widely utilised in the assessment of environmental impacts and toxicological testing. As an integral component of aquatic ecosystems, effects on macroinvertebrates are likely to have flow on effects throughout the ecosystem.

Hart *et al.* (1991) suggested that aquatic macroinvertebrates are likely to represent some of the most salinity sensitive components of freshwater aquatic ecosystems, with effects likely to occur at salinities as low as 1000 mg L^{-1} ($\approx 1470 \text{ } \mu\text{S cm}^{-1}$). Metzling (1993) observed clear salinity related differences in macroinvertebrate community structure in Victorian rivers across a salinity range of 51 to 1100 mg L^{-1} ($\approx 75\text{-}1600 \text{ } \mu\text{S cm}^{-1}$) and Horrigan *et al.* (2005) observed a reduction in sensitive taxa in Queensland streams at salinities of $800\text{-}1000 \text{ } \mu\text{S cm}^{-1}$. Kefford *et al.* (2006a) observed reduced macroinvertebrate species richness in Victorian rivers at salinities of $500\text{ to }999 \text{ } \mu\text{S cm}^{-1}$. A threshold of $1500 \text{ } \mu\text{S cm}^{-1}$ has been adopted by some environmental management organisations (e.g. Murray-Darling Basin Commission) as a salinity target for Australian rivers (Goss 2003; Nielsen *et al.* 2003).

While studies of changes in aquatic macroinvertebrate communities are a fundamental component to build understanding of the effects of increased salinity, the underlying mechanisms leading to the observed changes can be unclear. Often changes in salinity are accompanied by other environmental changes (e.g. increased sediment and nutrient loads,

altered temperature and hydrological regimes, reduced habitat quality etc.) which confound observed changes in community structure. It can also be difficult to determine if observed changes are due to direct or indirect effects of increased salinity. Indirect effects occur when other ecosystem components that macroinvertebrates rely on are adversely affected by salinity. For example reduced macrophyte abundance or diversity may affect populations of macroinvertebrates that rely on them for food and/or shelter. Direct effects are those that limit populations due to the direct physiological effects of increased salinity (e.g. increased mortality, reduced growth and reproduction etc.).

Scientists have long been aware that freshwater organisms (including macroinvertebrates) have physiological limits to the amount of salt they can tolerate. This is related to the osmoregulatory ability of a species. To enable cellular function, aquatic organisms need to maintain internal salt and water levels within an optimal osmotic range (Hart *et al.* 1991). This processes is referred to as osmoregulation. In freshwater macroinvertebrates optimal internal osmotic concentrations are higher than that of freshwater, resulting in an osmotic gradient favouring water gain and the loss of ions (Hart *et al.* 1991). Freshwater species have evolved a range of osmoregulatory mechanisms; primarily reduced permeability, production of hypotonic urine and active ion uptake in order to maintain an optimal internal osmolarity (Hart *et al.* 1991). Each species displays a salinity threshold up to which they can maintain constant optimal osmolarity of their body fluid, above this threshold more ions tend to be taken up increasing internal osmolarity (Hart *et al.* 1991). This leads to cell dehydration and ultimately death (Hart *et al.* 1991).

Significant research into the short-term lethal salinity tolerance of Australian freshwater macroinvertebrates has been conducted in recent years (e.g. Kefford *et al.* 2003a, 2006a; Dunlop *et al.* 2008) and the salinity level causing death has been found to vary greatly between different species. Kefford *et al.* (2003a) observed salinity 72-hour LC₅₀ values (concentration lethal to 50% of a test population) for macroinvertebrates from the Barwon River in Victoria ranging from 5500 $\mu\text{S cm}^{-1}$ (for a Baetid Mayfly species) to greater than 70000 $\mu\text{S cm}^{-1}$ (for Isopod and Decapod species). Generally non-arthropod macroinvertebrates (worms, leeches, flatworms, molluscs, etc.) are salinity sensitive (due to the lack of an exoskeleton) and crustacea display high tolerance. However, tolerant and sensitive taxa occur in both groups. Insects display a wide range of tolerances from some of the most sensitive species to highly salt tolerant (Kefford *et al.* 2003a). Beadle (1969) suggested that species salinity tolerance reflects to some extent the evolutionary pathway

taken for colonisation of freshwater systems (i.e. from marine systems via estuaries or via land) and the time elapsed since initial colonisation.

Investigations into the physiological salinity tolerance of freshwater macroinvertebrates have focused on short-term lethal effects and have largely been conducted with the older aquatic life stages of the investigated species (e.g. Kefford *et al.* 2003a; Kefford *et al.* 2005; Kefford *et al.* 2006a; Dunlop *et al.* 2008). Exposure periods have generally ranged from 48 to 96 hours. This follows toxicological methods and is based on logistical considerations rather than physiological responses of the test organisms. There has been little consideration of whether these exposure periods are sufficient to encompass the acute lethal effects of salinity on the investigated species. Different life stages of aquatic macroinvertebrate species have been found to display different salinity tolerances, with younger stages generally more sensitive (Hubschman 1975; Mills and Geddes 1980; Walsh 1994; Kefford *et al.* 2004a, 2007a). Thus investigations of only the older aquatic life stages may under or over estimate the physiological salinity tolerance of a species.

It is not only lethal effects that can limit a species ability to survive at increased salinities. Sublethal effects such as those affecting reproduction and growth have the potential to limit the viability of populations at salinities lower than those directly lethal to individuals. Various sublethal effects of salinity have been observed across a range of freshwater macroinvertebrate taxa. Styczynska-Jurewicz (1972) observed reduced fecundity of both a freshwater gastropod species (*Physa acuta*) and an oligochaete species (*Tubifex tubifex*) at salinities lower than those affecting adult survival. Kefford *et al.* (2004a) observed reduced egg hatching success of several species of gastropod and Kefford and Nugegoda (2005) observed effects on growth and reproduction of the gastropod species *Physa acuta* at salinities lower than those affecting adult survival. Clark *et al.* (2004) observed effects of salinity on the pupal mass and development time of a freshwater mosquito species. Effects of salinity on growth and development have been observed for other insect species (Kefford *et al.* 2006b; Hassell *et al.* 2006). Sublethal salinity effects have been observed for species of Hydra with reduced population growth (Kefford *et al.* 2007b; Zalizniak *et al.* 2006; Kefford *et al.* 2003b) and feeding (Kefford *et al.* 2003b) at salinities lower than those causing direct mortality.

While sublethal effects are likely to limit the survival of freshwater macroinvertebrate species at lower salinities than those affecting short-term survival, tests investigating these effects are more difficult and potentially expensive to conduct. The exposure times required can be

much longer than acute tests, knowledge of the species life history is required and techniques for keeping test organisms alive and in good health under laboratory conditions need to be developed. For these reasons conducting sublethal tests on a wide range of freshwater macroinvertebrate species to determine their salinity tolerances is not practicable. Elucidation of the relationship between a species acute salinity tolerance and its sublethal tolerance may enable the sublethal tolerance of a species to be inferred from the results of short-term acute tolerance tests. This may enable protection against sublethal effects to be factored into salinity related environmental management decisions.

1.3 This study

This study investigates the lethal and sublethal physiological salinity tolerance of four aquatic macroinvertebrate species commonly occurring in Victorian rivers and wetlands through a series of laboratory tests. Tests were designed to address the following research questions;

- 1.) Are standard 48-96 hr exposure periods adequate for the assessment of the acute physiological salinity tolerance of freshwater macroinvertebrate species?
- 2.) Do different life stages of freshwater macroinvertebrate species commonly display different physiological salinity tolerances and if so is one particular life stage regularly the most sensitive?
- 3.) Do sublethal effects commonly occur at salinities lower than those affecting short-term survival of freshwater macroinvertebrate species and if so at what salinities do they occur?
- 4.) Is there a correlation between the acute salinity tolerance and sublethal physiological salinity tolerance of freshwater macroinvertebrate species?

The species investigated were selected to represent the range of macroinvertebrate taxonomic groups occurring in Victorian lowland rivers and wetlands and to encompass a range of salinity sensitivities. It was necessary that the selected species be available in sufficient numbers to allow appropriate replication of tests, and that their biology enabled long-term maintenance under laboratory conditions. The species selected for testing were the introduced worm *Lumbriculus variegatus* Müller (Annelida: Oligochaeta), the introduced snail *Physa acuta* Draparnaud (Gastropoda: Physidae), the shrimp *Paratya australiensis* Kemp (Decapoda: Atyidae) and the midge *Chironomus tepperi* Skuse (Diptera: Chironomidae).

Two introduced species (i.e. *L. variegatus* and *P. acuta*) were selected as native species were unable to be sourced in great enough numbers to enable appropriate test replication. Both species are widespread in Australia and commonly occur in lowland rivers and wetlands (*L. variegatus*: Gooderham and Tsyrlin 2002, *P. acuta*: Smith 1996).

Sublethal tests focused on assessing the effects of salinity on growth, reproduction and recruitment. While there are a wide range of other sublethal end points that could be investigated (eg. behavioural, physiological, biochemical and histological, etc.), these end points were selected due to the clearer relationship between observed effects and potential implications for natural populations.

1.4 Broader research project

This study forms part of a broader project to develop a system to predict the loss of aquatic biodiversity from changes in salinity (Land and Water Australia (LWA) Project No. VCE 17, followed on by LWA Project No. RMI 12) (see Kefford *et al.* 2003b and Kefford *et al.* 2007c in Lovett *et al.* 2007). Acute salinity tolerance data are being collected for a wide selection of macroinvertebrate species occurring in Victorian lowland rivers (see Kefford *et al.* 2003a, 2003b, 2006a). These data have been used to construct species sensitivity distribution (SSD) models for aquatic macroinvertebrate communities to enable assessment of the potential loss of species for a given change in salinity. It is hoped that results from the current study can be used to apply a safety factor to the collected acute tolerance data to protect against the loss of species due to sublethal effects.

2.0 General Materials and Methods

2.1 Test organism sources

Test organisms used in this study were sourced from both natural populations and culture stock. The shrimp *Paratya australiensis* Kemp (Decapoda: Atyidae) and the introduced snail *Physa acuta* Draparnaud (Gastropoda: Physidae) were collected from populations in the Barwon River, Victoria. The midge *Chironomus tepperi* Skuse (Diptera: Chironomidae) was sourced from a laboratory culture maintained by the Department of Genetics at Latrobe University. The culture had been maintained in dechlorinated Melbourne tap water. The source stock for this culture was collected from wetland populations in southern New South Wales. The introduced worm *Lumbriculus variegatus* Müller (Annelida: Oligochaeta) was obtained from a culture at a commercial aquarium. The culture had been maintained in dechlorinated Melbourne tap water.

2.2 The Barwon River

The test organisms sourced from natural populations for this study, that is *P. australiensis* and *P. acuta*, were collected from the Barwon River in south west Victoria. The Barwon River was selected as it was from this catchment that the majority of species were collected for acute salinity tolerance assessment in the broader research project (Kefford *et al.* 2003a). The Barwon River is also typical of lowland rivers under threat from increased salinity. With the exception of forested areas in the river's headwaters, the Barwon catchment has been largely cleared and vegetation replaced with introduced pastures and, to lesser extent, crops. The river is also affected by schemes that divert saline water into the river from outside its catchment (Lough Calvert and Woody Yallock Diversion Schemes), which contribute to elevated salinities in the river's mid and lower reaches. The Barwon River catchment extends from the northern slopes of the Otway Ranges in its south and west, to Ballarat in the north, Geelong in the east and flows into Bass Strait at Barwon Heads. The catchment encompasses a total area of 388007 hectares and has a mean annual flow of 300000 megalitres (Department of Water Resources 1989).

Test organisms were collected from two sites on the Barwon River. One site was in the upper catchment on the West Branch of the Barwon River at the crossing of the Birregurra-Forrest Road, near the town of Forrest (38°30'36''S 143°43'12''E), hereafter referred to as the Forrest site. The other site was at Pollocksford Bridge (38°08'47''S 144°11'14''E) on the lower middle reaches of the Barwon River approximately 130 km from the rivers source and

upstream of the city of Geelong, here after referred to as the Pollocksford site. *P. acuta* was collected from both sites while *P. australiensis* was only collected from the Pollocksford site. A study of the Barwon catchment at 20 sites from Pollocksford Bridge and above identified 144 macroinvertebrate taxa (Canale *et al.* 2001). Studies of fish populations have identified nine native and six introduced species of freshwater fish in the Barwon River (Zampatti and Grgat 2000).

The two Barwon River collection sites for this study display different salinity regimes. The Pollocksford site, which is lower in the catchment and is also impacted by both the saline water disposal schemes, displayed higher salinity than the Forrest site. The average salinity for the Pollocksford site during the period of test organism collection from January 2001 to December 2003 was $1822 \pm 4 \mu\text{S cm}^{-1}$, with a minimum salinity of $419 \mu\text{S cm}^{-1}$ and a maximum salinity of $3788 \mu\text{S cm}^{-1}$ (N = 20296) (Victorian Water Resources Data Warehouse 2008). A similar salinity range was observed at this site during the five years prior to this study, with an average salinity of $1876 \pm 3 \mu\text{S cm}^{-1}$, a minimum salinity of $270 \mu\text{S cm}^{-1}$ and a maximum salinity of $4486 \mu\text{S cm}^{-1}$ (N = 32250) (Victorian Water Resources Data Warehouse 2008). Water temperature during the same period ranged from 7 to 35°C with a mean temperature of 17°C (N = 28012) (Victorian Water Resources Data Warehouse 2008). There is no water quality monitoring station located at the Forrest collection site. The nearest upstream monitoring site is at the Compensation Weir Spillway on the West Barwon Reservoir. This site displayed an average salinity of $156 \pm 10 \mu\text{S cm}^{-1}$, a minimum of $46 \mu\text{S cm}^{-1}$ and a maximum of $208 \mu\text{S cm}^{-1}$ (N = 21735) during the period of test organism collection from May 2002 until December 2003 (Victorian Water Resources Data Warehouse 2008).

2.3 Field collection methodology

Field sourced test organisms were collected from the field sites on several occasions, as they were required for testing. They were collected using hand sweep nets with a 250 μm mesh size. Net contents were transferred to white sorting trays and test organisms carefully removed and placed in plastic bags filled with river water. Air spaces in the bags were filled with oxygen and the bags sealed for transportation to the laboratory. The bags were kept cool and out of direct sunlight for the 2-3 hour journey to the laboratory.

2.4 Acclimation

Field collected test organisms and newly sourced culture organisms were acclimated to laboratory conditions for a period of at least 48 hours before use in tolerance tests. Field sourced organisms were maintained at the salinity of the river at time of collection for the duration of the acclimation period. Culture sourced test organisms were maintained in treated Melbourne tap water throughout culture and acclimation.

2.5 Laboratory conditions

Tests were conducted at 20 ± 2 °C with a 16-hour light: 8-hour dark photoperiod.

2.6 Test solution preparation

Test salinity solutions were prepared using aged, activated carbon and sand filtered Melbourne domestic supply water (hereafter referred to as filtered water) and the artificial sea salt Ocean Nature (Aquasonic, Wauchope, NSW, Australia). Test solutions were made up to the required salinity with the aid of a water quality meter (TPS FL90 Multimeter). Artificial sea salt was used, as while the proportions of ions contributing to salinity can vary in natural systems, in south-eastern Australia the ionic composition of surface waters commonly deviates little from that of sea water (Bayly and Williams 1973).

2.7 Water quality measurements

Water quality parameters; temperate, pH, dissolved oxygen and salinity were measured regularly throughout tests and in new test solutions. Water quality parameters were also measured at test organism field collection sites at the time of collection. All water quality measurements were made using a TPS FL90 Multimeter.

2.8 Salinity measurement and conversions

Salinity was measured as electrical conductivity ($\mu\text{S cm}^{-1}$) at 25°C (hereafter referred to as EC) as it is the most common method used for field salinity measurements. To enable comparison, salinity tolerance results from other studies reported as total soluble salts (TSS) or total dissolved solids (TDS) concentrations were converted to EC using the relationship $\text{TSS} = 0.68 \times \text{EC}$ (Hart *et al.* 1991). TDS was used interchangeably with TSS as there is commonly very little difference between these measures (Bailey *et al.* 2002). Converted values are indicated with an approximately equal symbol (i.e. \approx) or asterisk.

2.9 Test materials and methods

Materials and methods specific to individual tests are outlined in chapters 3 to 6, which deal individually with investigations of the salinity tolerance of each of the four test species.

2.10 Statistical analysis

Distribution of data was assessed using box plots. Any data considered to display excessive heterogeneity of variance or broadly different distributions were appropriately transformed and analysis performed on both transformed and untransformed data. Where outliers were identified analyses were performed with and without outliers to assess effects on analysis results. Proportional data were arcsine transformed prior to analysis.

Analysis of variance (ANOVA) tests were performed to detect significant differences in water quality parameters and test end points between replicates and test treatments. Unless otherwise stated, differences were considered significant at p-values ≤ 0.05 . Where significant differences were determined post hoc Tukey's pair-wise comparison tests were performed to discern differences (family error rate $\alpha = 0.05$). Differences between initial and final end points (e.g. initial versus final weight etc.) within treatments were assessed using paired t-tests.

Unless otherwise stated, standard logistic regression analyses were used to determine LC₅₀ effect levels for survival and other binomial end points (e.g. hatched/unhatched eggs). Rho-squared values for logistic regression analyses were calculated using the McFadden's method. Where linear relationships were evident, linear regressions were used to calculate LC₅₀ effect levels. Where necessary data were transformed to improve linearity. 95% confidence intervals (CI) were calculated using standard error (SE) of the slope and intercept estimates for each model (95% CI = 1.96 SE).

Where observed optimal values for proportional end points (e.g. survival, egg hatching success etc.) were less than 90% data were corrected to a percentage of the observed optimal using Abbott's Formula:

$$T^* = ((T - C)/(100 - C)) \times 100$$

Where;

T* = adjusted % test response

T = % test response

C = % control response

(Abbott 1925 cited by Chapman 1995 in Rand 1995).

Corrected data were used for the determination of LC_{50} and EC_{50} values.

Except where otherwise indicated statistical analyses were performed using SYSTAT Version 8.0 for Windows (SYSTAT Products, Chicago, IL, USA). Unless otherwise indicated, provided error estimates represent the standard error (SE) of the mean. Analyses specific to individual tests are outlined in Chapters 3 to 6.

3.0 Salinity tolerance of the aquatic oligochaete *Lumbriculus variegatus* Müller (Annelida: Oligochaeta)

3.1 Introduction

Oligochaete worms (Annelida: Oligochaeta) are an important component of freshwater ecosystems. They can be present in high densities (Dales 1963) and contribute significantly to biodiversity, with at least 90 species occurring in Australian freshwater ecosystems (Gooderham and Tsyrlin 2002). Oligochaetes can be an integral part of the sediment and nutrient dynamics of aquatic systems (Birtwell and Arthur 1980 in Brinkhurst and Cook 1980) and are important prey items for fish, birds, leeches and other invertebrates (Brinkhurst and Cook 1980; Pinder and Brinkhurst 1994). Two other classes of the phylum Annelida also occur in freshwater environments, these are the leeches (Hirudinea) and Polychaete worms (Polychaeta) a few species of which occur in more saline environments (Williams 1980).

Oligochaetes are found in osmotically diverse environments, from oceans and hyper saline lakes, to freshwaters and the fluctuating conditions of estuaries. Distributions within these environments reflect the salinity tolerance of individual species (Jansson 1962; Chapman and Brinkhurst 1980). Some species are able to tolerate broad salinity ranges, while others are adapted to low or high salinities. Marine species are osmoconformers and thus lack the ability to osmoregulate (Oglesby in Mill 1978). Adaptive radiation into environments of different salinities necessitated the development of mechanisms for osmoregulation.

There has been limited investigation of osmoregulation and salinity tolerance of oligochaetes. Research that has been conducted has focused largely on adaptations of marine species to reduced salinity (e.g. Jansson 1962; Tyene 1969; Ganapati *et al.* 1972). Very few studies have investigated the tolerance of freshwater oligochaete species to increased salinity. Notable exceptions are investigations of the effects of salinity on the survival, reproduction and haemolymph concentration of the Tubificid *Tubifex tubifex* (Styczynska-Jurewicz 1972) and the effects of salinity on the short-term survival of 9 species of freshwater oligochaete (Chapman *et al.* 1982). Berezina (2003) investigated four oligochaete species in a study of the short-term salinity tolerance of Russian freshwater invertebrates. Studies by Kefford *et al.* (2003a; 2006a) are the only investigations into the salinity tolerance of Australian oligochaete species.

There is very limited knowledge of the salinity tolerance of Australian freshwater oligochaete species. Very few studies investigating the field salinity tolerance of freshwater macroinvertebrates identify oligochaete species below Family level. The collation of salinity sensitivity data for organisms occurring in Australian inland waters by Bailey *et al.* (2002) reported field salinity records for only 10 species of aquatic oligochaete. The maximum field salinity recorded for oligochaetes was for the Tubificid *Rhyacodrilus* sp. which was found at a salinity of 58000 mg L⁻¹ (\approx 85300 μ S cm⁻¹) (Bailey *et al.* 2002). Half of the reported species occurred at maximum salinities less than 500 mg L⁻¹ (\approx 735 μ S cm⁻¹) (Bailey *et al.* 2002). The relationship between these field observations and the salinity tolerance of the species is unclear as values may be reflective of the range of water bodies sampled and the data provides no insight into the numbers present or the viability of the populations. Hart *et al.* (1991) concluded that most genera of freshwater annelid appear restricted to salinities less than 1000 mg L⁻¹ (\approx 1471 μ S cm⁻¹). Kefford *et al.* (2003a) observed a 96-hour salinity LC₅₀ (concentration lethal to 50% of test organisms) value of 9300 (95% CI 6000-15000) μ S cm⁻¹ for a mixture of species from the Barwon River. *Lumbriculus variegatus* from the Murray-Darling Basin displayed a 96-hour LC₅₀ value of 15000 μ S cm⁻¹ and it was determined that the 72-hour LC₅₀ value for *Branchiura sowerbyii* was greater than 6400 μ S cm⁻¹ (Kefford *et al.* 2006a).

In the current study *Lumbriculus variegatus* Müller (Oligochaeta: Lumbriculidae) was selected as a test species for investigation into the tolerance of a freshwater oligochaete to increased salinity. Species of the family Lumbriculidae are restricted to freshwater environments and occur in all types of freshwater ecosystems (Dales 1963). Distribution of the family is essentially holartic, however this species is widespread throughout the world, due in all likelihood to introductions through its use as live fish food in the aquarium industry (Williams 1980; Pinder and Brinkhurst 1994). The species is common and widespread throughout Australia (Gooderham and Tsyrlin 2002) and has been recorded from rivers, lakes and wetlands in the eastern states (Pinder and Brinkhurst 1994). Although the species is capable of sexual reproduction, sexually mature individuals are rare and the primary mode of reproduction is asexually by architomy (Williams 1980; Phipps *et al.* 1993). In this form of asexual reproduction new individuals arise through transverse fission of the parent with no prior morphological differentiation of individuals (Gibson and Harvey 2000). This mode of reproduction was no doubt a factor aiding the spread of the species (Williams 1980). *L. variegatus* has been widely adopted as a test organism for sediment toxicity and bioaccumulation studies (e.g. Phipps *et al.* 1993; Ankley *et al.* 1994; Ingersoll *et al.* 1995;

Kemble *et al.* 1999; Sibley *et al.* 1999). Despite its wide distribution and use as a test organism, the studies by Berezina (2003) and Kefford *et al.* (2006a) provide the only readily available information on the salinity tolerance of the species. In the collation of salinity sensitivity data for species occurring in Australian inland waters, information for *L. variegatus* is limited to one field record of occurrence at a salinity of 400 mg L⁻¹ ($\approx 670 \mu\text{S cm}^{-1}$) (Bailey *et al.* 2002).

This chapter investigates the tolerance of *L. variegatus* to increased salinity in laboratory experiments assessing short-term (acute) effects on survival and long-term effects on survival, reproduction and growth. In addition, the effects of salinity on the energy requirements of the species were investigated through evaluating the effect of salinity on starvation.

3.2 Materials and methods

3.2.1 Culture techniques

L. variegatus is commonly cultured in the aquarium industry for fish food. Stock for laboratory culture was obtained from Aquarium Industries Pty Ltd Melbourne, Australia and species identification verified using taxonomic keys (Pinder and Brinkhurst 1994). Culture stock was added to 20-L glass aquaria containing 15 L of unmodified filtered water and plastic fly mesh provided as substrate. Eighty-percent water changes were made every 2-3 days and small quantities of commercial trout pellets added as food.

3.2.2 Acute salinity tolerance

The acute (short-term) salinity tolerance of *L. variegatus* was investigated in two tests conducted in parallel, (1) without provision of food or substrate and (2) with the provision of food and substrate. The test with provision of food and substrate was conducted to determine if this provision (which was necessary for the investigation of the long-term effects of salinity) affects salinity tolerance. With the exception of food and substrate provision, methods and conditions were the same for both tests. *L. variegatus* were exposed to a test salinity range from 3000 to 18000 $\mu\text{S cm}^{-1}$ (treatment levels; 3000, 6000, 9000, 12000, 15000 and 18000 $\mu\text{S cm}^{-1}$) and unmodified filtered water ($\approx 140 \mu\text{S cm}^{-1}$), with 3 replicates of each treatment. 0.1 g L⁻¹ of ground commercial trout pellets and 2 g L⁻¹ of shredded unbleached tissue were added to the test solutions of replicates of test (2). No additional food was provided for the duration of the test. The tests were conducted in 440-mL plastic containers with 350 mL of test solution. Worms of a similar size were collected from the laboratory culture and randomly assigned to treatments, 10 individuals per replicate. Water quality measurements were taken at the beginning and end of the tests and water was not changed during the test. The number of worms alive (determined by failure to respond to gentle prodding) in each replicate was determined at 24-hour intervals and the test continued until mortality had stabilised. Test (2) with food was discontinued after 7-days exposure and Test (1) without food was continued to investigate the effects of salinity on starvation of *L. variegatus*.

3.2.3 Effects of salinity on starvation

Starvation of *L. variegatus* at different salinities was investigated through an extension of the short-term survival test conducted without feeding. The test was extended for a total exposure period of 6 weeks, with the number of worms alive determined at least once per week. Eighty

percent water changes were conducted weekly, and water quality measured before and after each water change. No food was added for the duration of the test.

3.2.4 Long-term effects of salinity

The long-term effects of salinity on the reproduction, survival and growth of *L. variegatus* were investigated over an experimental salinity range of 3000-15000 $\mu\text{S cm}^{-1}$ (treatment levels; 3000, 6000, 9000, 12000 and 15000 $\mu\text{S cm}^{-1}$) and in unmodified filtered water ($\approx 144 \mu\text{S cm}^{-1}$). Ten worms per replicate, with 5 replicates per treatment were maintained at the experimental salinity for 6 weeks. Worms of similar size were collected from the laboratory culture and placed in clean filtered water without food for 24 hours prior testing to allow evacuation of the digestive tract. Worms were then counted and the total wet weight added to each replicate determined. The mean wet weight of worms added to each replicate was 39.8 ± 0.7 mg. Three samples of 10 worms were wet weighed and then oven dried at 60°C to determine the initial wet:dry ratio.

The test was conducted in 440-mL plastic containers with 350 mL of test solution and 60 mL of fine gravel. Twenty milligrams of ground commercial trout pellets was added to each replicate at the start of the test and after each water change. Eighty percent of the water in each replicate was changed once a week, and water quality measurements taken before and after water changes. For the last 24 hours of the test, worms were placed in clean test solutions without food to allow evacuation of the digestive tract. After 6 weeks *L. variegatus* from each replicate were collected, counted and oven dried to constant weight at 60°C . Total dry weight was determined for each replicate and the change in biomass and number of individuals calculated for each replicate. The mode of reproduction of *L. variegatus* (primarily asexual by architomy) necessitated that the change in number of individuals be determined rather than reproduction and survival separately as original test organisms and newly separated individuals are indistinguishable. The change in number of individuals over the 6-week exposure period thus represents reproduction less mortality.

3.2.5 Statistics and calculations

Results for the short-term survival tests were analysed using SYSTAT Version 8.0 for Windows (SYSTAT Products, Chicago, IL, USA) and TOXCALC Version 5.0 (Tidepool Scientific Software). Data were analysed by ANOVA and LC_{50} values for each test estimated using the Spearman-Kärber method.

The rate of population increase at each test salinity during the long-term test was calculated in terms of percentage daily increase in number and biomass using the equation;

$$\% \text{ increase } (n \text{ or } w) = (\ln f - \ln i) \times 100t^{-1}$$

where;

n = number of individuals

w = total weight

i = initial number or weight

f = final number or weight

t = exposure period

(modified from Yeagers 1996 and Reynolds 1996).

These rates of increase were then used to calculate the time required for a doubling of the number of individuals and biomass at each test salinity using the equation;

$$T_2(n \text{ or } w) = \ln 2 \times (\% \text{ increase } (n \text{ or } w)/100)^{-1}$$

(modified from Yeagers 1996).

3.3 Results

3.3.1 Acute salinity tolerance

Water quality remained high during the acute tolerance Test (1) conducted without food addition (Table 3.1). Salinities varied less than 1% from treatment levels and the mean salinity of unmodified filtered water during the test was $135.8 \pm 2.7 \mu\text{S cm}^{-1}$ with a maximum variability of 2%. With the exception of salinity (ANOVA; $F_{6,6} = 2510380.608$, $P < 0.001$) there were no significant differences in mean water quality conditions between treatments (ANOVA; $F_{6,6} < 0.431$, $P > 0.835$ in all cases). Temperatures remained within the desired $20 \pm 2^\circ\text{C}$ range in all treatments, dissolved oxygen concentrations remained above 90% saturation and pH ranged between 7.31 and 9.08.

Table 3.1. Water quality during acute salinity tolerance Test (1) with *Lumbriculus variegatus* conducted without food provision. Mean \pm SE and range in parenthesis.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Laboratory water	135.8 ± 2.7 (133.1-138.5)	7.36 ± 0.04 (7.32-7.40)	20.0 ± 1.1 (18.9-21.0)	97.4 ± 4.7 (92.7-102.1)
3000	3015 ± 5 (3010-3020)	7.70 ± 0.39 (7.31-8.09)	19.9 ± 1.1 (18.8-21.0)	94.8 ± 2.9 (91.9-97.6)
6000	6025 ± 5 (6020-6030)	8.04 ± 0.68 (7.36-8.72)	19.6 ± 0.8 (18.8-20.4)	95.2 ± 3.9 (91.3-99.1)
9000	9005 ± 5 (9000-9010)	8.17 ± 0.74 (7.43-8.91)	19.6 ± 0.9 (18.9-20.5)	94.4 ± 3.5 (90.9-97.8)
12000	12010 ± 0 -	8.27 ± 0.77 (7.50-9.03)	19.5 ± 0.8 (18.7-20.3)	94.6 ± 3.0 (91.6-97.5)
15000	15040 ± 0 -	8.31 ± 0.77 (7.54-9.08)	19.6 ± 0.9 (18.7-20.4)	94.7 ± 4.1 (90.6-98.7)
18000	18010	8.8	18.6	96.9

The addition of food and substrate negatively impacted on water quality during Test (2) (Table 3.2). The greatest impact was on dissolved oxygen concentration, with levels being reduced to less than 20% saturation in some treatments (Table 3.2). Although this effect was

more pronounced at lower salinities, with the exception of salinity ($F_{6,6} = 639003.307$, $P < 0.001$) there were no significant differences in mean water quality conditions between treatments ($F_{6,6} < 0.286$, $P > 0.923$ in all cases). As for the acute tolerance test without food, temperatures remained within the desired range of 20 ± 2 °C, the pH range was slightly more acidic ranging from 6.41 to 9.08 and dissolved oxygen concentration ranged from 16.7 to 102.1% saturation (mean values ranged from 57.9 to 96.9% saturation) (Table 3.2). Salinities varied less than 1% from treatment levels and the mean salinity of unmodified filtered water was 143.0 ± 9.9 $\mu\text{S cm}^{-1}$ with a maximum variability of 7%.

Table 3.2. Water quality during acute salinity tolerance Test (2) with *Lumbriculus variegatus* conducted with food provision. Mean \pm SE and range in parenthesis.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Laboratory water	143.0 ± 9.9 (133.1-152.9)	6.95 ± 0.45 (6.51-7.40)	19.6 ± 0.7 (18.9-20.3)	61.7 ± 40.4 (21.3-102.1)
3000	3015 ± 5 (3010-3020)	7.25 ± 0.84 (6.41-8.09)	19.5 ± 0.7 (18.8-20.2)	59.4 ± 38.2 (21.2-97.6)
6000	6020 ± 0 -	7.65 ± 1.07 (6.58-8.72)	19.6 ± 0.8 (18.8-20.4)	57.9 ± 41.2 (16.7-99.1)
9000	9000 ± 10 (8990-9010)	7.87 ± 1.04 (6.83-8.91)	19.4 ± 0.7 (18.7-20.1)	75.5 ± 22.4 (53.1-97.8)
12000	12000 ± 10 (11990-12010)	7.95 ± 1.08 (6.87-9.03)	19.5 ± 0.8 (18.7-20.3)	74.5 ± 23.1 (51.4-97.5)
15000	15040 ± 0 -	8.01 ± 1.07 (6.94-9.08)	19.5 ± 0.8 (18.7-20.2)	80.1 ± 18.6 (61.5-98.7)
18000	18010	8.80	18.6	96.9

The survival of *L. variegatus* was significantly affected by salinity in both tests (ANOVA 96-hour survival; $F_{6,14} = 130.143$, $P < 0.001$ test (1), $F_{6,14} = 99.000$, $P < 0.001$ test (2)), with reduced survival evident in the 15000 $\mu\text{S cm}^{-1}$ and 18000 $\mu\text{S cm}^{-1}$ treatments (Tukey's tests; $\alpha = 0.05$, $P < 0.001$ in all cases when compared with unmodified filtered water and lower salinity treatments) (Figure 3.1). Survival displayed a typical threshold dose response to increased salinity, with 100% survival occurring at salinities up to and including 12000 $\mu\text{S cm}^{-1}$, intermediate survival at 15000 $\mu\text{S cm}^{-1}$ and 100% mortality at 18000 $\mu\text{S cm}^{-1}$ (Figure

3.1). In both tests all worms in the 18000 $\mu\text{S cm}^{-1}$ treatment died within the first 24 hours of exposure. Mortality in the 15000 $\mu\text{S cm}^{-1}$ treatments stabilised after 4 days (96 hours) of exposure at a mean survival $63.3 \pm 8.8\%$ in Test (1) and $70.0 \pm 10.0\%$ in Test (2). No further mortality occurred in either test for the remainder of the 7-day exposure period. Changes in numbers of *L. variegatus* in Test (1) for exposure periods longer than 7 days are presented in results for the starvation test (see 3.3.2).

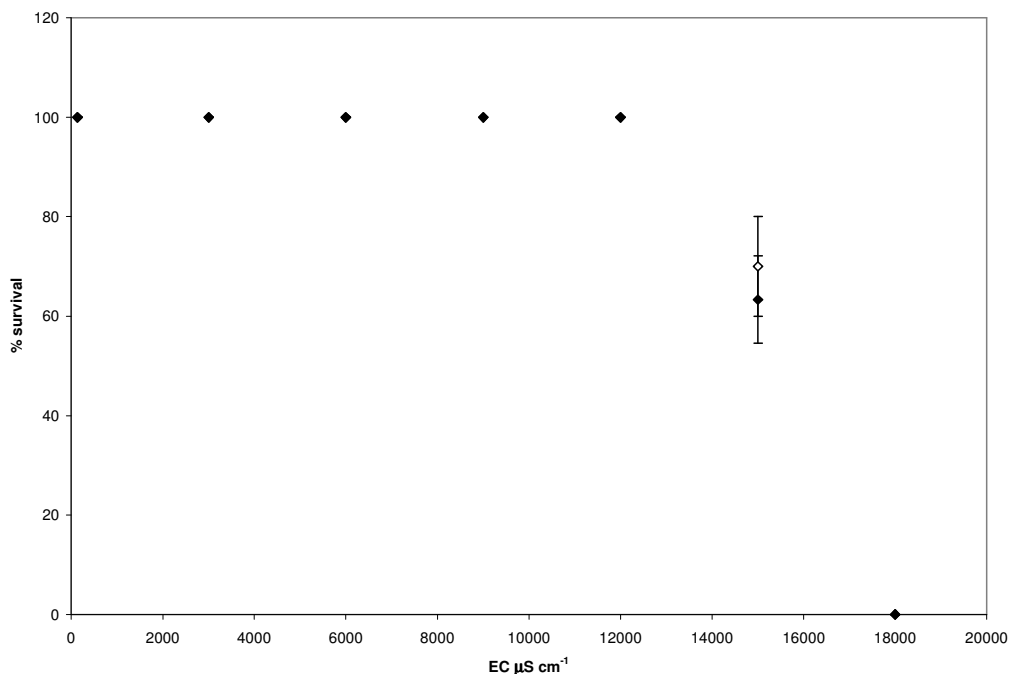


Figure 3.1. Survival (%) of *Lumbriculus variegatus* after 96 hours exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Without food (Test 1) (closed diamonds) and with food provision (Test 2) (open diamonds). Mean \pm SE.

LC₅₀ values calculated for each 24-hour exposure period for both tests reflect the stabilisation of mortality after 4 days exposure (Figure 3.2). The level of survival occurring after 4 days (96 hours) exposure (Figure 3.1) represents the time-independent acute salinity tolerance of *L. variegatus*, as the acute lethal mode of action ceased after this period of exposure (as evidenced by no further mortality). LC₅₀ values and 95% CI for each exposure period are presented in Appendix A Table A.1. Values were similar for tests with (Test 2) and without food (Test 1), the 96-hour (i.e. time-independent) LC₅₀ values were 15255 (14720-15809 95% CI) $\mu\text{S cm}^{-1}$ for Test (1) and 15462 (14946-15996 95% CI) $\mu\text{S cm}^{-1}$ for Test (2).

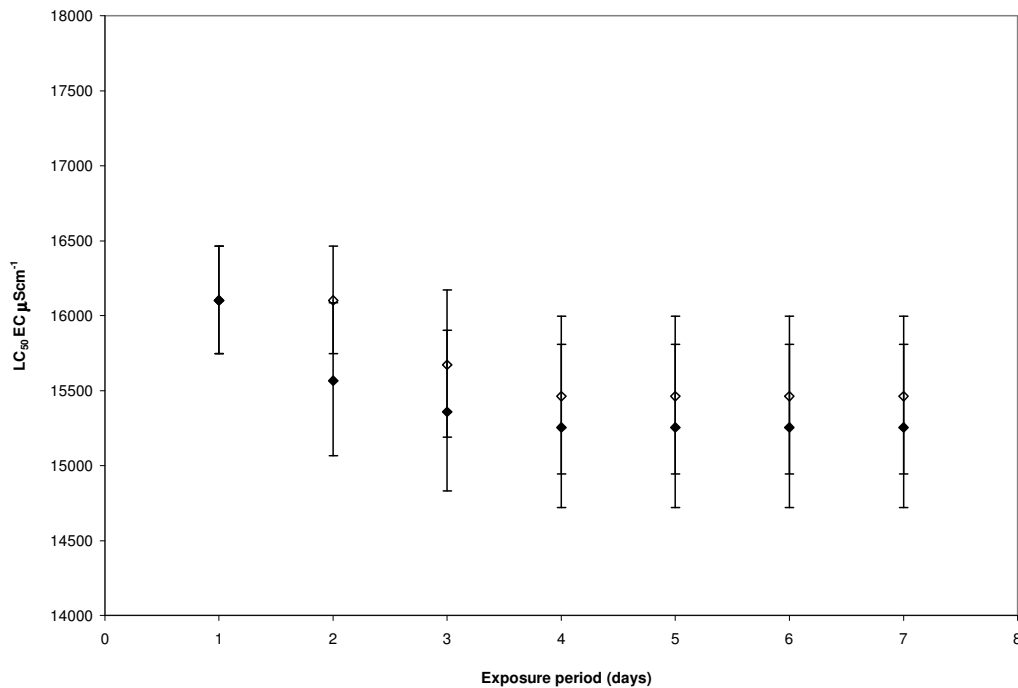


Figure 3.2 LC₅₀ values (EC $\mu\text{S cm}^{-1}$) for *Lumbriculus variegatus* after different periods (days) of salinity exposure. Without food (Test 1) (closed diamonds) and with food provision (Test 2) (open diamonds). Error bars indicate 95% CI.

There were no significant differences in survival of *L. variegatus* between the two test methodologies as indicated by two-way ANOVA of 96-hour survival data ($F_{6,28} = 0.250$, $P = 0.621$) and overlapping 95% confidence intervals of the LC₅₀ values.

3.3.2 Effects of salinity on starvation

Water quality in the starvation test remained high during the 30-day exposure period (Table 3.3), with salinities varying less than 1% from the treatment level. The mean salinity of unmodified filtered water during the test was 135.7 ± 1.1 EC $\mu\text{S cm}^{-1}$ with maximum variability of 2%. With the exception of salinity ($F_{6,24} = 1045245$, $P < 0.001$) there were no significant differences in mean water quality conditions between treatments ($F_{6,24} < 1.350$, $P > 0.274$ in all cases). Temperature remained within the desired $20 \pm 2^\circ\text{C}$ range in all treatments, dissolved oxygen concentration remained above 90% saturation and pH ranged between 7.31 and 9.08.

Table 3.3 Water quality during test investigating the effects of salinity on starvation of *Lumbriculus variegatus*. Mean \pm SE and range in parenthesis.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	135.7 \pm 1.1 (133.1-138.5)	7.35 \pm 0.03 (7.30-7.44)	19.9 \pm 0.4 (18.9-21.0)	97.3 \pm 2.3 (92.2-103.3)
3000	3008 \pm 4 (3000-3020)	7.51 \pm 0.15 (7.31-8.09)	19.9 \pm 0.4 (18.8-21.0)	95.6 \pm 1.5 (91.9-100.0)
6000	6014 \pm 7 (5990-6030)	7.69 \pm 0.26 (7.36-8.72)	19.8 \pm 0.4 (18.8-20.5)	95.1 \pm 1.6 (91.3-99.1)
9000	9000 \pm 5 (8990-9010)	7.78 \pm 0.29 (7.38-8.91)	19.8 \pm 0.4 (18.7-20.5)	94.4 \pm 1.6 (90.4-97.8)
12000	12002 \pm 5 (11990-12010)	7.84 \pm 0.3 (7.41-9.03)	19.8 \pm 0.4 (18.7-20.5)	94.8 \pm 1.4 (91.4-97.7)
15000	15022 \pm 8 (15000-15040)	7.92 \pm 0.29 (7.53-9.08)	19.8 \pm 0.4 (18.7-20.6)	94.4 \pm 1.6 (90.6-98.7)

There was little change in numbers of *L. variegatus* individuals during the 4 to 30 day exposure period (ie 30-day exposure excluding initial acute mortality)(Figure 3.3). Rather than decreasing due to additional mortality, the number of individuals increased during this period as a result of asexual fission (Figure 3.3). There was no change in the mean number of individuals in unmodified filtered water and only minimal increases in the 3000, 6000, 9000 $\mu\text{S cm}^{-1}$ and 15000 $\mu\text{S cm}^{-1}$ treatments (Figure 3.3). The number of individuals in the 12000 $\mu\text{S cm}^{-1}$ treatment increased by 2.7 ± 0.3 during the 4-30 day exposure period (Figure 3.3). Analysis indicated that the increase in number of individuals in the 12000 $\mu\text{S cm}^{-1}$ treatment was significantly greater than in unmodified filtered water and all other salinity treatments (ANOVA; $F_{5,12} = 10.280$, $P = 0.001$, Tukey's test; $\alpha = 0.05$, $P < 0.007$ for all comparisons with 12000 $\mu\text{S cm}^{-1}$ treatment, $P > 0.642$ for all other comparisons).

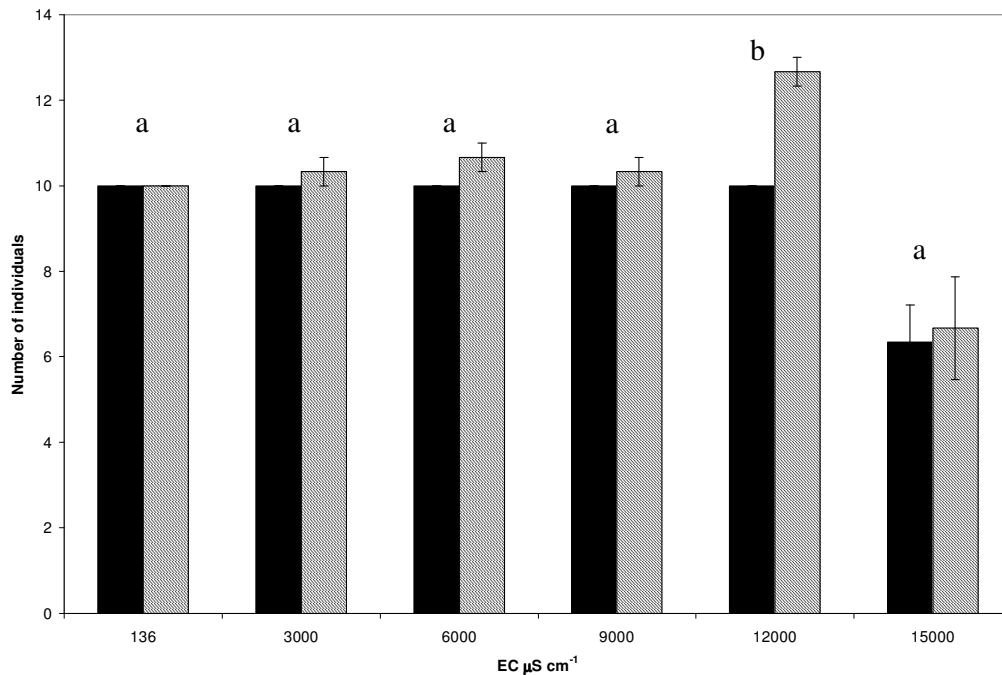


Figure 3.3 The number of *Lumbriculus variegatus* individuals surviving after acute salinity exposure (4-days) (solid) and after 30-days salinity exposure without food (hatched). Mean \pm SE. Letters indicate significant differences (between salinity treatments) in the change in number of individuals (within each treatment) between exposure periods ($P < 0.05$).

The test was conducted for a total exposure period of 6 weeks (42 days), however results are presented for only the exposure period up to 30 days as after this period it became difficult to define the number of individuals alive in the 15000 $\mu\text{S cm}^{-1}$ treatment. Individuals at this salinity had begun to disintegrate (yet continued to respond to stimuli) with no clear point at which to define death.

3.3.3 Long-term effects of salinity

Water quality remained high for the duration of the long-term test (Table 3.4). Mean salinities varied less than 1% from the nominal values and maximum differences were no greater than 4%, with the exclusion of unmodified filtered water which remained within 19% of the mean value ($144.4 \pm 3.5 \mu\text{S cm}^{-1}$). Dissolved oxygen levels remained in excess of 85% saturation and temperatures were within the $20 \pm 2^\circ\text{C}$ nominated range. There were no significant differences between treatments in either dissolved oxygen saturation (ANOVA; $F_{5,95} = 0.512$, $P = 0.767$) or temperature (ANOVA; $F_{5,95} = 0.118$, $P = 0.988$). There were significant differences in pH between treatments (ANOVA; $F_{5,95} = 2.773$, $P = 0.022$), Tukey's pair-wise comparisons test ($\alpha = 0.05$) indicated that the pH in the 15000 $\mu\text{S cm}^{-1}$ treatment

was significantly higher than in unmodified filtered water ($P = 0.028$). Differences in salinity (EC $\mu\text{S cm}^{-1}$) between treatments were highly significant (ANOVA; $F_{5,95} = 42777$, $P < 0.001$).

Table 3.4 Water quality during long-term salinity tolerance test with *Lumbriculus variegatus*. Mean \pm SE and range in parentheses. (EC to four significant figures.)

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	144.4 \pm 3.5 (128.0-171.3)	7.87 \pm 0.11 (7.42-9.04)	20.0 \pm 0.2 (18.3-21.5)	94.9 \pm 1.7 (88.5-115.1)
3000	3029 \pm 8 (2990-3110)	8.01 \pm 0.10 (7.62-8.81)	19.9 \pm 0.2 (18.3-21.4)	94.8 \pm 1.5 (86.8-107.4)
6000	6053 \pm 18 (5950-6200)	8.11 \pm 0.09 (7.55-8.83)	19.9 \pm 0.2 (18.3-21.0)	93.8 \pm 1.3 (86.9-103.1)
9000	9080 \pm 26 (8980-9370)	8.19 \pm 0.08 (7.69-8.79)	19.9 \pm 0.2 (18.3-21.1)	93.0 \pm 1.1 (87.5-101.6)
12000	12120 \pm 36 (11970-12450)	8.24 \pm 0.09 (7.71-8.86)	19.8 \pm 0.3 (18.0-21.2)	92.9 \pm 1.1 (85.8-102.3)
15000	15150 \pm 45 (14960-15560)	8.28 \pm 0.09 (7.74-8.84)	19.9 \pm 0.2 (18.0-21.1)	92.7 \pm 1.2 (86.8-102.1)

The relationship between the wet and dry weight of worms determined at the start of the test was;

$$\text{Dry Weight (mg)} = (0.148 \pm 0.006) \text{ Wet Weight (mg)}$$

($F_{1,2} = 582.313$, $P = 0.002$, $r^2 = 0.997$).

This relationship was used to calculate the initial dry weight of worms in each replicate for comparison with final dry weights. There were no significant differences in the initial dry weight of worms between treatments (ANOVA; $F_{5,24} = 1.245$, $P = 0.319$).

Over the six-week exposure period there were significant changes in the number of individuals (paired t -tests, $\alpha = 0.05$, $t_4 > |5.431|$, $P < 0.007$ in all cases) and the biomass (paired t -tests, $\alpha = 0.05$, $t_4 > |7.191|$, $P < 0.003$ in all cases) of *L. variegatus* in all treatments and in unmodified filtered water, with some treatments displaying increases while others

showed decreases. Salinity had a significant effect on both the change in the number of individuals (ANOVA; Rank (change in number + 10) transformed data, $F_{5,24} = 86.669$, $P < 0.001$) and the change in biomass (ANOVA; Log_{10} (change in biomass + 10) transformed data, $F_{5,24} = 183.887$, $P < 0.001$) over the six-week exposure period (see Figures 3.4 and 3.5 respectively). Both parameters displayed optimal mean values at $3000 \mu\text{S cm}^{-1}$, though these were not significantly higher than in unmodified filtered water (Tukey's tests; $P = 0.377$ number, $P = 0.903$ biomass). After six weeks exposure the number of individuals increased from 10 to 43.2 ± 2.8 ($332 \pm 28\%$ increase) in the $3000 \mu\text{S cm}^{-1}$ treatment and 37.6 ± 1.1 ($276 \pm 11\%$ increase) in unmodified filtered water. Worm biomass increased from 6.01 ± 0.28 mg to 22.64 ± 1.78 mg ($280 \pm 33\%$ increase) at $3000 \mu\text{S cm}^{-1}$ and from 6.20 ± 0.19 mg to 20.92 ± 0.42 mg ($240 \pm 16\%$ increase) in unmodified filtered water. At salinities greater than $3000 \mu\text{S cm}^{-1}$ the increase in number of individuals and biomass over the exposure period was reduced, with increasing magnitude of effect with increasing salinity (Figures 3.4 and 3.5 respectively). At $12000 \mu\text{S cm}^{-1}$ and $15000 \mu\text{S cm}^{-1}$ worm biomass after the six-week exposure period declined to below initial values ($28 \pm 2\%$ and $79 \pm 6\%$ reductions respectively). The number of individuals was reduced to below initial numbers ($68 \pm 10\%$ reduction) in the $15000 \mu\text{S cm}^{-1}$ treatment indicating that at this salinity mortality exceeded reproduction. With the exception of the $3000 \mu\text{S cm}^{-1}$ treatment, the increase in number of individuals and biomass in each salinity treatment was significantly less than in unmodified filtered water and all lower salinity treatments (Tukey's tests; $P < 0.026$ number, $P < 0.009$ biomass, in all cases). Significant differences between treatments are indicated in Figures 3.4 and 3.5.

Regression analysis indicated a very strong negative relationship between increasing salinity and the change in number and biomass of worms over the six-week exposure period. Analysis was performed on data excluding unmodified filtered water for both parameters. Change in biomass data was log_{10} transformed and the square root of EC (mS cm^{-1}) was used for the relationship between change in number of individuals and salinity. The relationships were best described by the following equations;

$$\text{Change in number of individuals} = -17.260 \times \sqrt{\text{EC (mS cm}^{-1})} + 62.079$$

$$(F_{1,23} = 261.933, P < 0.001, r^2 = 0.919, \text{SE slope} = 1.066, \text{SE constant} = 3.199)$$

$$\text{Log}_{10} (\text{change in dry weight (mg)} + 10) = (-55 \times 10^{-6}) \times \text{EC } (\mu\text{S cm}^{-1}) + 1.581$$

$$(F_{1,23} = 595.605, P < 0.001, r^2 = 0.963, \text{SE slope} = 2 \times 10^{-6}, \text{SE constant} = 0.022).$$

These relationships were used to calculate the salinity at which a 50% reduction (compared with observed optimal levels) in the increase in number of individuals and biomass of *L. variegatus* would occur after the six-week exposure period (i.e. EC₅₀ values). These EC₅₀ values were 6943 (SE 5323-9036) $\mu\text{S cm}^{-1}$ for increase in number of individuals and 5784 (95% CI 4668-7073) $\mu\text{S cm}^{-1}$ for increase in biomass. The salinity at which growth (biomass increase) would be reduced to zero (i.e. EC₁₀₀) after the six-week exposure period was also calculated using the regression relationship. This value was 10564 (95% CI 9129-12218) $\mu\text{S cm}^{-1}$. The salinity at which the increase in individuals would be reduced to zero (i.e. EC₁₀₀) was calculated as 12936 (SE 10323-16249) $\mu\text{S cm}^{-1}$. At salinities greater than this (i.e. $>12936 \mu\text{S cm}^{-1}$) mortality is predicted to exceed asexual reproduction of *L. variegatus*.

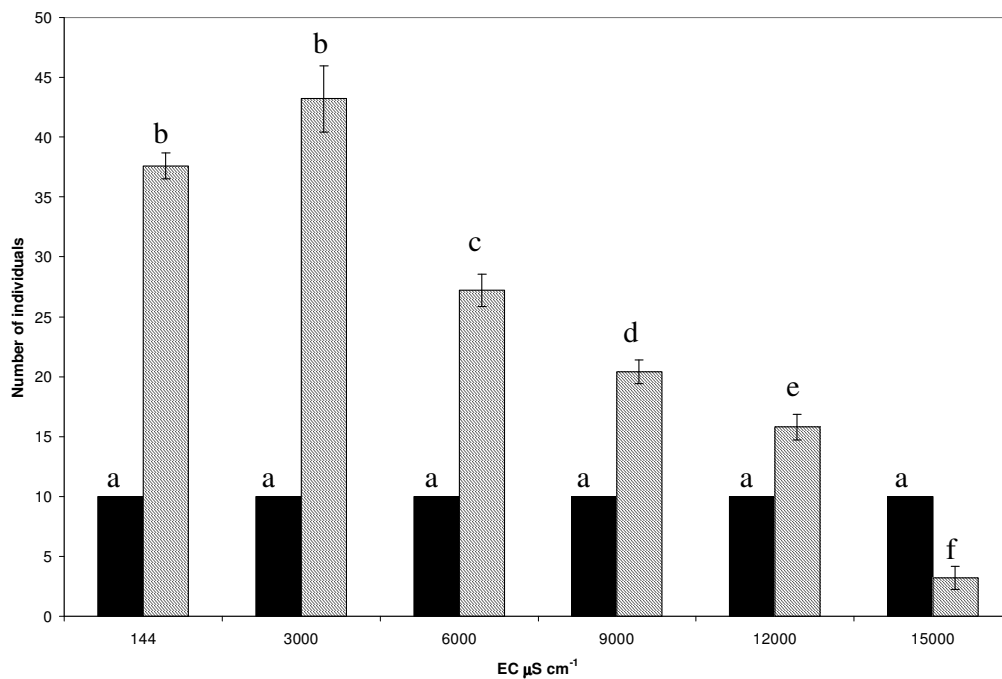


Figure 3.4 Initial (solid) and final (hatched) numbers of *Lumbriculus variegatus* individuals after six weeks exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE. Letters indicate statistically significant differences ($P < 0.05$). Statistical analysis performed on rank transformed data.

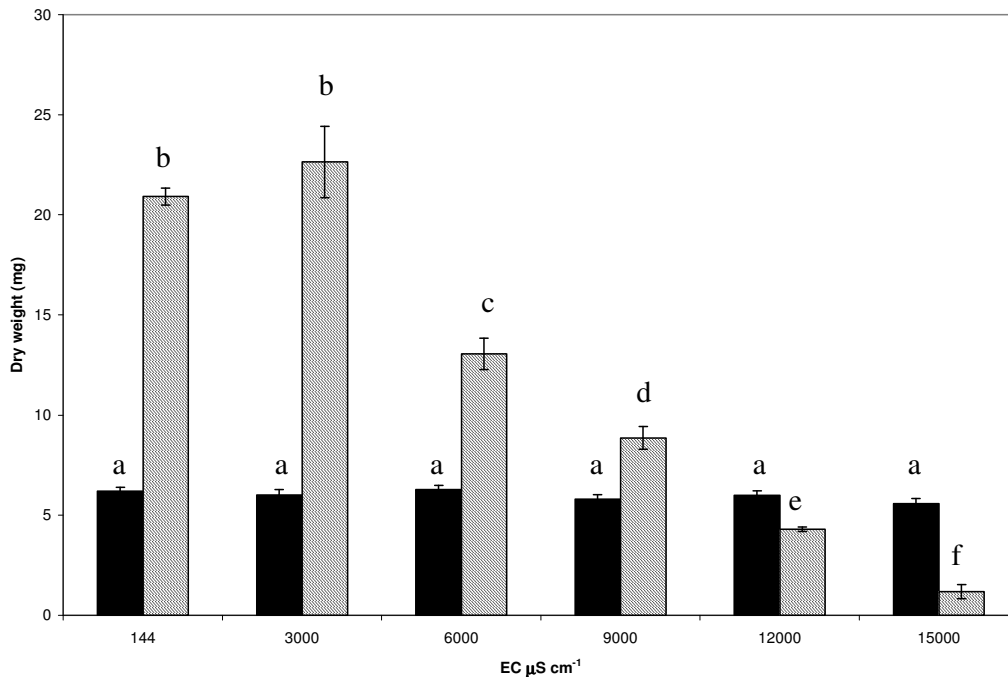


Figure 3.5 Initial and final biomass (mg dry weight) of *Lumbriculus variegatus* after six weeks exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE. Letters indicate statistically significant differences ($P < 0.05$). Statistical analysis performed on $\log_{10}(\text{dry weight} + 10)$ transformed data.

Increased salinity also affected the mean weight of individual worms (Figure 3.6). At the end of the six-week exposure period the mean weight of individual worms in all treatments and in filtered water had decreased from initial values (Figure 3.6) (paired t -tests; $\alpha = 0.05$, t_4 (3 for 15000 $\mu\text{S cm}^{-1}$ treatment) $> |5.630|$, $P < 0.042$ in all cases). With the exception of the 15000 $\mu\text{S cm}^{-1}$ treatment the final mean individual weight of worms decreased with increasing salinity (Figure 3.6). The final mean individual weight of worms in the 15000 $\mu\text{S cm}^{-1}$ treatment was greater than worms at 12000 $\mu\text{S cm}^{-1}$. This was due to a few larger individuals in replicates where worm numbers were greatly reduced. One 15000 $\mu\text{S cm}^{-1}$ replicate with only one worm present at the end of the exposure period was excluded as an extreme outlier. ANOVA of rank transformed data indicated a significant difference in mean individual worm size between treatments ($F_{5,23} = 17.147$, $P < 0.001$). With the exception of 3000 $\mu\text{S cm}^{-1}$ all treatments displayed lower final mean weights than worms in unmodified filtered water (Tukey's test; $\alpha = 0.05$, $P < 0.007$ in all cases). There were no differences in initial mean individual weight between treatments (ANOVA; $F_{5,24} = 1.241$, $P = 0.321$).

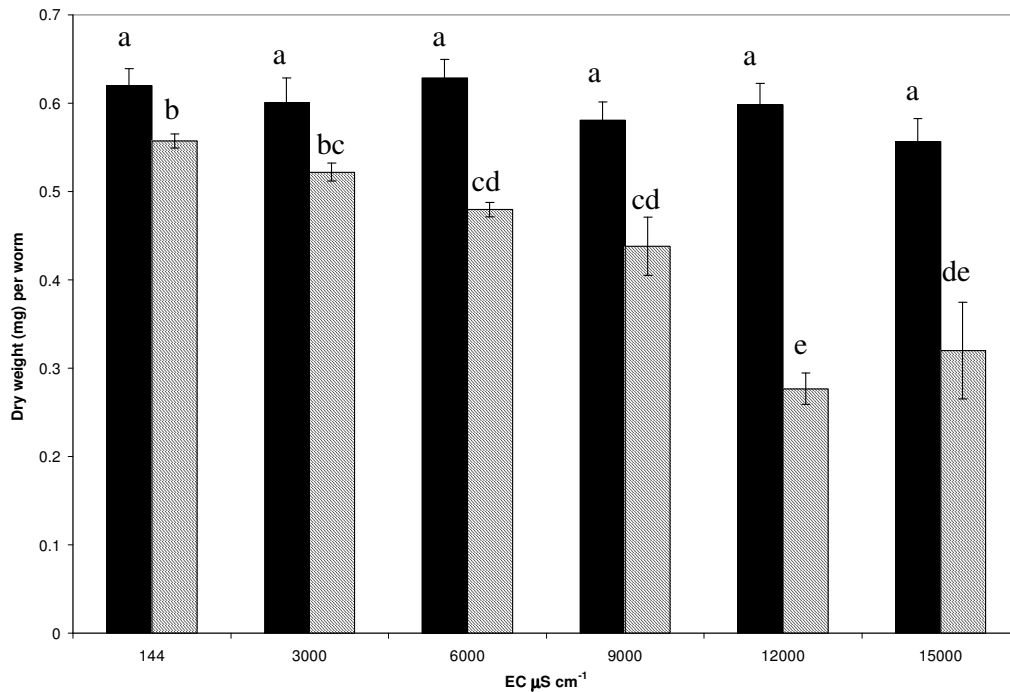


Figure 3.6 Initial (solid) and final (hatched) mean dry weight of *Lumbriculus variegatus* individuals after six weeks exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE. Extreme outlier excluded from final mean weight at 15000 EC $\mu\text{S cm}^{-1}$. Letters indicate statistically significant differences ($P < 0.05$). Statistical analysis performed on rank transformed data.

The daily rate of increase in the number of individuals and biomass that occurred in each replicate during the six-week exposure period were calculated. These rates were not calculated for biomass in the 12000 $\mu\text{S cm}^{-1}$ and 15000 $\mu\text{S cm}^{-1}$ treatments, or number of individuals in the 15000 $\mu\text{S cm}^{-1}$ treatment as values decreased rather than increased over the exposure period. As would be expected the effects of salinity on these rates reflect the effects of salinity on the number of individuals and biomass *per se*. ANOVA indicated significant effects of salinity on the rate of increase in both number and biomass ($F_{4,20} = 59.239$, $P < 0.001$ and $F_{3,16} = 40.728$, $P < 0.001$, respectively). Both rates displayed optimal mean values at 3000 $\mu\text{S cm}^{-1}$ though these were not significantly higher than rates observed in unmodified filtered water (Tukey's tests; $\alpha = 0.05$, $P = 0.466$ number, $P = 0.710$ biomass). Rates decreased significantly with increasing salinity with biomass the most strongly affected (Figure 3.7). Tukey's pair-wise comparisons indicated that the rate of increase in number of individuals and biomass at each salinity treatment, excluding 3000 $\mu\text{S cm}^{-1}$, were significantly lower than the rates in unmodified filtered water and all lower salinity treatments ($\alpha = 0.05$, $P < 0.023$ number, $P < 0.022$ biomass, in all cases). At each salinity the rate of increase in the

number of individuals was greater than the rate of increase in biomass (Figure 3.7, paired t -tests; $t_4 > 3.056$, $P < 0.039$ in each case). The optimal rate of increase in number of individuals observed at $3000 \mu\text{S cm}^{-1}$ was $3.5 \pm 0.2\%$ per day. This was reduced by approximately a third at $6000 \mu\text{S cm}^{-1}$ to $2.4 \pm 0.1\%$, by half at $9000 \mu\text{S cm}^{-1}$ to $1.7 \pm 0.1\%$ and by two thirds at $12000 \mu\text{S cm}^{-1}$ to $1.1 \pm 0.2\%$. The optimal rate of increase in biomass observed at $3000 \mu\text{S cm}^{-1}$ was $3.1 \pm 0.2\%$. This was reduced by approximately a third at $6000 \mu\text{S cm}^{-1}$ to $1.7 \pm 0.2\%$ and by two thirds at $9000 \mu\text{S cm}^{-1}$ to $1.0 \pm 0.1\%$.

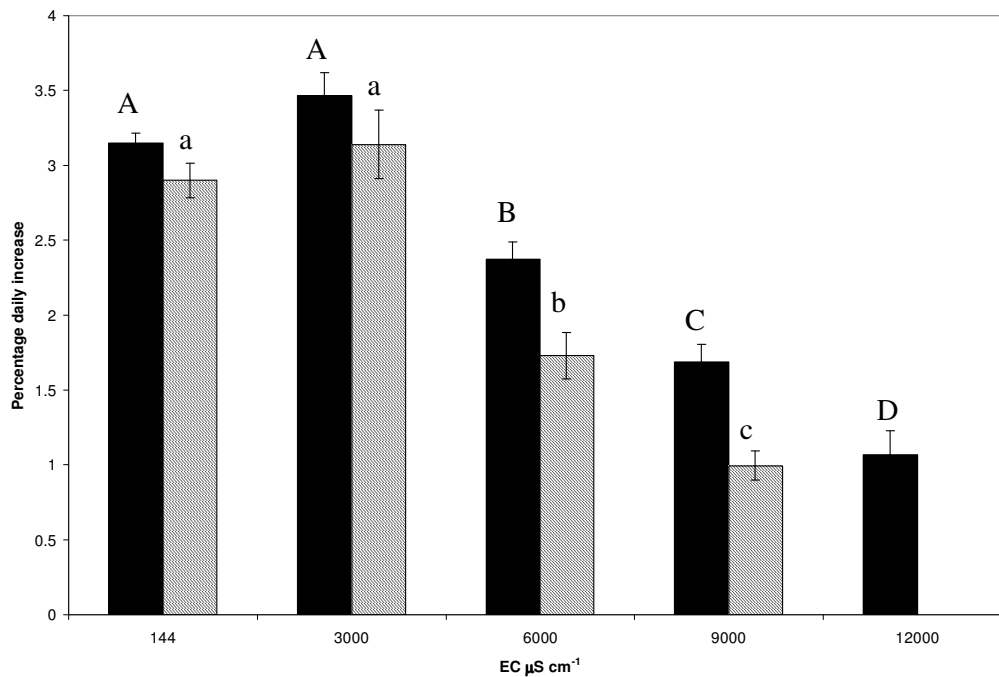


Figure 3.7 Daily rate of increase (%) in number of individuals (solid) and biomass (hatched) of *Lumbriculus variegatus* at different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE. Letters indicate statistically significant differences ($P < 0.05$).

The time required for doubling of the number of individuals and biomass in each replicate was calculated using the daily rates of increase. Again the effects of salinity on these rates reflect the effects of salinity on the number of individuals and biomass *per se*, with observed optimal doubling times at $3000 \mu\text{S cm}^{-1}$ decreasing with increasing salinity and biomass the most strongly affected (Figure 3.8). Salinity affected the doubling time for both number (ANOVA; rank transformed data, $F_{4,20} = 51.982$, $P < 0.001$) and biomass (ANOVA; \log_{10} transformed data, $F_{3,16} = 46.109$, $P < 0.001$). The doubling time for number of individuals was greater at each salinity (though not significant at $3000 \mu\text{S cm}^{-1}$) than the doubling time

for biomass (paired t -test; $t_4 > |3.711|$, $P < 0.022$ in all cases except $3000 \mu\text{S cm}^{-1}$; $t_4 = -2.100$, $P = 0.104$). The optimal doubling time for number of individuals observed at $3000 \mu\text{S cm}^{-1}$ was 20.2 ± 0.9 days which increased with increasing salinity to a maximum of 71.9 ± 11.9 days at $12000 \mu\text{S cm}^{-1}$. The optimal doubling time observed for biomass was 22.7 ± 2.0 days at $3000 \mu\text{S cm}^{-1}$ which increased to 72.3 ± 6.9 days at $9000 \mu\text{S cm}^{-1}$. Doubling times for worms exposed to salinities of $6000 \mu\text{S cm}^{-1}$ and greater were significantly longer than for those in unmodified filtered water and $3000 \mu\text{S cm}^{-1}$ (Tukey's tests; $P < 0.003$ and $P < 0.001$ number respectively, $P < 0.002$ and $P < 0.001$ biomass respectively).

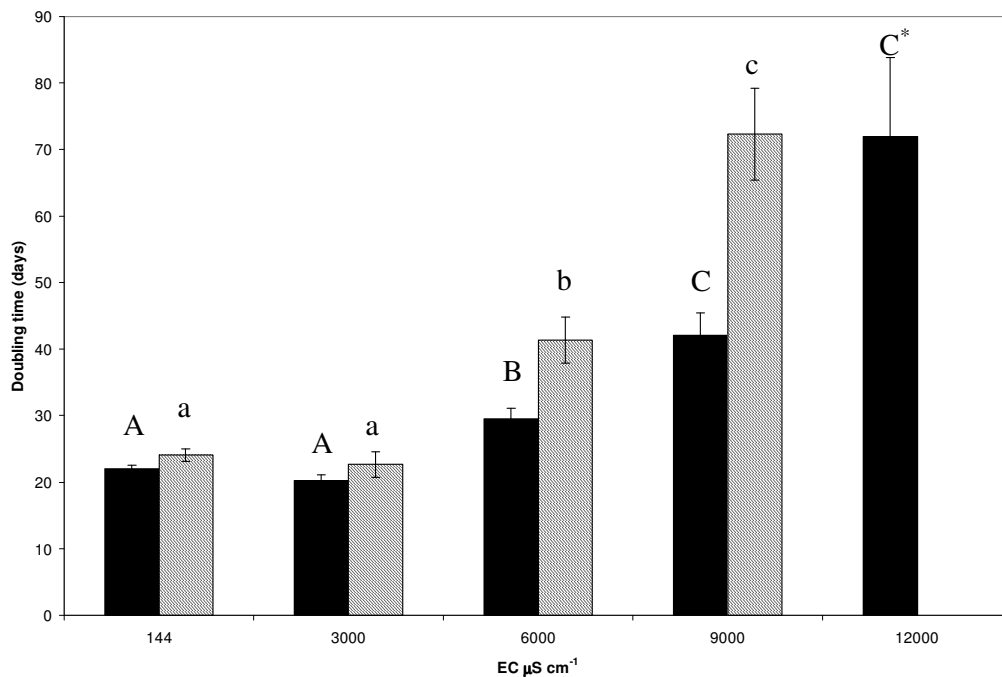


Figure 3.8 Time required for doubling of the number of individuals (solid) and biomass (hatched) of *Lumbriculus variegatus* at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE. Letters indicate statistically significant differences ($P < 0.05$). Statistical analyses performed on rank (number) and \log_{10} (biomass) transformed data. * Significantly greater than doubling time at $9000 \mu\text{S cm}^{-1}$ at a 90% confidence level ($P = 0.052$).

3.3.4 Relationship between lethal and sublethal salinity tolerance

The effects of salinity on the increase in number of individuals (survival/reproduction) and biomass (growth) of *L. variegatus* were evident at much lower salinities than levels affecting short-term survival (Figure 3.9). The EC₅₀ for biomass increase (which was the most sensitive sublethal end point) occurred at approximately 38% of the acute LC₅₀ of the species (EC₅₀:96-hour LC₅₀ = 0.38) (Table 3.5). That is, there was a 50% reduction in the growth rate of *L. variegatus* over the 6-week exposure period at a salinity approximately 62% (9500 $\mu\text{S cm}^{-1}$) lower than the 96-hour LC₅₀ value determined for this species. This ratio (i.e. EC₅₀:96-hour LC₅₀) was 0.45 for the increase in number of individuals (Table 3.5). That is, the survival/reproduction of *L. variegatus* was reduced by 50% at a salinity approximately 55% (8500 $\mu\text{S cm}^{-1}$) lower than the 96-hour LC₅₀.

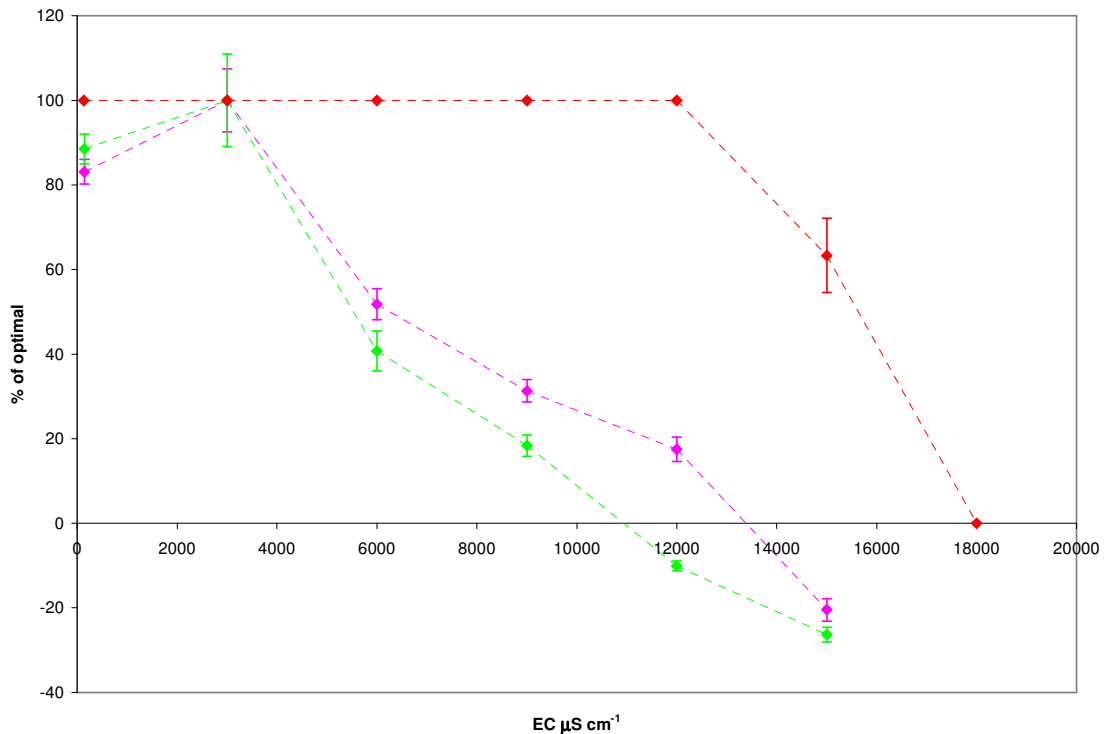


Figure 3.9 The proportional effects of salinity on 96-hour survival (red), growth (biomass) (green) and survival/reproduction (increase in number of individuals) (pink) of *Lumbriculus variegatus* after 6 weeks exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

Mortality was predicted to exceed reproduction at salinities greater than 12936 (SE 10323-16249) $\mu\text{S cm}^{-1}$, a salinity which results suggest would have minimal effects on short-term survival of the species (Figure 3.9). The biomass of *L. variegatus* was predicted to decline rather than increase at salinities greater than 10564 (95% CI 9129-12218) $\mu\text{S cm}^{-1}$, while no effects on short-term survival of the species were evident at salinities up to and including 12000 $\mu\text{S cm}^{-1}$ (Figure 3.9). Lethal and sublethal salinity tolerances of *L. variegatus* and other freshwater Oligochaete species observed in this and other studies are summarised in Table 3.6.

Table 3.5 Summary of the salinity tolerance of *Lumbriculus variegatus* and the ratio between sublethal EC_{50} values and the 96-hr LC_{50} (Test 1) for the species.

Endpoint	Observed optimal EC ($\mu\text{S cm}^{-1}$)	LOEC ($\mu\text{S cm}^{-1}$)	Salinity (EC $\mu\text{S cm}^{-1}$)	Ratio ($\text{EC}_{50}/96\text{-hr LC}_{50}$)
96 hr survival LC_{50} (Test 1)	136-12000	15000	15255 (95% CI 14720-15809)	-
96 hr survival LC_{50} (Test 2)	143-12000	15000	15462 (95% CI 14946-15996)	-
30 day starvation	136-9000	12000	-	-
42 day growth (biomass) EC_{50}	3000	6000	5784 (95% CI 4667-7072)	0.38
42 day growth (biomass) EC_{100}	-	-	10564 (95% CI 9129-12218)	-
42 day survival/reproduction EC_{50}	3000	6000	6943 (SE 5323-9036)	0.46
42 day survival/reproduction EC_{100}	-	-	12936 (SE 10323-16249)	-
42 day individual biomass	144	6000	-	-

Table 3.6 Summary of values for Oligochaete salinity tolerance. Bold indicated values converted from TSS to EC as per Hart *et al.* (1991). *Indicates estimated/recalculated values. # Indicates values converted from NaCl tolerance to “Ocean Nature equivalent salinity” as per Kefford *et al.* (2004b).

Family	Species	Endpoint	EC (mS cm ⁻¹)	Source
Lumbriculidae	<i>Lumbriculus variegatus</i>	Time-independent LC ₅₀ @ 20°C	15.3 (95% CI 14.7-15.8)	This study
		42-day survival/reproduction EC ₅₀ @ 20°C	6.9 (SE 5.3-9.0)	This study
		42-day growth EC ₅₀ @ 20°C	5.8 (95% CI 4.7-7.1)	This study
		96-hr LC ₅₀ @ 20°C	15	Kefford <i>et al.</i> (2006a)
		15-day LC ₅₀ @ 18°C	11.5*	Berezina (2003)
		15-day 90-100% survival @ 18°C	≥6.2,<9.3	Berezina (2003)
Naididae	<i>Stylogrilus heringianus</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
	<i>Nais variabilis</i>	NaCl 48-hr LC ₅₀ @ 12°C	3.8* (9.2#)	Hamilton <i>et al.</i> (1975)
	<i>Stylaria lacustris</i>	15-day 90-100% survival @ 18°C	≥11.9	Berezina (2003)
Tubificidae	<i>Branchiura sowerbyi</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
		72-hr LC ₅₀ @ 20°C	>6.4	Kefford <i>et al.</i> (2006a)
	<i>Ilyodrilus templetoni</i>	93-hr LC ₅₀ @ 10°C	11.0	Chapman & Brinkhurst (1980)
		15-day 90-100% survival @ 18°C	≥6.2,<11.9	Berezina (2003)
	<i>Limnodrilus hoffmeisteri</i>	96-hr LC ₅₀ @ 10°C	14.7	Chapman & Brinkhurst (1980)
		96-hr LC ₅₀ @ 10°C	14.7	Chapman <i>et al.</i> (1982)
		NaCl 96-hr LC ₅₀ @ 21°C	9.1 (14.1#)	Wurtz & Bridges (1961)
		NaCl time-independent (6-day) LC ₅₀ @ 21°C	8.5 (13.4#)	Wurtz & Bridges (1961)
		96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
		96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
		96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
	<i>Quistadrilus multisetosus</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
		96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
	<i>Rhyacodrilus montana</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)
96-hr LC ₅₀ @ 10°C		11.0	Chapman <i>et al.</i> (1982)	
<i>Spirosperma ferox</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)	
	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)	
<i>Spirosperma nikolskyi</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)	
	15-day 90-100% survival @ 18°C	≥9.3,<11.9	Berezina (2003)	
<i>Tubifex tubifex</i>	96-hr LC ₅₀ @ 10°C	13.2	Chapman <i>et al.</i> (1982)	
	72-hr LC ₅₀ @ 22°C	11.8*	Stycznska-Jurewicz (1972)	
	Egg production EC ₅₀ @ 22°C	5.9*	Stycznska-Jurewicz (1972)	
	NaCl 96-hr LC ₅₀ @ 30°C	2.9 (8.6#)	Khangarot (1991)	
	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)	
	<i>Varichaeta pacifica</i>	96-hr LC ₅₀ @ 10°C	11.0	Chapman <i>et al.</i> (1982)

3.4 Discussion

3.4.1 Acute salinity tolerance

The acute salinity tolerance of *L. variegatus* observed in this study was higher than that observed for the same species sourced from a population in Russia (Berezina 2003) but was highly comparable to that observed for *L. variegatus* from the Murray-Darling Basin (Kefford *et al.* 2006a) (see Table 3.6). The observed acute tolerance for *L. variegatus* was higher than values reported for other species of freshwater oligochaetes (see Table 3.6). This difference maybe attributed to differences in the pre-test salinity exposure of the test organisms. The study by Berezina (2003) was conducted with worms collected from low salinity sites in Russia, and with the exception of the study by Khangarat (1991) in India, all available data for other species of oligochaete are for worms collected in Europe and North America. The pre-test salinity exposure of *L. variegatus* used in this study is unconfirmed as specimens were obtained from a commercial source. However, the salinities of aquatic systems in Australia are generally higher than in Europe and North America (Williams and Wan 1972). Despite the test organisms being acclimated at a low salinity ($\approx 140 \mu\text{S cm}^{-1}$) for several weeks prior to testing, pre-test high salinity exposure may have influenced their salinity tolerance. Chapman and Brinkhurst (1980) found higher salinity tolerance in an estuarine population of *Limnodrilus hoffmeisteri* ($\text{LC}_{50} \approx 21300 \mu\text{S cm}^{-1}$) than in those collected from the lower salinity of the same river ($\text{LC}_{50} 14700 \mu\text{S cm}^{-1}$). Australian populations of *L. variegatus* may also be genetically adapted to higher salinities. Reynoldson *et al.* (1996) found significant genetic based variability between populations of *Tubifex tubifex* from Canada and Spain in their response to temperature and tolerance to toxicants.

The higher salinity tolerance of *L. variegatus* in this study compared to the study by Berezina (2003) and the high relative tolerance to values reported for other species (Table 3.6) may also be attributable to differences in test conditions. Factors such as test temperature and the type of salt used can affect salinity tolerance (James *et al.* 2003). This is supported by the similarity of tolerances for *L. variegatus* observed in this study and the study by Kefford *et al.* (2006a) who tested at the same temperature and used the same salt. These conditions may have been particularly favourable for the species in general and/or these individuals.

The addition of shredded tissue to the test chambers did not appear to provide any benefit for increasing salinity tolerance. This is despite several other studies finding increased tolerance

of oligochaetes to salinity and toxicants in the presence of natural sediments (Chapman *et al.* 1982). Likewise the addition of food did not appear to increase survival. It was evident from the results of the starvation test that *L. variegatus* can survive long periods without food. *L. variegatus* was able to tolerate the reduced water quality (primarily reduced dissolved oxygen concentration) that resulted from tissue and food addition without a reduction in salinity tolerance. This can be attributed to the capability of the species to tolerate low oxygen conditions (V. Mattson pers. comm. in Sibley *et al.* 1999). Although the poor water quality did not affect short-term survival, fine gravel was substituted for shredded tissue in the long-term tolerance test to avoid any confounding effects of poor water quality on sublethal parameters.

Partial survival in only one salinity treatment reduced precision of LC₅₀ estimation and prohibited calculation of other estimates (e.g. LC₀₁, LC₁₀, etc.). This distribution necessitated the use of a non-parametric method (i.e. Spearman-Kärber) for calculation of the LC₅₀. Inclusion of additional treatments between 12000 and 18000 $\mu\text{S cm}^{-1}$ would reduce this problem.

In this study, most mortality occurred within the first 96 hours of exposure. It was therefore unnecessary to extend the exposure period beyond the standard 96 hours to ensure the full acute mode of toxicity was accounted for and not erroneously attributed to longer-term effects. Wurtz and Bridges (1961) found acute toxicity of salinity to *Limnodrilus hoffmeisteri* to stabilise over the slightly longer period of 144 hours (6 days).

3.4.2 Starvation test

Although there appeared to be some effect of salinity evident at 12000 $\mu\text{S cm}^{-1}$, the starvation test with *L. variegatus* was largely unsuccessful. This was due to the biology of the species being unsuitable for this type of test. The ability of the species to withstand long periods without food resulted in an excessively long test duration before any effects became evident and slow body degeneration rather than outright mortality made end points extremely difficult to define. Worms in unmodified filtered water survived without mortality and without any increase in numbers for up to 42 days at which point the test was terminated. Phipps *et al.* (1993) also observed long survival of *L. variegatus* without food with worms maintained in lake water surviving for 35 days. Berezina (2003) found *L. variegatus* to survive in distilled water without food for 30 days. Many authors have observed the type of body degeneration observed in this study in other oligochaete species when exposed to toxicants and

environmental stressors (Styczynska-Jurewicz 1972; Chapman *et al.* 1982; Khangarot 1991). Chapman *et al.* (1982) observed that this effect was not stressor specific.

The significantly greater increase in number of individuals in the 12000 $\mu\text{S cm}^{-1}$ treatment during the starvation test appears to indicate salinity-related stress. It is likely that the increase in numbers was a result of fission due to weight loss rather than increased reproductive effort in response to stress. This effect was not evident at 15000 $\mu\text{S cm}^{-1}$, this was perhaps due to the least tolerant individuals dying at this salinity during acute exposure and also due to difficulties in counting the number of individuals due to body degeneration. Weight measurements of worms in addition to monitoring mortality may have been useful in elucidating response mechanisms and in further detection of salinity effects.

It is interesting to note that the high levels of reproduction that occurred at lower salinities during the long-term tolerance test were not observed during the starvation test. This perhaps suggests that while *L. variegatus* is able to tolerate long periods without food it is unable to reproduce under these conditions. The lack of reproduction may also have been related to the absence of substrate.

3.4.3 Sublethal salinity tolerance

The sublethal effects of salinity on *L. variegatus* were evident at much lower salinities than those affecting short-term survival. The study of *T. tubifex* by Styczynska-Jurewicz (1972) also found sublethal effects to occur at salinities much lower than those affecting short-term survival. The number of eggs produced per individual decreased rapidly from a maximum at a salinity of 1 g L^{-1} ($\approx 1471 \mu\text{S cm}^{-1}$) and approached zero at 5 g L^{-1} ($\approx 7353 \mu\text{S cm}^{-1}$) with the EC_{50} at approximately 4 g L^{-1} ($\approx 5882 \mu\text{S cm}^{-1}$), while the 72-hour LC_{50} for the species was approximately 7 g L^{-1} ($\approx 10294 \mu\text{S cm}^{-1}$) (see Table 3.6) (Styczynska-Jurewicz, 1972). The effect of salinity on reproduction of *T. tubifex* (Styczynska-Jurewicz, 1972) displayed a similar pattern of effect to that observed for *L. variegatus* during this study, with optimal values at salinities slightly higher than tap water and steadily decreasing values with increasing salinity rather than a sharp threshold effect as was observed for short-term survival.

The continuous nature of the effect of salinity on the investigated sublethal end points, suggests that any change in salinity is likely to affect reproduction/survival and growth of this species. Therefore it is necessary to consider the magnitude of effect occurring at any given salinity.

Whilst the observed optimal salinity for reproduction/survival and growth of *L. variegatus* observed in this study was 3000 $\mu\text{S cm}^{-1}$, the actual optimal salinity for these parameters may occur anywhere between the next lowest and highest treatment levels. That is, between the salinity of unmodified filtered water ($\approx 144 \mu\text{S cm}^{-1}$) and 6000 $\mu\text{S cm}^{-1}$. It is likely that the true optimal salinity for reproduction/survival and growth of *L. variegatus* is lower than 3000 $\mu\text{S cm}^{-1}$ as lower salinity is more reflective of levels that occur in freshwater ecosystems. Several other studies have observed optimal growth, development and reproduction of macroinvertebrate species at slightly elevated salinities (see Kefford and Nuggeoda 2005; Kefford *et al.* 2006b; Hassell *et al.* 2006). As proportional effects (e.g. EC_{50} values) were calculated in relation to the observed optimal at 3000 $\mu\text{S cm}^{-1}$, occurrence of the true optimal at a lower salinity would mean that the calculated proportional effects underestimated the sublethal impacts of salinity. As optimal conditions could occur at lower salinity levels it is possible that significant effects of salinity were occurring at the 3000 $\mu\text{S cm}^{-1}$ treatment level. Inclusion of more treatments at lower salinities would provide greater insight to these issues. Additional low salinity treatments were not included in this study due to a primary focus on determining sublethal effects at upper tolerance limits and logistical limitations.

While there were differences in pH between treatments (in addition to differences in salinity), these differences were only significant between the highest and lowest salinity treatments (i.e. unmodified filtered water and 15000 $\mu\text{S cm}^{-1}$). pH alone does not account for the observed differences in survival/reproduction and growth between treatments as effects were evident in treatments without significant differences in pH. However increased pH may have contributed to the observed effects. pH and salinity are interrelated, as salinity increases so does pH. This is observed in natural water bodies (Environment Protection Authority Victoria, unpublished data). Allowing pH to increase in conjunction with salinity more accurately reflects natural conditions.

The magnitude of the effect of salinity on reproduction/survival and growth of *L. variegatus* observed during this study was related to the duration of salinity exposure. The length of the test was a compromise between attempting to minimise the influence of exposure period and logistical considerations. It is unclear if some recovery would occur if the period of exposure were extended. It seems more likely however that the magnitude of effect would increase with exposure period. If this were the case the sublethal effects of salinity would be greater than the results of this study indicate. Further tests of varied duration are required to investigate the effect of exposure period on the magnitude of sublethal salinity stress.

The observed optimal doubling time for *L. variegatus* observed at 3000 $\mu\text{S cm}^{-1}$ during this study (20 ± 1 days) was longer than that of 10 to 14 days observed by Phipps *et al.* (1993) for the same species under culture conditions in lake water at 20°C. This was most likely due to differing conditions and the smaller average size of worms used in this study (0.6 mg average worm size for this study versus a range of 0.5 to 2.2 mg in that of Phipps *et al.* 1993). It may however, also suggest that this salinity (i.e. 3000 $\mu\text{S cm}^{-1}$) was less than optimal for growth.

Despite some methodological considerations it seems clear that the sublethal effects of salinity observed in this study have the ability to greatly reduce fitness of *L. variegatus* populations, potentially rendering populations non-viable at salinities much lower than those affecting mortality in the short term. Both growth and reproduction were reduced to zero at salinities having minimal effect on short-term survival of the species.

3.4.4 Relative salinity tolerance

The relative salinity tolerance of *L. variegatus* compared to other Australian freshwater oligochaetes is unclear, as there are no tolerance data available for other species. With the exception of one species from a hypersaline lake, the maximum field salinities observed for Australian oligochaetes by Bailey *et al.* (2002) were well below those affecting the short-term survival of *L. variegatus* during this study. Berezina (2003) found *L. variegatus* to be the least tolerant of acute salinity exposure of the four Russian species of oligochaete investigated (see Table 3.6). However, *L. variegatus* were collected from a lower salinity site than the other species investigated in the study (Berezina 2003). Endemic Australian species may be expected to be more salt tolerant than *L. variegatus* (an introduced species from the Northern Hemisphere) due to evolutionary adaptation to historical periods of high salinity in Australia (Hart *et al.* 1991). However, *L. variegatus* may display a higher sublethal salinity tolerance than other species of freshwater oligochaete due to its primarily asexual mode of reproduction. Kinne (1964) found sexual reproduction in aquatic invertebrates to be more affected by increased salinity than asexual reproduction.

As highlighted by the differences in acute salinity tolerance of *L. variegatus* between the study by Berezina (2003) and this study, different populations of the same species may be expected to display differences in salinity tolerance. However, the degree of variation in acute salinity tolerance for the same species of freshwater oligochaetes observed in different studies (Table 3.6) is quite limited. Without data for comparison it is difficult to predict the amount of variation in sublethal responses to salinity that may be expected between

populations and whether the relationship between sublethal effects and acute toxicity remains constant. The EC₅₀/LC₅₀ ratio displayed by *T. tubifex* (0.57) (Styczniska-Jurewicz 1972) was comparable to those observed in this study for *L. variegatus* (0.38 and 0.46).

3.5 Conclusions

Results of this study suggest that any change in salinity is likely to affect the population dynamics (growth, reproduction etc.) of *L. variegatus* and that significant effects are likely to occur at salinities much lower than those affecting short-term survival of the species. The magnitude of effects may vary with duration of salinity exposure, environmental conditions and previous salinity exposure of the population. The acute salinity tolerance displayed by *L. variegatus* during this study and comparison with published data for other species (see Table 3.6) suggest that freshwater oligochaetes may be more salt tolerant than suggested by Hart *et al.* (1991) (i.e. limited to salinities $\leq 1000 \text{ mg l}^{-1}$ ($\approx 1471 \text{ }\mu\text{S cm}^{-1}$). However, sublethal effects may reduce fitness of populations at salinities lower than those causing immediate mortality. Further research into the acute and sublethal salinity tolerances of other oligochaete species occurring in Australian freshwater ecosystems is required.

4.0 Salinity tolerance of the aquatic snail *Physa acuta* Draparnaud (Mollusca: Gastropoda: Physidae)

4.1 Introduction

The phylum Mollusca is represented in inland waters by the classes Bivalvia (clams and mussels) and Gastropoda (snails). These groups evolved in marine systems and progressively radiated into freshwater systems via estuaries (Gooderham and Tsyrlin 2002). In freshwater systems, gastropods are more diverse and widely distributed than bivalves. Gastropods are found in a wide range of inland aquatic habitats and can represent a large component of the macroinvertebrate communities of these systems. They can be an important food item for fish, waterfowl and other predators and can influence macrophyte and algal communities through grazing pressure (Dillon *et al.* 2002). While some genera such as *Coxiella* are characteristically found in salt lakes at salinities as high as three times that of seawater ($>150000 \mu\text{S cm}^{-1}$) (Williams 1980; Bailey *et al.* 2002; Pinder 2005), other gastropods are generally restricted to a more freshwater salinity range. Forty-eight percent of reported Australian Mollusca genera were found at salinities less than 3000 mg L^{-1} ($\approx 4400 \mu\text{S cm}^{-1}$) (Bailey *et al.* 2002). Other genera (*Isodorella*, *Physa* and *Potamopygrus*) occurred across a broader salinity range, from fresh to moderately saline (up to $\approx 10500 \mu\text{S cm}^{-1}$) (Bailey *et al.* 2002).

There are two evolutionary distinct groups of gastropods that inhabit inland waters. These are the Prosobranchs that possess gills and an operculum and the Pulmonates, which have evolved a lung for gas exchange. Many freshwater Pulmonates have re-evolved gills. Pulmonate gastropods are considered to be less salinity tolerant than the Prosobranchs (Williams *et al.* 1991).

While there are considerable records of the field distribution of gastropod species with respect to salinity, there have been very few studies of their physiological salinity tolerances. Of the few studies that have investigated the physiological salinity tolerance of freshwater gastropods the majority have focused on short-term lethal effects with very few considering chronic and sublethal effects or the tolerance of potentially sensitive life stages.

Short-term exposure to elevated salinities has been shown to reduce the survival of freshwater gastropods. Kefford *et al.* (2003a, 2005, 2006a) investigated the short-term effects of increased salinity on the survival of several Australian and South African freshwater

gastropod species. Seventy-two hour LC₅₀ values ranged from 9000 $\mu\text{S cm}^{-1}$ for the freshwater limpet *Ferrissia petterdi* (Ancyliidae) up to 15000 $\mu\text{S cm}^{-1}$ for the Lymnaeid snail *Austropeplea tomentosa* (Lymnaeidae). Investigations by Berezina (2003) and Stycznska-Jurewicz (1972) found a similar short-term tolerance range for five freshwater gastropod species from Russia and one from Poland, respectively. Dunlop *et al.* (2008) observed a 72-hour LC₅₀ value of 30600 $\mu\text{S cm}^{-1}$ for the gastropod *Thiara plotiopsis* (Thiridae) collected from populations in Queensland. Seventy-two hour LC₅₀ values of >10000, >15000 and >20000 $\mu\text{S cm}^{-1}$ were observed for snails from the genus *Helicorbis* and two species of Hydrobiidae respectively (Dunlop *et al.* 2008).

Several studies have observed reduced egg hatching success for freshwater gastropod species, at salinities lower than those affecting adult survival (Madsen 1990; Zukowski pers. comm. in Clunie *et al.* 2002; Kefford *et al.* 2004a, 2007a). Fifty percent reduction in hatching success has been found to occur at salinities as much as 79% lower than the adult LC₅₀ of a species (Kefford *et al.* 2007a). The few studies that have investigated the sublethal effects of salinity on freshwater gastropods have observed reduced growth (Madsen 1990; Forshaw 1994 in Marshall and Bailey 2004; Kefford and Nugegoda 2005) and fecundity (Remane and Schlieper 1958 in Kinne 1964; Stycznska-Jurewicz 1972; Madsen 1990; Forshaw 1994 in Marshall and Bailey 2004; Peska 2003; Kefford and Nugegoda 2005) at salinities lower than those affecting adult survival.

This study investigates the acute, chronic and sublethal effects of increased salinity on the freshwater pulmonate gastropod *Physa acuta* Draparnaud (Gastropoda: Physidae). Variability in sublethal responses between populations with different salinity exposure history is also investigated. Thought to have originated in North America, *P. acuta* has become invasive throughout Europe, Africa, South Asia, Japan and Australia (Dillon *et al.* 2002). Its spread has been attributed to the aquarium and aquatic plant industries (Dillon *et al.* 2002). Given the species wide distribution it could “reasonably be nominated as the worlds most cosmopolitan freshwater gastropod” (Dillon *et al.* 2002). As is generally true of pulmonate gastropods *P. acuta* are preferentially outcrossing simultaneous hermaphrodites that are also capable of self-fertilisation (Dillon *et al.* 2002). Fertilisation is internal and eggs are laid within a gelatinous egg mass (Dillon *et al.* 2002). Development is direct, that is, there is no larval phase (Dillon *et al.* 2002). The species is a grazer, feeding on macrophytes and epibenthic algae (Dillon *et al.* 2002). *P. acuta* is widespread throughout lowland rivers, lakes and wetlands of south-eastern Australia (Smith 1996) and is often present in large numbers.

In Australia it has been recorded from salinities up to 11700 $\mu\text{S cm}^{-1}$ (Goonan *et al.* 1992). Kefford *et al.* (2003a, 2006a respectively) has observed 72-hour LC_{50} values of 14000 and 12600 $\mu\text{S cm}^{-1}$ for adult *P. acuta* collected from the Barwon River and a range of Victorian rivers respectively. Dunlop *et al.* (2008) observed a 72-hour LC_{50} value of 15700 $\mu\text{S cm}^{-1}$ for *P. acuta* collected from south east Queensland and Styczniska-Jurewicz (1972) observed a 10-day LC_{50} of $\approx 13200 \mu\text{S cm}^{-1}$ for specimens collected from a population in Poland. Elevated salinity has been shown to affect the growth (Kefford and Nugegoda 2005), fecundity (Styczniska-Jurewicz 1972; Peska 2003; Kefford and Nugegoda 2005) and egg hatching (Zukowski pers. comm. in Clunie *et al.* 2002; Kefford *et al.* 2004a) of *P. acuta*. Styczniska-Jurewicz (1972) investigated osmoregulation of *P. acuta* across a salinity range from freshwater to $\approx 13200 \mu\text{S cm}^{-1}$ finding it to be a hyperosmotic regulator across the investigated salinity range.

4.2 Materials and Methods

4.2.1 Field collection

P. acuta were collected from the Pollocksford and Forrest sites on the Barwon River (see Materials and methods 2.2). Snails were collected from the Forrest site on several occasions and the Pollocksford site one occasion, as required for testing. River salinities at the time of collection of *P. acuta* ranged from 155 to 217 $\mu\text{S cm}^{-1}$ at the Forrest site and was 2365 $\mu\text{S cm}^{-1}$ at the Pollocksford site.

4.2.2 Long-term tolerance

4.2.2.1 Adult survival

The long-term survival of adult *P. acuta* collected from the Forrest site on the West-Barwon River was investigated over a salinity range of 3000 to 17000 $\mu\text{S cm}^{-1}$ (treatment levels; 3000, 7000, 12000, 15000 and 17000 $\mu\text{S cm}^{-1}$), in unmodified filtered water ($137.3 \pm 3.5 \mu\text{S cm}^{-1}$) and at the salinity of the river at time of collection (155 $\mu\text{S cm}^{-1}$). The test was conducted in 5-L glass aquaria containing 4 L of aerated test solution. Snails were randomly distributed between 3 replicates of each treatment, with 15 snails per replicate. Water changes were made once a week and snails were fed frozen lettuce throughout the test. Survival was monitored at 24-hour intervals over a 30-day period. Results were analysed to assess both the acute and longer-term effects of salinity on the survival of *P. acuta*.

4.2.2.2 Egg production

The effect of salinity on *P. acuta* egg production was investigated during the long-term survival test (outlined above). Egg masses produced in each replicate over the 30-day test period were collected and the number of eggs in each mass counted. To correct for variable survival, the number of eggs laid per snail per day was calculated for each replicate.

4.2.3 Egg hatching success

Adult *P. acuta* collected from the Forrest site on the West-Barwon River were maintained in the laboratory at the same salinity as the river at time of collection (165 $\mu\text{S cm}^{-1}$). Egg masses were collected daily and transferred to a test salinity range of 1000 to 15000 $\mu\text{S cm}^{-1}$ (treatment levels; 1000, 3000, 7000, 10000, 12000 and 15000 $\mu\text{S cm}^{-1}$). Eggs were also transferred to a salinity treatment of the same salinity as the river at the time of test specimen collection (165 $\mu\text{S cm}^{-1}$), river water collected at the time of test specimen collection ($182.7 \pm$

2.9 $\mu\text{S cm}^{-1}$) and unmodified filtered water ($137.3 \pm 3.5 \mu\text{S cm}^{-1}$). Hatching success was assessed for three egg masses for each treatment. The test was conducted in multi-well plates. Each plate contained 6 wells of 16-mL volume. Ten millilitres of test solution was added to each well. Egg masses were monitored daily for the number of eggs hatched, time to first hatching and percentage hatching was determined. Hatching was considered to have occurred when juvenile snails had emerged from both the egg and the egg mass. Test solutions were changed every two to three days. Water quality was measured before and after water changes, with test solutions pipetted to a measuring cylinder to enable measurement. Results from this test and the long-term survival test were used to determine the salinity range for a test further investigating the effects of salinity on the reproductive success of *P. acuta*.

4.2.4 Reproductive success

Testing was conducted to further investigate the effects of salinity on the reproductive success of *P. acuta* and to also investigate the influence of past salinity history on salinity tolerance. Adult *P. acuta* were collected from the two sites on the Barwon River, which display different salinity ranges. The Pollocksford site had higher a mean salinity of $1822 \pm 4 \mu\text{S cm}^{-1}$ (min. = 419, max. = $3788 \mu\text{S cm}^{-1}$) during the period of test organism collection (Victorian Water Resources Data Warehouse 2008). Mean salinity at a monitoring site close to the Forrest site was $156 \pm 0.1 \mu\text{S cm}^{-1}$ (min. = 46, max. = $208 \mu\text{S cm}^{-1}$) during the period of test organism collection (Victorian Water Resources Data Warehouse 2008). (See Materials and methods 2.2 for further information on test organism field collection sites.) The effects of salinity on egg production and egg hatching were investigated for snails from both sites. The test salinity range was 1000 to $12000 \mu\text{S cm}^{-1}$ (treatment levels; 1000, 5000, 7000, 9000 and $12000 \mu\text{S cm}^{-1}$) and the salinity of each site at time of collection ($217 \mu\text{S cm}^{-1}$ and $2365 \mu\text{S cm}^{-1}$ for Forrest and Pollocksford sites respectively).

4.2.4.1 Egg production

The number of eggs produced by *P. acuta* at each test salinity was investigated over a 30-day period. Snails from both sites were placed in clear polyethylene bags containing 2 litres of aerated water. Each treatment was conducted in triplicate with 9 snails randomly assigned to each replicate. Inspections were made daily to determine the number of egg masses laid and the number of surviving snails. Snails were fed frozen lettuce and algal fish food flakes throughout the test. Water was changed weekly and egg masses from each replicate collected. Water quality was measured before and after each water change. Egg masses not used for the egg hatching test (see 4.2.4.2) were preserved in 70% ethanol for later determination of the

number of eggs in each mass. Egg counting was conducted with the aid of a stereo microscope. The number of eggs laid per snail per day was calculated for each replicate.

4.2.4.2 Egg hatching success

The hatching success of eggs laid at each test salinity by snails from both collection sites was investigated. Three egg masses from each replicate were collected and transferred to 6 by 16-mL multi-well plates with each well containing 10 mL of test solution of the same salinity at which eggs were laid. To ensure acclimation to the test salinity egg masses laid during the first 5 days of salinity exposure were excluded from the investigation. Water was changed once a week and egg masses monitored daily to determine the number of eggs hatched. Hatching was considered to have occurred when juvenile snails had emerged from both the egg and the egg mass.

4.2.4.3 Cumulative affects on reproduction success

The cumulative effects of salinity on the reproduction success of *P. acuta* from each of the collection sites was assessed by taking into account effects on both egg production and the hatching success of the produced eggs. Egg production for each replicate was converted to a percentage of the mean observed optimal for *P. acuta* from each site and then these values multiplied by the mean proportional hatching success of eggs from that replicate.

4.2.5 Adult growth

The effects of salinity on the growth of adult *P. acuta* from the Forrest site on the West-Barwon River was investigated over a test salinity range of 500 to 10000 $\mu\text{S cm}^{-1}$ (treatment levels; 500, 1000, 3000, 5000, 7000 and 10000 $\mu\text{S cm}^{-1}$), at the salinity of the river at time of collection (188 $\mu\text{S cm}^{-1}$) and in unmodified filtered water (135.2 \pm 4.0 $\mu\text{S cm}^{-1}$). After a 24-hour period without food, snails were blotted dry with tissue and weighed to the nearest 0.1 of a milligram using an analytical balance, before distribution to salinity treatments. A sample of ten individuals was oven dried to constant weight at 60°C to determine the initial wet to dry weight ratio. Eight snails for each salinity treatment were distributed to separate 440-mL plastic containers containing 350 mL of aerated test solution. Water was changed every two to three days and water quality measured before and after water changes. Snails were fed frozen lettuce in excess throughout the test and monitored daily to determine survival. After 30-days exposure at the test salinities snails were wet weighed, shell lengths measured using calipers and dry weight determined. Snails were not fed for the last 24 hours of the exposure period to allow evacuation of the digestive tract.

4.3 Results

4.3.1 Adult survival

Water quality values during the 30-day survival test with *P. acuta* are presented in Table 4.1. Salinity (EC $\mu\text{S cm}^{-1}$) varied significantly between all treatments with the exception of unmodified filtered water and the river salinity treatment (ANOVA; $F_{6,182} = 74111$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in all cases except unmodified filtered water versus river salinity treatment $P = 0.929$). The mean salinity of unmodified filtered water was $137.3 \pm 3.5 \mu\text{S cm}^{-1}$. The mean salinity of the river salinity treatment ($172.7 \pm 3.6 \mu\text{S cm}^{-1}$) was 11% higher than the intended salinity of $155 \mu\text{S cm}^{-1}$ and maximum variation was 41% higher. With the exception of the river salinity treatment mean salinity levels in all other treatment were within 1% of the nominated salinities, with maximum variation less than 5.5%.

Table 4.1 Water quality during 30-day survival test with adult *Physa acuta*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	137.3 ± 3.5 (117.3-181.5)	7.36 ± 0.06 (6.72-7.88)	19.0 ± 0.1 (18.0-19.7)	92.4 ± 0.8 (85.7-100.5)
155	172.7 ± 3.6 (154.8-218.8)	7.30 ± 0.06 (6.84-7.90)	19.0 ± 0.1 (18.2-19.6)	86.6 ± 1.9 (54.9-98.1)
3000	3009 ± 7.9 (2920-3090)	7.24 ± 0.04 (6.88-7.54)	18.9 ± 0.1 (18.2-19.6)	91.6 ± 0.7 (82.8-97.6)
7000	7023 ± 18.3 (6860-7310)	7.40 ± 0.03 (7.00-7.61)	19.0 ± 0.1 (18.3-19.6)	89.9 ± 1.5 (64.5-99.1)
12000	12060 ± 30 (11750-12420)	7.72 ± 0.03 (7.45-8.07)	19.0 ± 0.1 (18.3-19.6)	93.1 ± 0.7 (87.8-101.3)
15000	15140 ± 40 (14760-15810)	7.88 ± 0.02 (7.61-8.06)	19.0 ± 0.1 (18.3-19.7)	92.6 ± 0.8 (85.0-100.2)
17000	17070 ± 30 (17010-17270)	8.01 ± 0.03 (7.83-8.07)	19.3 ± 0.2 (18.7-19.8)	96.1 ± 1.5 (89.8-101.3)

Mean percent oxygen saturation in each treatment was greater than 85%, however levels fell as low as 54.9% saturation in the river salinity treatment. This treatment displayed significantly lower oxygen saturation levels than all other treatments with the exception of the 7000 $\mu\text{S cm}^{-1}$ treatment (ANOVA; $F_{6, 182} = 4.653$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.039$ in all cases).

Mean pH in each treatment increased with increasing salinity (Table 4.1) with significant differences evident between treatments (ANOVA; $F_{6, 182} = 38.640$, $P < 0.001$). The pH levels of unmodified filtered water and the river salinity treatment were significantly lower than that of treatments $\geq 12000 \mu\text{S cm}^{-1}$ (Tukey's pair-wise comparisons $P < 0.001$ in all cases) and differences were also significant between treatments separated by a few salinity levels ($P < 0.05$).

There were no significant differences in water temperature between treatments (ANOVA; $F_{6, 182} = 1.271$, $P = 0.273$). However, mean temperature levels were closer to 19°C than the intended 20°C, though still within the desired $20 \pm 2^\circ\text{C}$ range.

Survival of adult *P. acuta* displayed a typical threshold response to increased salinity (Figure 4.1). After 96 hours exposure, survival remained high in all salinity treatments up to and including 7000 $\mu\text{S cm}^{-1}$, intermediate survival occurred at 12000 and 15000 $\mu\text{S cm}^{-1}$ and was reduced to only $2.2 \pm 2.2\%$ at 17000 $\mu\text{S cm}^{-1}$ (Figure 4.1). All individuals at 17000 $\mu\text{S cm}^{-1}$ had died after 6 days of exposure. ANOVA indicated significantly lower survival in the 15000 and 17000 $\mu\text{S cm}^{-1}$ treatments compared to survival at the salinity of river water at the time of collection (155 $\mu\text{S cm}^{-1}$) at each 24 hour exposure period (ANOVAs; $F_{6,14} = 10.000-91.038$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.05$ in all cases). Mortality generally gradually decreased in each treatment with increasing exposure period. There did appear to be an initial acute phase of mortality up until seven days exposure, after which survival decreased more gradually. This is evident in the plot of LC_{50} with increasing exposure period (Figure 4.2). The time independent acute salinity tolerance of adult *P. acuta* was therefore considered to occur after 7 days exposure. Survival in the river salinity treatment (173 $\mu\text{S cm}^{-1}$) fell below 90% after 10 days exposure and was reduced to $48.9 \pm 5.9\%$ after 30 days exposure. For this reason all logistic regressions for LC_{50} determination were conducted with Abbott's corrected data (see Materials and methods 2.10). The 96-hour salinity LC_{50} value for *P. acuta* was determined as 13171 (95% CI 12816-13529) $\mu\text{S cm}^{-1}$ ($r^2 = 0.410$), the time independent acute

LC₅₀ after 7 days exposure was 11815 (95% CI 11379-12258) $\mu\text{S cm}^{-1}$ ($r^2 = 0.316$) and the 30-day LC₅₀ was 10539 (95% CI 10133-10945) $\mu\text{S cm}^{-1}$ ($r^2 = 0.380$). An increase in LC₅₀ values for exposure periods between 25 and 30 days is evident in Figure 4.3. This was related to correction for high mortality in the low salinity treatments during this period using Abbott's formula. LC₅₀ values for all exposure periods are presented in Appendix A Table A.2.

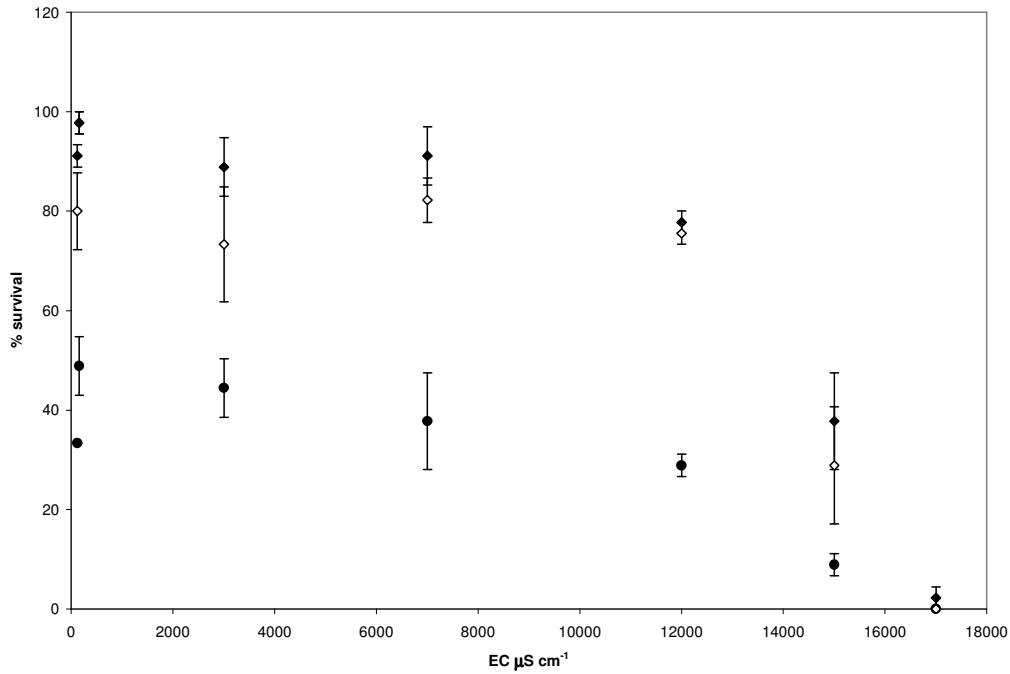


Figure 4.1 Survival of adult *Physa acuta* at different salinities (EC $\mu\text{S cm}^{-1}$) after 4 days (96 hrs) (closed diamonds), 7 days (open diamonds) and 30 days (closed circles) exposure. Mean \pm SE.

For each exposure period, survival in unmodified filtered water ($137.3 \pm 3.5 \mu\text{S cm}^{-1}$) was lower than in the river salinity treatment ($172.7 \pm 3.6 \mu\text{S cm}^{-1}$). T-tests found this difference to be significant ($P < 0.05$) on only 5 of the 24-hourly exposure periods.

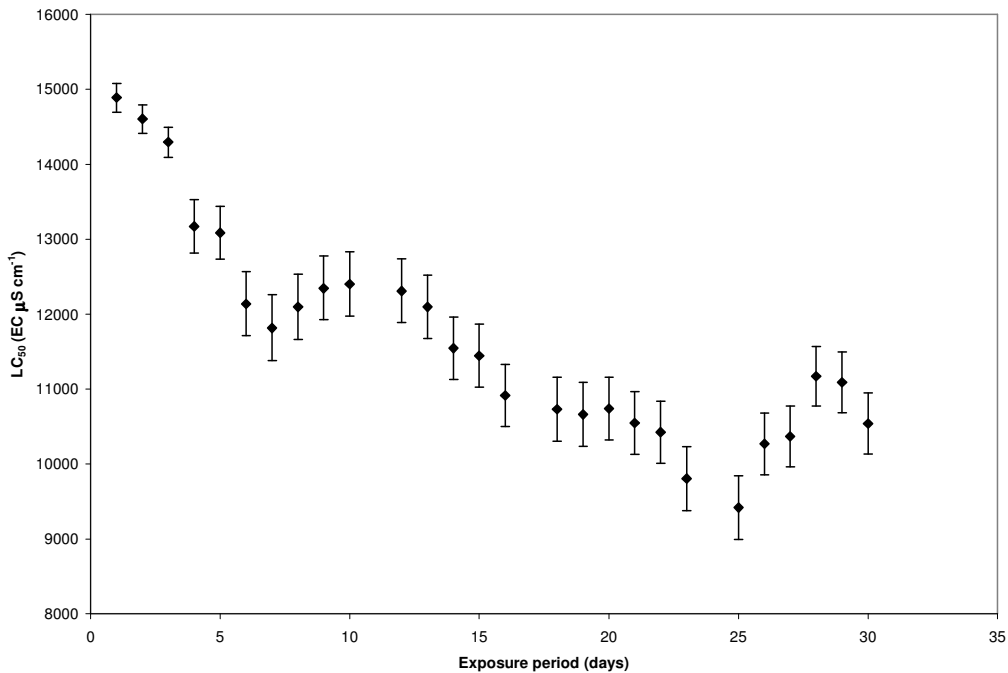


Figure 4.2 Salinity LC₅₀ values (EC μS cm⁻¹) for adult *Physa acuta* after different periods of exposure. Error bars indicate 95% confidence intervals.

4.3.2 Egg production during 30-day survival test

The mean number of eggs produced per individual per day by *P. acuta* during the first 29 days of the 30-day survival test decreased dramatically with increasing salinity (Figure 4.3). Maximum egg production of 8.85 ± 1.08 eggs per snail, per day was observed in the river water salinity treatment ($173 \mu\text{S cm}^{-1}$), with a comparable value of 8.87 ± 0.64 eggs per snail per day observed in unmodified filtered water. No eggs were produced in the 15000 and 17000 $\mu\text{S cm}^{-1}$ treatments and egg production was less than 0.1 eggs per snail per day in the 12000 $\mu\text{S cm}^{-1}$ treatment.

ANOVA indicated significant differences in egg production (number of eggs per snail per day) between salinity treatments (ANOVA; $F_{6, 14} = 55.509$, $P < 0.001$), with production at the river salinity and in unmodified filtered water higher than all other salinities (Tukey's pairwise comparisons $P < 0.003$ in all cases). Linear regression analysis indicated a strong negative relationship between egg production per snail per day and the square root of EC. Analysis was performed with only the linear data range from salinities 173 to 12000 $\mu\text{S cm}^{-1}$. The relationship was best described by the following equation;

Egg production per snail, per day = $-0.095 \times \sqrt{\text{EC}} (\mu\text{S cm}^{-1}) + 9.900$
 ($F_{1, 10} = 107.722$, $P < 0.001$, $r^2 = 0.915$, SE slope = 0.009, SE constant = 0.679).

Using this equation and 50% of the optimum egg production observed at the river water salinity (i.e. 50% egg production per snail, per day = 4.425), the salinity EC_{50} value of *P. acuta* egg production was calculated as 3321 (SE 2127-5121) $\mu\text{S cm}^{-1}$.

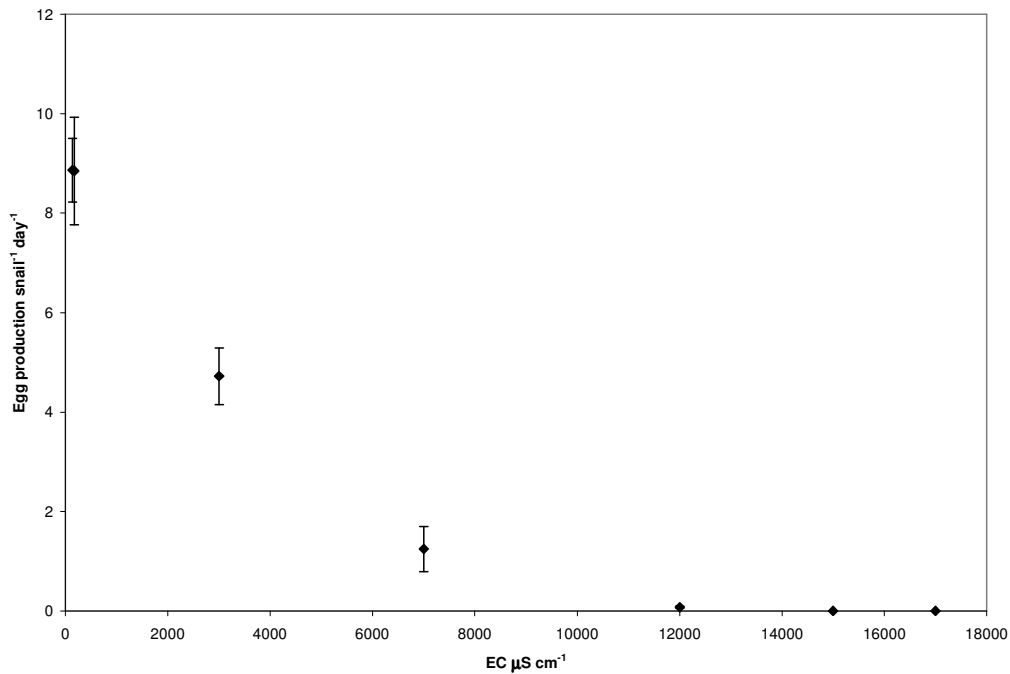


Figure 4.3 Egg production ($\text{snail}^{-1} \text{ day}^{-1}$) by adult *Physa acuta* at different salinities (EC $\mu\text{S cm}^{-1}$) during the first 29 days of the 30-day survival test. Mean \pm SE.

4.3.3 Hatching success of eggs transferred to salinity treatments

With the exception of EC, there were no significant differences in measured water quality parameters between salinity treatments of the transferred egg hatching test with *P. acuta* (ANOVAs; DO $F_{8, 18} = 1.218$, $P = 0.344$, Temperature $F_{8, 45} = 0.113$, $P = 0.999$ and pH $F_{8, 18} = 0.197$, $P = 0.988$). There were no significant differences in salinity between the river salinity treatment and river water or unmodified filtered water (Tukey's pair-wise comparisons $P > 0.063$ in both cases), all other salinities were significantly different (ANOVA; $F_{8, 45} = 349305$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.004$ in all cases). Dissolved oxygen remained above 70% saturation in all treatments (Table 4.2). Despite occasional low values, mean water temperatures were within the 20 ± 2 °C desired range (Table 4.2). pH values ranged between 6.80 and 7.65 (Table 4.2).

Table 4.2 Water quality during egg hatching test with transferred *Physa acuta* eggs. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
River water	182.7 \pm 2.9 (176.0-192.6)	7.11 \pm 0.15 (6.80-7.27)	19.7 \pm 0.2 (18.8-20.0)	90.2 \pm 1.4 (87.8-92.7)
Untreated	124.1 \pm 4.6 (109.4-138.8)	7.16 \pm 0.16 (6.86-7.42)	19.5 \pm 0.3 (18.3-20.1)	84.3 \pm 5.1 (74.4-91.0)
165	167.7 \pm 1.2 (165.0-172.0)	7.08 \pm 0.15 (6.79-7.25)	19.5 \pm 0.3 (18.4-20.1)	88.9 \pm 1.6 (85.8-90.8)
1000	1007 \pm 3 (1000-1020)	7.15 \pm 0.08 (6.99-7.25)	19.4 \pm 0.3 (18.2-20.0)	90.2 \pm 1.2 (88.4-92.4)
3000	2993 \pm 7 (2960-3010)	7.02 \pm 0.16 (6.75-7.30)	19.5 \pm 0.4 (18.1-20.1)	89.0 \pm 0.9 (87.1-90.2)
7000	6995 \pm 4 (6980-7010)	7.11 \pm 0.18 (6.75-7.29)	19.4 \pm 0.5 (17.7-20.3)	91.3 \pm 0.7 (90.3-92.6)
10000	10000 \pm 0 (9990-10020)	7.18 \pm 0.20 (6.77-7.41)	19.4 \pm 0.4 (17.9-20.1)	89.9 \pm 0.2 (89.6-90.2)
12000	12000 \pm 0 (11990-12020)	7.24 \pm 0.23 (6.79-7.55)	19.3 \pm 0.4 (17.7-20.1)	92.2 \pm 2.4 (89.6-97.0)
15000	14970 \pm 30 (14840-15020)	7.28 \pm 0.25 (6.80-7.65)	19.3 \pm 0.4 (18.0-20.0)	91.4 \pm 0.9 (90.3-93.2)

The hatching success of transferred *P. acuta* eggs displayed a typical threshold response to increased salinity, with high hatching success at salinities up to and including 7000 $\mu\text{S cm}^{-1}$, greatly reduced hatching success at 10000 $\mu\text{S cm}^{-1}$ (34.4 \pm 6.6% hatching) and no hatching occurring at 12000 and 15000 $\mu\text{S cm}^{-1}$ (Figure 4.4). ANOVA indicated significant differences in hatching success between treatments, with hatching significantly lower at salinities 10000 $\mu\text{S cm}^{-1}$ and higher (ANOVA; $F_{8, 17} = 40.641$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.001$ in each case). There were no significant differences in hatching success between the river salinity treatment and river water or unmodified filtered water (t -test $P > 0.568$ in both cases). The salinity EC_{50} for hatching success of transferred *P. acuta* eggs determined by logistic regression was 7985 (95% CI 7694-8282) $\mu\text{S cm}^{-1}$ ($r^2 = 0.491$).

Replicate 3 of the 1000 $\mu\text{S cm}^{-1}$ treatment was excluded from all analyses as an outlier as no hatching occurred compared with 100% hatching success for the other replicates for this treatment.

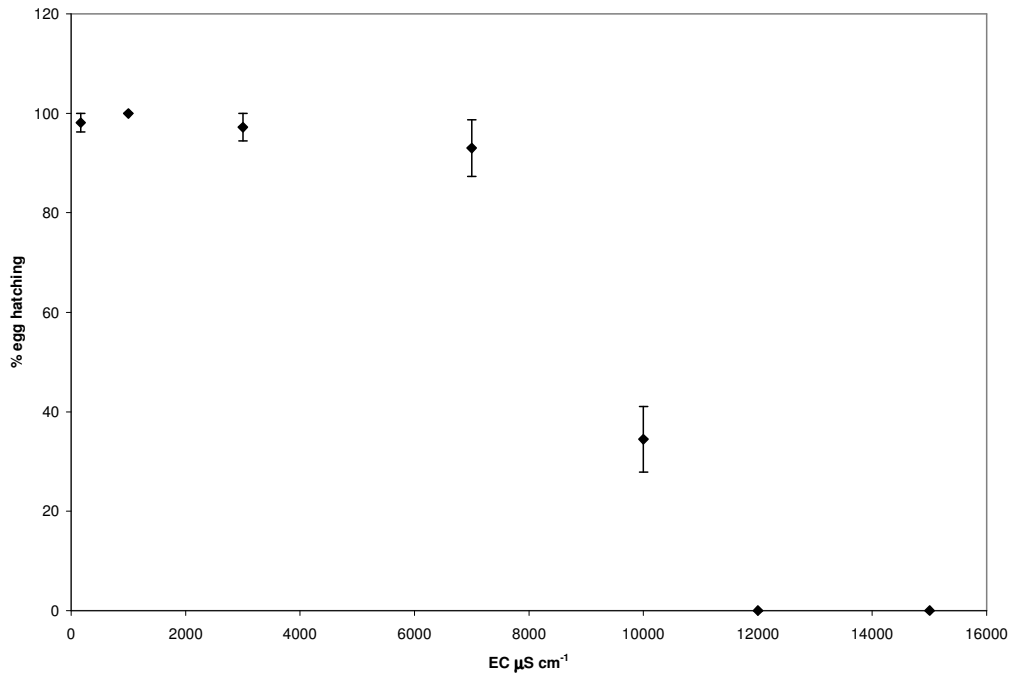


Figure 4.4 Hatching success of *Physa acuta* eggs laid in unmodified filtered water and transferred to different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

The development time of *P. acuta* eggs was also affected by increased salinity, with the time to first hatch increasing from an average of 9 days at lower salinities to 12.7 ± 0.3 days at 10000 $\mu\text{S cm}^{-1}$ (Figure 4.5), an increase of 41%. There was no variability in the time to first hatch between replicates in unmodified filtered water and the 3000 and 7000 $\mu\text{S cm}^{-1}$ treatments, as indicated by the absence of error bars in Figure 4.5. ANOVA indicated that time to first hatch was significantly longer at 10000 $\mu\text{S cm}^{-1}$ than all lower salinities (ANOVA; $F_{8,13} = 62.462$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.001$ in all cases).

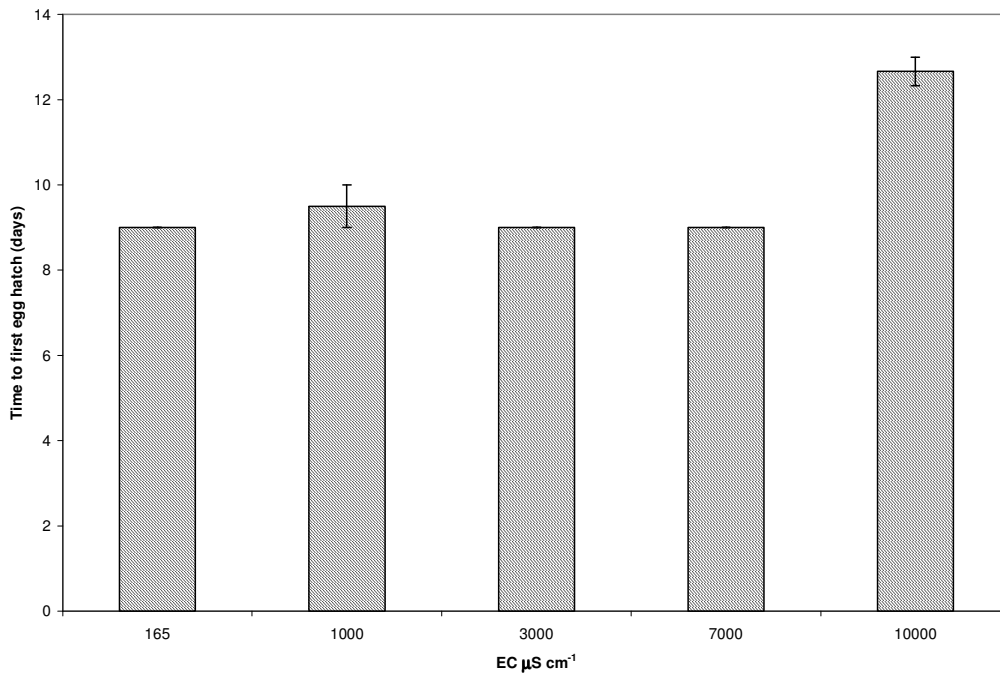


Figure 4.5 Time to first hatch (days) of *Physa acuta* eggs transferred to different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

4.3.4 Reproduction success

There were no significant differences in water quality values for treatments with *P. acuta* from the two different collection sites (t -tests; $t_{12-14} < |0.8|$, $P > 0.437$ in all cases). Values presented in Table 4.3 are for treatments with snails from both sites. Mean treatment salinities were higher than nominal values (Table 4.3). With the exception of the lowest salinity treatment (i.e. river salinity at the Forrest site) mean EC values were within 4% of nominal values and maximum variability was within 10%. Mean EC for the Forrest salinity treatment was $240.5 \mu\text{S cm}^{-1}$, 11% higher than the river EC at time of test specimen collection (i.e. $217 \mu\text{S cm}^{-1}$). The mean EC for the Pollocksford salinity treatment was $2415 \mu\text{S cm}^{-1}$, 2% higher than the river EC at time of test specimen collection (i.e. $2365 \mu\text{S cm}^{-1}$). Differences in salinity between treatments were significant in all cases (ANOVA; $F_{6, 105} = 12906.813$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.001$ all each cases).

pH values ranged from 6.99 to 9.35 and mean values increased with increasing salinity, ranging from 7.40 to 8.35 (Table 4.3). pH values for the Forrest salinity treatment and the $1000 \mu\text{S cm}^{-1}$ treatment were significantly lower than values for treatments $7000 \mu\text{S cm}^{-1}$ and higher (ANOVA; $F_{6, 97} = 6.021$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.029$ in each case).

Mean water temperatures were higher than the desired 20°C, being closer to 22°C but were still within 2°C of the desired temperature (Table 4.3). Maximum water temperatures reached as high as 23.9°C (Table 4.3). There were no significant differences in temperature between salinity treatments (ANOVA; $F_{6, 105} = 0.137$, $P = 0.991$). Dissolved oxygen levels remained above 82% saturation with mean values greater than 87% (Table 4.3). There were no significant differences in dissolved oxygen levels between salinity treatments (ANOVA; $F_{6, 100} = 0.125$, $P = 0.993$).

Table 4.3 Water quality during the reproduction success test with *P. acuta* from the Forrest and Pollocksford sites on the Barwon River. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
217	240.5 \pm 6.9 (216.9-298.0)	7.40 \pm 0.06 (7.10-7.94)	22.0 \pm 0.2 (21.1-23.5)	87.3 \pm 0.8 (82.9-92.5)
1000	1034 \pm 9 (999-1096)	7.44 \pm 0.06 (6.99-7.90)	21.8 \pm 0.2 (21.0-23.6)	87.8 \pm 0.7 (84.4-93.0)
2365	2415 \pm 18 (2364-2576)	7.76 \pm 0.13 (7.23-8.57)	21.8 \pm 0.2 (20.9-23.5)	87.4 \pm 0.6 (84.7-90.6)
5000	5066 \pm 25 (4980-5280)	8.05 \pm 0.17 (7.40-8.97)	21.9 \pm 0.2 (20.8-23.8)	87.1 \pm 0.7 (83.5-91.2)
7000	7124 \pm 37 (6990-7340)	8.14 \pm 0.19 (7.46-9.12)	21.9 \pm 0.2 (20.9-23.7)	87.6 \pm 0.5 (85.3-90.8)
9000	9148 \pm 46 (8990-9520)	8.19 \pm 0.18 (7.36-9.18)	21.9 \pm 0.2 (20.9-23.6)	87.4 \pm 0.7 (82.0-90.5)
12000	12210 \pm 80 (11990-13110)	8.35 \pm 0.21 (7.45-9.35)	22.0 \pm 0.2 (21.0-23.9)	87.3 \pm 0.7 (84.1-95.4)

4.3.4.1 Egg production

Egg production by *P. acuta* from the Forrest site of the Barwon River displayed a sigmoidal decrease with increasing salinity (Figure 4.6). Observed optimal egg production of 6.5 ± 0.9 eggs per snail per day occurred at the river salinity from which they were collected (i.e. 217 $\mu\text{S cm}^{-1}$). A dramatic decrease in egg production was evident between 217 and 1000 $\mu\text{S cm}^{-1}$, with mean egg production at 1000 only $41 \pm 14\%$ of that at 217 $\mu\text{S cm}^{-1}$. Across the salinity

range from 1000 to 5000 moderate egg production was observed (Figure 4.6). Egg production in the 2365 and 5000 $\mu\text{S cm}^{-1}$ treatments was $50 \pm 15\%$ and $52 \pm 9\%$ of the observed optimal respectively. A further decrease in egg production with increasing salinity was observed at salinities of 7000 $\mu\text{S cm}^{-1}$ and higher. Egg production decreased from $32 \pm 3\%$ of the observed optimal at 7000 $\mu\text{S cm}^{-1}$ to only 0.9 ± 0.4 eggs per snail per day at 12000 $\mu\text{S cm}^{-1}$, a reduction of 86%. Variability between replicates was high for salinity treatments of 5000 $\mu\text{S cm}^{-1}$ and lower.

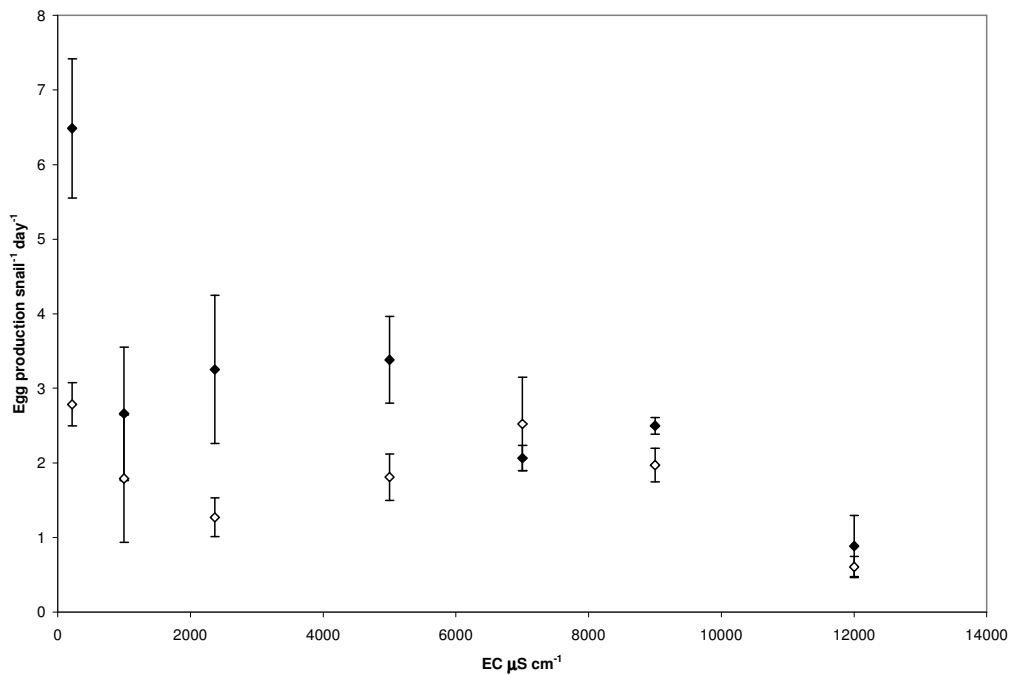


Figure 4.6 Egg production (snail $^{-1}$ day $^{-1}$) by *Physa acuta* collected from the Forrest (closed diamonds) and Pollocksford (open diamonds) sites on the Barwon River during 30 days exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

Egg production by snails from the Forrest site at 217 $\mu\text{S cm}^{-1}$, was significantly higher than at all other salinities at a 90% confidence level (ANOVA; $F_{6,14} = 6.578$, $P < 0.002$ Tukey's pair-wise comparisons $P < 0.069$ in all cases). Egg production at salinities of 1000 and ≥ 5000 $\mu\text{S cm}^{-1}$ were significantly lower than at 217 $\mu\text{S cm}^{-1}$ a 95% confidence level (Tukey's pair-wise comparisons $P < 0.018$ in each case).

The relationship between egg production by snails from the Forrest site and salinity was best described by the equation:

$$\text{Egg production snail}^{-1} \text{ day}^{-1} = -1.651 \times \sqrt{(\text{EC mS cm}^{-1})} + 6.766$$

($F_{1, 16} = 38.293$, $P < 0.001$, $r^2 = 0.705$, SE of slope = 0.267, SE of constant = 0.650).

This equation and the optimal egg production observed at 217 $\mu\text{S cm}^{-1}$ were used to calculate the EC_{50} for egg production by *P. acuta* from the Forrest site of 4535 (SE 2233-9061) $\mu\text{S cm}^{-1}$.

Egg production by *P. acuta* from the Pollocksford site of the Barwon River was generally lower than that of snails from the Forrest site (Figure 4.6). As for snails from the Forrest site, egg production was highest at 217 $\mu\text{S cm}^{-1}$ ($2.8 \pm 0.3 \text{ eggs}^{-1} \text{ snail}^{-1} \text{ day}^{-1}$) and lowest at 12000 $\mu\text{S cm}^{-1}$ ($0.6 \pm 0.1 \text{ eggs}^{-1} \text{ snail}^{-1} \text{ day}^{-1}$). However, the pattern of effect between these salinities was difficult to discern due to high within treatment variability (Figure 4.6). ANOVA determined a significant (at a 90% confidence level) reduction in egg production between only the 217 and 12000 $\mu\text{S cm}^{-1}$ treatments (ANOVA; $F_{6, 14} = 2.617$, $P = 0.065$ Tukey's pairwise comparison $P = 0.051$). A *t*-test of data from these treatments indicated a difference in egg production at a 99% confidence level ($t_4 = 6.784$, $P = 0.002$). The second lowest egg production ($1.3 \pm 0.3 \text{ eggs}^{-1} \text{ snail}^{-1} \text{ day}^{-1}$) was observed at 2365 $\mu\text{S cm}^{-1}$, the salinity from which these snails were collected. Egg production more than doubled from this level when snails were transferred to the lower salinity of the Forrest site (i.e. 217 $\mu\text{S cm}^{-1}$). A *t*-test indicated a significant difference in egg production between these treatments ($t_4 = 3.890$, $P = 0.018$).

Given the unusual shape and uncertainty of the relationship between egg production and salinity for *P. acuta* collected from the Pollocksford site, it was not appropriate to fit a regression to the whole data set to determine the EC_{50} . Although egg production fell below 50% of the observed optimal (at 217 $\mu\text{S cm}^{-1}$) in the 2365 $\mu\text{S cm}^{-1}$ treatment, it was greater than 50% at higher salinities. At salinities of 7000 $\mu\text{S cm}^{-1}$ and higher, egg production displayed a consistent decrease with increasing salinity. Regression analysis of data from this salinity range was used to estimate the EC_{50} . The relationship was best described by the equation;

$$\text{Egg production snail}^{-1} \text{ day}^{-1} = (-0.389 \times 10^{-3}) \times (\text{EC } \mu\text{S cm}^{-1}) + 5.328$$

($F_{1, 7} = 14.036$, $P = 0.007$, $r^2 = 0.667$, SE of slope = 0.104×10^{-3} , SE of constant = 0.992).

Using this relationship and the optimal egg production observed at $217 \mu\text{S cm}^{-1}$ the salinity EC_{50} for egg production by *P. acuta* collected from the Pollocksford site of the Barwon River was calculated as 10098 (5955-17263 SE) $\mu\text{S cm}^{-1}$. Given the associated uncertainties, this value should be considered a preliminary estimate and confirmed by further research.

The number of egg masses laid per snail per day displayed a similar decreasing trend with increasing salinity to that of total egg production (Figure 4.7). The number of egg masses laid by snails collected from the Forrest site was significantly higher at $217 \mu\text{S cm}^{-1}$ than all other salinities (ANOVA; $F_{6, 14} = 11.630$, $P < 0.001$ Tukey's pair-wise comparisons $P < 0.023$ in all cases). The number of egg masses laid by *P. acuta* collected from the Pollocksford site was significantly higher at $217 \mu\text{S cm}^{-1}$ than at 9000 and 12000 $\mu\text{S cm}^{-1}$ (ANOVA; $F_{6, 14} = 6.992$, $P = 0.001$ Tukey's pair-wise comparisons $P < 0.012$ in each case).

The number of eggs laid per egg mass by *P. acuta* displayed a similar response to increased salinity for snails from both collection sites (Figure 4.8). The number of eggs per egg mass was generally higher for snails collected from the Forrest site, however snails from both sites displayed an increase in the number of eggs per mass in the 9000 $\mu\text{S cm}^{-1}$ treatment (Figure 4.8). The number of eggs per mass was lower at 12000 $\mu\text{S cm}^{-1}$ than at 9000 $\mu\text{S cm}^{-1}$ for snails from both collection sites. There was little difference in the number of eggs per mass in the 217-7000 $\mu\text{S cm}^{-1}$ salinity range. Due to high variability between replicates differences in the number of eggs per egg mass were not significant for snails collected from the Forrest site (ANOVA; $F_{6, 14} = 1.881$, $P = 0.115$). Although variability was still high between treatment replicates the number of eggs per mass was significantly higher at 9000 $\mu\text{S cm}^{-1}$ than at salinities of 5000 $\mu\text{S cm}^{-1}$ and lower for snails collected from the Pollocksford site (ANOVA; $F_{6, 14} = 5.304$, $P = 0.005$ Tukey's pair-wise comparisons $P < 0.051$ for treatments $\leq 5000 \mu\text{S cm}^{-1}$, $P > 0.163$ for all other comparisons).

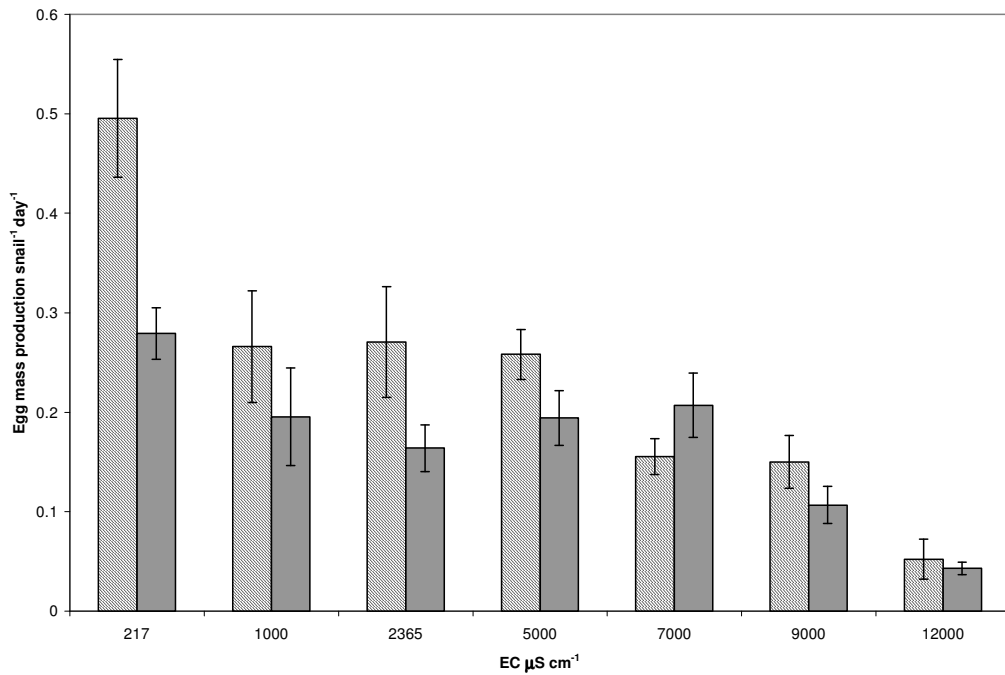


Figure 4.7 Egg mass production (snail⁻¹ day⁻¹) by *Physa acuta* collected from the Forrest (hatched bars) and Pollocksford (solid bars) sites on the Barwon River during 30 days exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

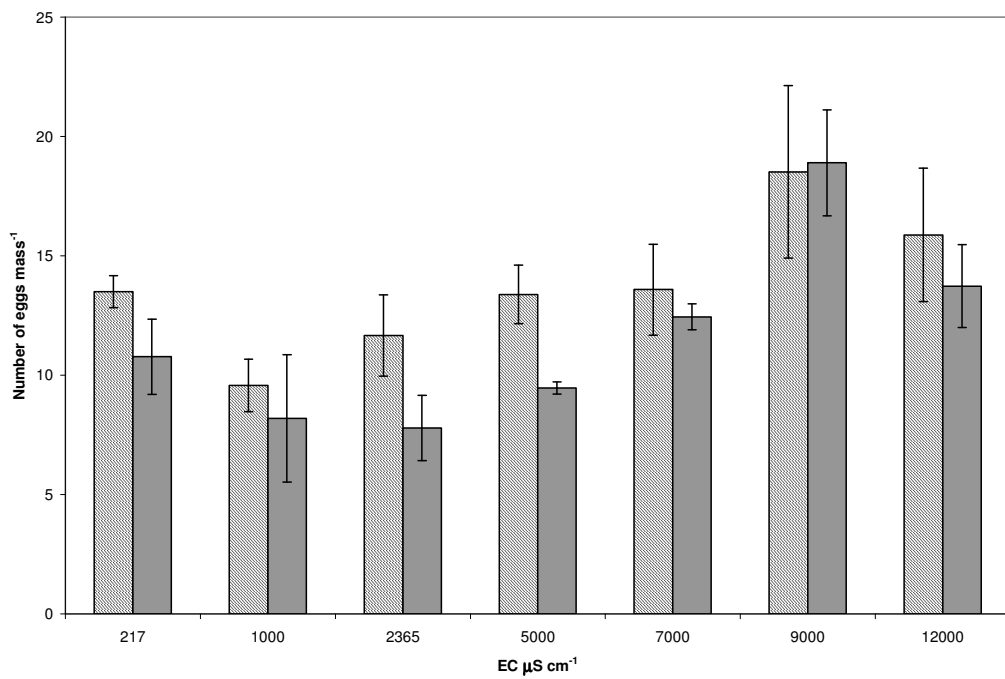


Figure 4.8 Number of *Physa acuta* eggs per egg mass laid by snails collected from the Forrest (hatched bars) and Pollocksford (solid bars) sites on the Barwon River during 30 days exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

4.3.4.2 Egg hatching success

Three egg masses from each of the three egg production treatment replicates were collected for assessment of hatching success. With the exception of the 7000 $\mu\text{S cm}^{-1}$ treatment for *P. acuta* from the Pollocksford site there were no significant differences in the hatching success of egg masses from different treatment replicates (ANOVA; $F_{2, 5-6} < 3.828$, $P > 0.084$ in all cases). Therefore, statistical analyses were performed on hatching data pooled for the three treatment replicates, as variability was greatest between individual masses rather than between treatment replicates. There was significant variability in the hatching success of egg masses laid in the 7000 $\mu\text{S cm}^{-1}$ treatment by *P. acuta* from the Pollocksford site, with mean hatching success in replicate 1 ($23.9 \pm 12.1\%$) significantly lower than the other two replicates which displayed 100% hatching success (ANOVA; $F_{2, 6} = 115.338$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in both cases). Results for replicate 1 were excluded from analyses as outliers. One egg mass from replicate 3 of the 7000 $\mu\text{S cm}^{-1}$ treatment for *P. acuta* from the Forrest site displayed much lower hatching success than other egg masses from this salinity (18% versus a mean of 85% at this salinity). It was identified as an extreme outlier and was excluded from analyses.

The hatching success of *P. acuta* eggs laid at test salinities displayed a typical threshold response to increased salinity (Figure 4.9). Hatching success was high at salinities up to and including 5000 $\mu\text{S cm}^{-1}$ for snails from both the Forrest and Pollocksford sites, but was reduced to $39.7 \pm 10.4\%$ and $33.0 \pm 15.2\%$ respectively at 9000 $\mu\text{S cm}^{-1}$. None of the eggs laid at 12000 $\mu\text{S cm}^{-1}$ hatched successfully. At 7000 $\mu\text{S cm}^{-1}$ hatching success of eggs laid by snails from the Pollocksford site displayed 100% hatching success while eggs laid by snails from the Forrest site displayed only $85.4 \pm 5.0\%$ hatching success.

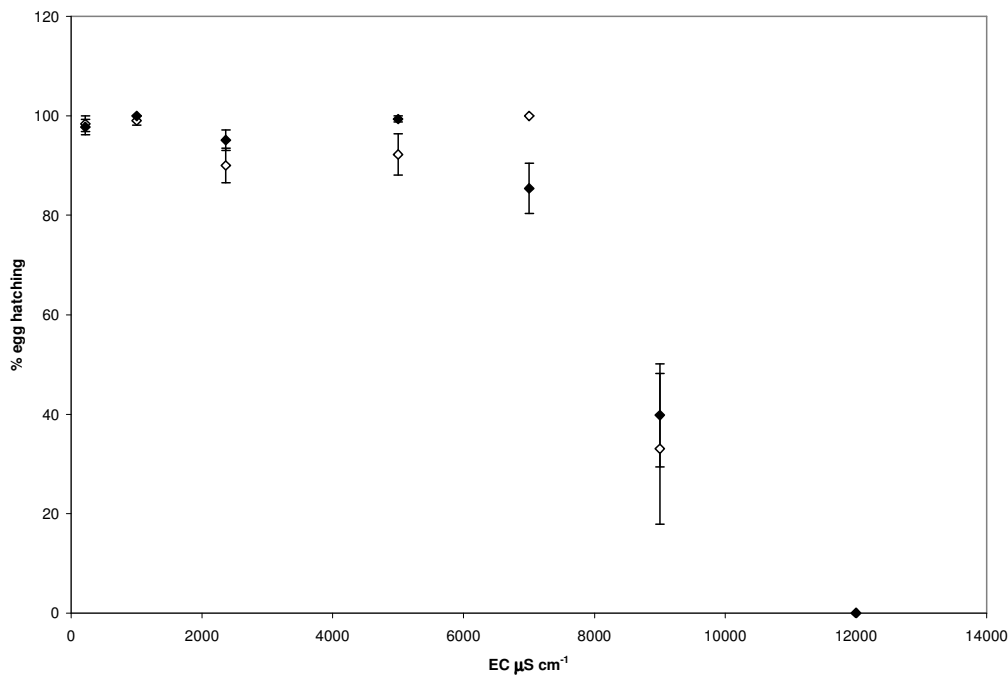


Figure 4.9 Hatching success of *Physa acuta* eggs laid and hatched at different salinities (EC $\mu\text{S cm}^{-1}$) by individuals collected from the Forrest (closed diamonds) and Pollocksford (open diamonds) sites on the Barwon River. Mean \pm SE.

The hatching success of eggs laid by snails from the Forrest site was significantly reduced at salinities of $7000 \mu\text{S cm}^{-1}$ and higher (ANOVA; $F_{6, 55} = 67.292$, $P < 0.001$). Hatching success of eggs laid at $9000 \mu\text{S cm}^{-1}$ and $12000 \mu\text{S cm}^{-1}$ were significantly lower than all lower salinities (Tukey's pair-wise comparisons $P < 0.003$ in all cases). The hatching success of eggs laid at $7000 \mu\text{S cm}^{-1}$ was significantly lower than the Forrest site river salinity (i.e. $217 \mu\text{S cm}^{-1}$) and the 1000 and $5000 \mu\text{S cm}^{-1}$ treatment salinities (Tukey's pair-wise comparisons $P < 0.028$ in each case), but not the Pollocksford site river salinity (i.e. $2365 \mu\text{S cm}^{-1}$) (Tukey's pair-wise comparison $P = 0.265$). Hatching success of eggs laid by snails from the Pollocksford site was significantly reduced at 9000 and $12000 \mu\text{S cm}^{-1}$ (ANOVA; $F_{6, 52} = 35.771$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in all cases). Hatching success of eggs laid by snails from the Pollocksford site was not significantly reduced at $7000 \mu\text{S cm}^{-1}$ (Tukey's pair-wise comparisons $P > 0.371$ in all cases). The significance of analyses was not altered by the exclusion of outliers.

Salinity EC_{50} values for *P. acuta* egg hatching success determined by logistic regression were 8345 (95% CI 8069 - 8619) $\mu\text{S cm}^{-1}$ ($r^2 = 0.602$) for eggs laid by snails from the Forrest site

and 8291 (95% CI 7925-8651) $\mu\text{S cm}^{-1}$ ($r^2 = 0.572$) for eggs laid by snails from the Pollocksford site.

4.3.4.3 Cumulative effects on reproductive success

The cumulative effects of salinity on the reproductive success of *P. acuta* from both of the Barwon River collection sites mirrored the effects of egg production up to salinity levels that affected egg hatching success, that is 7000 $\mu\text{S cm}^{-1}$ for *P. acuta* from the Forrest site and 9000 $\mu\text{S cm}^{-1}$ for *P. acuta* from the Pollocksford site (Figure 4.10). Significant effects of salinity on reproductive success were evident for snails from both collection sites (ANOVAs; Forrest: $F_{6, 14} = 9.077$, $P < 0.001$ and Pollocksford: $F_{6, 13} = 4.661$, $P = 0.010$). Despite a small number of eggs being produced at 12000 $\mu\text{S cm}^{-1}$, egg hatching success at this salinity suggested that none of these eggs would hatch successfully and therefore reproductive success for snails from both sites was zero at this salinity. A significant decrease in reproductive success was evident between 217 and 1000 $\mu\text{S cm}^{-1}$ for snails from the Forrest site (Tukey's pair-wise comparison $P = 0.022$). At 1000 $\mu\text{S cm}^{-1}$ mean reproductive success was only $41.0 \pm 13.8\%$ for *P. acuta* from the Forrest site. There was little variation in reproductive success for snails from the Forrest site across the 1000 to 5000 $\mu\text{S cm}^{-1}$ salinity range (Figure 4.10). Reproductive success was reduced to $27.0 \pm 1.4\%$ at 7000 $\mu\text{S cm}^{-1}$ and $15.5 \pm 3.6\%$ at 9000 $\mu\text{S cm}^{-1}$ for snails from the Forrest site.

Reproductive success was significantly lower at 9000 $\mu\text{S cm}^{-1}$ ($23.4 \pm 2.5\%$) than at 217 $\mu\text{S cm}^{-1}$ for snails from the Pollocksford site. As was the case for egg production, reproductive success was considerably lower (only $41.0 \pm 8.5\%$ of the observed optimal) at the salinity from which these snails were collected than at 217 $\mu\text{S cm}^{-1}$. Although there were no significant differences in reproductive success in the 217 to 7000 $\mu\text{S cm}^{-1}$ salinity range for snails from Pollocksford, there was a trend for increasing reproductive success with both an increase and decrease in salinity from the river salinity (i.e. 2365 $\mu\text{S cm}^{-1}$) within this range (Figure 4.10). High variability between replicates and unusually high mean reproductive success at 7000 $\mu\text{S cm}^{-1}$ for *P. acuta* from the Pollocksford site was due to one replicate displaying both high egg production and hatching success.

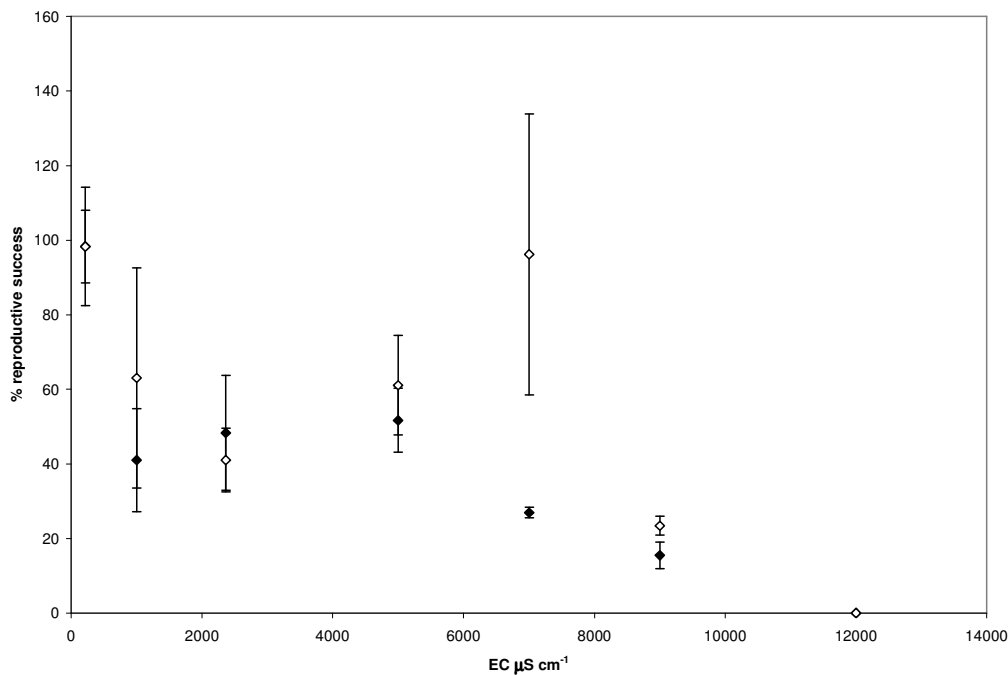


Figure 4.10 Reproductive success (combined effects on egg production and hatching success) of *Physa acuta* collected from the Forrest (closed diamonds) and Pollocksford (open diamonds) sites of the Barwon River at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

Due to high within treatment variability and the unusual shape of the responses to increased salinity EC_{50} values for reproductive success for *P. acuta* were not calculated. However, EC_{50} values for reproductive success could be expected to be similar to those for egg production as egg hatching success only influenced reproductive success at higher salinities.

4.3.5 Growth

Water quality did not display any marked deterioration during the 30-day growth test with adult *P. acuta*. Mean values and ranges for each of the measured parameters are presented in Table 4.4. Mean EC values were within 8% of nominal values with maximum variability less than 18%. The mean EC for the river salinity treatment was $202.4 \pm 3.7 \mu\text{S cm}^{-1}$ which was 8% higher than the desired EC of $188 \mu\text{S cm}^{-1}$. The mean salinity of unmodified filtered water was $135.3 \pm 4.0 \mu\text{S cm}^{-1}$, with a maximum variability of 20%. EC was significantly different between all treatments (ANOVA; $F_{7, 120} = 145775.495$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in all cases). pH ranged from 6.80 to 8.60 and significant differences were evident between lower and higher salinity treatments (ANOVA; $F_{7, 120} = 9.154$, $P < 0.001$). Temperature remained within one degree of the desired 20°C and, with the exception of unmodified filtered water, dissolved oxygen remained above 70% saturation.

Table 4.4 Water quality during 30-day growth test with adult *Physa acuta*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	135.2 \pm 4.0 (112.0-162.0)	7.26 \pm 0.05 (6.92-7.57)	20.0 \pm 0.1 (19.2-20.9)	90.4 \pm 2.4 (67.0-100.4)
188	202.4 \pm 3.7 (187.4-221.3)	7.23 \pm 0.02 (7.10-7.43)	19.9 \pm 0.1 (18.8-20.8)	91.1 \pm 2.1 (74.2-101.6)
500	516.0 \pm 5.1 (500.0-578.0)	7.30 \pm 0.02 (7.18-7.43)	19.9 \pm 0.2 (18.8-20.9)	91.9 \pm 1.9 (78.2-102.0)
1000	1018 \pm 6 (1000-1066)	7.31 \pm 0.03 (7.14-7.53)	19.8 \pm 0.1 (18.7-20.7)	91.0 \pm 1.9 (78.0-101.1)
3000	3009 \pm 8 (2960-3090)	7.42 \pm 0.04 (7.08-7.55)	19.8 \pm 0.2 (18.7-20.7)	91.1 \pm 2.2 (71.8-102.0)
5000	5011 \pm 11 (4970-5120)	7.57 \pm 0.07 (6.98-8.01)	19.8 \pm 0.2 (18.7-20.9)	90.6 \pm 2.3 (70.5-100.2)
7000	7003 \pm 10 (6950-7110)	7.71 \pm 0.09 (7.22-8.18)	19.8 \pm 0.2 (18.7-20.7)	90.3 \pm 2.1 (71.2-99.8)
10000	10000 \pm 20 (9940-10220)	7.76 \pm 0.14 (6.80-8.60)	19.9 \pm 0.2 (18.8-20.9)	91.4 \pm 1.9 (73.9-99.5)

The following regression derived from a sample of 10 snails at the beginning of the test was used to calculate the initial dry weights of snail used in the growth test.

$$\text{Dry weight (mg)} = 0.265 \times \text{wet weight (mg)}$$

$$(F_{1,9} = 1148.177, P < 0.001, r^2 = 0.992, \text{SE of slope} = 0.008)$$

Mean initial wet weight of snails used in the growth test was 9.5 ± 0.3 mg with a maximum weight of 17.5 mg and a minimum of 4.9 mg. There were no significant difference between salinity treatments in initial wet weights or calculated initial dry weights of snails for which growth data were obtained (ANOVA; $F_{4,19} = 0.720, P = 0.589$).

Mortality during the 30-day exposure period was high in many of the treatments (12.5-75%). While survival was highest at salinities of $500 \mu\text{S cm}^{-1}$ and lower, there was no pattern of

reduced survival with increasing salinity in treatments of 1000 to 10000 $\mu\text{S cm}^{-1}$. Results for the 1000, 3000 and 7000 $\mu\text{S cm}^{-1}$ treatments were excluded as only two of the initial eight snails survived until the end of the exposure period and there was high variability in growth between individuals. Results for a very large individual in unmodified filtered water were also excluded, as it's initial size was considerably larger than the other surviving snails and therefore may have displayed a different pattern of growth.

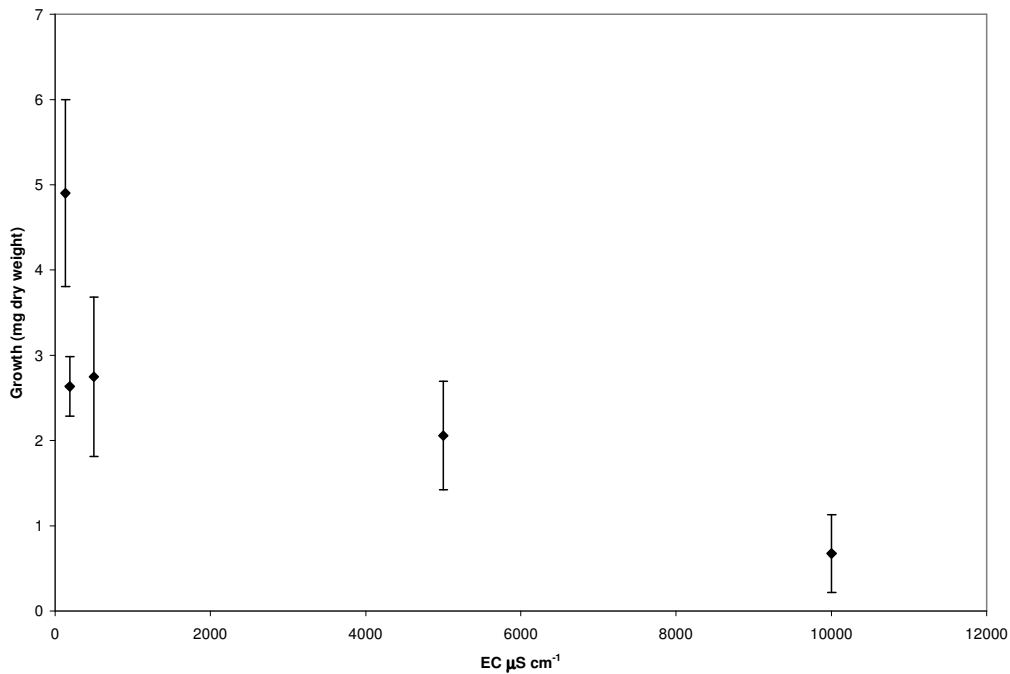


Figure 4.11 Growth (mg dry weight) of adult *Physa acuta* from the Forrest site after 30 days exposure at different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE.

Mean increases in dry weight of *P. acuta* at each salinity over the 30-day exposure period are presented in Figure 4.11. A maximum mean growth of 4.9 ± 1.1 mg dry weight was observed in unmodified filtered water, this decreased with increasing salinity to 0.7 ± 0.5 mg dry weight at $10000 \mu\text{S cm}^{-1}$. This represents a $84 \pm 11\%$ reduction in growth. Mean growth in the river salinity ($188 \mu\text{S cm}^{-1}$) and $500 \mu\text{S cm}^{-1}$ treatments was reduced by 46 and 44% respectively and was less than 50% of the observed optimal at $5000 \mu\text{S cm}^{-1}$. As indicated by the error bars in Figure 4.11 there was high within treatment variability in growth at all treatment levels. ANOVA indicated a significant difference in growth between only unmodified filtered water and the $10000 \mu\text{S cm}^{-1}$ treatment (ANOVA; $F_{4, 19} = 2.911$, $P =$

0.049, Tukey's pair-wise comparison $P = 0.025$). Regression analyses suggested little variability in the final wet:dry ratio of snails from different salinity treatments.

Although the graph of mean values (Figure 4.11) suggests a non-linear negative relationship between growth and salinity, due to the high degree of within treatment variability there was little difference in the strength of regressions using untransformed data and the square root of EC ($r^2 = 0.224$ versus 0.251 respectively). As transformation increased the error range of the calculated EC_{50} , regression analysis of untransformed data were used. The relationship was described by the equation:

$$\text{Growth (mg dry weight)} = (-25.8 \times 10^{-3}) \times \text{EC } (\mu\text{S cm}^{-1}) + 3.263$$

$$(F_{1, 23} = 6.626, P = 0.017, r^2 = 0.224, \text{SE slope} = 10.0 \times 10^{-3}, \text{SE constant} = 0.449).$$

This equation and the mean optimal growth observed in unmodified filtered water were used to calculate the salinity EC_{50} value for adult *P. acuta* growth of 3151 (SE 1017-7987) $\mu\text{S cm}^{-1}$.

4.3.6 Lethal vs sublethal salinity tolerance

Sublethal effects of salinity on *P. acuta* were evident at salinities much lower than those affecting short-term adult survival (Figure 4.12). A summary of the salinity sensitivity of *P. acuta* during this study is presented in Table 4.5 together with the sublethal to lethal ratios for the investigated end points. All of the investigated sublethal end points for *P. acuta* displayed 50% effect levels at salinities considerably lower than the 96-hour LC_{50} for this species. Growth was the most salinity sensitive of the investigated effects, with a sublethal to lethal ratio of 0.24 (Table 4.5). Egg production by snails from the Forrest site of the Barwon River was the second most sensitive endpoint to the affects of salinity with the sublethal to lethal ratio ranging from 0.25 to 0.34 for the different tests in which this was investigated. Egg production by *P. acuta* from the Pollocksford site of the Barwon River displayed the least difference between the EC_{50} and the LC_{50} with a ratio of 0.77.

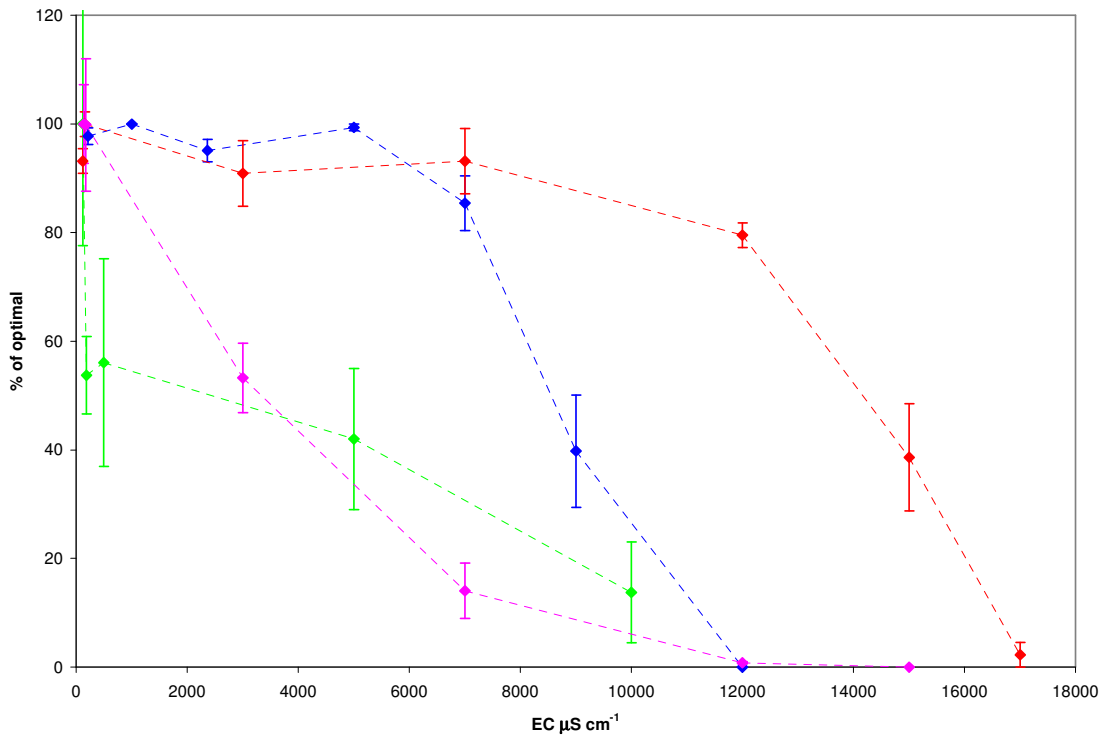


Figure 4.12 The proportional effects of salinity ($\text{EC } \mu\text{S cm}^{-1}$) on the 96-hr adult survival (red), egg hatching success (blue), egg production (pink) and adult growth (green) of *Physa acuta* collected from the Forrest site on the Barwon River. Mean \pm SE.

Table 4.5 Summary of the salinity tolerance of *Physa acuta* and the ratio between sublethal EC₅₀ values and the 96-hr LC₅₀ for the species. * Indicates results excluding identified outliers.

<i>End point</i>	Observed optimal EC ($\mu\text{S cm}^{-1}$)	LOEC ($\mu\text{S cm}^{-1}$)	EC₅₀ ($\mu\text{S cm}^{-1}$)	Ratio (EC ₅₀ /96-hr LC ₅₀)
96 hr survival	173	15000	13171 (95% CI 12816-13529)	-
7 day survival	173	15000	11815 (95% CI 11379-12258)	0.90
30 day survival	173	15000	10539 (95% CI 10133-10945)	0.90
Egg production (survival test)	173	3000	3321 (SE 2127-5121)	0.25
Egg hatch success (transferred)	1000	10000	7985* (95% CI 7694-8282)	0.61
Time to egg hatch (transferred)	112-7000	10000	-	-
Egg production <i>Forrest snails</i>	217	1000	4535 (SE 2233-9061)	0.34
Egg production <i>Pollocksford snails</i>	217	2365	10098 (SE 5955-17263)	0.77
Egg hatch success <i>Forrest snails</i>	1000	7000	8345* (95% CI 8069-8619)	0.63
Egg hatch success <i>Pollocksford snails</i>	1000	9000	8291* (95% CI 7925-8651)	0.63
Cumulative reproduction effects <i>Forrest snails</i>	217	1000	-	-
Cumulative reproduction effects <i>Pollocksford snails</i>	217	9000	-	-
Growth	135	10000	3151* (SE 1017-7987)	0.24

4.4 Discussion

4.4.1 Effects of salinity on adult survival

The 96-hour LC₅₀ for adult *P. acuta* observed in this study was comparable to the combined 72-hour LC₅₀ of 12600 $\mu\text{S cm}^{-1}$ observed by Kefford *et al.* (2006a) for *P. acuta* from a number of Victorian streams. It is also comparable to the 10-day LC₅₀ value of approximately $\approx 13200 \mu\text{S cm}^{-1}$ observed by Stycznska-Jurewicz (1972) for *P. acuta* from a population in Poland. This high comparability suggests that perhaps there is little variability in the short-term salinity tolerance of adults between different populations of this species. However, Kefford *et al.* (2003a) found the slightly higher 72-hour LC₅₀ of 14000 (95% CI 13000-15000) $\mu\text{S cm}^{-1}$ for *P. acuta* from the Pollocksford site of the Barwon River and Dunlop *et al.* (2008) observed a 72-hour LC₅₀ of 14700 (95% CI 15400-16000) $\mu\text{S cm}^{-1}$ for *P. acuta* from south east Queensland.

Salinity dependent mortality did not increase substantially with extended exposure period. This was evident through the only modest reductions in LC₅₀ values after 7 and 30 days exposure. Although significantly lower, the 30-day LC₅₀ was only 2632 $\mu\text{S cm}^{-1}$ lower than the 96-hour LC₅₀. The initial acute toxicity phase did appear to cease after the first 7 days of exposure.

The high mortality across all salinities over the 30-day exposure period may reflect the natural age dependent rate of mortality of the selected test specimens or may be a result of unfavourable test conditions. The low dissolved oxygen levels in the river salinity treatment were unlikely to have affected survival as *P. acuta* can air breath (Gooderham and Tsyrlin 2002). Given this high mortality, the 30-day LC₅₀ value should be considered a preliminary estimate and reviewed when culture techniques for *P. acuta* are improved.

The lower survival of *P. acuta* observed in unmodified filtered water in comparison to survival in the river salinity treatment is likely to reflect reduced tolerance of lower salinities. Kefford and Nugegoda (2005), Kefford *et al.* (2006b) and Hassell *et al.* (2006) have observed reduced tolerance of several macroinvertebrate species (including *P. acuta*) to low as well as high salinities. Although this difference in survival was not significant for all exposure periods during this study, this is most likely due to low statistical power (type II error) and is likely to reflect a real response.

4.4.2 Effects of salinity on egg production

In both the reproduction test and the adult survival test, egg production by *P. acuta* was severely reduced at increased salinity. At 12000 $\mu\text{S cm}^{-1}$ egg production was reduced to very low levels in both tests and for snails from both Barwon River collection sites. This salinity coincides with the salinity range over which adult survival began to be affected for this species. Peska (2003) observed very limited egg production by *P. acuta* collected from a tributary of the Barwon River where salinity was 11100-11400 $\mu\text{S cm}^{-1}$.

In both tests, a significant reduction in egg production by snails from the Forrest collection site occurred between the river salinity treatment and the lowest salinity treatment. This was 1000 $\mu\text{S cm}^{-1}$ for the reproduction test and 3000 $\mu\text{S cm}^{-1}$ for the survival test. Kefford and Nugegoda (2005) did not find a significant reduction in egg production by *P. acuta* at 1000 $\mu\text{S cm}^{-1}$ for snails collected from the Campaspe River but did find a significant reduction at 3000 $\mu\text{S cm}^{-1}$. This may be due to the higher salinity (and therefore exposure of snails to higher salinities) of the Campaspe River in comparison to the Forrest site of the Barwon River. Stycznaska-Jurewicz (1972) observed an increase in egg production by *P. acuta* between $\approx 590 \mu\text{S cm}^{-1}$ and $\approx 1471 \mu\text{S cm}^{-1}$ followed by a large decrease at $\approx 2941 \mu\text{S cm}^{-1}$, egg production then plateaued until a further decrease at $\approx 7353 \mu\text{S cm}^{-1}$. This pattern of response is similar to that observed in this study for egg production by snails from the Pollocksford site.

The observed decreases in the number of eggs laid per snail per day with increasing salinity were due to decreases in the number of egg masses produced rather than decreases in the number of eggs laid in each egg mass. This response was also observed by Stycznaska-Jurewicz (1972) for *P. acuta*. The increase in the number of eggs per mass at 9000 $\mu\text{S cm}^{-1}$ would appear to be an attempt to compensate for the reduction in the number of egg masses produced. The production of fewer egg masses containing a greater number of eggs may represent a more energy efficient strategy but poses the disadvantages of “putting all your eggs in one basket” (i.e increased risk of proportionally high losses due predation, exposure to unfavourable conditions etc.).

Egg production by *P. acuta* collected from the Forrest site of the Barwon River displayed lower egg production across the low salinity range (i.e. river salinity to 2000 $\mu\text{S cm}^{-1}$) during the reproduction test than during the adult survival test but similar if not higher production at

the higher salinities (i.e. 4000 to 12000 $\mu\text{S cm}^{-1}$). Lower egg production in the low salinity range during the reproduction test may have reflected seasonal differences in reproduction rates, different nutritional states and/or differences in the size of snails used in the different tests. Snails used for the survival test were collected in October of 2001 while those used for the reproduction test were collected in January 2003. The slightly higher salinity tolerance of egg production displayed by snails during the reproduction test may have been a result of recent exposure to higher river salinities over the summer months. Snails used in the survival test would have been exposed to lower spring salinities before test commencement. Observed differences between the two tests may also have been related to differences in mean water temperatures between the two tests (19°C during the survival test and 22°C during the reproduction test).

4.4.3 Effects of salinity on egg hatching success

There were no significant differences in the salinity EC_{50} values for *P. acuta* egg hatching success between the different exposure methods (i.e. transferred eggs versus eggs laid at test salinities) or snails from the different Barwon River collection sites. However, a significant reduction in hatching success was evident at a lower salinity for eggs laid at the test salinity by snails from the Forrest collection site (LOEC 7000 $\mu\text{S cm}^{-1}$) than for transferred eggs and eggs laid by snails from the Pollocksford collection site (LOEC 10000 and 9000 $\mu\text{S cm}^{-1}$ respectively).

It is unclear why egg masses from one replicate of the 7000 treatment for snails from the Pollocksford site displayed much lower hatching success (0-38.5% hatching success) than egg masses from the other replicates of this treatment (100% hatching success). No differences in water quality were detected between replicates.

That only a limited reduction in egg salinity tolerance was observed when snails were exposed for the entire reproductive process suggests a lack of a highly salt sensitive stage during the period up until early embryo development. Kefford *et al.* (2007a) observed increased tolerance of *Glyptophysa gibbosa* (Gastropoda: Planorbidae) eggs when exposed at only the final stages of development. This suggests that although there may not be an early salt sensitive stage, embryo tolerance may increase with development.

The salinity tolerance of *P. acuta* eggs observed during this study reflects that observed by Kefford *et al.* (2004a) for *P. acuta* collected from the Pollocksford site of the Barwon River.

They observed high hatching success at salinities up to and including $6400 \mu\text{S cm}^{-1}$ and no hatching at 12000 and $15000 \mu\text{S cm}^{-1}$ (Kefford *et al.* 2004a). The tolerance of *P. acuta* eggs is comparable to that of the South African freshwater limpet *Burnupia stenochorias* (Gastropoda: Ancyliidae) which displayed an egg hatching EC_{50} value of 9200 (95% CI 8300 - 10000) $\mu\text{S cm}^{-1}$ (Kefford *et al.* 2004a), but is high compared to the tolerance of three Australian gastropod species which displayed egg hatching EC_{50} values of between 2600 and $6100 \mu\text{S cm}^{-1}$ (Kefford *et al.* 2007a). One unidentified Australian Lymnaeid species possibly displayed a higher tolerance than *P. acuta* with greater than 90% egg hatching success occurring at $10000 \mu\text{S cm}^{-1}$ (Kefford *et al.* 2004a). However the eggs of this species were exposed in the late stages of embryonic development.

Although optimal egg hatching success was observed at $1000 \mu\text{S cm}^{-1}$ in each of the tests, there was no significant reduction in hatching success evident at the lower salinity of unmodified filtered water. A reduction in gastropod egg hatching success at low salinities has been observed by Kefford *et al.* (2007a). This may occur for *P. acuta* at salinities lower than those of this study.

The observed increase in time to first hatch of *P. acuta* eggs at $10000 \mu\text{S cm}^{-1}$ was presumably due to slowed development as a result of increased energy requirements for osmoregulation at this salinity. An increase in egg development time with increased salinity has also been observed for *Burnupia stenochorias* (Kefford *et al.* 2004a) but was not evident in the three Australian gastropod species studied by Kefford *et al.* (2007a).

4.4.4 Cumulative effects of salinity on reproduction success

Although some eggs were laid at $12000 \mu\text{S cm}^{-1}$ by *P. acuta* from both the Pollocksford and Forrest collection sites during the reproduction test no egg hatching was observed at this salinity. Egg hatching success therefore represented the upper limiting factor for reproduction by *P. acuta* with increased salinity. At salinities lower than those affecting egg hatching, reproductive success was limited by the effects of salinity on egg production. In the intermediate salinity range effects on both factors combined to reduce reproductive success. No other studies investigating the effects of salinity on gastropod egg production and the hatching success of the produced eggs could be found for comparison.

While consideration of the combined effects of salinity on egg production and egg hatching provides a good indication of potential effects on reproductive success, this does not take into

account the effects of salinity on snail growth. As the number of eggs produced by gastropods is linked to adult size, reductions in growth due to salinity are likely to impact on reproduction success. The number of individuals successfully reaching reproductive maturity may also be limited by the effects of salinity on juvenile survival. An attempt was made to assess the survival of hatchlings from the reproductive test however the test was abandoned when it was discovered that hatchlings were escaping and moving between different salinity treatments. However, hatchlings were observed alive at all salinities where they hatched successfully. Results by Kefford *et al.* (2007a) suggest that gastropod hatchlings may display higher salinity tolerance than eggs.

4.4.5 Effects of salinity on growth

High mortality and high within treatment variability hampered investigation of the effects of salinity on the growth of *P. acuta*. Results suggest a large reduction in growth with only a small increase in salinity. However, due to the high within treatment variability a statistically significant reduction in growth was only evident between the highest and lowest salinities. The calculated salinity EC₅₀ value for growth does, however, adequately reflect the observed reduction in growth with increased salinity. The decrease in growth with increased salinity observed in this study reflects the results of Kefford and Nugegoda (2005) who observed a significant reduction in the growth rate of *P. acuta* between 1000 and 5000 $\mu\text{S cm}^{-1}$.

It is unclear why such high levels of mortality were experienced during the test period. However, similar levels were observed during the 30-day survival test. While there would appear to be naturally quite high variability in growth between individuals this may have been exacerbated by the wide size range of snails used for the test. Measurement of snail growth is made more difficult by the additional factor of shell weight. Changes in weight can be a result of changes in both the weight of the shell and changes in body mass. Given that the shell weight represents a high proportion of overall weight, changes in the body mass of snails without corresponding changes in shell weight may be hard to detect. That shell weight is unlikely to display a decrease adds to these difficulties. Measurement of shell length to assess growth is potentially more problematic for these reasons. A combination of both measurements perhaps provides the best method with which to detect changes in snail growth. For this study initial shell length was only measured for the subset of snails used to determine the wet weight:dry weight ratio. The initial size of snails used in the growth test was calculated using a regression derived from the subset of snails and the change in shell length over the test period calculated. These results suffered from the same problem of high within

treatment variability and failed to detect any additional significant effects. An increased number of snails at each salinity and a narrower range of initial snail sizes may reduce the problems experienced in this study.

4.4.6 Effects of salinity history on salinity tolerance

A history of higher salinity exposure appears to have a limited effect on the hatching success of eggs of *P. acuta* from the Pollocksford site of the Barwon River. The only observable difference in egg hatching success between snails from the different collection sites was a higher tolerance at 7000 $\mu\text{S cm}^{-1}$ of eggs laid by snails from the Pollocksford site. Eggs laid by snails from the Pollocksford site displayed 100% hatching success at this salinity while those laid by snails from the Forrest site displayed 85% hatching success.

Although not investigated directly in this study, there would appear to be limited difference in the short-term salinity tolerance of adult snails from the different collection sites. Kefford *et al.* (2004a) found a 72-hour LC_{50} of 14000 (95% CI 13000-15000) $\mu\text{S cm}^{-1}$ for *P. acuta* from the Pollocksford site, which is comparable to the 72-hour LC_{50} of 14296 (95% CI 14091-14492) $\mu\text{S cm}^{-1}$ observed in this study for *P. acuta* from the Forrest collection site.

Salinity history would appear, however, to have an effect on egg production by *P. acuta*. Snails from the Pollocksford site displayed a broad salinity range over which egg production remained high in comparison to the observed optimal. Higher salinity tolerance for egg production increases the range of favourable conditions for reproduction. The EC_{50} value for egg production by snails from Pollocksford was 5000 $\mu\text{S cm}^{-1}$ higher than that for snails from the Forrest collection site. However, given the uncertainty associated with the EC_{50} estimate for *P. acuta* from the Pollocksford site (see Section 4.3.4.1), this difference should be confirmed by further studies.

The overall lower egg production by *P. acuta* from the Pollocksford site may indicate reduced fitness of this population in comparison to snails from the Forrest collection site. This may be due to genetic differences, differences in nutritional state or indicate increased stress from environmental factors such as salinity. Differences in size distribution of snails from the different collection sites may also have resulted in different rates of egg production. That optimal egg production was observed when snails were transferred to the lowest salinity treatment may indicate salinity limitation of reproduction for this species at the Pollocksford

site. Peska (2003) also observed increased egg production by *P. acuta* collected from the Barwon catchment when transferred to lower salinity.

P. acuta from the Pollocksford site have a history of exposure to a moderate salinity range in comparison to that experienced by some populations. For example Peska (2003) collected *P. acuta* from a salinity of 11400 $\mu\text{S cm}^{-1}$ and Goonan *et al.* (1992) has recorded *P. acuta* at a salinity of 11700 $\mu\text{S cm}^{-1}$. Snails from higher salinities may display more extreme effects of previous salinity exposure than those observed for *P. acuta* from the Pollocksford site of the Barwon River.

4.4.7 Lethal vs sublethal salinity tolerance

All of the investigated sublethal effects of salinity were found to occur at salinities much lower than those affecting adult survival. These effects have obvious implications for the fitness of *P. acuta* populations at increased salinities. Populations are not sustainable at salinities where reproduction and/or growth have ceased, therefore salinities at which this occurs represents the upper limit for viable populations.

With the exception of adult survival, egg hatching success was the least sensitive to increased salinity of the investigated end points. The ratio of the egg hatching success EC_{50} to the LC_{50} was 0.63. This is comparable to the ratio of 0.46 to 0.90 for the results of Kefford *et al.* (2004a) for *P. acuta* from the Pollocksford site. Ratios for egg hatching success of 0.84 and 0.21 have been observed for the South African freshwater limpet *Burnupia stenochorias* (Kefford *et al.* 2004a) and the Australian Planorbid species *Glyptophysa gibbosa* (Kefford *et al.* 2007a) respectively.

Large reductions in egg production and growth were observed with increases in salinity within the fresh to 1000 $\mu\text{S cm}^{-1}$ range. Unlike the threshold type effect observed for egg hatching success these factors displayed a more continuous response to changes in salinity, with the magnitude of effect generally increasing with increasing salinity. Fifty percent effect levels for growth and egg production were experienced at 24% and 34% of the adult 96-hour LC_{50} respectively.

4.4.8 Further research

A test investigating the combined effects of reduced growth and reproduction would provide further insight to the potential impacts of salinity on populations of this species. Given that individuals are likely to live for up to two years a full life-cycle test is probably not feasible. However, investigation of effects from egg production through egg hatching, juvenile survival and growth may be plausible. To further investigate the effects of salinity history on the salinity tolerance of *P. acuta*, investigations with individuals from a higher salinity range than that of the Pollocksford site would be insightful. Investigation of the variability of sublethal salinity tolerances between populations from similar salinities would enable better interpretation of research findings.

5.0 Salinity tolerance of the Atyid shrimp *Paratya australiensis* Kemp 1917 (Crustacea: Decapoda: Atyidae)

5.1 Introduction

Crustacea are a common component of freshwater ecosystems throughout the world. Evolutionary radiation from the ocean, through estuaries and into freshwater systems necessitated the development of osmoregulatory mechanisms. These mechanisms have included a reduction of cuticle permeability, active uptake of salts and production of dilute urine (M^cMahon in Brinck 1986).

Both micro and macro Crustacea occur in freshwater systems. Microcrustacea include; water fleas (Order: Cladocera), copepods (Subclass: Copepoda), clam shrimp (Order: Conchostraca) and seed shrimp (Class: Ostracoda). Macrocrustacea include; amphipods (Order: Amphipoda), isopods (Order: Isopoda), syncarids (Superorder: Syncarida), shield shrimp (Order: Notostraca), fairy shrimp (Order: Anostraca) and the decapod crustacea (which are considered the most highly evolved order (Williams 1980)) encompassing; crabs (Family: Hymenosomatidae), crayfish (Family: Parastacidae), prawns (Family: Palaemonidae) and shrimp (Family: Atyidae) (Gooderham and Tsyrlin 2002).

Freshwater shrimp (Family: Atyidae) occur in all tropical regions of the world and are also common in many temperate regions including Australia and New Zealand (Born 1968). Atyids are almost exclusively restricted to freshwater environments (Born 1968; Walsh and Mitchell 1995) and are often a highly abundant component of these systems (Shaw 1981; Boulton and Lloyd 1991; Hart 2001). They are an important food source for fish (A. King DSE pers. comm.) and other predators, and as detrital feeders (Gooderham and Tsyrlin 2002) play a vital role in nutrient cycling.

Paratya australiensis Kemp is the most common atyid shrimp in south-eastern Australia, occurring from south-eastern South Australia to southern Queensland with some northern Queensland populations (Williams 1977). The species also occurs in Tasmania (Walker 1972; Williams 1977). Recent genetic research suggests that *P. australiensis* may represent a complex of species, some widely distributed and others geographically restricted (Cook *et al.* 2006). *P. australiensis* occurs most commonly in lowland rivers and streams but is also found in non flowing freshwaters (Williams and Smith 1979) and in upland streams in southern Queensland (Hancock and Bunn 1997). Walsh and Mitchell (1995) found all life stages of *P.*

australiensis occurring in estuaries in south western Victoria. Studies into the ecology of the *P. australiensis* have found that individuals live between one and two years (Walker 1972; Williams 1977; Hancock and Bunn 1997; Richardson *et al.* 2004). Breeding occurs during spring and summer, with young hatching as free living larvae from eggs brooded on the female (Williams 1977).

Investigations into the salinity tolerance of *P. australiensis* have found all life stages to be highly salt tolerant, being able to tolerate salinities in excess of 30000 $\mu\text{S cm}^{-1}$ for at least short periods of time (Adults: Walker 1972; Williams 1984; Morris 1991; Walsh 1994; Kefford *et al.* 2003a, Juveniles: Walsh 1994 and Larvae: Kefford *et al.* 2004a). Williams (1984) investigated the osmoregulation abilities of *P. australiensis* finding adults able to maintain constant hyperosmotic regulation up to a salinity of approximately 19000 $\mu\text{S cm}^{-1}$, which was isosmotic to the haemolymph. Above this salinity internal osmolarity increased with increasing salinity at levels slightly higher than the external media (Williams 1984). Studies with other freshwater decapods have observed similar osmoregulatory patterns (Mills and Geddes 1980; Williams 1984) and characteristically high salinity tolerances (Mills and Geddes 1980; Williams 1984; Kefford *et al.* 2003a, 2004a).

Despite the short-term salinity tolerance of *P. australiensis* being widely studied, there have been no investigations into the long-term tolerance of the species or the sublethal effects of salinity. In this study, the long-term salinity tolerance of adults and larvae and the effects of salinity on juvenile growth were investigated. The effects of salinity on egg hatching and breeding success were also investigated.

5.2 Materials and methods

5.2.1 Test specimen field collection

P. australiensis used for testing were collected from the Pollocksford site of the Barwon River (see Materials and methods 2.2). Shrimp were collected on several occasions, as required for testing. River salinities at the time of collection of *P. australiensis* ranged from 1149 to 2280 $\mu\text{S cm}^{-1}$.

5.2.2 Adult acute salinity tolerance

The salinity tolerance of adult *P. australiensis* was investigated across an experimental salinity range of 1000 to 50000 $\mu\text{S cm}^{-1}$ (treatment levels; 1000, 10000, 20000, 25000, 30000, 35000, 40000 and 50000 $\mu\text{S cm}^{-1}$) and in unmodified filtered water ($134.8 \pm 2.8 \mu\text{S cm}^{-1}$). The 1000 $\mu\text{S cm}^{-1}$ treatment was representative of the salinity of the river water at the time of test specimen collection. The test was conducted in 5-L glass aquaria containing 4 L of aerated test solution. Treatments were conducted in triplicate and 10 individuals randomly allocated to each replicate. The number of *P. australiensis* surviving in each replicate was determined at 24-hour intervals for the first ten days of exposure and every two to three days until termination of the test after sixteen days of exposure. Death was defined as failure to respond to gentle prodding and any dead animals removed. Test solutions were renewed every 2 to 3 days and water quality measure before and after water changes. Shrimp were fed commercial trout pellets throughout the test.

5.2.3 Larval salinity tolerance

Gravid female *P. australiensis* collected from the field were placed individually into 440-mL plastic containers containing 350 mL of aquarium water made up to the salinity of the river at time of collection (2280 $\mu\text{S cm}^{-1}$). Water was changed every 2 to 3 days and water quality measured before and after water changes. Gravid females were fed commercial trout food throughout the test. Containers were inspected daily to determine when egg hatching occurred. One day after initial egg hatching was observed larvae (up to 48 hrs old) were collected and distributed to salinity treatments. The salinity tolerance of larvae was investigated across a salinity range of 2280 to 50000 $\mu\text{S cm}^{-1}$ (treatment levels; 2280, 10000, 20000, 25000, 30000, 40000, 50000 $\mu\text{S cm}^{-1}$), with 2280 $\mu\text{S cm}^{-1}$ representative of the salinity of river water at the time of collection of females. As larval hatching occurred on different days, larvae from one female were distributed across treatments (15 larvae per treatment) to form one replicate. The larvae from 3 females were distributed across the treatments

providing 3 replicates of each treatment. The larval tolerance test was conducted in glass dishes containing 250 mL of test solution. Replicates were examined using a dissection microscope at 24-hour intervals to determine the number of surviving larvae. Larvae were considered dead if they failed to respond to gentle prodding. Larvae were fed standardised quantities of ground commercial trout food throughout the test and were transferred to fresh test solutions every 48-hours. Water quality was measured before and after water changes. Larval survival was monitored in each replicate for an 11-day period.

5.2.4 Egg hatching salinity tolerance

The hatching success of *P. australiensis* eggs was investigated across an experimental salinity range of 2000 to 30000 $\mu\text{S cm}^{-1}$ (treatment levels; 2000, 10000, 15000, 20000 and 30000 $\mu\text{S cm}^{-1}$) and in unmodified filtered water ($67.3 \pm 1.6 \mu\text{S cm}^{-1}$). The 2000 $\mu\text{S cm}^{-1}$ treatment was representative of the salinity of the river water at the time of collection of gravid females used for the test. Gravid female *P. australiensis* collected from the field were placed individually into 440-mL plastic containers containing 350 mL of salinity solution. Five females were held at each test salinity. Water was changed every 2 to 3 days and water quality measured before and after water changes. Females were fed commercial trout food throughout the test. Containers were inspected daily to determine when egg hatching occurred and the number of hatched/unhatched eggs.

5.2.5 Effects of salinity on juvenile growth

Juvenile *P. australiensis* less than one year old (based on size at time of collection) were collected from the Barwon River at the Pollocksford site in late Autumn. After acclimation to laboratory conditions for 10 days, shrimp were wet weighed (after two days starvation to allow gut evacuation) to the nearest 0.1 of a milligram using an analytical balance and placed individually into 440-mL plastic containers containing 350 mL of test solution. Thirty-five individuals were wet weighed and then oven dried to constant weight at 60°C to determine the initial wet to dry weight ratio of test specimens. The effects of salinity on the growth of juvenile *P. australiensis* was assessed over a salinity range of 2200 to 30000 $\mu\text{S cm}^{-1}$ (treatment levels; 2200, 5000, 10000, 20000 and 30000 $\mu\text{S cm}^{-1}$) with 2200 $\mu\text{S cm}^{-1}$ representative of the salinity of the river at time of collection. Ten individuals were maintained at each of the test salinities. Test solutions were changed every 2 to 3 days and water quality was measured before and after changes. Test solutions were aerated and shrimp fed daily a combination of Spirulina fish food flakes and commercial trout food pellets. Containers were inspected daily for moulted exoskeletons to enable determination of moulting

frequencies for each individual. After two months shrimp were wet weighed (after two days starvation to allow gut evacuation) and then oven dried to constant weight at 60°C and dry weights determined.

5.2.6 Effects of salinity on breeding success

The effects of salinity on the breeding success of *P. australiensis* were investigated across an experimental salinity range of 1767 to 30000 $\mu\text{S cm}^{-1}$ (treatment levels; 1767, 5000, 10000, 20000 and 30000 $\mu\text{S cm}^{-1}$) with the 1767 $\mu\text{S cm}^{-1}$ treatment representing the salinity of the river water at the time of test specimen collection. Adult *P. australiensis* for the test were collected from the field in late October, acclimated to laboratory conditions for two weeks and the test commenced in mid November. The sex of individuals was determined using a stereomicroscope, with projections from the sternal plate a defining character of males (C. Walsh CRCFE Pers. Comm.). Five individuals of each sex were placed together in 5-L glass aquaria containing 4 L of aerated test solution with three replicates of each test salinity. Test solutions were renewed every 2 to 3 days and water quality measured before and after changes. Shrimp were fed commercial trout pellets and Spirulina fish flakes through out the test. Shrimp were monitored daily for signs of breeding.

5.3 Results

5.3.1 Adult acute salinity tolerance

Water quality remained high during the acute tolerance test with adult *P. australiensis* (Table 3.1). Mean percent oxygen saturation in each treatment was 90% or higher and mean temperatures were within the $20 \pm 2^\circ\text{C}$ desired range. Mean salinities during the test were lower than the nominated treatment levels, however variability was no greater than 1% from the treatment levels and maximum variability was less than 10% for all treatments with the exception of the $10000 \mu\text{S cm}^{-1}$ treatment which displayed a maximum variability of 11%. The mean salinity for filtered water during the test was $134.8 \pm 2.8 \mu\text{S cm}^{-1}$ with a maximum variability of 20%.

Table 5.1 Water quality for acute tolerance test with adult *Paratya australiensis*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature $^\circ\text{C}$	D. O. %
Untreated	134.8 ± 2.8 (121.4-162.2)	7.68 ± 0.05 (7.33-8.02)	18.8 ± 0.1 (18.4-19.2)	89.7 ± 1.7 (75.6-98.7)
1000	1003 ± 6 (946-1074)	7.51 ± 0.02 (7.38-7.60)	18.5 ± 0.1 (17.3-19.3)	90.4 ± 2.3 (75.5-100.6)
10000	9955 ± 75 (8940-10300)	8.04 ± 0.16 (7.36-9.21)	18.7 ± 0.1 (17.8-19.3)	89.82 ± 2.4 (69.8-97.8)
20000	19870 ± 130 (18150-20350)	8.21 ± 0.12 (7.61-9.15)	18.6 ± 0.1 (17.4-19.4)	90.7 ± 2.3 (71.9-102.2)
25000	24770 ± 160 (22760-25240)	8.13 ± 0.09 (7.59-8.69)	18.6 ± 0.1 (17.5-19.3)	90.0 ± 3.1 (65.3-104.7)
30000	29880 ± 190 (27600-30700)	8.17 ± 0.07 (7.81-8.65)	18.6 ± 0.1 (17.5-19.3)	91.2 ± 2.3 (75.4-102.5)
35000	34660 ± 240 (31700-35400)	8.29 ± 0.08 (7.96-8.76)	18.7 ± 0.1 (17.7-19.4)	92.5 ± 2.5 (78.4-102.7)
40000	39590 ± 280 (35900-40600)	8.36 ± 0.08 (7.95-9.27)	18.7 ± 0.1 (17.5-19.4)	93.0 ± 2.3 (75.6-103.8)
50000	49850 ± 180 (49200-50500)	8.25 ± 0.10 (8.03-8.64)	18.6 ± 0.1 (18.2-18.9)	97.7 ± 0.8 (95.6-100.2)

Salinity was significantly different between all treatments (ANOVA; $F_{8,165} = 8393.968$, $P < 0.001$, Tukey's pair-wise comparisons Tukey's $P < 0.001$ in all cases except 135 vs 1000 $P = 0.006$). Temperature and dissolved oxygen percent saturation did not vary significantly between salinity treatments (ANOVA; Temperature $F_{8,165} = 0.410$, $P = 0.914$ and DO $F_{8,116} = 0.657$, $P = 0.728$). There were significant difference in pH between treatments (ANOVA; $F_{8,128} = 9.474$, $P < 0.001$), with 135 and 1000 $\mu\text{S cm}^{-1}$ treatments displaying significantly lower pH values than all higher salinity treatments (Tukey's pair-wise comparisons; $P < 0.036$ in all cases, not significantly different to each other $P > 0.126$, all other comparisons $P > 0.235$). There were no significant differences in water quality conditions between treatment replicates (ANOVA; $F_{2, 3-18} < 4.868$, $P > 0.113$ in all cases).

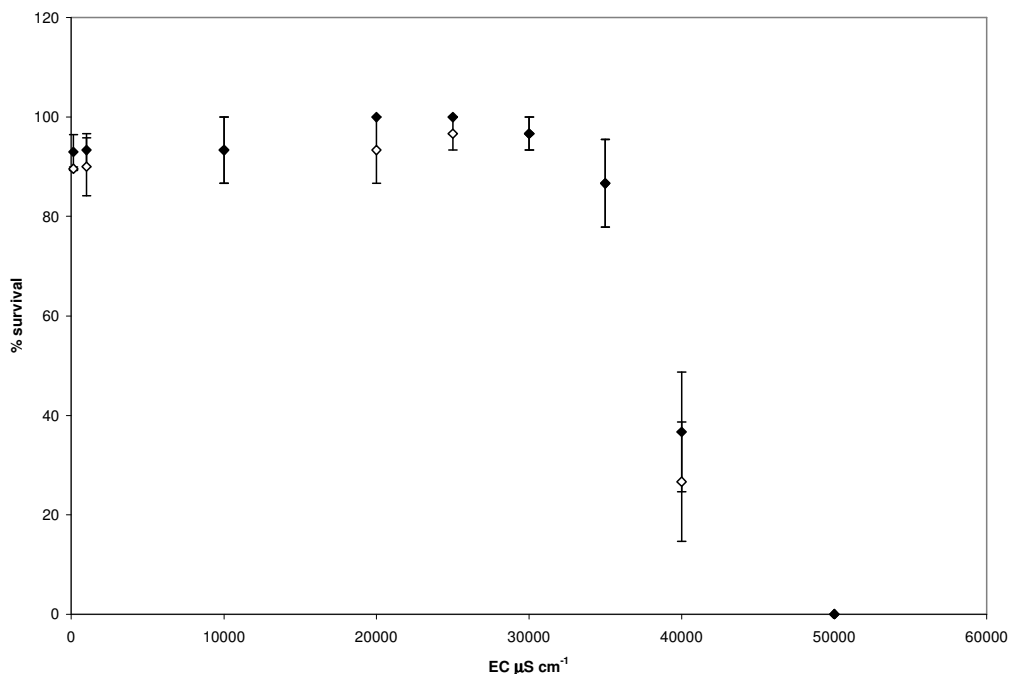


Figure 5.1 Survival (%) of adult *Paratya australiensis* after 4 days (closed diamonds) and 7 days (open diamonds) exposure at different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE.

The survival of adult *P. australiensis* was significantly affected by salinity (ANOVA for each exposure period; $F_{8,18} > 22.956$, $P < 0.001$ in all cases), with reduced survival evident in the 40000 and 50000 $\mu\text{S cm}^{-1}$ treatments after the first 24 hours of exposure. Survival displayed a typical threshold dose response to salinity, with high survival up to and including salinities of 35000 $\mu\text{S cm}^{-1}$ for the duration of the test, 100% mortality at 50000 $\mu\text{S cm}^{-1}$ occurring within the first 24 hours of exposure and partial survival at 40000 $\mu\text{S cm}^{-1}$ (from 63 ± 3 after 1-day exposure to 13 ± 9 after 16-days exposure). Mortality in the 40000 $\mu\text{S cm}^{-1}$ treatment

stabilised after 7 days exposure. Figure 5.1 displays survival at the different salinity levels after 4 days and 7 days exposure.

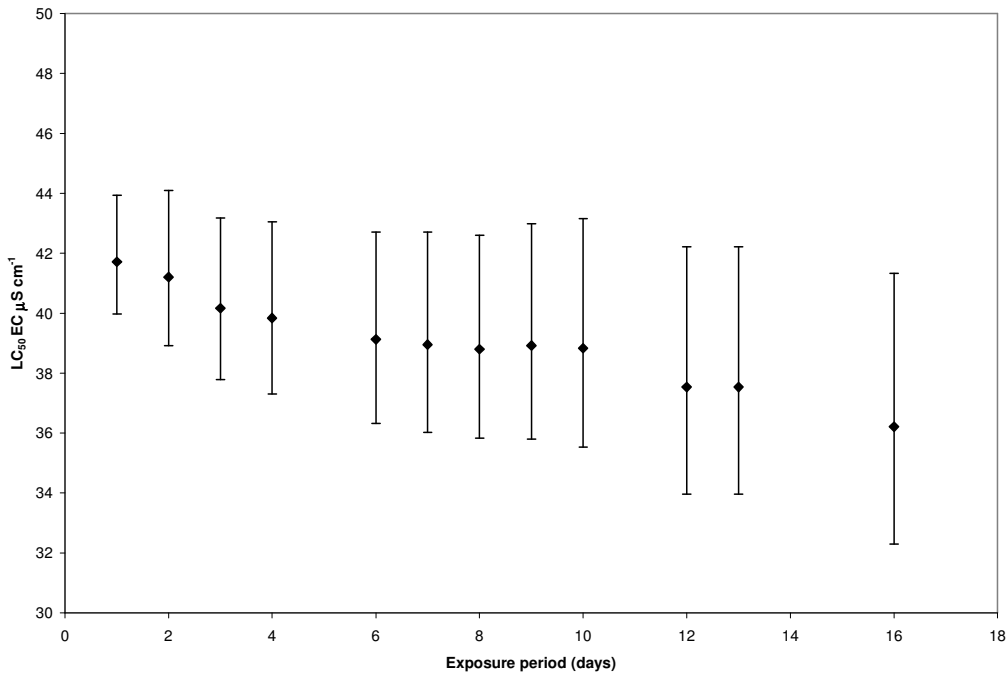


Figure 5.2 LC₅₀ values (EC μS cm⁻¹) for adult *Paratya australiensis* after different periods of salinity exposure. Error bars indicate 95% CI.

There was little change in LC₅₀ values throughout the exposure period and 95% CI indicate that there were no significant differences in LC₅₀ values for different exposure periods (Figure 5.2 and Appendix A Table A.3). Lower LC₅₀ values and broader 95% confidence intervals during the latter stages of the test (12 to 16 days exposure) were due to mortality occurring in lower salinity treatments. The 4-day (96-hour) LC₅₀ value for *P. australiensis* during this test was 39844 (95% CI 37308-43055) μS cm⁻¹ ($r^2 = 0.40$). The period of acute salinity toxicity did not extend beyond 7 days exposure when mortality stabilised in the 40000 μS cm⁻¹ treatment. The time independent LC₅₀ value after 7 days exposure was 38957 (95% CI 36021-42714) μS cm⁻¹ ($r^2 = 0.32$) (although this was not significantly different to the 96-hour value). The salinity LC₅₀ values determined for adult *P. australiensis* in this study and those observed in other studies are presented in Table 5.2.

Table 5.2 LC₅₀ values for adult *Paratya australiensis* determined by this and other studies. * Indicates values have been converted from TSS and ≈ indicates value was estimated from presented data.

Study	Source population	Temperature (°C)	Exposure period (hours)	LC ₅₀ value (μS cm ⁻¹)	95% confidence intervals
This study	Barwon River, Victoria	20	96	39844	37308-43055
This study	Barwon River, Victoria	20	Time independent	38957	36021-42714
Dunlop <i>et al.</i> (2008)	Murray-Darling Basin, Queensland	25	72	34200	31200-37200
Kefford <i>et al.</i> (2006a)	Murray-Darling Basin, Victoria	20	72	29100	-
Kefford <i>et al.</i> (2003a)	Barwon River, Victoria	20	72	38000	37000-41000
Walsh (1994)	Hopkins River, Victoria	20	96	33700*	32500-34700*
Bacher and Garnham (1992)	Yarra River, Victoria	15	96	6600*	4600-9900*
Morris (1991)	Murray River, South Australia	20	96	31600*	30900-32400*
Williams (1984)	Torrens River, Victoria	20	96	≈38000*	-
Walker (1972)	Tasmania	12-13	72	32500*	-

5.3.2 Larval salinity tolerance

Water quality remained high during the salinity tolerance test with larval *P. australiensis*. Mean percent oxygen saturation in each treatment was greater than 95% and mean temperatures were within the $20 \pm 2^\circ\text{C}$ desired range. Mean salinities during the test were higher than the nominated treatment levels, however variability was less than 1% for all treatments with the exceptions of the 10000 and 20000 $\mu\text{S cm}^{-1}$ treatments which were less than 2%. Maximum salinity ranges were all within 5% of the nominated treatment levels. Mean pH ranged from 7.36 to 8.70 and increased with increasing salinity.

There were no significant differences in water quality parameters between replicates of each treatment (ANOVA; $F_{2, 3-27} < 0.592$, $P > 0.566$). There were however significant differences in pH (ANOVA; $F_{6, 167} = 19.109$, $P < 0.001$) and temperature (ANOVA; $F_{6, 167} = 3.257$, $P = 0.005$) between salinity treatments. pH increased with increasing salinity, with differences significant between treatments separated by one to two salinity levels ($P < 0.040$ where significant differences occurred). Temperatures in the 50000 $\mu\text{S cm}^{-1}$ treatments were significantly higher than those of the 2280, 10000 and 40000 $\mu\text{S cm}^{-1}$ treatments ($P < 0.029$ in each case). However, mean temperature differences were less than 0.2 °C. Salinity (EC

$\mu\text{S cm}^{-1}$) was significantly different between all treatments (ANOVA; $F_{6, 167} = 98516.777$, $P < 0.001$).

Table 5.3 Water quality for salinity tolerance test with larval *Paratya australiensis*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
2280	2300 \pm 4 (2278-2350)	7.36 \pm 0.02 (7.20-7.58)	20.3 \pm 0.1 (20.0-20.5)	96.7 \pm 1.1 (90.2-110.4)
10000	10130 \pm 30 (9970-10500)	7.61 \pm 0.08 (7.29-8.78)	20.2 \pm 0.1 (20.0-20.5)	97.9 \pm 0.9 (93.4-111.0)
20000	20240 \pm 50 (19950-20800)	7.90 \pm 0.08 (7.62-9.04)	20.3 \pm 0.1 (20.1-20.5)	98.0 \pm 1.0 (93.0-111.1)
25000	25170 \pm 45 (24900-25800)	8.02 \pm 0.09 (7.69-9.33)	20.3 \pm 0.1 (20.1-20.6)	99.0 \pm 0.9 (93.4-110.3)
30000	30266 \pm 53 (30000-30900)	8.09 \pm 0.08 (7.63-9.24)	20.3 \pm 0.1 (20.1-20.3)	98.6 \pm 0.8 (94.3-110.6)
40000	40160 \pm 50 (39900-40600)	8.27 \pm 0.11 (7.89-9.20)	20.2 \pm 0.1 (20.0-20.5)	97.2 \pm 0.5 (94.1-100.7)
50000	50270 \pm 120 (50000-50600)	8.70 \pm 0.21 (8.21-9.18)	20.4 \pm 0.1 (20.3-20.6)	95.5 \pm 1.0 (93.3-97.6)

Larval survival was significantly affected by salinity (ANOVAs for each exposure period; $F_{6, 13-14} > 8.750$, $P < 0.001$ in each case) displaying a typical dose response (Figure 5.3). Survival was reduced to zero in the 50000 $\mu\text{S cm}^{-1}$ treatment after 3 days exposure and after 8 days in the 40000 $\mu\text{S cm}^{-1}$ treatment. Survival in the 10000 and 20000 $\mu\text{S cm}^{-1}$ treatments remained high until 11 days of exposure when it fell below 90% at which point the test was terminated. Survival in the 2280 $\mu\text{S cm}^{-1}$ treatment decreased with exposure period due to suspected fungal infection, being reduced to $48 \pm 22\%$ after 11 days of exposure. For this reason the 2280 $\mu\text{S cm}^{-1}$ treatment was excluded from logistic regressions to determine LC_{50} values. LC_{50} values for each 24-hour exposure period indicate that mortality did not stabilise during the 11-day test duration but decreased steadily after the initial 48 hours exposure (Figure 5.4). The 4-day (96-hour) LC_{50} for larval *P. australiensis* during this test was 39944 (95% CI 38153-41881) $\mu\text{S cm}^{-1}$ ($r^2 = 0.603$), this decreased to 27551 (95% CI 26084-29134) $\mu\text{S cm}^{-1}$

($r^2 = 0.538$) after 10 days exposure (See Appendix A Table A.4 for LC_{50} values for all exposure periods). Survival curves for 96-hour and 10-day exposure periods are presented in Figure 5.3.

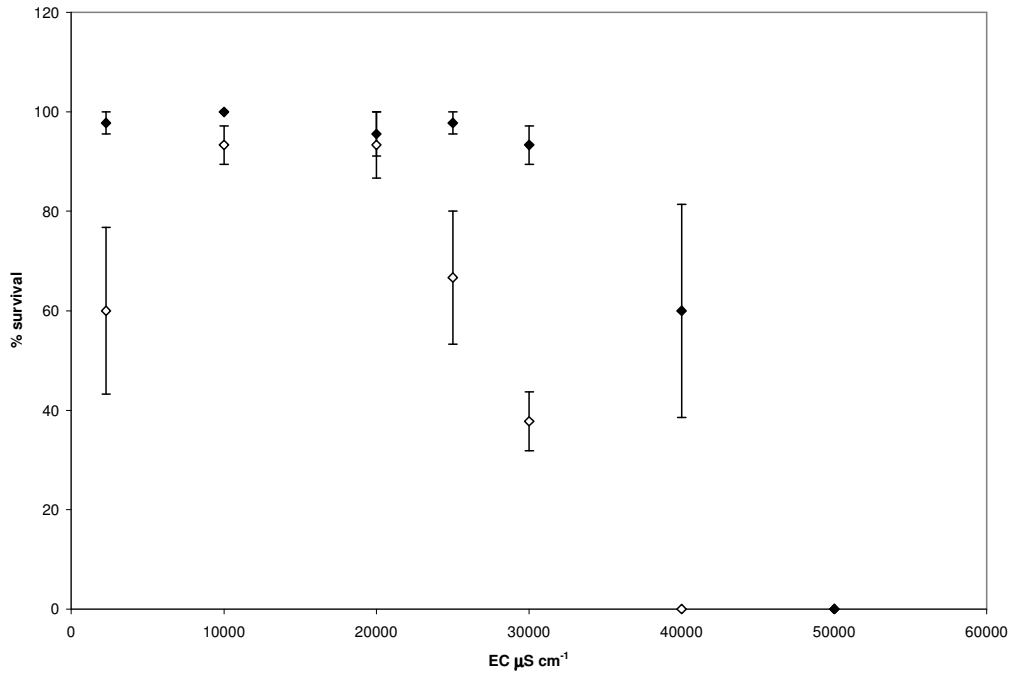


Figure 5.3 Survival (%) of larval *Paratya australiensis* after 4 days (96-hours)(closed diamonds) and 10 days (open diamonds) exposure at different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE.

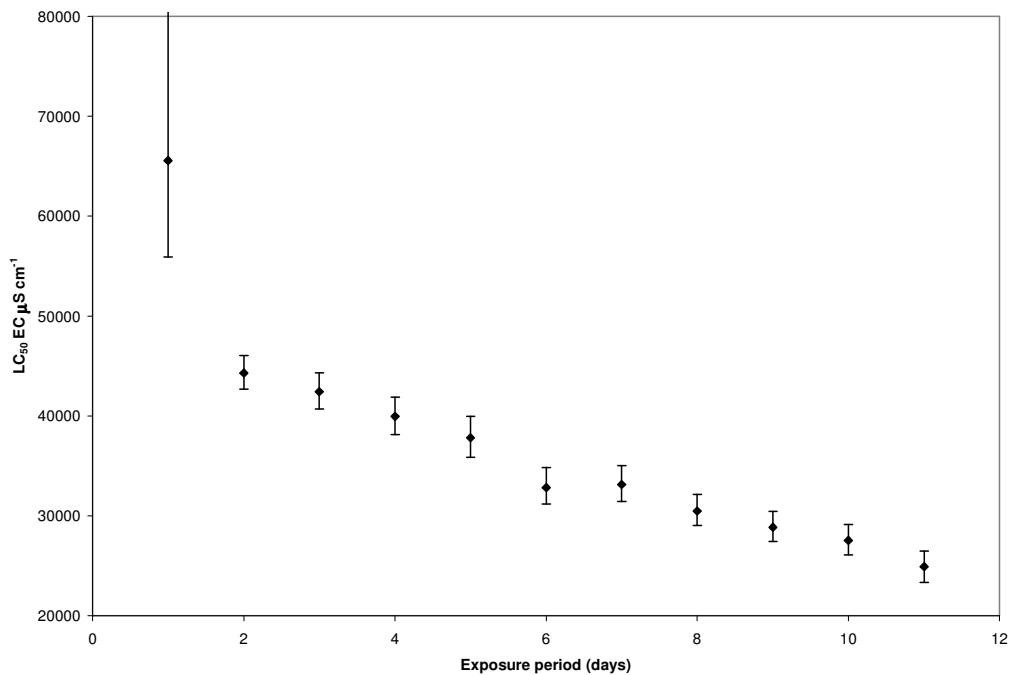


Figure 5.4 LC₅₀ values (EC μS cm⁻¹) for larval *Paratyta australiensis* after different periods of salinity exposure. Error bars indicate 95% CI.

5.3.3 Effects of salinity on egg hatching success

Close to 100% hatching success of *P. australiensis* eggs was observed at all of the test salinities (i.e. 67-30000 μS cm⁻¹), with no more than 2-3 individual eggs, of often greater than one hundred eggs in a clutch, failing to hatch. The only occasions where egg hatching did not occur was where females died before eggs had hatched. Survival of newly hatched larva appeared to decrease with increasing salinity, however this was not directly investigated in this test.

5.3.4 Effects of salinity on juvenile growth

Water quality remained high throughout the two-month test period (Table 5.4). Mean salinities were within 0.5% of the nominated treatment salinity, with maximum variability less than 8%. Differences in salinities between treatments were highly statistically significant (ANOVA; $F_{4, 335} = 125404.353$, $P < 0.001$). Mean temperatures were within the 20 ± 2 desired range, with the maximum variability only 0.2°C outside the range. Mean dissolved oxygen percent saturation values were greater than 89%, with all measured values greater than 50%. There were no significant differences in temperature or dissolved oxygen saturation between treatments (ANOVA; $F_{4, 335} = 0.156$, $P = 0.960$ and ANOVA; $F_{4, 115} = 0.129$, $P = 0.972$ respectively). pH ranged between 6.99 and 9.20 during the test and was

generally higher with increasing salinity. Mean values ranged from 7.53 ± 0.03 in the 2200 $\mu\text{S cm}^{-1}$ treatment to 8.14 ± 0.05 in the 30000 $\mu\text{S cm}^{-1}$ treatment. Differences in pH were significant between treatments (ANOVA; $F_{4, 253} = 28.955$, $P < 0.001$) with the 10000, 20000 and 30000 $\mu\text{S cm}^{-1}$ treatments displaying higher pH values than the 2200 and 5000 $\mu\text{S cm}^{-1}$ treatments (Tukey's pair-wise comparisons; $P < 0.01$ in all cases).

Table 5.4 Water quality for test investigating the effects of salinity on the growth of juvenile *Paratya australiensis*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
2200	2210 \pm 4 (2128-2356)	7.53 \pm 0.03 (6.99-8.20)	20.5 \pm 0.1 (19.4-22.2)	90.8 \pm 1.4 (72.4-99.7)
5000	5010 \pm 9 (4870-5270)	7.66 \pm 0.05 (7.08-8.91)	20.5 \pm 0.1 (19.6-22.1)	90.2 \pm 1.5 (69.7-99.4)
10000	9999 \pm 16 (9680-10450)	7.95 \pm 0.06 (7.21-9.20)	20.5 \pm 0.1 (19.5-21.9)	90.7 \pm 1.4 (71.8-100.5)
20000	20014 \pm 33 (19470-20850)	8.10 \pm 0.06 (7.28-9.14)	20.4 \pm 0.1 (19.0-21.9)	90.2 \pm 2.1 (54.1-103.0)
30000	29916 \pm 61 (27600-31000)	8.14 \pm 0.05 (7.53-9.12)	20.5 \pm 0.1 (19.4-22.0)	89.2 \pm 2.4 (50.5-104.9)

There were no significant differences in initial wet weights of juvenile *P. australiensis* between salinity treatments (ANOVA; $F_{4, 45} = 0.541$, $P = 0.707$). The initial size of shrimp ranged from between 20.4 and 39.3 mg wet weight, with a mean wet weight of 29.4 ± 0.8 mg. The relationship between the wet and dry weight of juvenile shrimp collected at the same time as test specimens was;

$$\text{Dry Weight (mg)} = (0.188 \pm 0.002) \times \text{Wet Weight (mg)}$$

$$(F_{1,32} = 10380.463, P < 0.001, r^2 = 0.997)$$

This relationship was used to calculate the initial dry weights of test specimens (Figure 5.5). Individuals died in the 2200 and 10000 $\mu\text{S cm}^{-1}$ treatments during the test period leaving 9 individuals in these treatments.

The growth (increase in dry weight) of juvenile *P. australiensis* was significantly affected by salinity over the two-month exposure period (ANOVA; $F_{4, 43} = 4.250$, $P = 0.005$). Growth was greatest in the 5000 $\mu\text{S cm}^{-1}$ treatment with individuals increasing in dry weight by an average of 3.44 ± 0.55 mg (Figures 5.5 and 5.6). The least growth was observed in the 30000 $\mu\text{S cm}^{-1}$ treatment with a mean increase of only 0.69 ± 0.23 mg (Figures 5.5 and 5.6). Tukey's pair-wise comparisons indicate that growth was significantly higher in the 5000 $\mu\text{S cm}^{-1}$ treatment than in both the 2200 $\mu\text{S cm}^{-1}$ ($P = 0.002$) and 30000 ($P = 0.002$) treatments, which displayed $46.5 \pm 13.5\%$ and $20.2 \pm 6.6\%$ of the growth that occurred at 5000 $\mu\text{S cm}^{-1}$ respectively. Shrimp in the 10000 and 20000 $\mu\text{S cm}^{-1}$ treatments also displayed lower mean growth than those at 5000 $\mu\text{S cm}^{-1}$ (Figures 5.5 and 5.6), however these difference were not statistically significant. A two-way *t*-test between the observed optimal growth at 5000 $\mu\text{S cm}^{-1}$ and growth at 10000 $\mu\text{S cm}^{-1}$ indicated a significant difference at a 90% confidence level ($t_{17,0}=1.740$, $P = 0.100$). However, comparison between growth at 5000 $\mu\text{S cm}^{-1}$ and that at 20000 $\mu\text{S cm}^{-1}$ was not significant ($t_{17,8}=1.318$, $P = 0.204$). There was a high degree of variability in the amount of growth between individuals at the same salinity and some individuals lost weight during the test period. There was no significant effect of initial size on weight increase ($r^2 < 0.16$).

Due to the high within treatment variability and the unusual pattern of response with increased salinity an EC_{50} value for juvenile *P. australiensis* growth could not be determined through analysis of the whole data set. As evident in Figure 5.6, growth of juvenile *P. australiensis* fell below 50% of the optimal observed at 5000 $\mu\text{S cm}^{-1}$ at salinities between 20000 and 30000 $\mu\text{S cm}^{-1}$. Regression analysis of growth occurring at these salinities was used to enable determination of the EC_{50} value. The relationship was best described by the equation;

$$\text{Growth (mg dry weight)} = (-0.165 \times 10^{-3}) \times (\text{EC } \mu\text{S cm}^{-1}) + 5.646$$

$$(F_{1, 18} = 6.289, P = 0.022, r^2 = 0.259, \text{SE of slope} = 0.066 \times 10^{-3}, \text{SE of constant} = 1.678).$$

Using this relationship and the optimal growth observed at 5000 $\mu\text{S cm}^{-1}$ the salinity EC_{50} for growth of juvenile *P. australiensis* was calculated as 23794 (9732-56606 SE) $\mu\text{S cm}^{-1}$.

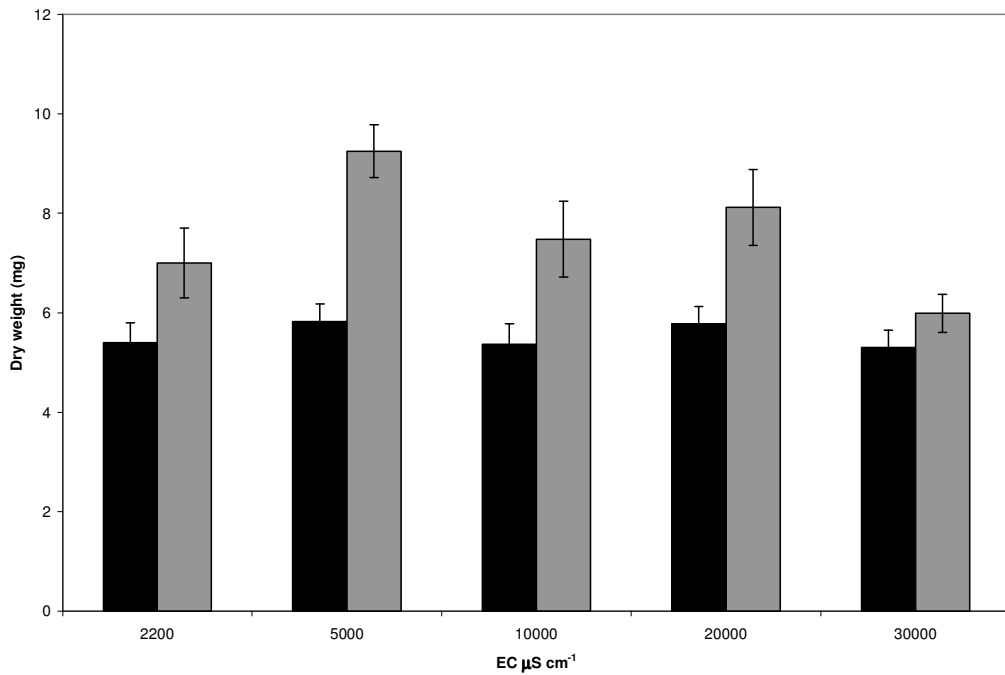


Figure 5.5 Calculated initial dry weight (mg) (black) and final dry weight (mg) (grey) of juvenile *Paratya australiensis* after two months exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

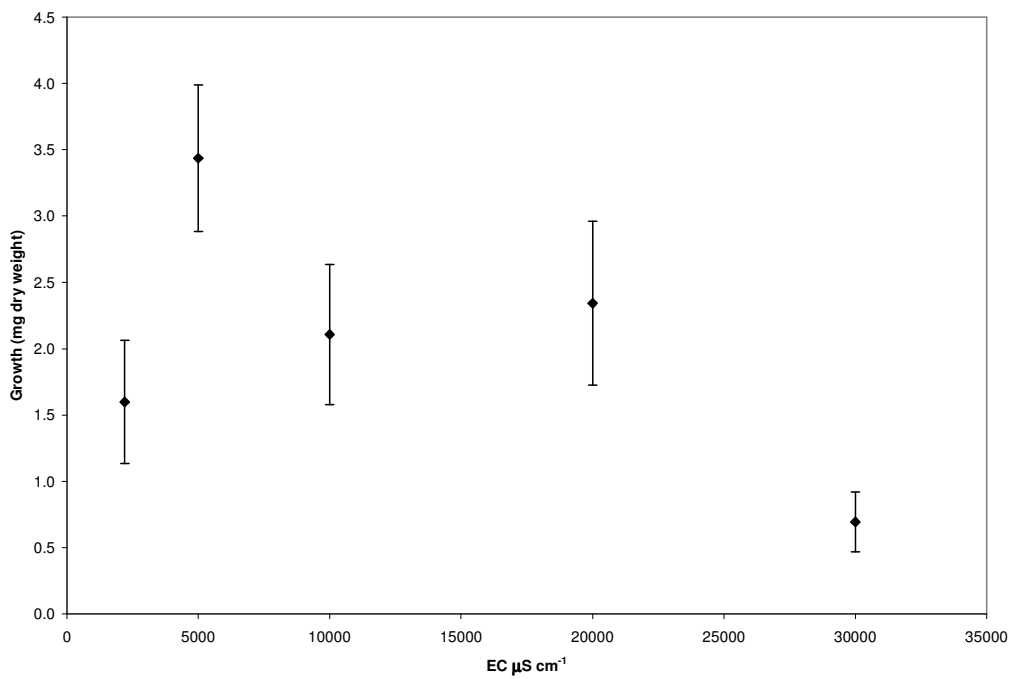


Figure 5.6 The increase in dry weight (mg) of juvenile *Paratya australiensis* after two months exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

As would be expected given the differences in growth, there was a significant effect of salinity on the absolute final dry weight of shrimp (ANOVA; $F_{4, 43} = 3.830$, $P = 0.009$) (Figure 5.5). The mean dry weight of shrimp was greatest at 5000 (9.25 ± 0.53 mg) and smallest at 30000 (5.99 ± 0.38 mg) (Figure 5.5). Although the mean values for dry weight varied between all treatments (Figure 5.5), only the difference between these two treatments was significant (Tukey's pair-wise comparison; $P = 0.005$) due to the variability in this measure.

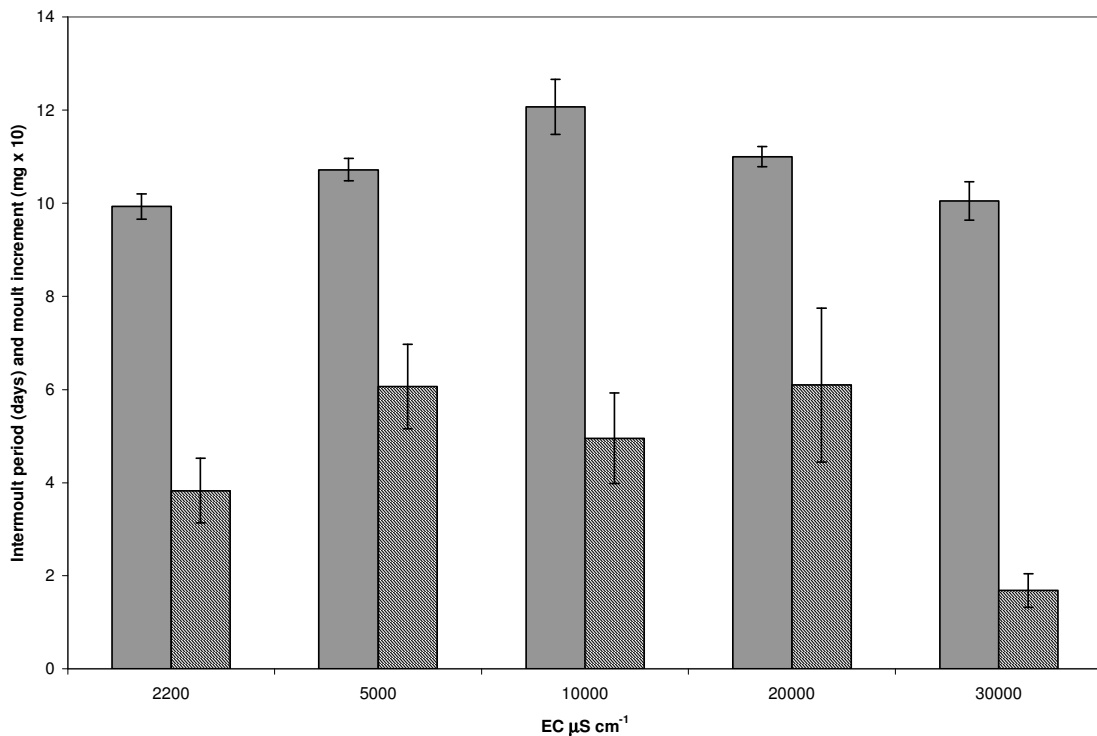


Figure 5.7 The mean intermoult period (days) (solid bars) and mean moult weight increment (dry weight mg per moult $\times 10$) (hatched bars) for juvenile *Paratya australiensis* during two months exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

There were significant differences in the intermoult period (number of days between moults excluding initial period until first moult) of juvenile *P. australiensis* at different salinities (ANOVA; $F_{4, 42} = 5.369$, $P = 0.001$) (Figure 5.7). Intermoult period was significantly longer at 10000 $\mu\text{S cm}^{-1}$ (12.07 ± 0.59 days) than at 2200 $\mu\text{S cm}^{-1}$ (9.93 ± 0.27 days) and 30000 $\mu\text{S cm}^{-1}$ (10.05 ± 0.41 days) (Tukey's pair-wise comparison; $P < 0.01$ in both cases). Extreme outliers were excluded from data for the 5000 and 20000 $\mu\text{S cm}^{-1}$ treatments. The mean weight increment increase per moult also varied significantly between salinity treatments

(ANOVA; $F_{4, 38} = 3.118$, $P = 0.026$) (Figure 5.7). The weight increment increase per moult was significantly less at $30000 \mu\text{S cm}^{-1}$ (0.17 ± 0.04 mg per moult) than at 5000 (0.61 ± 0.09 mg per moult) and $20000 \mu\text{S cm}^{-1}$ (0.61 ± 0.17 mg per moult) (Tukey's pair-wise comparison; $P < 0.039$ in both cases). Replicates displaying negative growth were excluded from analysis.

The final dry:wet weight ratio was significantly affected by salinity (ANOVA; $F_{4, 43} = 4.534$, $P = 0.004$) (Figure 5.8). Shrimp in the $5000 \mu\text{S cm}^{-1}$ treatment displayed the lowest proportional water content (dry:wet = 0.238 ± 0.004), significantly lower than those in the 2200 (dry:wet = 0.211 ± 0.007) and $30000 \mu\text{S cm}^{-1}$ (dry:wet = 0.205 ± 0.004) treatments (Tukey's pair-wise comparison; $P = 0.032$ and 0.004 respectively). Mean dry:wet weight ratios in all treatments after two months exposure indicated lower water content than shrimp at the beginning of the test (i.e. dry:wet = 0.188 ± 0.002).

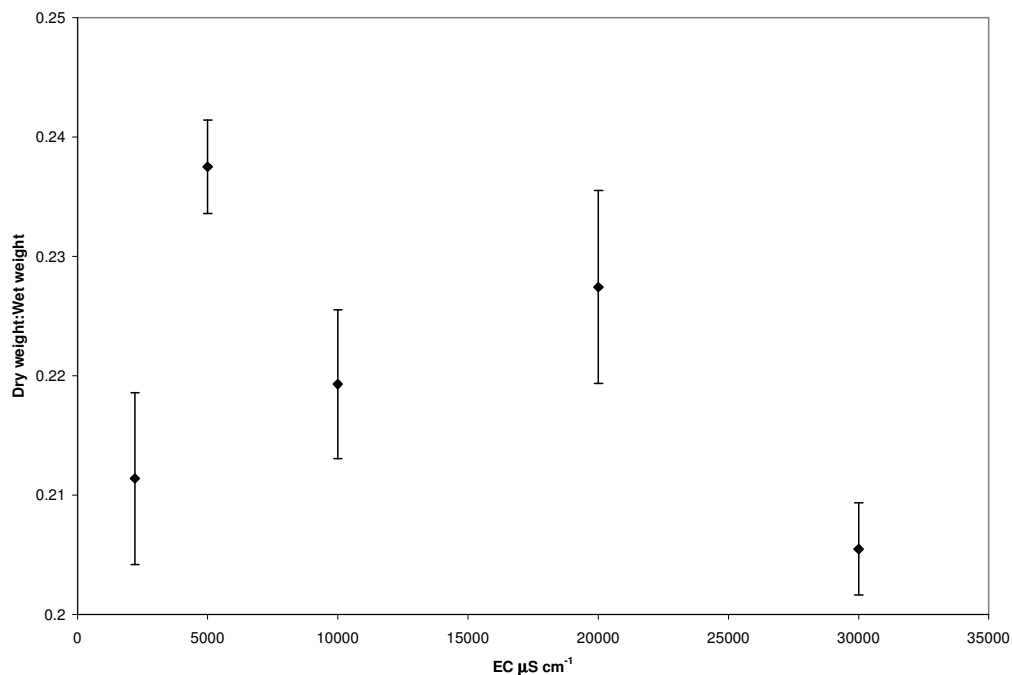


Figure 5.8 Dry:wet weight ratios of juvenile *Paratya australiensis* after two months exposure at different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE.

5.3.5 Effects of salinity on breeding success

No signs of breeding behaviour or of females becoming gravid were observed at any of the test salinities (i.e. including the salinity of river water at the time of test specimen collection) during 30 days of exposure. The test was deemed unsuccessful and terminated at this point.

5.3.6 Relationship between lethal and sublethal salinity tolerance

Significant effects on both 10-day larval survival and juvenile growth occurred at salinities having no effect on acute adult survival (Figure 5.9). The ratio between the EC₅₀ values for these end points and the 96-hour LC₅₀ for adult survival were 0.69 and 0.60 respectively (Table 5.5).

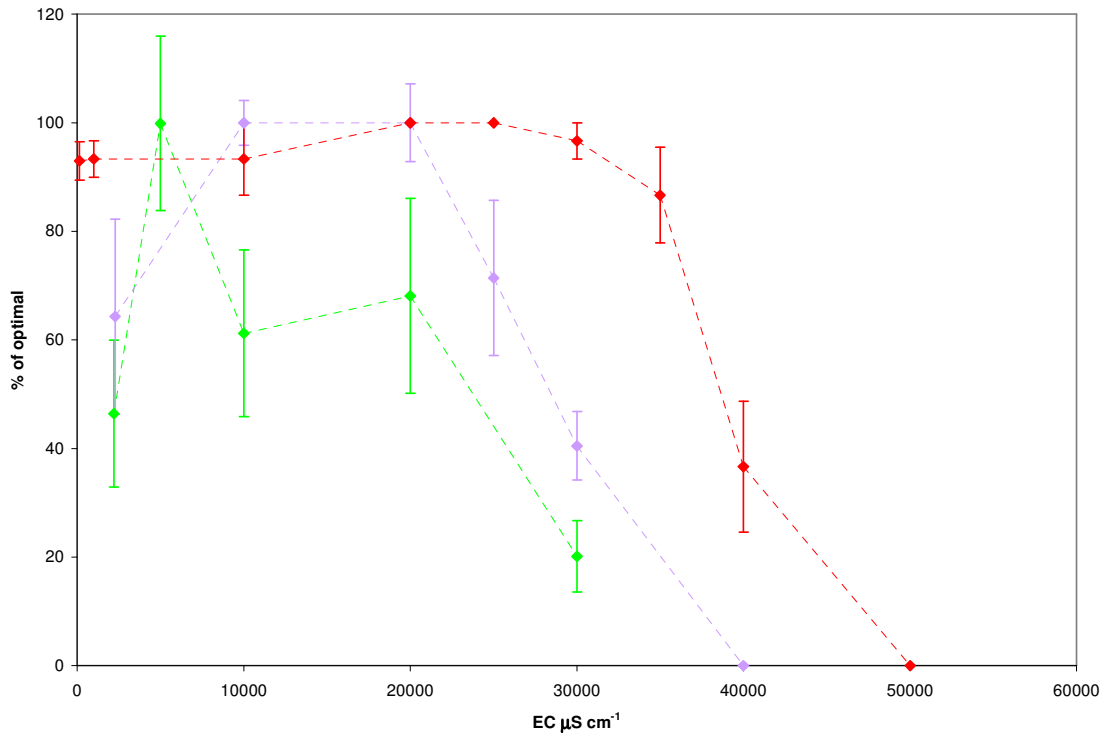


Figure 5.9 The proportional effects of salinity (EC $\mu\text{S cm}^{-1}$) on the 96-hr adult survival (red), 10-day larval survival (purple) and juvenile growth (green) of *Paratya australiensis* collected from the Barwon River. Mean \pm SE.

Table 5.5 Summary of the salinity tolerance of *Paratya australiensis* and the ratio between sublethal EC₅₀ values and the 96-hr LC₅₀ for the species.

<i>End point</i>	Observed optimal EC ($\mu\text{S cm}^{-1}$)	LOEC ($\mu\text{S cm}^{-1}$)	EC₅₀ ($\mu\text{S cm}^{-1}$)	Ratio (EC₅₀/96-hr LC₅₀)
96-hr adult survival	135-35000	40000	39844 (95% CI 37308-43055)	-
Time independent adult survival	135-35000	40000	38957 (95% CI 36021-42714)	-
96-hr larval survival	2280-30000	40000	39944 (95% CI 38153-41881)	1.00
10-day larval survival	10000-20000	25000	27551 (95% CI 26084-29134)	0.69
Juvenile growth (60-days)	5000	30000	23794 (SE 9732-56606)	0.60

5.4 Discussion

5.4.1 Adult acute salinity tolerance

Quite clearly adult *P. australiensis* have very high acute salinity tolerance, however they were unable to survive 24-hours at 50000 $\mu\text{S cm}^{-1}$ which is close to the salinity of sea water. This high tolerance probably reflects the marine ancestry of the species.

While comparable to the tolerance observed by Williams (1984) and Kefford *et al.* (2003a), the 96-hour LC_{50} value determined for adult *P. australiensis* by this study was higher than tolerances observed for this species by other studies (see Table 5.2). The LC_{50} values determined by Walker (1972), Morris (1991), Walsh (1994), Kefford *et al.* (2006a) and Dunlop *et al.* (2008) are lower again compared to the value determined by this study (see Table 5.2). The LC_{50} value determined by Bacher and Garnham (1992) for adult *P. australiensis* collected from the Yarra River, is much lower than all other determinations for adults of this species. These differences may be attributable to differences in condition of the test specimens, different test conditions, the past salinity exposure of test specimens and/or seasonal effects. That this species is likely a complex of species with some geographically isolated populations (Cook *et al.* 2006) indicates the potential for differences in salinity tolerances to develop between populations with different historical exposure to salinity. Studies into the genetics (Hughes *et al.* 1995) and movement (Hancock and Hughes 1999) of populations of *P. australiensis* in upland streams in south east Queensland have found very little movement and interbreeding between populations.

Other species of freshwater crustacea display characteristic high salinity tolerance, with the majority displaying higher acute salinity tolerance than that of adult *P. australiensis*. The freshwater shrimp *Macrobrachium australiense* (Decapoda: Palaemonidae) which often occurs in the same rivers as *P. australiensis* (Richardson *et al.* 2004) displays higher tolerance than *P. australiensis* (96-hour LC_{50} approximately 59000 $\mu\text{S cm}^{-1}$ (Williams 1984)), while *Caridina nilotica* (Decapoda: Atyidae) a freshwater Atyid shrimp from South Africa displays slightly lower tolerance (96-hour LC_{50} 34000 95% CI 33000-36000 $\mu\text{S cm}^{-1}$ (Kefford *et al.* 2004a)). Australian species of freshwater crayfish (Decapoda: Parastacidae) display slightly higher acute tolerance than *P. australiensis* (*Cherax destructor* 96-hour LC_{50} approximately 44000 $\mu\text{S cm}^{-1}$ (Mills and Geddes 1980) and *Geocherax gracilis* 72-hour LC_{50} 40000-50000 $\mu\text{S cm}^{-1}$ (Kefford *et al.* 2003a)). The crab *Amarinus lacustris* (Decapoda: Hymenosomatidae) displays the highest acute salinity tolerance of studied freshwater decapods, with Williams

(1984) observing close to 100% survival after 96 hours exposure at approximately 67000 $\mu\text{S cm}^{-1}$ and Kefford *et al.* (2003a) estimating the 72-hour LC_{50} for the species as between 70000 and 85000 $\mu\text{S cm}^{-1}$. Non decapod freshwater crustacea also display very high salinity tolerance, with the amphipod species *Austrochiltonia australis* and *Austrochiltonia subtenuis* (Amphipoda: Ceinidae) displaying 96-hour LC_{50} values of approximately 46000 and 53000 $\mu\text{S cm}^{-1}$ respectively (Walker 1984) and the isopod *Cymodetta* sp. (Isopoda: Sphaeromatidae) displaying a 72-hour LC_{50} of between 70000 and 75000 $\mu\text{S cm}^{-1}$ (Kefford *et al.* 2003a). Riverine microcrustacea species from the Murray-Darling Basin in Victoria display generally lower acute salinity tolerance than macroinvertebrate crustacea taxa (Kefford *et al.* 2007b).

That there was little change in LC_{50} values for *P. australiensis* between 4 days (96 hours) of exposure and 10 days of exposure (when mortality stabilised) indicates that the standard 96 hours of exposure sufficiently encompasses the acute phase of salinity toxicity to adults of this species. There was quite high error associated with LC_{50} determinations, which increased with increasing exposure period. This was due to variability between replicates and mortality at lower salinities. This could be reduced by increasing replication and excluding lower treatments from LC_{50} determinations. The precision of LC_{50} determinations may be increased by the inclusion of more treatments within the salinity range where partial survival occurred (i.e. between 30000 and 50000 $\mu\text{S cm}^{-1}$).

5.4.2 Larval salinity tolerance

The salinity tolerance of larval *P. australiensis* observed in this study after 96 hours exposure (96-hour LC_{50} 39944 95% CI 38153-41881 $\mu\text{S cm}^{-1}$) was comparable to that of the adult life stage (96-hour LC_{50} 39844 95% CI 37308-43055 $\mu\text{S cm}^{-1}$). However, after 10 days exposure, salinity tolerance was greatly reduced (10-day LC_{50} 27551 95% CI 26084-29134 $\mu\text{S cm}^{-1}$). The 48-hour salinity LC_{50} for 1-15 day old larval *P. australiensis* determined by Kefford *et al.* (2004a) (33000 95% CI 26000-44000 $\mu\text{S cm}^{-1}$) is intermediate between the 96-hour and 10-day LC_{50} values observed during this study, with both of these values falling within the 95% confidence range. Walsh (1994) observed lower salinity tolerance for juvenile *P. australiensis* (96-hour LC_{50} 31500 95% CI 30400-32400 $\mu\text{S cm}^{-1}$) than the adult stage but suggested that larval tolerance was higher than that of adults. This was based on an observed optimal salinity of approximately 22000 $\mu\text{S cm}^{-1}$ for rearing of larvae (Walsh 1994). This observed optimal may, however, have been strongly influenced by methodology, in particular the survival of *Artemia salina* (Crustacea: Anostraca) which was provided as food (Walsh

1993). The high survival of larval *P. australiensis* at a salinity of 20000 $\mu\text{S cm}^{-1}$ observed after 10 days exposure during this test reflects the tolerance observed by Walsh (1993).

The 48-hour LC_{50} for the larval stage of *Caridina nilotica* (Decapoda: Atyidae), a freshwater Atyid shrimp from South Africa, (37000 95% CI 33000-44000 $\mu\text{S cm}^{-1}$) (Kefford *et al.* 2004) was very similar salinity to that observed for larval *P. australiensis*. The salinity tolerance of larval *Macrobrachium australiense* (Decapoda: Palaemonidae) from south east Queensland is also similar to that of *P. australiensis*, with 50% or higher survival to post larval stage at a salinity of approximately 22000 $\mu\text{S cm}^{-1}$ but 100% mortality at approximately 29400 $\mu\text{S cm}^{-1}$ (Lee and Fielder 1981). The salinity tolerance of larvae of *Palaemonetes kadiakensis* (Decapoda: Palaemonidae), a freshwater shrimp from north America, seems to be much lower than that of *P. australiensis* and the other studied shrimp species with Hubschman (1975) observing a reduction in survival at a salinity of approximately 5100 $\mu\text{S cm}^{-1}$. In contrast the salinity tolerance of larvae of the freshwater crab *Amarinus lacustris* appears to be much higher than that of the freshwater shrimp species with 100% survival observed after 48 hrs exposure at a salinity of 47000 $\mu\text{S cm}^{-1}$ (Kefford *et al.* 2004a).

The significantly higher temperature observed in the 50000 $\mu\text{S cm}^{-1}$ treatment during this study was due to slightly higher temperatures in all treatments at the start of the test. As mortality occurred within the first three days of the test at 50000 $\mu\text{S cm}^{-1}$, the higher temperature during this test period resulted in higher average temperature in this treatment. The difference in mean temperatures was only small (i.e. no greater than 0.2°C) and the range of temperatures observed was well within the $20 \pm 2^\circ\text{C}$ desired range. Therefore, it is unlikely that the slightly higher average temperature in the 50000 $\mu\text{S cm}^{-1}$ treatment would have affected larval survival.

The inability to maintain high survival of larval *P. australiensis* at any salinity for more than 11 days suggests that test conditions were unsuitable for long term larval survival. Several attempts to raise larvae through to the juvenile stage were unsuccessful. It is unclear which aspects of the test conditions were unfavourable. Walsh (1993) successfully reared *P. australiensis* larvae from hatching through to the juvenile stage in the laboratory at a salinity of $\approx 22000 \mu\text{S cm}^{-1}$. Attempts to rear larvae at salinities less than $\approx 7400 \mu\text{S cm}^{-1}$ were unsuccessful, however this was thought to be due to unviability of the food source (live *Artemia salina*) at this salinity (Walsh 1993). Attempts to use live *Artemia* as a food source

in larval salinity tolerance investigations during this study were also unsuccessful due to the inability of this species to survive at low salinities. The ground commercial trout pellets provided as food during the test may have been unsuitable. Walsh (1993) found that larvae at salinities where *Artemia* did not survive did not develop past stage III. It took *P. australiensis* larvae 9-12 days to develop to stage IV at a salinity of $\approx 22000 \mu\text{S cm}^{-1}$ at 18-22°C (Walsh 1993). This suggests that the food source may have been unsuitable during this study as wide spread mortality was observed after 12 days. Unfavourable conditions may have had some influence on observed patterns of mortality with increased salinity during this study. Further investigations of the salinity tolerance of larval *P. australiensis* with a known suitable food source are required.

Walsh and Mitchell (1995) suggested movement of larvae between estuaries via the ocean as a possible (although rare) mode of dispersal for *P. australiensis*. The results of this study suggest that larval *P. australiensis* would be unable to tolerate salinities as high as that of sea water (approximately $51500 \mu\text{S cm}^{-1}$) for any extended period of time, with 100% mortality occurring at a salinity of $50000 \mu\text{S cm}^{-1}$ after 3 days exposure. There may however be differences in salinity tolerance between different populations of the species.

5.4.3 Effects of salinity on egg hatching success

The observation during this study that high egg hatching success occurred where adult females remained alive suggests that egg hatching success may display similar salinity tolerance to long term adult survival. However, eggs were only exposed during the later stages of development. Further research exposing eggs for the full development period is required to effectively assess the salinity tolerance *P. australiensis* eggs.

5.4.4 Effects of salinity on juvenile growth

Growth of juvenile *P. australiensis* did not display a threshold response with increased salinity as was observed for adult and larval survival of the species. Observed optimal growth occurred at $5000 \mu\text{S cm}^{-1}$, which was higher than the salinity from which the test specimens were collected ($2200 \mu\text{S cm}^{-1}$). Higher growth at slightly elevated salinities has been observed for a number of freshwater macroinvertebrate species (see Kefford and Nugegoda 2005; Hassell *et al.* 2006; Kefford *et al.* 2006b). At higher salinities there was a range (10000 to $20000 \mu\text{S cm}^{-1}$) across which growth was reduced by a similar level and above this growth was further reduced (e.g. $30000 \mu\text{S cm}^{-1}$). The observed effects on growth at $30000 \mu\text{S cm}^{-1}$ represent an 80% reduction in growth. That all 10 juvenile shrimp survived at this salinity for

the two-month test period indicated they are able to tolerate this salinity for extended periods. At this salinity, survival of adult *P. australiensis* was unaffected after 16 days exposure in the acute survival test and 30 days in the reproduction test. However, larval survival at this salinity was reduced to 38% after 10 days (Figure 5.3). Results suggest some reduction in growth at 10000 and 20000 $\mu\text{S cm}^{-1}$, however, the high variability in individual growth throughout the test reduced the ability to statistically discern differences.

Growth in Crustacea reflects the interaction of both moult frequency and the increment of size increase per moult (Hart 1980). At the observed optimal salinity for growth during this study (i.e. 5000 $\mu\text{S cm}^{-1}$) growth was the result of short periods between moults and large weight increases with each moult. At 30000 $\mu\text{S cm}^{-1}$ where the lowest growth was observed the intermoult period was also short but weight increases with each moult were very small. Hart (1980) observed longer intermoult duration with increasing size and greatest moult increments in intermediate sizes in the freshwater Atyid shrimp *Caridina nilotica* (Decapoda: Atyidae).

The observed pattern of dry:wet weight ratios of *P. australiensis* after two months salinity exposure may reflect changes in internal water balance as a result of osmoregulation. Williams (1984) investigated osmoregulation in *P. australiensis* from the Torrens River (South Australia) and found effective hyperosmotic regulation up to approximately 18000-19000 $\mu\text{S cm}^{-1}$ with a constant haemolymph salinity of approximately 19000 $\mu\text{S cm}^{-1}$ maintained. At salinities greater than 19000 $\mu\text{S cm}^{-1}$ haemolymph salinity increased at slightly hypertonic concentrations with increasing salinity (Williams 1984). If the pattern of osmoregulation observed by Williams (1984) reflects that occurring during this study, decreases in the salinity of the external media below approximately 19000 $\mu\text{S cm}^{-1}$ would result in an increasing osmotic gradient across the cuticle favouring the inflow of water. This may explain why *P. australiensis* displayed higher water content in the 10000 and 2200 $\mu\text{S cm}^{-1}$ treatments, as there may have been an imbalance between the influx of water and the excretion efficiency of the renal organs. At salinities above 19000 $\mu\text{S cm}^{-1}$ the osmotic gradient would be reversed, favouring loss of water from the body. The higher water content of *P. australiensis* at 30000 $\mu\text{S cm}^{-1}$ may reflect overcompensation for water loss by increased drinking or may also be related to the smaller final size of shrimp at this salinity. The higher dry:wet weight ratio observed for *P. australiensis* at 5000 $\mu\text{S cm}^{-1}$ may be related to the larger final size of individuals at this salinity as there was a trend for lower proportional water content with increased size. This was evident in the higher dry:wet weight ratio for shrimp at

the end of the test than at the start. Despite statistical significance, the maximum difference in mean dry:wet weight ratios between treatments was only small, representing only a 3% difference in water content.

The effects of salinity on growth of juvenile *P. australiensis* maybe expected to be different under natural conditions compared to laboratory conditions. This test was designed to investigate the relative effects of salinity on growth rather than determine the absolute effects expected in natural populations. The observed effects maybe more extreme under natural conditions due to the higher energy requirements required for movement, biotic interactions, feeding and tolerating physico-chemical fluctuations. The nutritional state of individuals would also differ between natural and laboratory conditions. Given that individuals were fed to satiation in the laboratory, it could be assumed that they were in a strong nutritional state, providing that the food supplied was suitable. Saturation feeding may have allowed any increased energy costs required for osmoregulation to be off set by increased food intake; under field conditions this would require greater feeding effort and unlimited food availability. Another potentially confounding factor relates to the observation that more highly stressed individuals displayed reduced movement (personal observation). This may result in increased effects in the field due to reduced capacity for feeding and also result in higher predation.

Variability was high in all end points measured in this test. This high variability may have been due to differences in growth due to differences in size, moult stage or sex (Hart 1980), errors involved in weighing of shrimp and natural variability. Using a smaller range of initial sizes of shrimp and increasing replication may potentially reduce this variability. Sexing of shrimp at this size class is not easy while they are alive (personal observation). If these errors were reduced it may be possible to discern effects of salinity at salinities lower than 30000 $\mu\text{S cm}^{-1}$.

5.4.5 Effects of salinity on breeding success

The failure of *P. australiensis* to breed during the breeding test may have been due to a number of reasons. Both photoperiod and temperature have been found to be important factors in the timing of *P. australiensis* breeding (Hancock 1995; Hancock and Bunn 1997). The conditions within the laboratory (16:8 light:dark and $20 \pm 2^\circ\text{C}$) may not have been favourable for breeding. Other factors such as stocking density and the lack of vegetative cover may also have been unfavourable.

The size of the females used in the test may also have been a factor in the failure to breed. The breeding population of females in the Barwon River observed over a number of seasons appeared to be comprised of two age classes, with the smaller of the two classes breeding later in the season (personal observation). Hancock and Bunn (1997) also observed this pattern in populations of *P. australiensis* in south-east Queensland. Williams (1977) and Hancock (1995) observed a two-year life-cycle for female *P. australiensis* with a small proportion reproducing in their first year but most not until their second breeding season. This appears to reflect observations of the source population of test specimens from in the Barwon River. Given that the breeding season in Victoria is from mid-spring until late summer (Williams 1977; Richardson et al. 2004), when females were collected for this test in October many of the larger females were already berried. This resulted in smaller females being collected for the test. These females may not have been ready to breed at this point in the season or may have been first-year females not ready to breed until the following season. Conducting the test earlier in the breeding season with larger females and providing test conditions more reflective of natural conditions may improve success.

5.4.6 Relationship between lethal and sublethal salinity tolerance

Both larval survival and juvenile growth of *P. australiensis* were found to be significantly affected by salinities lower than those affecting the acute survival of the adult life stage of the species. The ratio between the EC₅₀ values for these end points and the 96-hour LC₅₀ for the adult life stage were 0.69 and 0.60 respectively. It is likely, however, that juvenile growth is more sensitive than was determined in this study as effects were obscured by high within treatment variability.

Few studies have investigated both the acute salinity tolerance of the adult life stage and the tolerance of sensitive life stages or sublethal end points for freshwater macrocrustacea. Both Walsh (1994) and Kefford *et al.* (2003a, 2004a) observed lower salinity tolerance of younger life stages of *P. australiensis* than the adult stage. However, tolerances observed were not as sensitive as 10-day larval survival observed in this study (EC₅₀:LC₅₀ 0.88 and 0.93 respectively). Kefford *et al.* (2004a) found the larval stage of the South African shrimp *Caridina nilotica* to be as salinity tolerant as the adult life stage. Mills and Geddes (1980) investigated the salinity tolerance of the yabby *Cherax destructor* finding behavioural responses and juvenile survival to be more sensitive than adult survival (EC₅₀:LC₅₀ 0.60 and 0.86 respectively). Kefford *et al.* (2007b) observed a similar relationship between the sensitivity of egg hatching and adult survival for the microcrustacea *Newnhamia fenestra*

(Ostracoda: Notodromadidae) as the sensitivity of sublethal end points observed in this study (EC₅₀:LC₅₀ 0.65).

This study indicates that even for highly salt tolerant species it is important to consider the sensitivity of different life stages and sublethal effects when assessing the tolerance of a species. Further research into the sublethal tolerance of freshwater macrocrustacea is clearly needed. It is likely that further investigation will indicate even greater sensitivities to those observed in this study.

6.0 Salinity tolerance of the midge *Chironomus tepperi* Skuse (Insecta: Diptera: Chironomidae)

6.1 Introduction

Insects form a major component, in both diversity and abundance, of the macroinvertebrate communities of lowland streams. Some groups are fully aquatic while others complete only early life stages in the aquatic environment. Dipterans are often the most diverse insect order in these ecosystems, with the family Chironomidae making a large contribution to species diversity (Bunn and Davies 1992; Metzeling 1993; Marshall and Bailey 2004).

Chironomids are found in a wide range of aquatic environments and are often present in high abundance. All life stages except the adult stage are aquatic, with eggs being oviposited into water by the flying adult stage. Chironomids are an important food source for fish, water birds (Usinger 1956) and predatory macroinvertebrates, and also play an important role in nutrient cycling (Webster 1983 and Fisher and Gray 1983 in Huryn and Wallace 1986). The family has global distribution represented by over 5000 species (Oliver 1971).

Dipterans are the only insect order to successfully colonise highly saline waters (Hart *et al.* 1991; Williams *et al.* 1991), with some Chironomid species adapted to living in hypersaline lakes (Timms 1983; Williams *et al.* 1990; Pinder *et al.* 2005), peripheral marine habitats and intertidal rock pools (Oliver 1971; Kokkinn and Williams 1988). Other dipteran families occurring in highly saline environments include; Ceratopogonidae, Culicidae, Ephydriidae and Empididae (McGinnis and Brust 1983, Williams *et al.* 1990, Kay *et al.* 2001, Bailey *et al.* 2002). While some species of Chironomidae are highly salt tolerant, others are more sensitive. While much attention has been paid to the osmoregulatory adaptations of those species occurring at high salinities, little research has been conducted into the salinity tolerances of freshwater species (Greenaway 1986, Hart *et al.* 1991). The sublethal and long-term effects of salinity on freshwater Chironomidae species have attracted even less attention.

Berezina (2003) studied the salinity tolerance of six species of Russian chironomid larvae, finding maximum tolerances (i.e. 90 to 100% survival) after 15 days exposure ranging between approximately 3000 and 5000 $\mu\text{S cm}^{-1}$. Kefford *et al.* (2003a) investigated the short-term larval tolerance of a mixture of Chironomidae species from the Barwon River in Victoria, determining a 48hr LC_{50} of 10000 (95% CI 6800-15000) $\mu\text{S cm}^{-1}$. Chironomidae from the Murray-Darling basin in Victoria displayed moderate salinity tolerance with most of

the investigated species displaying 72-hour LC₅₀ values greater than 12800 µS cm⁻¹ (Kefford *et al.* 2006a). Dunlop *et al.* (2008) observed a 72-hour LC₅₀ of 14700 (95% CI 9600-19900) µS cm⁻¹ for mixed species of Chironomidae from the upper Murray-Darling Basin in Queensland. Investigation into the hatching success of unidentified *Chironomus* species eggs from a Victorian River found 50% percent hatching success (EC₅₀) at a salinity of 17700 (95% CI 15400-20500) µS cm⁻¹ (Kefford *et al.* 2007a), with a 10 day LC₅₀ for the hatched larvae of 16200 µS cm⁻¹. Hassell *et al.* (2006) investigated the emergence success, growth and development of an unidentified *Chironomus* species collected from the King Parrot Creek in Victoria, finding a reduction in emergence success and increased development time with increasing salinity. Chapman *et al.* (2000) found *Chironomus tentans* (Diptera: Chironomidae) to be less salt tolerant than embryos and fry of Rainbow trout (*Oncorhynchus mykiss*) and the green alga *Selenastrum capricornutum*.

Chironomus tepperi Skuse is a common colonist species in newly inundated habitats and as such is a significant pest species in rice plantations in NSW (Stevens 1993). The species was identified from a Murray River floodplain wetland with a salinity of 39800 µS cm⁻¹ (Goonan *et al.* 1992). It has also been recorded at a salinity of ≈19559 µS cm⁻¹ in the Paroo Lakes in NSW (Timms 1983) and ≈8088 µS cm⁻¹ in the Glenelg River in Victoria (Williams *et al.* 1991). Kefford *et al.* (2004a) investigated the hatching success of eggs of this species transferred to a range of test salinities, finding an EC₅₀ of 18000 (95% CI 17000-20000) µS cm⁻¹. Five days after hatching all larvae from these eggs had died at a salinity of 15000 µS cm⁻¹ while there was some survival at 12600 µS cm⁻¹ (Kefford *et al.* 2004a).

Chironomid species have been widely employed to test sediment toxicity, with standard test procedures developed for *Chironomus tentans* and *Chironomus riparius* (USEPA 1994; ASTM 1997). These methods along with a full life-cycle test for *Chironomus tentans* developed by Benoit *et al.* (1997) were adapted to investigate the salinity tolerance of the freshwater midge *C. tepperi*. This chapter investigates the acute, sublethal and full life-cycle effects of salinity on *C. tepperi*.

6. 2 Materials and methods

6.2.1 Culture techniques

C. tepperi eggs and culture methods were provided by the Genetics Department of Latrobe University, Bundoora, Victoria. Stock for this culture was originally sourced from areas surrounding the Yanco Agricultural Institute near Griffith in central New South Wales. Eggs and larvae were raised in mesh-covered 20-L plastic containers. Containers were filled with 4 L of unmodified filtered water ($\approx 133 \mu\text{S cm}^{-1}$) and quantities of shredded paper tissue and ground commercial trout food. The cultures were continuously aerated. Emerged adults were transferred to mesh covered 20-L plastic containers for breeding. Small dishes containing unmodified filtered water were provided for egg deposition. The culture was restocked as required with eggs collected from the reproduction chambers. The culture and all tests were maintained at a 16:8 hour light:dark photoperiod.

6.2.2 Larval acute salinity tolerance

The acute salinity tolerance of final instar (i.e. 4th instar) *C. tepperi* larvae was investigated using larvae from the laboratory culture. The test salinity range was 5000 to 25000 $\mu\text{S cm}^{-1}$ (treatment levels; 5000, 10000, 15000, 20000 and 25000 $\mu\text{S cm}^{-1}$) and unmodified filtered water ($146.1 \pm 18.5 \mu\text{S cm}^{-1}$) with 3 replicates of each treatment. The test was conducted in 440-mL plastic containers with 300 mL of test solution and standardised quantities of shredded paper tissue and ground commercial trout food (0.8 g tissue paper and 0.2 g food per litre). Earlier attempts to conduct the test without food and tube building material were marred by high levels of cannibalism. Eight day old larvae were collected from the laboratory culture and randomly distributed (10 per replicate) between the treatment replicates. Water quality was measured at the beginning and end of the test and water was not changed for the test duration. Larval survival was determined at 24-hour intervals for a period of 96 hours.

6.2.3 Egg hatching success

The effects of salinity on the hatching success of *C. tepperi* eggs were investigated using two methodologies. One method used eggs laid in unmodified filtered water, transferring them to test salinities, while the other used eggs laid at the test salinity. To collect egg masses, test solutions ranging from 3000 to 12000 $\mu\text{S cm}^{-1}$ (treatment levels; 3000, 5000, 7000, 10000 and 12000 $\mu\text{S cm}^{-1}$) and unmodified filtered water ($133 \mu\text{S cm}^{-1}$) were added to dishes in the *C. tepperi* culture reproduction chambers. Egg masses (between 2 and 14) from each salinity were collected, the masses broken up and the eggs mixed. Twenty eggs per replicate with 6

replicates per treatment were transferred to 6 by 16-mL multi-well plates containing 10 mL of test solution per well. Eggs laid at the test salinities were maintained at the salinity at which they were laid. Eggs laid in unmodified filtered water were distributed across a test salinity range of 5000 to 30000 $\mu\text{S cm}^{-1}$ (treatment levels; 5000, 10000, 15000, 20000, 25000 and 30000 $\mu\text{S cm}^{-1}$) and unmodified filtered water. A small amount of ground commercial trout food was added to each replicate. Eggs were monitored daily and the number of hatched eggs counted. Water quality was measured before and after water changes, with water pipetted from well plates to a measuring cylinder to enable water quality measurements. pH was not measured due to equipment failure. Results from this test were used to determine the salinity range for the full life-cycle test with *C. tepperi*.

6.2.4 Oviposition preference

To determine if *C. tepperi* displays a salinity preference for egg oviposition, females were provided with four different salinities (138, 5000, 15000 and 25000 $\mu\text{S cm}^{-1}$) in which to lay their eggs. Adult *C. tepperi* were collected from the culture chambers and distributed randomly to 3 mesh covered oviposition chambers. Each chamber contained four shallow dishes filled with the test salinity solutions. The test solutions were assigned to the dishes so that the position of each test salinity was different in each chamber. For six consecutive mornings the number of egg masses oviposited into each salinity was determined for each chamber. Egg masses and salinity solutions were pipetted from the dishes and fresh test solutions added each morning. Water quality of the fresh salinity solutions was measured daily, with the exclusion of pH, which was not measured due to equipment failure.

6.2.5 Full life-cycle effects

The long-term effects of salinity on *C. tepperi* were investigated by maintaining test specimens at test salinities of 3000, 7000 and 10000 $\mu\text{S cm}^{-1}$ and unmodified filtered water ($144.5 \pm 3.7 \mu\text{S cm}^{-1}$) for an entire life-cycle; from egg deposition to hatching of the second generation. Survival and growth of the second generation was also investigated. The effects of salinity on growth, development and reproductive success were investigated.

Over a number of days adult *C. tepperi* were collected from the laboratory culture and transferred randomly to 4 mesh covered 20-L plastic containers. When sufficient numbers of adults had been collected, a small dish within each container provided for egg deposition was filled with one of each of the test salinity solutions. Water quality of test solutions was determined before addition to containers.

The day following addition of test solutions egg masses were collected from each of the tanks. Egg masses from the same salinity (between 6 and 19 masses per treatment) were broken up and the eggs mixed together. Eggs were transferred to 6 by 16-mL multi-well plates containing 10 mL of test solution per well. Eight replicates of 40 eggs were established for each test salinity, with each treatment spread over two plates. A small amount of ground commercial trout food was added to each replicate. Eggs were examined daily to determine time till first hatch, hatching success and larval survival. Water quality of test solutions was determined before addition to multi-well plates and after larvae were transferred to the next test stage.

Five days after the eggs were laid (at which point egg hatching had ceased), 11 larvae per replicate were transferred to 440-mL plastic containers, containing 300 mL of test solution and standardised quantities of shredded paper tissue (1.6 g l^{-1}) and ground commercial trout food (0.2 g l^{-1}). Solutions were continuously aerated and fifty percent water changes were conducted twice weekly. Water quality was measured before and after water changes.

Thirteen days after the eggs were laid (when larvae were 10 to 12 days old) 3 replicates from each treatment were used to determine larval growth. Surviving larvae from each replicate were collected, counted and oven dried at 60°C . The pooled dried weight of each replicate was determined and mean dry weight of larvae calculated.

Remaining replicate containers (5 per treatment) were placed inside 5-L glass tanks covered with mesh to prevent escape of emerging adults. Tanks were monitored daily and emerged adults counted, sexed and transferred to reproduction tanks. Emergence was monitored until all larvae had emerged or 7 days had passed since last emergence, at which point emergence was deemed to have ceased. Mean number of days until emergence, emergence success and sex ratios were determined for each replicate.

Five-litre mesh-covered glass tanks containing small dishes filled with test solution for egg deposition were used to monitor reproductive success of the emerged adults from each replicate. Tanks were monitored daily, deposited egg masses counted and collected and dead adults removed. Water quality of old and fresh test solutions were measured throughout this test stage.

Collected egg masses were transferred to 6 by 16-mL multi-well plates containing 10 mL of test solution per well. The number of eggs in each mass was counted and eggs monitored for hatching. The number of eggs per mass was determined using a stereomicroscope and the ring count method described by Benoit *et al.* (1997) for larger egg masses. Time to first hatch and hatching success of eggs was determined. The number of eggs and egg masses produced per female was also determined for each replicate.

The growth of second generation larvae was investigated for a subset of egg masses. Methodology differed slightly to that for first generation larvae. Hatched larvae were maintained in the multi-well plates in which they hatched until 6 days after first hatching. 11 larvae per replicate were collected from separate egg masses and transferred to 440-mL plastic containers, containing 300 mL of test solution and the same standardised quantities of shredded paper tissue and ground commercial trout food as for first generation larvae. Due to too few numbers of surviving larvae this was not possible for the 10000 $\mu\text{S cm}^{-1}$ treatment. Three replicates were established for each of the other salinity treatments (i.e. 3000 and 7000 $\mu\text{S cm}^{-1}$) and unmodified filtered water. Larvae were maintained in these containers following the same methods for the first generation up until 12 days post hatching when surviving larvae were counted and dried for growth determination. Survival of second generation larvae was only assessed during the period encompassed by the growth test (i.e. from 6 to 12 days post hatch). This period was 2 days shorter than the second stage of larval survival assessment for the first generation (day 6 to 12 post egg oviposition versus day 5 to 13 post egg oviposition respectively).

6.3 Results

6.3.1 Larval acute salinity tolerance

Water quality remained high during the acute tolerance test with final (4th) instar *C. tepperi* larvae (Table 6.1). Mean percent oxygen saturation in each treatment was greater than 90% and mean temperatures were within the 20 ± 2 °C desired range. Mean salinities during the test were slightly higher than the nominated values, however maximum variability was less than 1%. The mean salinity for filtered water during the test was 146.1 ± 18.5 $\mu\text{S cm}^{-1}$ with a maximum variability of 13%. Mean pH ranged from 7.77 in unmodified filtered water to 8.34 in the 25000 $\mu\text{S cm}^{-1}$ treatment. With the exception of salinity (ANOVA; $F_{5, 6} = 195656.949$, $P < 0.001$, Tukey's pair-wise comparisons all $P < 0.001$) there were no significant differences in water quality parameters between treatments (ANOVA; $F_{5, 6} < 0.334$, $P > 0.876$ in all cases).

Table 6.1 Water quality for the acute tolerance test with final instar *Chironomus tepperi* larvae. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	146.1 ± 18.5 (127.6-164.5)	7.77 ± 0.50 (7.27-8.26)	20.6 ± 0.1 (20.5-20.7)	93.2 ± 12.8 (80.4-106.0)
5000	5015 ± 15 (5000-5030)	7.82 ± 0.58 (7.24-8.40)	20.6 ± 0.1 (20.5-20.7)	91.3 ± 11.2 (80.1-102.4)
10000	10030 ± 30 (10000-10060)	8.00 ± 0.57 (7.43-8.57)	20.6 ± 0.1 (20.5-20.6)	91.6 ± 11.4 (80.2-102.9)
15000	15010 ± 20 (14990-15030)	8.11 ± 0.50 (7.61-8.61)	20.6 ± 0.1 (20.5-20.6)	93.7 ± 10.3 (83.4-104.0)
20000	20020 ± 20 (20000-20040)	8.25 ± 0.44 (7.81-8.69)	20.6 ± 0.1 (20.5-20.6)	96.0 ± 9.2 (86.8-105.2)
25000	25030 ± 20 (25010-25050)	8.34 ± 0.38 (7.96-8.71)	20.7 ± 0.1 (20.6-20.7)	96.7 ± 11.3 (85.4-107.9)

Survival of larvae was significantly affected by salinity with effects evident at each 24-hour exposure period (ANOVA; $F_{5, 12} > 3.999$, $P < 0.024$ in all cases). In the first 24 hours of exposure, a small reduction in survival was evident in only the highest salinity treatment, 25000 $\mu\text{S cm}^{-1}$. However, after 48 hours exposure all larvae in the 25000 $\mu\text{S cm}^{-1}$ treatment had died and survival was only $16.7 \pm 6.7\%$ in the 20000 $\mu\text{S cm}^{-1}$ treatment (Figure 6.1). After 96 hours exposure all larvae in the 20000 $\mu\text{S cm}^{-1}$ treatment had also died. Figure 6.1 shows survival of larvae at each salinity after 48 and 96 hours exposure. There was some mortality in the lower salinity treatments during the test period, however, survival in filtered water did not fall below $86.7 \pm 8.8\%$. The test was terminated after 96 hours exposure as many larvae were beginning to pupate.

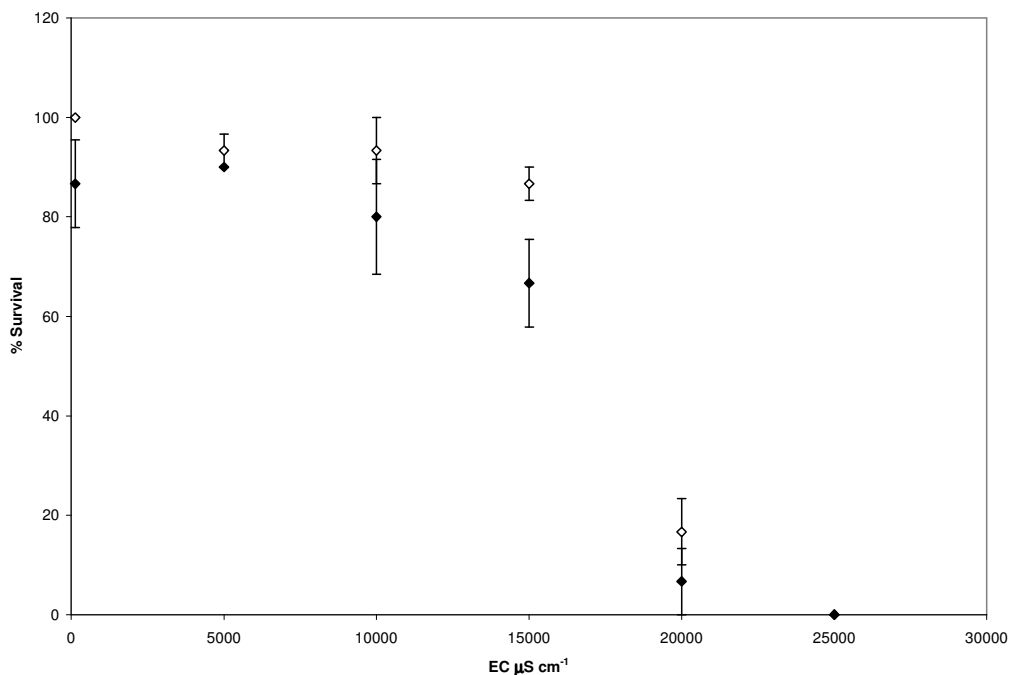


Figure 6.1 Survival (%) of final instar *Chironomus tepperi* larvae after 48 hours (open diamonds) and 96 hours (closed diamonds) exposure at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

The high level of survival in all treatments during the first 24 hours of salinity exposure indicates that the LC_{50} for this period was higher than the salinity range of the test (i.e. greater than 25000 $\mu\text{S cm}^{-1}$). LC_{50} values determined by logistic regression for each of the other 24-hour interval exposure periods are presented in Figure 6.2. LC_{50} values decreased with increasing exposure period without reaching a stable value. LC_{50} values for 48 and 96 hour exposure periods were 17025 (95% CI 15726-18357) $\mu\text{S cm}^{-1}$ ($r^2 = 0.600$) and 14077 (95% CI

12387-15814) $\mu\text{S cm}^{-1}$ ($r^2 = 0.437$) respectively. These values and the 72-hour LC_{50} can be found in Appendix A Table A.5.

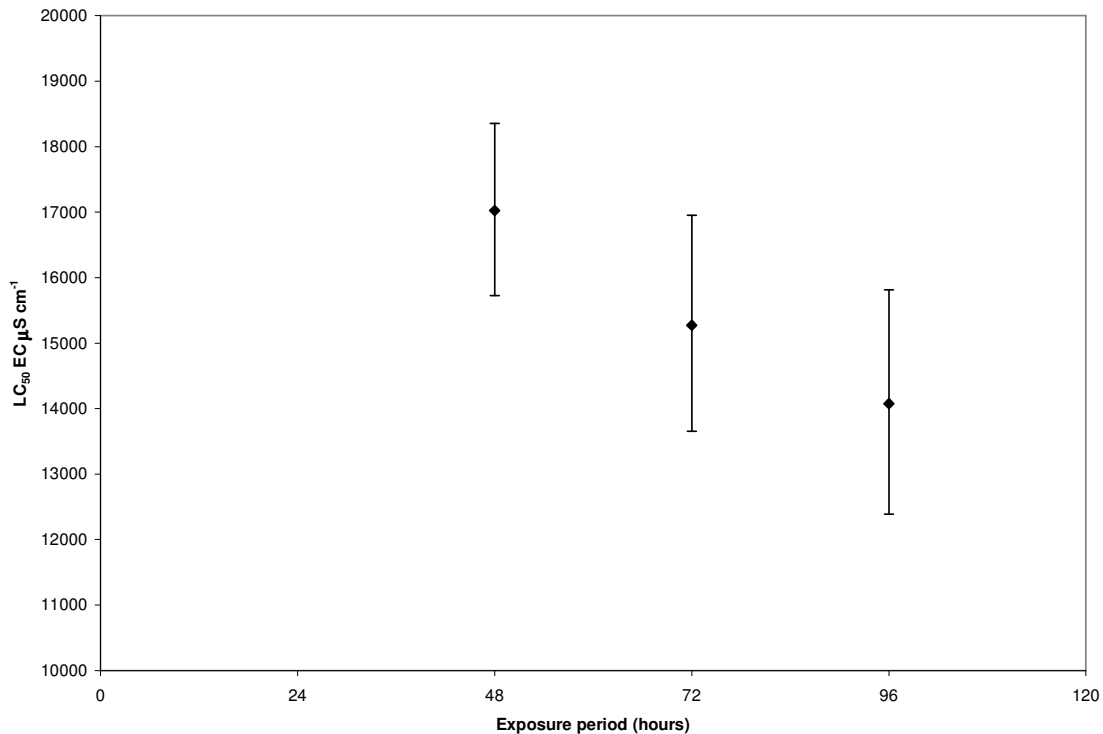


Figure 6.2 LC_{50} values ($\text{EC } \mu\text{S cm}^{-1}$) for final instar *Chironomus tepperi* larvae after different periods of salinity exposure. Error bars indicate 95% CI.

6.3.2 Egg hatching success

Water quality remained high in all treatments during both egg hatch tests (Tables 6.2 and 6.3) with the exception of replicates 1 to 3 of the $3000 \mu\text{S cm}^{-1}$ treatment of the direct oviposition test. In these replicates, excessive addition of food resulted in fungal growth and low dissolved oxygen levels. These replicates were excluded from analysis. Dissolved oxygen levels remained above 90% in all other replicates (Tables 6.2 and 6.3). Temperatures remained within the $20 \pm 2^\circ\text{C}$ desired range throughout both tests (Tables 6.2 and 6.3). The mean salinity of untreated filtered water was $137.9 \pm 2.3 \mu\text{S cm}^{-1}$ in both tests with maximum variability of less than 6%. Mean treatment values were no greater than 0.6% from nominal treatment levels for both tests and maximum variability was less than 1% in the transferred egg test and less than 1.5% in the direct oviposition test. Difference in water salinity were highly significant between all treatments for both tests (transfer test: ANOVA $F_{6, 35} = 1088708.621$, $P < 0.001$ and direct oviposition test: ANOVA $F_{5, 30} = 603574.421$, $P < 0.001$). There were no significant differences in dissolved oxygen levels or temperature between

treatments of each test (transfer test: ANOVAs, temperature: $F_{6, 35} = 1.791$, $P = 0.130$, DO: $F_{6, 35} = 0.582$, $P = 0.742$ and direct oviposition test: ANOVAs, temperature: $F_{5, 30} = 0.385$, $P = 0.855$, DO: $F_{5, 30} = 0.633$, $P = 0.676$).

Table 6.2 Water quality during *Chironomus tepperi* egg hatch test with eggs transferred to test salinities. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	Temperature °C	D. O. %
Untreated	137.9 \pm 2.3 (133.1-146.0)	20.5 \pm 0.1 (20.2-20.8)	93.0 \pm 1.0 (90.3-95.3)
5000	5030 \pm 4 (5020-5040)	20.3 \pm 0.2 (19.8-20.8)	93.5 \pm 0.8 (91.2-95.3)
10000	10017 \pm 8 (10000-10040)	20.3 \pm 0.2 (19.8-20.8)	93.3 \pm 1.0 (90.7-95.6)
15000	14993 \pm 7 (14980-15020)	20.3 \pm 0.2 (19.8-20.8)	94.0 \pm 1.1 (91.0-96.4)
20000	20002 \pm 5 (19990-20020)	20.3 \pm 0.2 (19.8-20.8)	93.1 \pm 0.7 (90.3-94.3)
25000	25032 \pm 2 (25030-25040)	20.7 \pm 0.1 (20.6-20.8)	95.1 \pm 1.2 (92.0-97.7)
30000	29953 \pm 24 (29900-30020)	20.8 \pm 0.1 (20.7-20.8)	94.3 \pm 1.0 (91.8-96.5)

Table 6.3 Water quality during *Chironomus tepperi* egg hatch test with eggs laid at test salinities. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	Temperature °C	D. O. %
Untreated	137.9 \pm 2.3 (133.1-146.0)	20.5 \pm 0.1 (20.2-20.8)	93.0 \pm 1.0 (90.3-95.3)
3000	3018 \pm 5 (3010-3040)	20.5 \pm 0.1 (20.2-20.8)	92.8 \pm 0.9 (90.2-94.9)
5000	5030 \pm 4 (5020-5040)	20.3 \pm 0.2 (19.8-20.8)	93.5 \pm 0.8 (91.2-95.3)
7000	7008 \pm 4 (7000-7020)	20.5 \pm 0.1 (20.2-20.8)	91.7 \pm 0.2 (90.8-92.0)
10000	10017 \pm 8 (10000-10040)	20.3 \pm 0.2 (19.8-20.8)	93.3 \pm 1.0 (90.7-95.6)
12000	11997 \pm 8 (11980-12020)	20.5 \pm 0.1 (20.2-20.8)	92.5 \pm 0.2 (92.0-93.0)

Egg hatching success displayed a threshold type response to increased salinity in both tests (Figure 6.3). The observed optimal hatching success of *C. tepperi* eggs was 70.8 \pm 4.9% in unmodified filtered water (137.9 \pm 2.3 $\mu\text{S cm}^{-1}$) in the transferred eggs test and 75.0 \pm 2.9% in the 3000 $\mu\text{S cm}^{-1}$ treatment for the direct oviposition test (Figure 6.3). Poor hatching success (33.3 \pm 6.0%) occurred in replicates 1 to 3 in the 3000 $\mu\text{S cm}^{-1}$ treatment of the direct oviposition test due to fungal growth and reduced oxygen levels as a result of excess food addition. These replicates were therefore excluded from analysis. Egg hatching success in the direct oviposition test was reduced at salinities that had little effect on transferred eggs (Figure 6.3). Hatching success of eggs laid at 12000 $\mu\text{S cm}^{-1}$ was less than 1% (0.8 \pm 0.8%) while hatching success of eggs transferred to 15000 $\mu\text{S cm}^{-1}$ was only 7% lower than the observed optimal. Hatching success of transferred eggs was reduced to below 1% (0.8 \pm 0.8%) at 25000 $\mu\text{S cm}^{-1}$ and no hatching occurred at 30000 $\mu\text{S cm}^{-1}$. The effects of salinity on egg hatching success were highly significant for each test (transferred test: ANOVA; $F_{6, 35} = 114.182$, $P < 0.001$ and direct oviposition test: ANOVA; $F_{5, 27} = 28.114$, $P < 0.001$). Hatching success of eggs laid at 10000 $\mu\text{S cm}^{-1}$ and 20000 $\mu\text{S cm}^{-1}$ was significantly lower than the observed optimal for eggs laid at 3000 $\mu\text{S cm}^{-1}$ (Tukey's pair-wise comparisons $P <$

0.05 and $P < 0.001$ respectively). Hatching success of eggs transferred to salinities of 20000 $\mu\text{S cm}^{-1}$ and greater (i.e. 250000 and 30000 $\mu\text{S cm}^{-1}$) were significantly lower than the observed optimal hatch rate for eggs in unmodified filtered water ($137.9 \pm 2.3 \mu\text{S cm}^{-1}$) (Tukey's pair-wise comparisons $P < 0.001$ in each case).

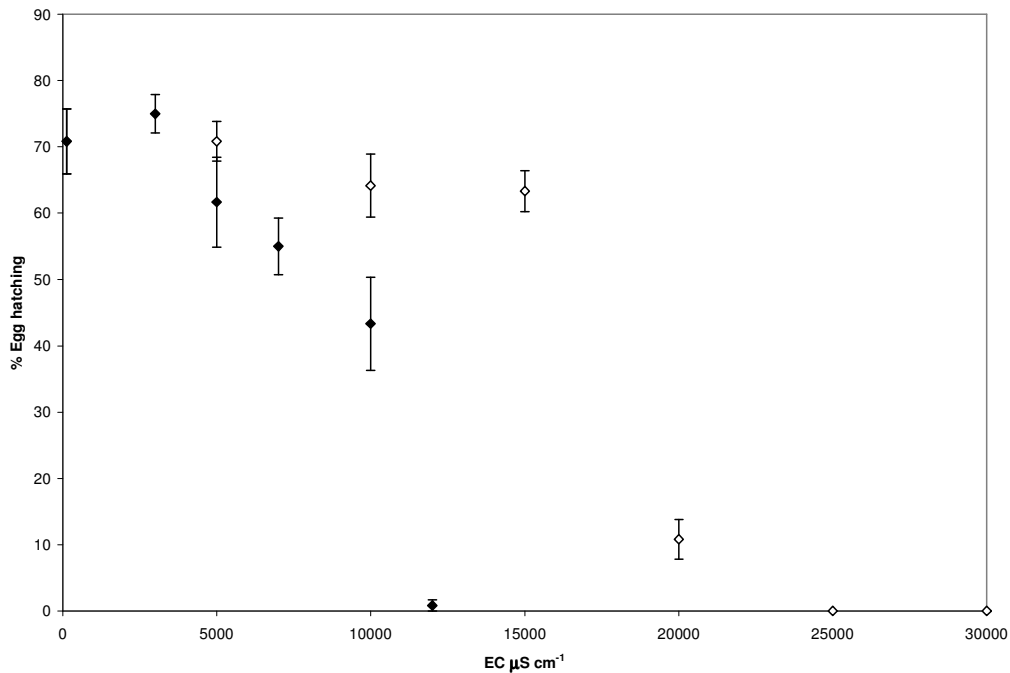


Figure 6.3 Hatching success (%) of *Chironomus tepperi* eggs laid at (closed diamonds) and transferred to (open diamonds) different salinities ($\text{EC } \mu\text{S cm}^{-1}$). Mean \pm SE.

As the observed optimal hatching success in both tests was lower than 100%, data were corrected to a proportion of the observed optimal using Abbott's formula (see Materials and Methods 2.10). Corrected data were used in logistic regression analysis for determination of EC_{50} values. As egg hatch success was higher at 3000 $\mu\text{S cm}^{-1}$ than in unmodified filtered water during the direct oviposition test, egg hatching data for unmodified filtered water were excluded from the logistic regression for determination of the EC_{50} value for this test to reduce error.

The EC_{50} value for hatching of transferred *C. tepperi* eggs was 17381 (95% CI 17058-17705) $\mu\text{S cm}^{-1}$ ($r^2 = 0.568$) while that for eggs laid at the test salinity was 8294 (95% CI 7979-8621) $\mu\text{S cm}^{-1}$ ($r^2 = 0.180$).

6.3.3 Oviposition preference

Water quality of test solutions added daily to the oviposition dishes during the oviposition preference test was high through out the test (Table 6.4). Salinity was the only water quality variable to vary significantly between treatments (ANOVA; EC: $F_{3, 20} = 19915.587$, $P < 0.001$, Temp and DO: $F_{3, 20} < 0.712$, $P > 0.556$). DO concentrations were greater than 95% saturation throughout the test and temperatures were within the $20 \pm 2^\circ\text{C}$ desired range (Table 6.4).

Table 6.4 Water quality during *Chironomus tepperi* oviposition salinity preference test. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	Temperature °C	D. O. %
Untreated	138.0 \pm 0.1 (137.6-138.4)	19.9 \pm 0.1 (19.7-20.1)	97.0 \pm 0.4 (96.0-98.2)
5000	4857 \pm 156 (4080-5040)	20.0 \pm 0.1 (19.8-20.2)	97.1 \pm 0.2 (96.4-97.8)
15000	15000 \pm 9 (14980-15040)	20.0 \pm 0.1 (19.6-20.1)	96.7 \pm 0.4 (95.9-98.0)
25000	25000 \pm 15 (24930-25040)	20.1 \pm 0.1 (19.9-20.3)	97.0 \pm 0.3 (95.9-97.9)

Over the six days of collection the total number of egg masses laid in each oviposition chamber ranged from 12 to 36. A clear downward trend was observed in the percentage of *C. tepperi* egg masses oviposited with increasing salinity (Figure 6.4). $39.0 \pm 10.5\%$ of egg masses were laid into the lowest salinity ($138.0 \pm 0.1 \mu\text{S cm}^{-1}$) while only $14.4 \pm 3.1\%$ were laid at the highest salinity ($25000 \mu\text{S cm}^{-1}$). Regression analyses using the square root of EC to improve linearity indicated a negative relationship between oviposition preference and increasing salinity. The relationship was best described by the equation:

$$\text{Arcsine proportion egg mass oviposition} = (-2 \times 10^{-3}) \times \sqrt{\text{EC}} (\mu\text{S cm}^{-1}) + 0.422$$

($F_{1, 10} = 8.781$, $P = 0.014$, $r^2 = 0.468$, SE slope = 1×10^{-3} , SE constant = 0.066).

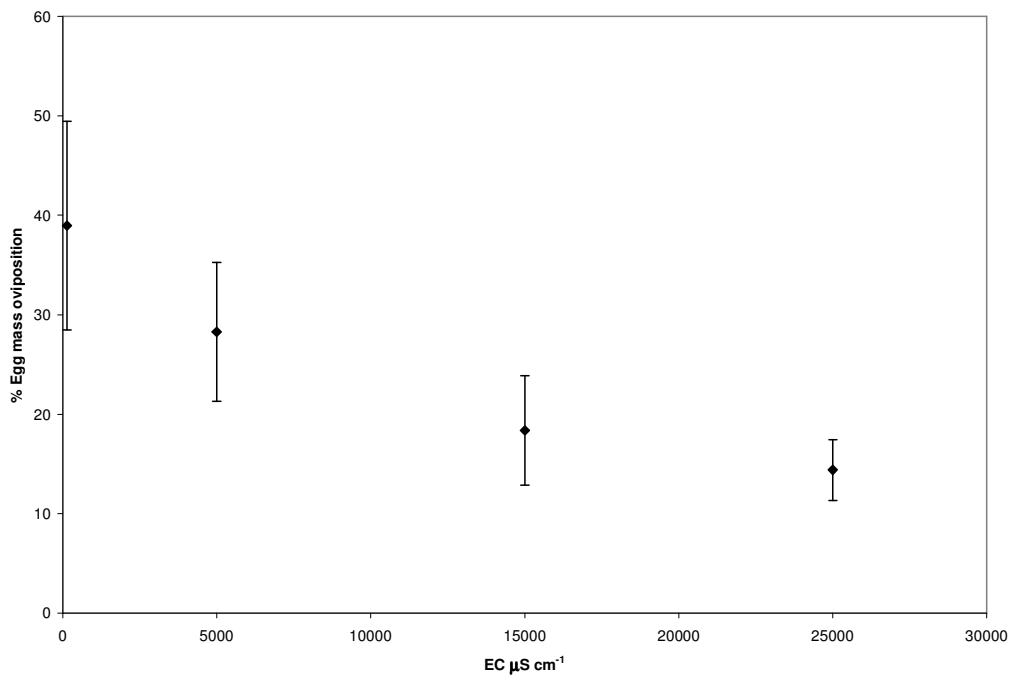


Figure 6.4 Percentage of *Chironomus tepperi* egg masses oviposited at different salinities (EC $\mu\text{S cm}^{-1}$). Mean \pm SE.

Despite the clear trend, chi-square analysis of data pooled from all oviposition chambers failed to indicate a significant deviation from even distribution of egg masses between the four test salinities ($\chi^2 = 6.062$, $P > 0.10$). This was due to the relatively small number of egg masses laid and high variability between replicates. However, when data were combined for the lower salinity treatments (i.e. 138 and 5000 $\mu\text{S cm}^{-1}$) and compared to combined data for the higher salinity treatments (i.e. 15000 and 25000 $\mu\text{S cm}^{-1}$), chi-square analysis indicated a significantly lower proportion of egg masses were oviposited at the higher salinities ($\chi^2 = 5.444$, $P < 0.025$). $67.3 \pm 8.5\%$ of eggs were oviposited at salinities $\leq 5000 \mu\text{S cm}^{-1}$ compared to $32.7 \pm 8.5\%$ at salinities $\geq 15000 \mu\text{S cm}^{-1}$. Although not measured directly due to time constraints, the number of eggs per egg mass was observed to be affected by oviposition salinity, with egg masses laid at 25000 $\mu\text{S cm}^{-1}$ containing visibly fewer eggs.

6.3.4 Full life-cycle effects

Water quality remained high in all treatments throughout the full life-cycle test with *C. tepperi* (Table 6.5). The mean salinity for untreated filtered water was $144.5 \pm 3.7 \mu\text{S cm}^{-1}$, with a maximum variation of 25.6% (Table 6.5). Treatment salinities varied less than 10% from the nominated treatment levels and mean values were within 2.5% of the nominated salinities (Table 6.5). Temperature remained within the $20 \pm 2^\circ\text{C}$ desired range and dissolved

oxygen levels remained above 70% saturation throughout the test with mean values from 94.0 to 95.2% saturation. Mean pH levels ranged from 7.40 to 7.81 increasing with increasing salinity (Table 6.5).

Table 6.5 Water quality during full life-cycle salinity tolerance test with *Chironomus tepperi*. Mean \pm SE and range in parenthesis. EC to four significant figures.

Salinity treatment	EC $\mu\text{S cm}^{-1}$ @ 25°C	pH	Temperature °C	D. O. %
Untreated	144.5 \pm 3.7 (122.9-181.6)	7.40 \pm 0.06 (6.57-7.69)	19.4 \pm 0.1 (18.3-20.3)	95.2 \pm 2.2 (75.6-109.0)
3000	3066 \pm 26 (2990-3300)	7.59 \pm 0.04 (7.14-7.80)	19.5 \pm 0.2 (18.3-20.3)	95.0 \pm 2.3 (70.4-105.6)
7000	7106 \pm 46 (7000-7670)	7.72 \pm 0.07 (7.19-8.23)	19.6 \pm 0.2 (18.0-21.5)	94.0 \pm 2.5 (70.4-105.6)
10000	10140 \pm 60 (10000-10830)	7.81 \pm 0.08 (7.28-8.44)	19.5 \pm 0.2 (18.3-20.4)	94.1 \pm 2.4 (72.7-105.1)

There were significant differences in salinity between test treatments (ANOVA; $F_{3, 61} = 12027.740$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in all cases) and no significant differences in temperature or dissolved oxygen saturation between treatments ($F_{3, 61} = 0.189$, $P = 0.904$ and $F_{3, 61} = 0.068$, $P = 0.977$ respectively). There were significant differences in pH between the higher and lower salinity treatments (ANOVA; $F_{3, 61} = 8.241$, $P < 0.001$), with pH significantly lower in unmodified filtered water than in the 7000 and 10000 $\mu\text{S cm}^{-1}$ treatments ($P < 0.01$ in both cases) and pH lower in the 3000 $\mu\text{S cm}^{-1}$ treatment than in the 10000 $\mu\text{S cm}^{-1}$ treatment ($P = 0.027$).

6.3.4.1 1st generation egg hatching success

The majority of *C. tepperi* eggs hatched 2 days after they were laid, with some eggs hatching a day earlier and a day later. There were significant differences in the hatching success of first generation *C. tepperi* eggs between salinity treatments of the full life-cycle test (ANOVA; $F_{3, 27} = 20.260$, $P < 0.001$). The observed optimal hatching success of $67.9 \pm 2.3\%$ in unmodified filtered water ($144.5 \pm 3.7 \mu\text{S cm}^{-1}$) was significantly higher than in all salinity treatments (Tukey's pair-wise comparisons: $P < 0.002$ in all cases) (Figure 6.5). Unusually, hatching success was higher at 10000 $\mu\text{S cm}^{-1}$ ($52.5 \pm 2.8\%$) than at 3000 and 7000 $\mu\text{S cm}^{-1}$

(48.4 ± 1.8 and $39.7 \pm 0.3\%$ respectively) (Figure 6.5). This difference in hatching success was statistically significant between the $10000 \mu\text{S cm}^{-1}$ and $7000 \mu\text{S cm}^{-1}$ treatments (Tukey's pair-wise comparison: $P = 0.011$). The poor hatching success of eggs in replicate 6 of unmodified filtered water (22.5%) was excluded from analysis as an outlier. Analyses were performed on arcsine transformed data.

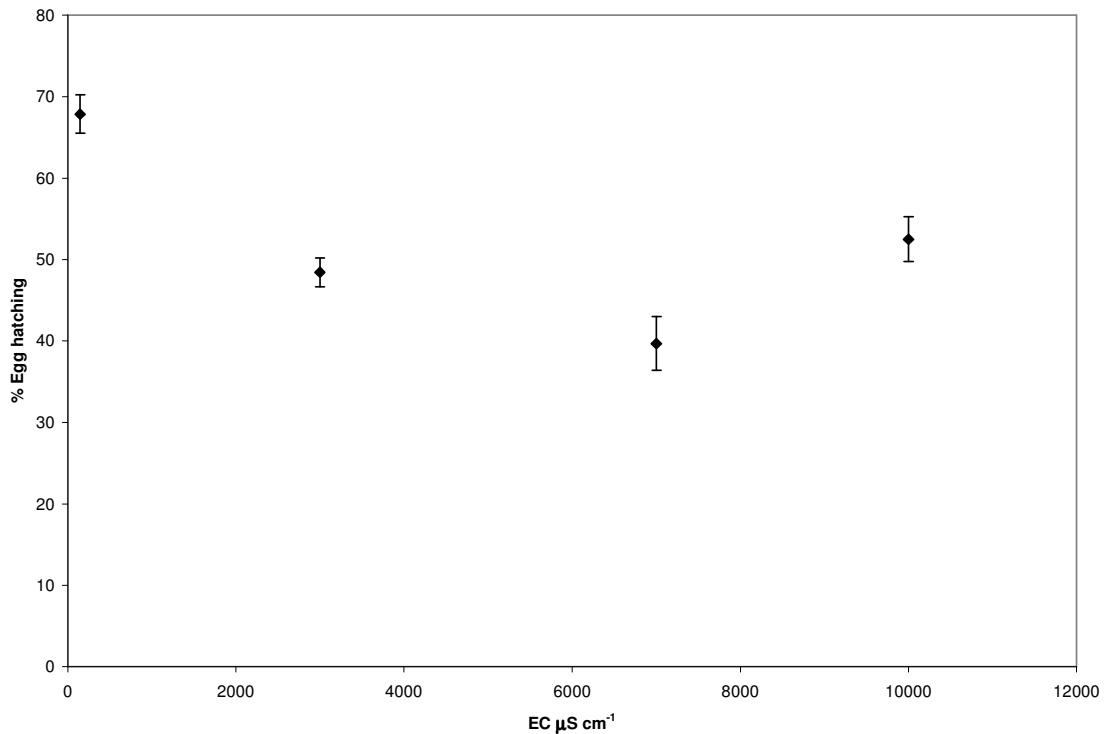


Figure 6.5 Hatching success (%) of 1st generation *Chironomus tepperi* eggs laid and hatched at different salinities ($\text{EC } \mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

Linear regression analysis of data indicated a poor relationship between hatching success and salinity when data for the $10000 \mu\text{S cm}^{-1}$ treatment were included. The relationship was best described by the equation:

$$\text{Arcsine (proportion egg hatch)} = (-19 \times 10^{-6}) \times \text{EC } (\mu\text{S cm}^{-1}) + 0.647$$

$$(F_{1, 29} = 8.999, P = 0.006, r^2 = 0.237, \text{SE slope} = 6 \times 10^{-6}, \text{SE constant} = 0.040).$$

Using this equation and the arcsine of 50% of the optimal egg hatch success observed in unmodified filtered water (33.9%, Arcsine 0.346) The EC_{50} for 1st generation *C. tepperi* egg hatching success was calculated as 15834 (SE 10434-26219) $\mu\text{S cm}^{-1}$.

The relationship was improved with the exclusion of results for the 10000 $\mu\text{S cm}^{-1}$ treatment.
Equation:

$$\text{Arcsine (proportion egg hatch)} = (-47 \times 10^{-6}) \times \text{EC } (\mu\text{S cm}^{-1}) + 0.712$$

($F_{1,21} = 43.062$, $P < 0.001$, $r^2 = 0.672$, SE slope = 7×10^{-6} , SE constant = 0.032).

The relationship excluding results for the 10000 $\mu\text{S cm}^{-1}$ treatment yielded the EC_{50} for 1st generation egg hatching of 7784 (SE 6182-9946) $\mu\text{S cm}^{-1}$, which was more in keeping with results of earlier egg hatching tests. However, this estimate is outside the data range used for the regression analysis (i.e. maximum salinity of 7000 $\mu\text{S cm}^{-1}$). This was a more significant issue for the EC_{50} value calculated with the inclusion of data for the 10000 $\mu\text{S cm}^{-1}$ treatment. Given these issues, further testing is required to confirm the EC_{50} estimate for hatching success of 1st generation *C. tepperi* eggs. Hatching success of 2nd generation *C. tepperi* eggs during the full life exposure test is presented in section 6.3.4.9.

6.3.4.2 1st generation larval survival

The survival of first generation larvae was assessed at two stages during their development; 1.) between egg hatching and transfer to the next test stage 5 days after eggs were laid (i.e. larvae 2 to 4 days old), and 2.) from this stage until 13 days after eggs were laid (i.e. larvae 10 to 12 days old).

There were no significant differences in larval survival between salinity treatments during either stage, nor when survival was assessed over the combined period (ANOVA; Stage 1.) $F_{3,28} = 1.403$ $P = 0.263$, stage 2.) $F_{3,8} = 1.454$, $P = 0.298$ and combined $F_{3,8} = 1.870$, $P = 0.213$). Larval survival up until 5 days post egg laying ranged from $98.5 \pm 0.7\%$ in unmodified filtered water to $92.1 \pm 2.5\%$ at 7000 $\mu\text{S cm}^{-1}$ (Figure 6.6). Mortality was higher during the second stage, with survival in unmodified filtered water and the 7000 $\mu\text{S cm}^{-1}$ treatment decreasing to 78.8 % (SE 3.0 and 16.9 respectively) (Figure 6.6). Reduce survival at 7000 $\mu\text{S cm}^{-1}$ was due largely to high mortality in replicate 2 (54.5%), mortality in other replicates for this treatment was no greater than 10%. Removal of this replicate from analysis did not alter the non-significance of results for this stage or the combined period (ANOVA; $F_{3,7} = 3.074$, $P = 0.100$ and $F_{3,7} = 1.598$, $P = 0.274$ respectively).

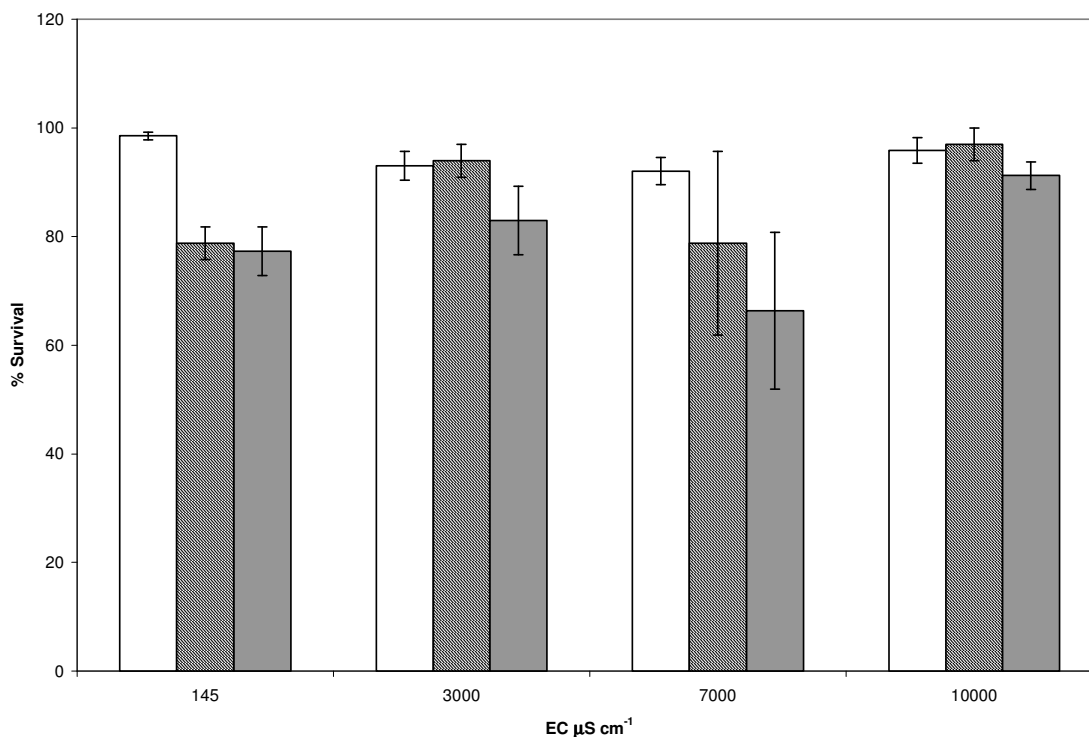


Figure 6.6 Survival (%) of first generation *Chironomus tepperi* larvae raised at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Open bars indicate survival during the period from egg hatch until 5 days after eggs were laid, hatched bars indicate survival during the period from 5 to 13 days after eggs were laid, and solid bars indicates survival during these combined periods (i.e. from egg hatch until 13 days post egg laying). Mean \pm SE.

6.3.4.3 1st generation larval growth

Salinity significantly affected the growth (mean dry weight per individual) of first generation *C. tepperi* larvae during the full life-cycle test (ANOVA; $F_{3, 8} = 22.582$, $P < 0.001$). Observed optimal growth occurred at 3000 $\mu\text{S cm}^{-1}$ with a mean dry weight per larva of 0.68 ± 0.04 mg (Figure 6.7). At salinities greater than 3000 $\mu\text{S cm}^{-1}$ growth decreased with increasing salinity, with a 42% reduction at 7000 $\mu\text{S cm}^{-1}$ and a 79% reduction at 10000 $\mu\text{S cm}^{-1}$ (Figure 6.7). Growth at these salinities was significantly lower than at 3000 $\mu\text{S cm}^{-1}$ (Tukey's pair-wise comparisons $P < 0.05$ in both cases) and growth at 10000 $\mu\text{S cm}^{-1}$ was also significantly lower than growth in unmodified filtered water (Tukey's pair-wise comparison $P = 0.001$). Despite a lower mean weight per larva there was no significant difference in growth in unmodified filtered water compared to growth at 3000 $\mu\text{S cm}^{-1}$ (Tukey's pair-wise comparison $P = 0.683$).

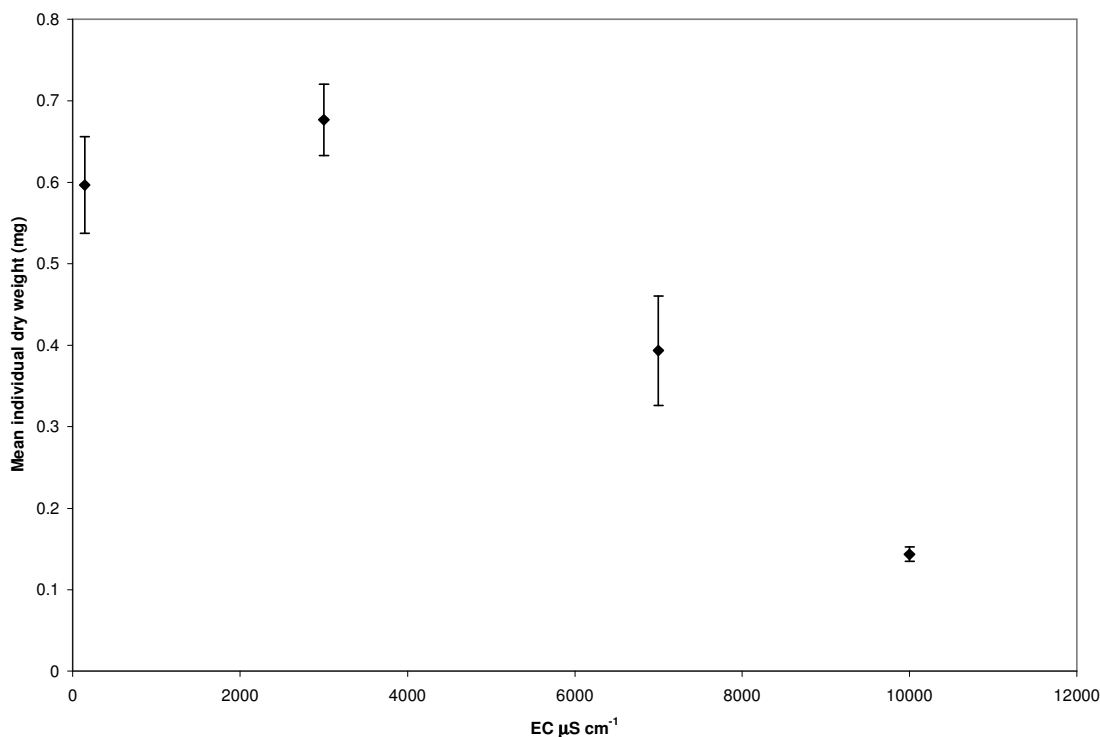


Figure 6.7 Mean individual dry weight (mg) of 1st generation *Chironomus tepperi* larvae raised at different salinities (EC μS cm⁻¹) 13 days post eggs oviposition during the full life-cycle test. Mean ± SE.

Regression analysis indicated a strong negative relationship between increasing salinity and larval growth. To enable consideration of only the linear range of the relationship, data for growth in untreated filtered water were excluded from the regression analysis. The relationship was best described by the equation;

$$\text{Mean individual dry weight} = (-76 \times 10^{-6}) \times \text{EC} (\mu\text{S cm}^{-1}) + 0.910$$

($F_{1,7} = 74.396$, $P < 0.001$, $r^2 = 0.914$, SE slope = 9×10^{-6} , SE constant = 0.064).

Using this equation and 50% of the observed optimal growth at 3000 μS cm⁻¹ (i.e. 0.34 mg dry weight) the EC₅₀ for 1st generation *C. tepperi* larval growth was calculated as 7522 (SE 5973-9488) μS cm⁻¹.

6.3.4.4 Adult emergence success

Adult emergence success was greatest for *C. tepperi* raised in unmodified filtered water (EC $144.5 \pm 3.7 \mu\text{S cm}^{-1}$), with a mean emergence success of $86.4 \pm 7.9\%$ (Figure 6.8). There was no change in emergence success at $3000 \mu\text{S cm}^{-1}$ ($85.5 \pm 3.6\%$) compared with unmodified filtered water. However, at salinities greater than $3000 \mu\text{S cm}^{-1}$ emergence success decreased with increasing salinity, being reduced to $45.5 \pm 8.6\%$ at $10000 \mu\text{S cm}^{-1}$ (Figure 6.8). This was a greater than 60% reduction in emergence success. ANOVA indicated a significant effect of salinity on emergence success (ANOVA; $F_{3, 15} = 8.930$, $P = 0.001$), with emergence success at $10000 \mu\text{S cm}^{-1}$ significantly lower than in unmodified filtered water and $3000 \mu\text{S cm}^{-1}$ (Tukey's pair-wise comparisons $P = 0.002$ and $P = 0.004$ respectively). Emergence success was also significantly lower at $7000 \mu\text{S cm}^{-1}$ than in unmodified filtered water at a 90% confidence level (Tukey's pair-wise comparison $P = 0.085$). Analysis was performed on arcsine transformed data and replicate 6 of unmodified filtered water were excluded as an outlier with an emergence success of only 22.2%. Logistic regression analysis of data excluding replicate 6 of unmodified filtered water, was used to determine an EC_{50} value of 9784 (95% CI 8237-12659) $\mu\text{S cm}^{-1}$ ($r^2 = 0.150$) for emergence success of 1st generation *C. tepperi*.

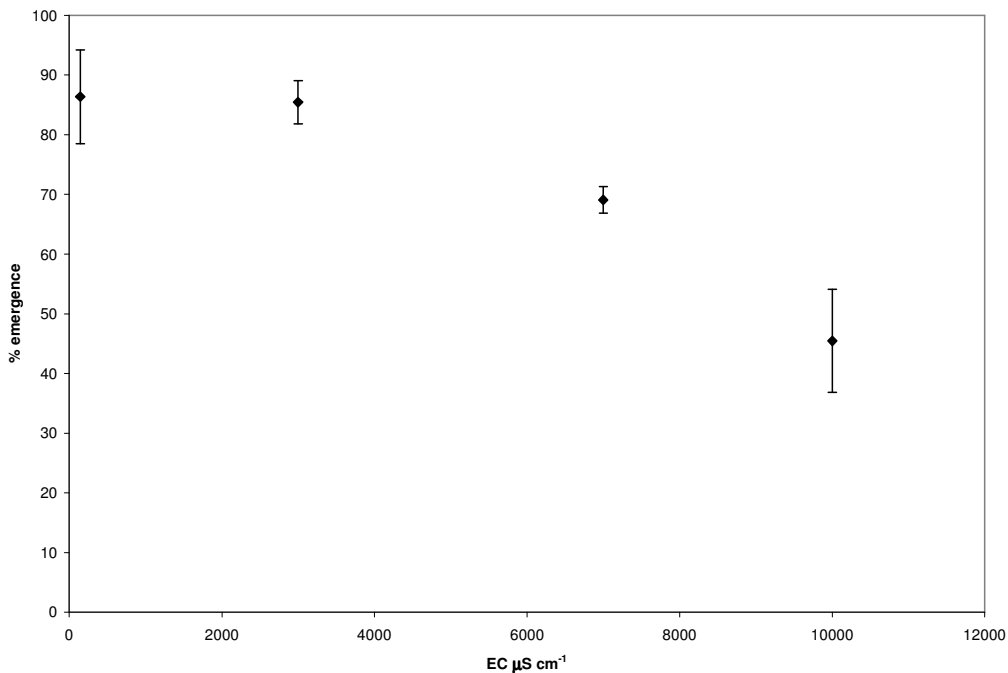


Figure 6.8 Adult emergence success (% of hatched larvae) of *Chironomus tepperi* raised at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

6.3.4.5 Time to emergence

C. tepperi began emerging as adults 17 days after egg oviposition, with males raised in unmodified filtered water (EC $144.5 \pm 3.7 \mu\text{S cm}^{-1}$) the first to emerge. The last emergence occurred 27 days after egg oviposition and was a female from the $10000 \mu\text{S cm}^{-1}$ treatment. The mean number of days until emergence increased with increasing salinity, increasing from 19.5 ± 0.3 days in unmodified filtered water to 23.8 ± 0.4 days at $10000 \mu\text{S cm}^{-1}$ (Figure 6.9). There was a general trend for males to emerge faster at each salinity than females. *T*-tests on mean emergence times for each replicate indicated that the difference between sexes was only significant in unmodified filtered water (EC $144.5 \pm 3.7 \mu\text{S cm}^{-1}$) ($t_{5,1} = -2.711$, $P = 0.041$ separate variances). The mean time to emergence at this salinity was 18.7 ± 0.4 days for males and 20.1 ± 0.3 for females.

There were significant differences in the mean time to emergence of adult *C. tepperi* between the salinity treatments (ANOVA; $F_{3, 15} = 24.402$, $P < 0.001$). Emergence time was significantly longer at $10000 \mu\text{S cm}^{-1}$ than at all other salinities (Tukey's pair-wise comparisons $P < 0.025$ in each case) and emergence time at $7000 \mu\text{S cm}^{-1}$ significantly longer than in unmodified filtered water (Tukey's pair-wise comparison $P = 0.002$). As differences in emergence time between sexes were only significant in unmodified filtered water, analysis was performed on data for combined sexes. Analysis of data for the separate sexes indicated the same significant effects as combined data with the exception that the time to emergence of females at $7000 \mu\text{S cm}^{-1}$ was not significantly longer than in unmodified filtered water (Tukey's pair-wise comparison $P = 0.164$).

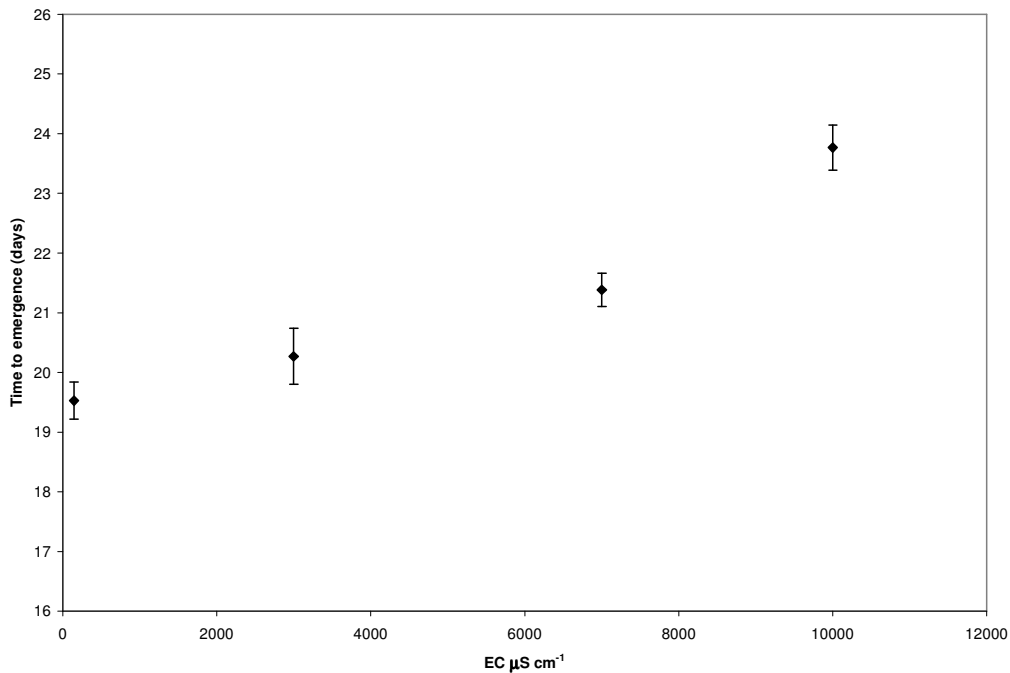


Figure 6.9 Time (number of days from egg oviposition) to emergence of adult *Chironomus tepperi* raised at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

There was a significant difference in emergence time between replicates of the 3000 $\mu\text{S cm}^{-1}$ treatment (ANOVA; $F_{4, 42} = 4.534$, $P = 0.004$) with replicate 7 having significantly longer time to emergence than replicates 1 and 3 (Tukey's pair-wise comparisons $P = 0.024$ and $P = 0.003$ respectively). There were no significant differences between replicates of the other treatments ($P > 0.101$ in each case).

Regression analysis indicated a strong positive relationship between salinity ($\text{EC}(\mu\text{S cm}^{-1})^2$) and time to emergence, with the relationship best described by the following equation:

$$\text{Time to emergence (days)} = (40 \times 10^{-6}) \times \text{EC} (\mu\text{S cm}^{-1})^2 + 19.658$$

($F_{1, 17} = 76.283$, $P < 0.001$, $r^2 = 0.818$, SE slope = 5×10^{-6} , SE constant = 0.263).

While time to emergence was delayed there was no protraction of the period over which emergence occurred (ANOVA; $F_{3, 16} = 1.471$, $P = 0.260$), with the mean time from first to last emergence at each salinity between 3 and 5 days. Data for replicate 6 of unmodified filtered water were excluded from all analyses.

6.3.4.6 Adult sex ratio

The highest proportion of emergent females occurred at 3000 $\mu\text{S cm}^{-1}$, with $61.4 \pm 8.5\%$ of *C. tepperi* adults emerging at this salinity being female. Above 3000 $\mu\text{S cm}^{-1}$ the proportion of females decreased with increasing salinity, with more males than females emerging at both 7000 and 10000 $\mu\text{S cm}^{-1}$ (Figure 6.10). At 10000 $\mu\text{S cm}^{-1}$ the number of females emerging was reduced to $38.1 \pm 13.1\%$. There was a high degree of variability in sex proportions within each of the salinity treatments (Figure 6.10). The proportion of females emerging in replicate 6 of the 10000 $\mu\text{S cm}^{-1}$ treatment (80%) was much higher than the other replicates of this treatment. ANOVA and box plots of the data identified this replicate as an extreme outlier. The mean proportion of females emerging at 10000 $\mu\text{S cm}^{-1}$ with the value for replicate 6 excluded was $27.7 \pm 10.1\%$ (Figure 6.10). Due to the small number of adults emerging in replicate 6 of unmodified filtered water (compared to other replicates of this treatment) this replicate was excluded from analysis.

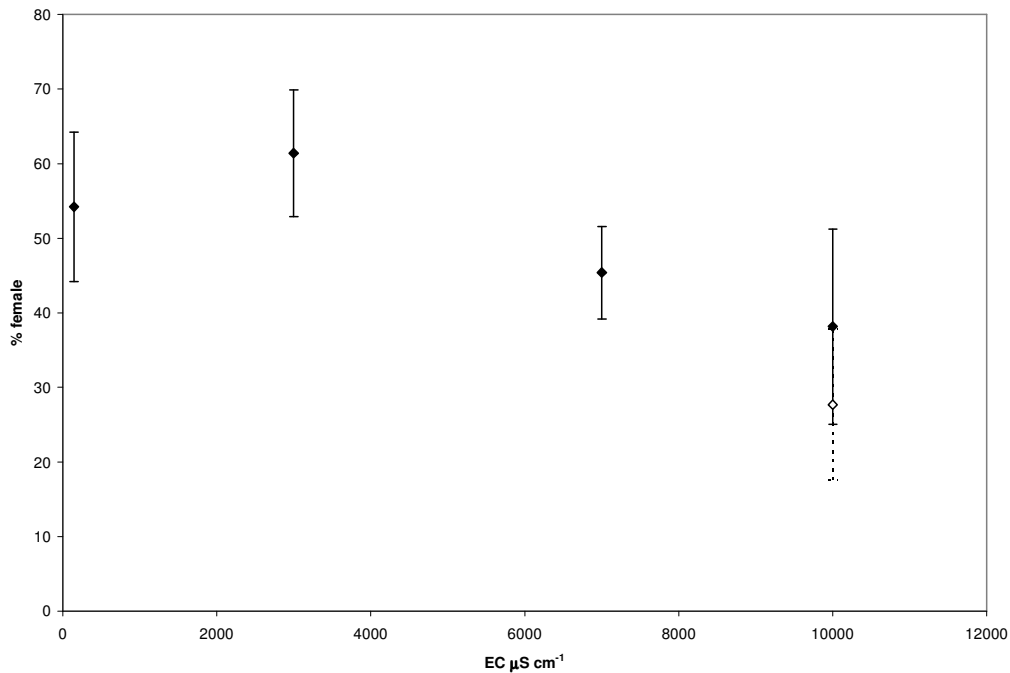


Figure 6.10 The percentage of female emergent *Chironomus tepperi* adults at different salinities ($\text{EC } \mu\text{S cm}^{-1}$) during the full life-cycle test. Open diamond indicates data excluding outlier at 10000 $\mu\text{S cm}^{-1}$. Mean \pm SE.

ANOVA of arcsine transformed data with replicate 6 of the 10000 $\mu\text{S cm}^{-1}$ treatment included failed to indicate a significant difference in the proportion of females emerging at the different

salinity levels (ANOVA; $F_{3, 15} = 1.071$, $P = 0.391$). With the outlier at $10000 \mu\text{S cm}^{-1}$ excluded there was a significant difference at 90% confidence level (ANOVA; $F_{3, 14} = 2.743$, $P = 0.082$), with pair-wise comparison indicating a higher proportion of females emerging at $3000 \mu\text{S cm}^{-1}$ than at $10000 \mu\text{S cm}^{-1}$ (Tukey's pair-wise comparison $P = 0.066$). *T*-test analysis of data for the 3000 and $10000 \mu\text{S cm}^{-1}$ treatments indicated a significant difference in the proportion of females emerging at these salinities with a 95% confidence level ($t_7 = 2.550$, $P = 0.038$ separate variances) when the outlier at $10000 \mu\text{S cm}^{-1}$ was excluded.

6.3.4.7 Generation time

The time required to complete a full generation by *C. tepperi* at each of the test salinities displayed a similar but more exaggerated effect to that of the time until adult emergence (Figure 6.11). The mean number of days from the oviposition of initial egg masses until egg oviposition by first generation adults ranged from 25 ± 1.0 days in unmodified filtered water and $3000 \mu\text{S cm}^{-1}$ (SE 1.0 and 0.9 respectively) to 34 ± 0.6 days at $10000 \mu\text{S cm}^{-1}$ (Figure 6.11). This represents a 36% increase in generation time. ANOVA indicated that generation time was significantly longer at $10000 \mu\text{S cm}^{-1}$ than at all other salinities (ANOVA; $F_{3, 13} = 24.471$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in each case). Regression analysis indicated a strong positive relationship between generation time and salinity ($\text{EC} (\mu\text{S cm}^{-1})^2$), which was best described by the following equation:

$$\text{Total generation time (days)} = (89 \times 10^{-6}) \times \text{EC} (\mu\text{S cm}^{-1})^2 + 23.925$$

($F_{1, 15} = 47.939$, $P < 0.001$, $r^2 = 0.818$, SE slope = 13×10^{-6} , SE constant = 0.639).
EC was squared to enable linear regression analysis.

The period between adult emergence (mean time to emergence) and egg oviposition (mean time until egg oviposition) was significantly longer at $10000 \mu\text{S cm}^{-1}$ than all other salinities (ANOVA; $F_{3, 12} = 19.493$, $P < 0.001$, Tukey's pair-wise comparisons $P < 0.001$ in each case). The mean time between emergence and egg oviposition was approximately five days in unmodified filtered water and the 3000 and $7000 \mu\text{S cm}^{-1}$ salinity treatments, while, this period was 10.9 ± 0.6 days at $10000 \mu\text{S cm}^{-1}$. Replicate 5 of the $3000 \mu\text{S cm}^{-1}$ treatment was excluded from analysis as an outlier.

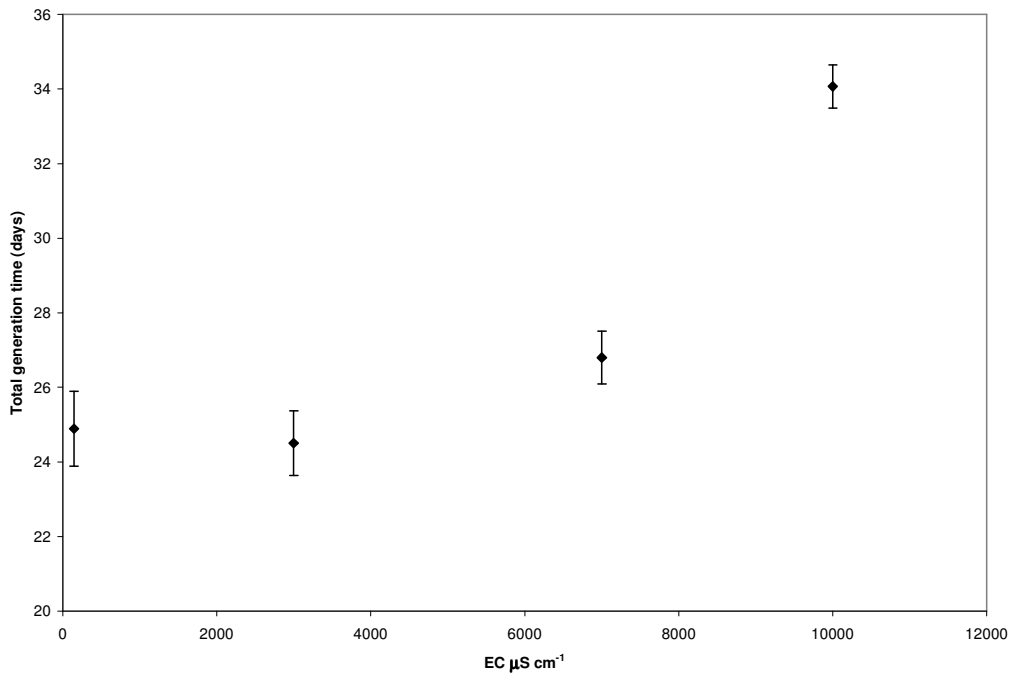


Figure 6.11 The total generation time (days) for first generation *Chironomus tepperi* raised at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

6.3.4.8 Reproductive effort

The number of egg masses laid per female decreased with increasing salinity (Figure 6.12). Females in unmodified filtered water produced close to three (2.9 ± 0.1) egg masses each while at $10000 \mu\text{S cm}^{-1}$ females produced only one (1.1 ± 0.4) egg mass each (Figure 6.13). Replicate 5 of the $3000 \mu\text{S cm}^{-1}$ treatment was identified as an outlier. Much lower egg mass production per female was observed in this replicate, with only 0.5 egg masses per female compared with 2.0 to 2.6 egg masses per female.

The differences between salinity treatments in the number of egg masses laid per female were significant both with and without the inclusion of the outlier at $3000 \mu\text{S cm}^{-1}$ (ANOVA including 3000 replicate 5: $F_{3, 14} = 5.434$, $P = 0.011$; excluding 3000 replicate 5: $F_{3, 13} = 10.685$, $P = 0.001$). With inclusion of the outlier only the $10000 \mu\text{S cm}^{-1}$ treatment displayed significantly lower egg mass production per female than unmodified filtered water (Tukey's pair-wise comparison $P = 0.006$). With the outlier excluded, egg mass production per female was also significantly lower at 7000 than in unmodified filtered water (Tukey's pair-wise comparisons $P = 0.001$ and 0.036 respectively). Replicate 1 of the $10000 \mu\text{S cm}^{-1}$ treatment was excluded from analyses as no females emerged in this replicate.

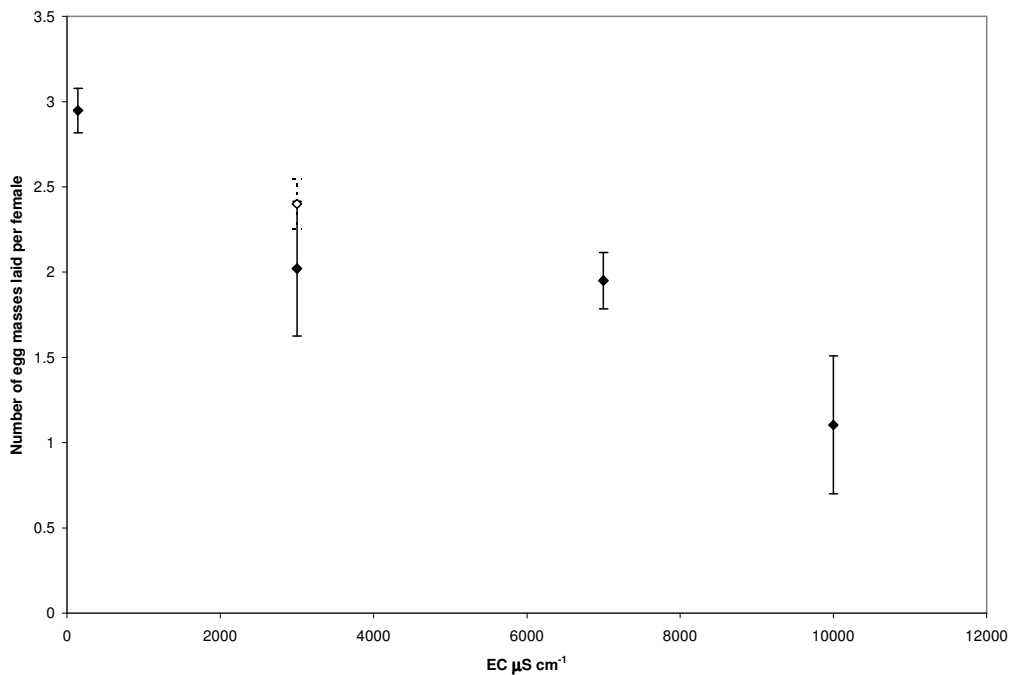


Figure 6.12 The number of egg masses oviposited per female by first generation *Chironomus tepperi* at each salinity (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Open diamond indicates data excluding an outlier at 3000 $\mu\text{S cm}^{-1}$. Mean \pm SE.

Delays in determining the number of eggs in each egg mass due to the large number of masses produced resulted in egg hatching occurring in some masses before the eggs had been counted. An accurate assessment of the number of eggs could not be made after hatching occurred due to difficulties in counting living larvae and potential error due to cannibalism. This only occurred for a small proportion of egg masses in unmodified filtered water and the 3000 $\mu\text{S cm}^{-1}$ treatment. With the exception of replicate 7 of the 3000 $\mu\text{S cm}^{-1}$ treatment, egg numbers were determined for greater than 85% of egg masses in each replicate. Replicate 7 of 3000 $\mu\text{S cm}^{-1}$ was excluded from analysis, as egg number was determined for only 7 (54%) of the 13 egg masses laid in this replicate.

The number of eggs per mass varied greatly in unmodified filtered water and the 3000 $\mu\text{S cm}^{-1}$ treatment, ranging from close to 500 to less than ten eggs per mass. Investigation of when masses of different sizes were oviposited suggested that females in these treatments laid one very large egg mass followed by several smaller masses. This resulted in bimodal distribution of the number of eggs per mass in unmodified filtered water and the 3000 $\mu\text{S cm}^{-1}$ treatment. While the mean number of eggs per egg mass peaked at 3000 $\mu\text{S cm}^{-1}$ and decreased with increasing salinity (Figure 6.13), there were no significant differences in mean, median or

rank number of eggs per mass between salinity treatments detected due to this high variability (ANOVA; mean: $F_{3, 12} = 2.407$, $P = 0.118$, median: $F_{3, 12} = 0.676$, $P = 0.583$, rank: $F_{3, 12} = 0.350$, $P = 0.790$).

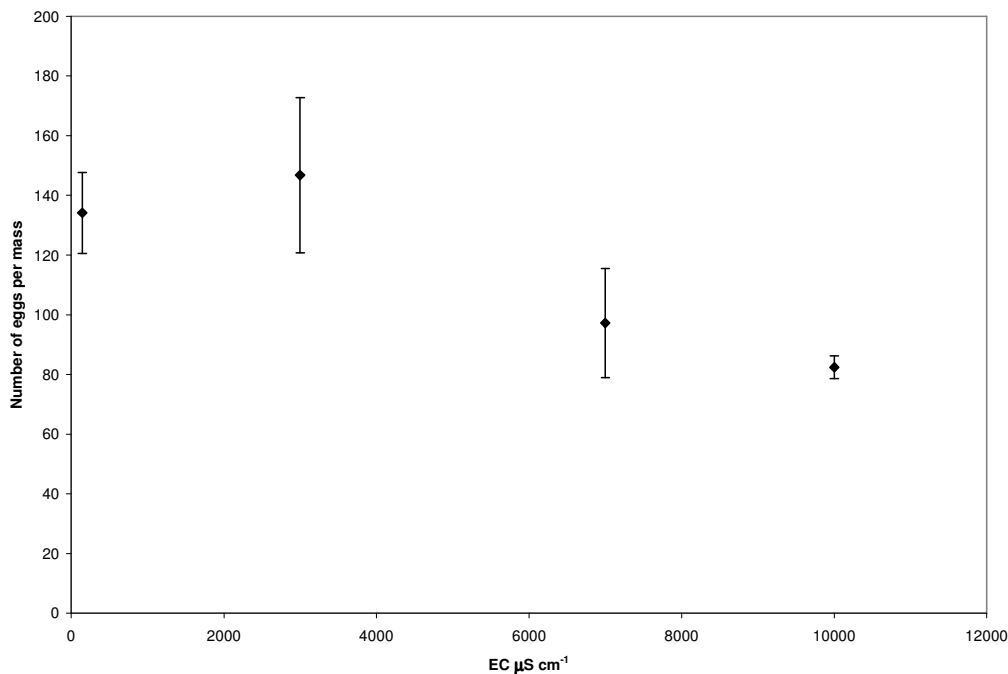


Figure 6.13 The number of eggs per egg mass laid by first generation *Chironomus tepperi* at each salinity (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

To determine total egg production for each replicate the mean number of eggs per mass for that replicate was substituted for masses for which egg number was not determined. Mean values were used as the masses for which egg number was not determined tended to be the larger egg masses. Use of median or mean rank number of eggs per mass had greater potential to underestimate the number of eggs in these masses.

Egg production per female decreased dramatically with increasing salinity (Figure 6.14). The mean number of eggs laid per female decreased from 393 ± 33 in unmodified filtered water to 90 ± 32 at $10000 \mu\text{S cm}^{-1}$, a greater than 90% reduction (Figure 6.14). Replicate five of the $3000 \mu\text{S cm}^{-1}$ treatment was again evident as an outlier with mean egg production per female more than 65% lower than the other replicates of this treatment. ANOVA analyses both including and excluding the outlier in the $3000 \mu\text{S cm}^{-1}$ treatment indicated that egg production per female was significantly lower at both 7000 and $10000 \mu\text{S cm}^{-1}$ than in unmodified filtered water (ANOVA including replicate 5 of the $3000 \mu\text{S cm}^{-1}$ treatment: $F_{3, 13}$

= 7.739, $P = 0.003$, excluding replicate 5 of the 3000 $\mu\text{S cm}^{-1}$ treatment: $F_{3, 12} = 11.770$, $P = 0.001$ Tukey's pair-wise comparisons $P < 0.027$ in all cases).

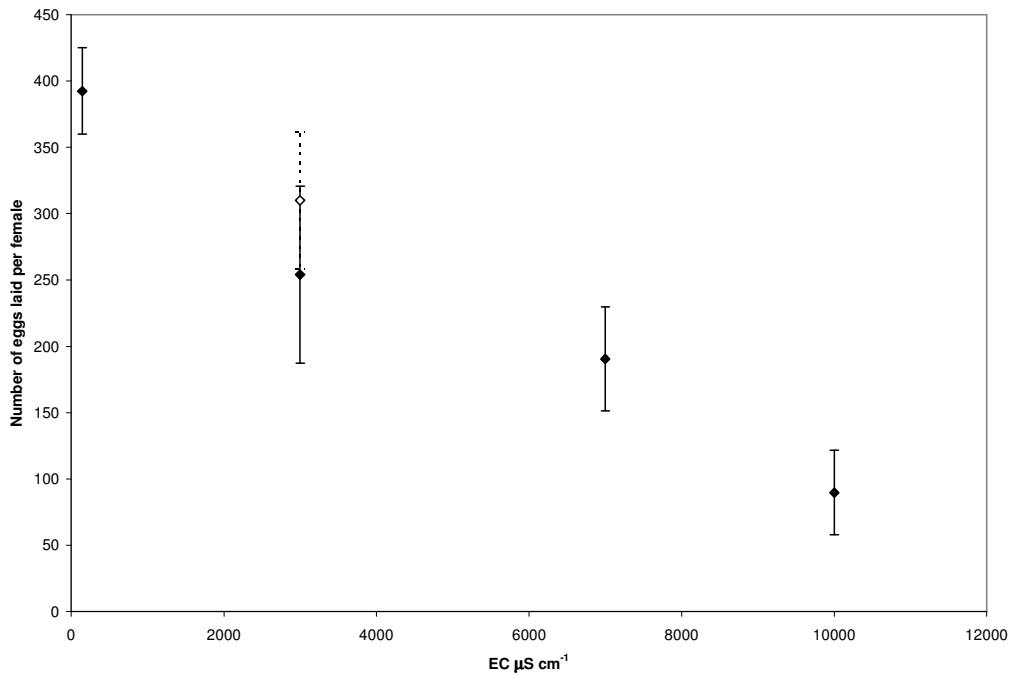


Figure 6.14 The number of eggs laid per female by first generation *Chironomus tepperi* at each test salinity (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Open diamond indicates data excluding an outlier at 3000 $\mu\text{S cm}^{-1}$. Mean \pm SE.

Linear regression analyses both including and excluding the outlier at 3000 indicated a negative relationship between egg production per female and salinity. The relationship was best described by the equations:

Including outlier:

$$\text{Number of eggs laid per female} = (-21.484 \times 10^{-3}) \times \text{EC } (\mu\text{S cm}^{-1}) + 375.991$$

($F_{1, 15} = 23.934$, $P < 0.001$, $r^2 = 0.615$, SE slope = 5.822×10^{-3} , SE constant = 36.852).

Excluding outlier:

$$\text{Number of eggs laid per female} = (-30.557 \times 10^{-3}) \times \text{EC } (\mu\text{S cm}^{-1}) + 399.703$$

($F_{1, 14} = 41.019$, $P < 0.001$, $r^2 = 0.746$, SE slope = 4.771×10^{-3} , SE constant = 30.922).

Using these equations and 50% the observed optimal egg production per female in unmodified filtered water (i.e. 196.25) the EC_{50} for egg production per female was calculated as 8366 (SE 5233-13829) $\mu\text{S cm}^{-1}$ with the inclusion of the outlier at 3000 $\mu\text{S cm}^{-1}$ and 6658 (SE 4884-6762) $\mu\text{S cm}^{-1}$ with the outlier excluded.

6.3.4.9 2nd generation egg hatching success

Due to the large number of egg masses laid, not all masses could be monitored for second generation egg hatching success. A random sample of between 33 and 100% of egg masses from each replicate were monitored for egg hatching, with the exception of replicate 5 of the 3000 $\mu\text{S cm}^{-1}$ treatment for which no egg masses were monitored. Replicate 7 of the 3000 $\mu\text{S cm}^{-1}$ treatment was excluded from analyses as only 23% of egg masses were monitored for egg hatching and replicate 1 of unmodified filtered water was excluded as monitoring of egg masses was skewed towards masses with low viability.

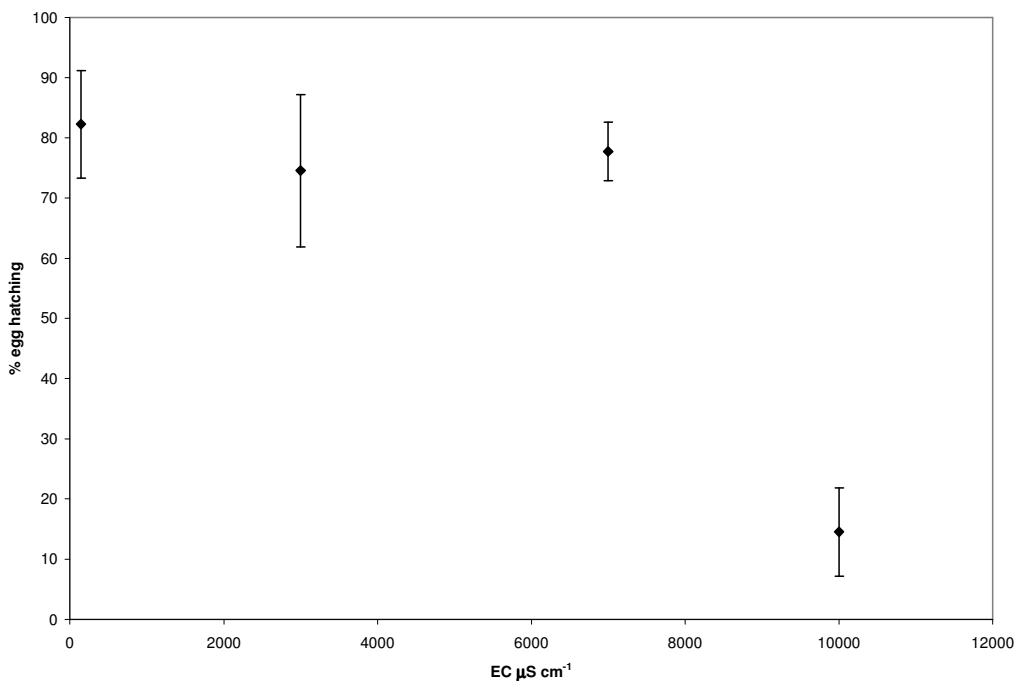


Figure 6.15 Hatching success (%) of 2nd generation *Chironomus tepperi* eggs at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

Egg hatching success was highest in unmodified filtered water with a mean hatching success of 82.3 \pm 8.9% (Figure 6.15). Hatching success of eggs in the 3000 and 7000 $\mu\text{S cm}^{-1}$ treatments was comparable to that of unmodified filtered water, while hatching success of eggs in the 10000 $\mu\text{S cm}^{-1}$ treatment was much lower with only 14.5 \pm 7.3% of eggs hatching

(Figure 6.15). The hatching success of eggs at $10000 \mu\text{S cm}^{-1}$ was significantly lower than that in unmodified filtered water and both lower salinity treatments (ANOVA; $F_{3, 10} = 7.689$, $P = 0.006$, Tukey's pair-wise comparisons $P < 0.021$ in all cases).

Logistic regression analysis of Abbott's corrected data estimated the EC_{50} value for 2nd generation egg hatching success as 9072 (95% CI 8728-9468) $\mu\text{S cm}^{-1}$ ($r^2 = 0.244$).

At each salinity there were egg masses that displayed very low egg viability, with hatching successes of less than 5%. Although not significantly different due to high variability, there was a greater proportion of low viability egg masses laid in the $10000 \mu\text{S cm}^{-1}$ treatment (Figure 6.16)(ANOVA; $F_{3, 10} = 2.013$, $P = 0.176$).

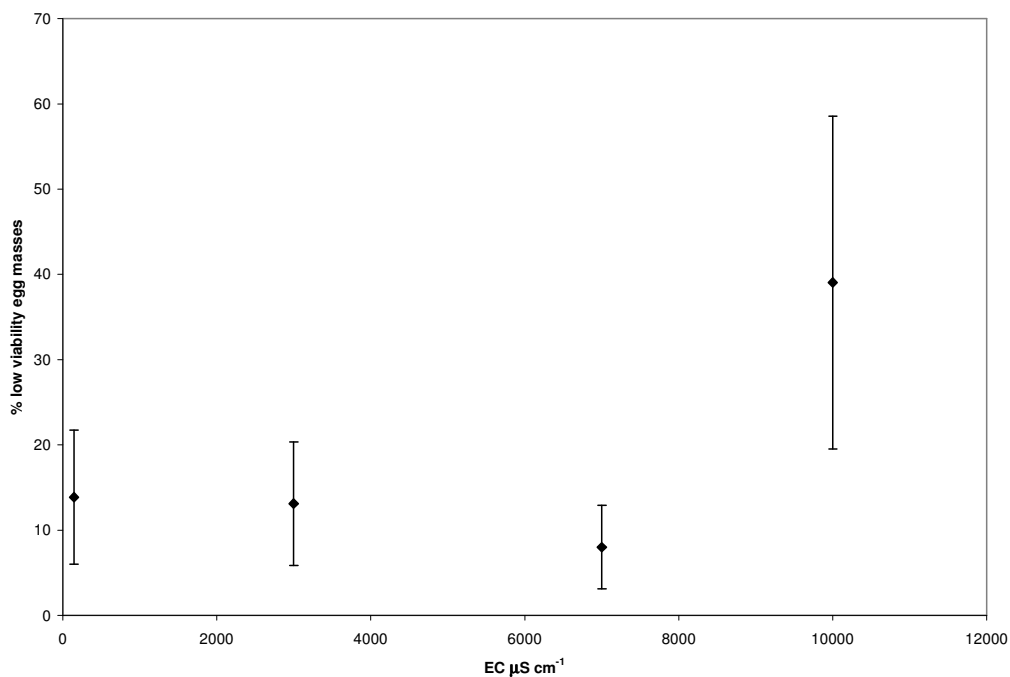


Figure 6.16 The proportion of low viability (<5% hatch success) 2nd generation *Chironomus tepperi* egg masses at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

6.3.4.10 2nd generation larval recruitment

The combined effects of full life-cycle salinity exposure on both egg production and egg hatching success were assessed to determine the number of *C. tepperi* larvae that would successfully recruit to the second generation per female. There was a dramatic reduction in recruitment success with increasing salinity (Figure 6.17). The number of larvae recruited to

the second generation decreased from 343 ± 42 per female in unmodified filtered water to 14 ± 9 at $10000 \mu\text{S cm}^{-1}$ (Figure 6.17). The number of larvae recruited per female was significantly lower at 7000 and 10000 than in unmodified filtered water (ANOVA; $F_{3, 11} = 12.918$, $P = 0.001$, Tukey's pair-wise comparisons $P = 0.014$ and $P = 0.001$ respectively).

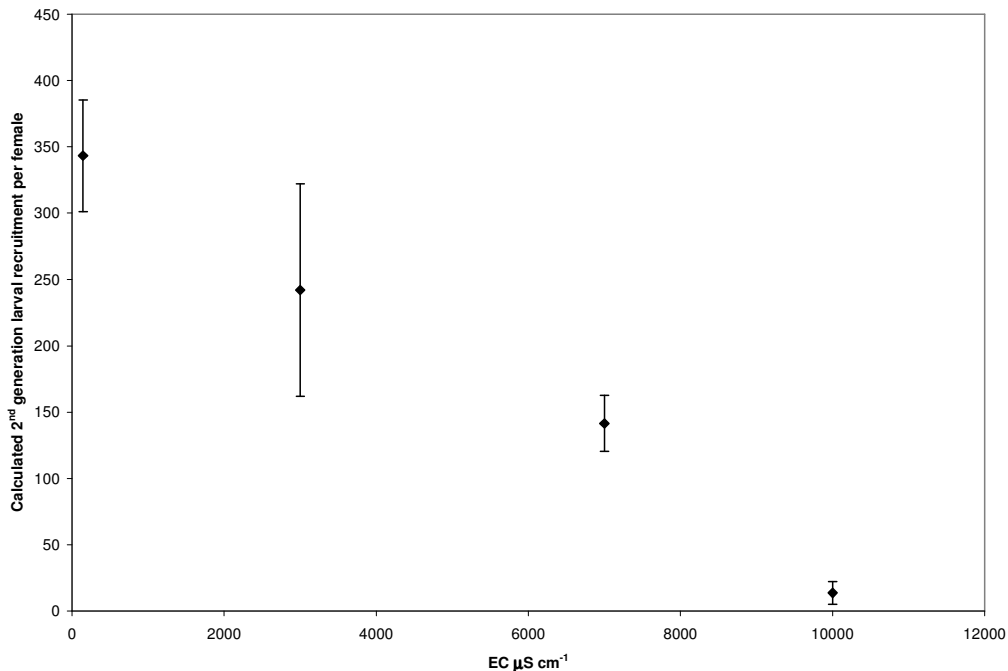


Figure 6.17 Calculated 2nd generation *Chironomus tepperi* larval recruitment per female at different salinities ($\text{EC } \mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

Regression analysis indicated a strong negative linear relationship between the square root of EC and 2nd generation larval recruitment per female. The relationship was best described by the equation:

$$\text{Number larvae recruited per female} = -3.556 \times \sqrt{\text{EC } (\mu\text{S cm}^{-1})} + 409.342$$

$$(F_{1, 13} = 34.011, P < 0.001, r^2 = 0.723, \text{SE slope} = 0.610, \text{SE constant} = 45.745).$$

Using this equation and 50% of the observed optimal 2nd generation larval recruitment per female in unmodified filtered water the EC_{50} for larval recruitment per female was calculated as 4470 (SE 2124-9260) $\mu\text{S cm}^{-1}$.

6.3.4.11 2nd generation larval growth

There were significant differences between salinity treatments in the growth (mean individual dry weight) of second generation *C. tepperi* larvae (ANOVA; $F_{2, 6} = 7.808$, $P = 0.021$). The

pattern of effect was the same as for first generation larval growth, however mean individual weights were lower at each salinity (Figure 6.18). Observed optimal growth occurred in the 3000 $\mu\text{S cm}^{-1}$ treatment (mean individual dry weight 0.50 ± 0.06 mg), with mean individual larval dry weights in unmodified filtered water and 7000 $\mu\text{S cm}^{-1}$ significantly lower (Tukey's pair-wise comparison $P = 0.040$ and 0.027 respectively) (Figure 6.18). Growth of second generation larvae could not be assessed for the 10000 $\mu\text{S cm}^{-1}$ treatment, as there were too few surviving individuals to establish replicates.

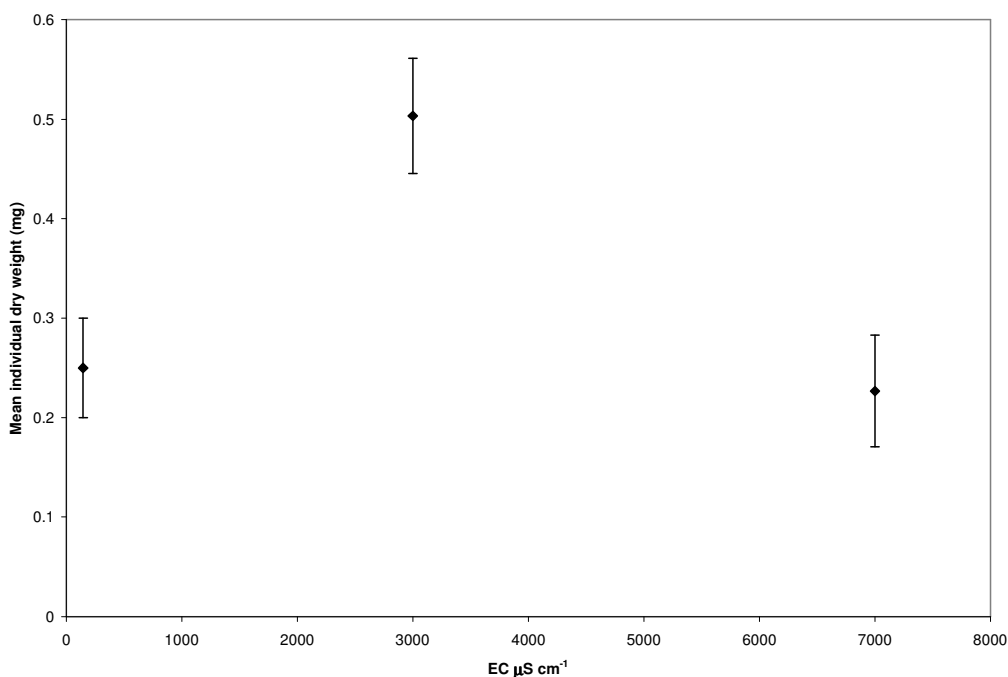


Figure 6.18 Mean individual dry weight (mg) of 2nd generation *Chironomus tepperi* larvae 13 days post eggs oviposition at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

Regression analysis excluding data for unmodified filtered water yielded the following equation:

$$\text{Mean individual dry weight} = (-69 \times 10^{-6}) \times \text{EC} (\mu\text{S cm}^{-1}) + 0.710$$

$$(F_{1,4} = 11.725, P = 0.027, r^2 = 0.746, \text{SE slope} = 20 \times 10^{-6}, \text{SE constant} = 0.109).$$

Using this equation and 50% of observed optimal growth, the EC_{50} value for second generation larval growth can be calculated as 6638 (SE 3921-11571) $\mu\text{S cm}^{-1}$.

6.3.4.12 2nd generation larval survival

Survival of second generation larvae was only assessed during the period encompassed by the growth assessment test, that is from 6 days post egg oviposition to 12 days post egg oviposition. As for growth, the effects at 10000 $\mu\text{S cm}^{-1}$ could not be assessed due to too few numbers of hatched larvae. Data for this period indicates that, as for 1st generation larvae, there were no significant differences evident in 2nd generation larval survival between salinity treatments (ANOVA; $F_{2,6} = 1.764$, $P = 0.250$). Survival ranged from $97.0 \pm 3.0\%$ at 3000 $\mu\text{S cm}^{-1}$ to $84.8 \pm 3.0\%$ in unmodified filtered water (Figure 6.19). These values were highly comparable to survival of first generation larval survival during the second stage of assessment (see Figure 6.6).

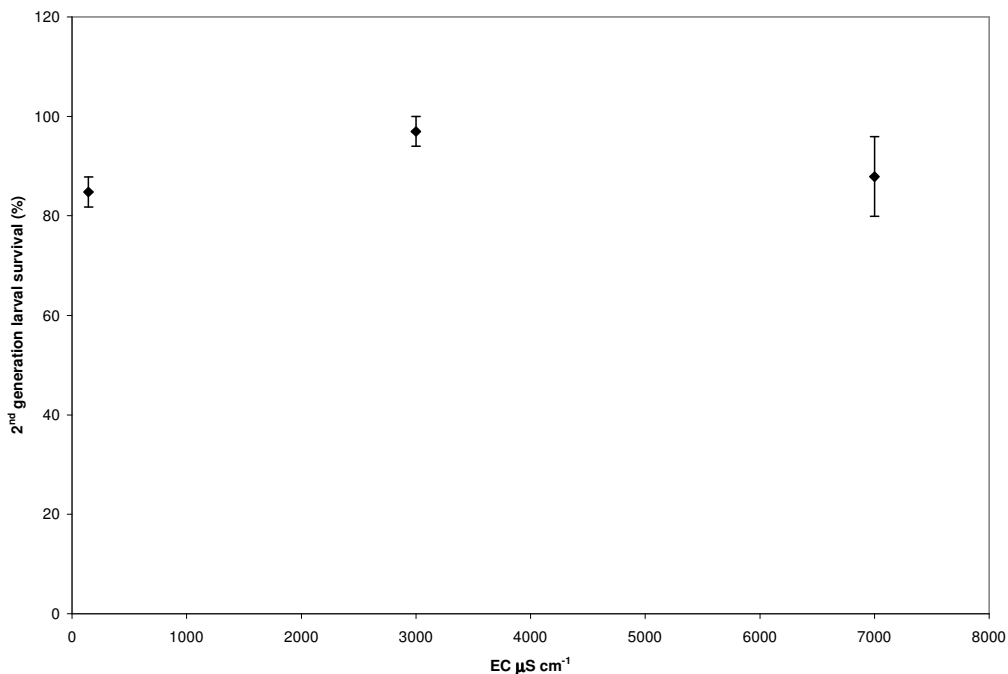


Figure 6.19 Survival (%) of 2nd generation *Chironomus tepperi* larvae between 6 and 12 days post egg oviposition at different salinities (EC $\mu\text{S cm}^{-1}$) during the full life-cycle test. Mean \pm SE.

6.3.4.13 Cumulative full life-cycle effects

The cumulative full life-cycle effects of salinity on the recruitment success of second generation *C. tepperi* larvae were calculated using the effect levels determined for each life stage. This method allowed the effects of salinity on the hatching success of first generation eggs to be factored into the total affect of salinity over the full life-cycle. Calculations started with an initial egg number of 100 and used the mean effect levels at each life stage for each treatment replicate. That is, the observed effects of salinity for each replicate on 1st

generation egg hatching success, early larval survival, proportion of emergent females (sex ratio), egg production and 2nd generation egg hatching success, were used to determine how many second generation larvae would result from an initial 100 1st generation eggs. Calculations were only made for replicates that were successfully assessed for a full life-cycle. The second stage of larval survival was not included in calculations as mortality during this stage was encompassed by emergence success results. From an initial egg number of 100, the mean number of second generation *C. tepperi* larvae calculated to hatch successfully in unmodified filtered water was 10830 ± 3256 . This decreased to just 178 ± 150 in the $10000 \mu\text{S cm}^{-1}$ treatment. This represents a greater than 98% reduction in recruitment. These values were converted to percent recruitment success and are presented in Figure 6.20. The calculated cumulative effects of salinity over a full life-cycle dramatically decrease recruitment success of 2nd generation larvae with increasing salinity (Figure 6.20).

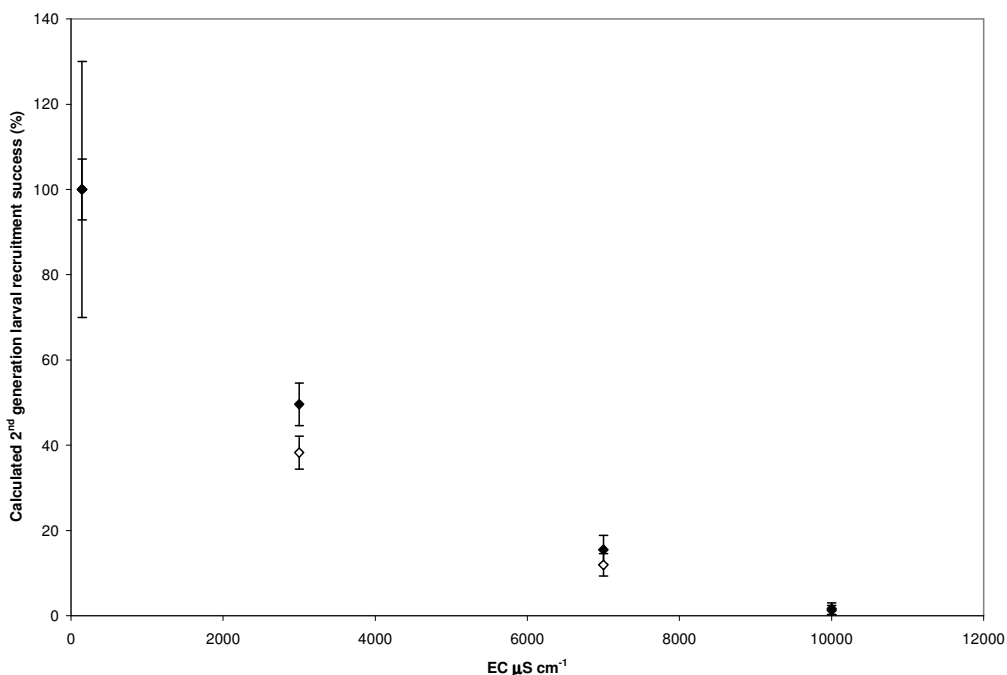


Figure 6.20 The calculated cumulative effect of full lifecycle salinity exposure ($\text{EC } \mu\text{S cm}^{-1}$) on the recruitment success of 2nd generation *Chironomus tepperi* larvae (% recruitment). Open diamonds indicate data excluding an outlier in unmodified filtered water. Mean \pm SE.

There was a high degree of variability in the calculated larval recruitment for unmodified filtered water replicates, with replicate 3 displaying approximately 70% lower recruitment than replicates 5 and 7. This replicate was identified as an outlier during regression analysis. ANOVA of calculated second generation larval recruitment indicated highly significant

differences between salinity treatments (ANOVA; $F_{3, 12} = 14.210$, $P < 0.001$). Tukey's pair-wise comparisons indicated that recruitment numbers were significantly lower at 7000 and 10000 ($P < 0.002$ in both cases) than in unmodified filtered water ($P < 0.002$ in both cases) and that recruitment at 3000 $\mu\text{S cm}^{-1}$ was significantly lower than in unmodified filtered water at a 90% confidence level ($P = 0.067$). ANOVA excluding replicate 3 of unmodified filtered water (ANOVA; $F_{3, 11} = 162.381$, $P < 0.001$) indicated a greater number of significant pair-wise differences. All salinity treatments displayed significantly lower recruitment than in unmodified filtered water ($P < 0.001$ in all cases) and recruitment at 3000 $\mu\text{S cm}^{-1}$ was significantly higher than at 7000 and 10000 $\mu\text{S cm}^{-1}$ ($P < 0.001$ in each case), recruitment at 7000 $\mu\text{S cm}^{-1}$ was significantly higher than at 10000 $\mu\text{S cm}^{-1}$ at a 90% confidence level ($P = 0.052$).

To enable linear regression analysis, EC was square-rooted. This produced a strong linear relationship between $\sqrt{\text{EC}}$ ($\mu\text{S cm}^{-1}$) and recruitment success of 2nd generation larvae (n) best described by the equation:

$$\text{Recruitment success of 2}^{\text{nd}} \text{ generation larvae (n)} = -122.3 \times \sqrt{\text{EC}} (\mu\text{S cm}^{-1}) + 12170.2$$

($F_{1, 14} = 49.074$, $P < 0.001$, $r^2 = 0.778$, SE slope = 17.5, SE constant = 1341.8).

Using this equation and the 50% level of mean larval recruitment in unmodified filtered water (i.e. 50% of 10830) the EC_{50} value for the cumulative effects of salinity on second generation larval recruitment was calculated as 3048 (SE 1499-5906) $\mu\text{S cm}^{-1}$.

Regression analysis excluding replicate 3 of unmodified filtered water, which was identified as an outlier, produced the following equation:

$$\text{Recruitment success of 2}^{\text{nd}} \text{ generation larvae (n)} = -158.0 \times \sqrt{\text{EC}} (\mu\text{S cm}^{-1}) + 14851.8$$

($F_{1, 13} = 251.225$, $P < 0.001$, $r^2 = 0.951$, SE slope = 9.7, SE constant = 765.4).

This equation using 50% of mean larval recruitment in unmodified filtered water excluding the value for replicate 3 (i.e. 50% of 14035) yielded the EC_{50} value of 2458 (SE 1777-3360) $\mu\text{S cm}^{-1}$.

6.3.5 Relationship between lethal and sublethal salinity tolerance

Each of the sensitive life stage and sublethal end points investigated for *C. tepperi* displayed effects at salinities lower than those affecting acute survival of final instar larvae. Figure 6.21 presents the proportional effects of salinity on selection of these end points. Significant effects are evident at salinities having minimal effect on acute larval survival.

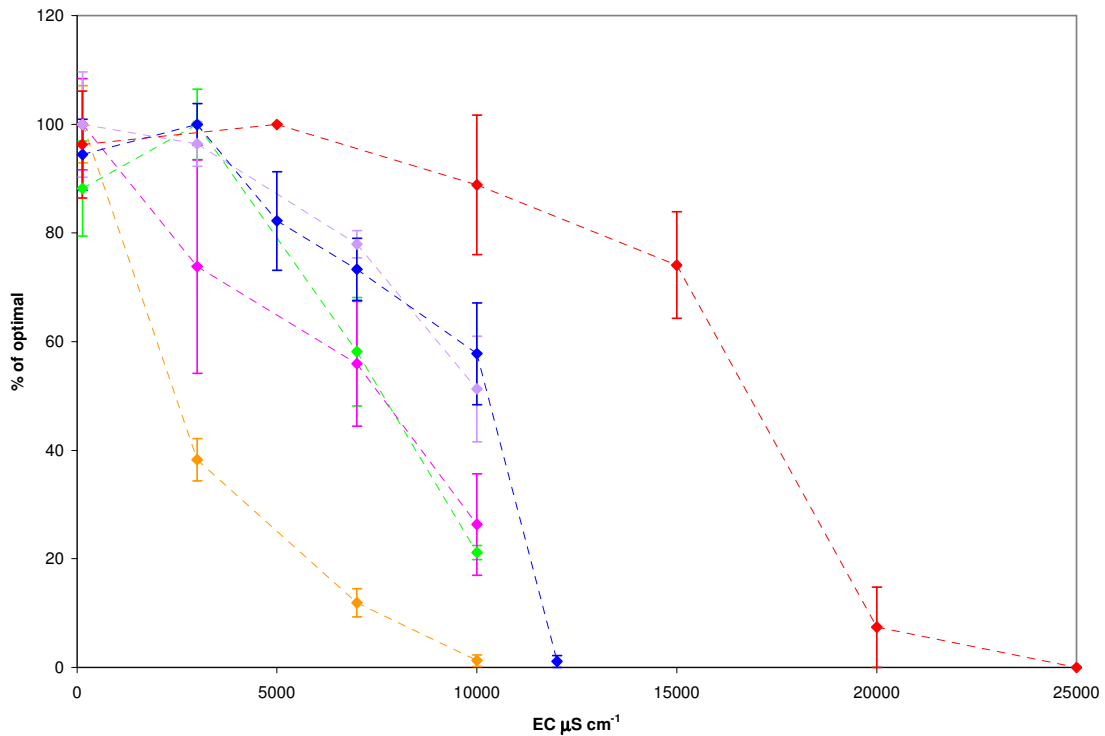


Figure 6.21 The proportional effects of salinity ($\text{EC } \mu\text{S cm}^{-1}$) on the 96-hr larval survival (red), egg hatching success (blue), egg production (pink), larval growth (green) and full life-cycle 2nd generation recruitment (gold) of *Chironomus tepperi*. Mean \pm SE.

Table 6.6 summarises salinity tolerance results for *C. tepperi* and relates sublethal salinity tolerances to the acute lethal tolerance of final instar larvae, through the ratio of sublethal EC_{50} to the 96-hour LC_{50} . With the exception of hatching success of transferred eggs, all sublethal EC_{50} values were lower than the 96-hour LC_{50} . The least sensitive sublethal endpoint was emergence success which was only 24% lower than the 96-hour LC_{50} (ratio = 0.76) while the most sensitive sublethal endpoint was the calculated cumulative life-cycle effects on 2nd generation larval recruitment, which was 83% lower than the 96-hour LC_{50} (ratio = 0.17). Of the individual life stage end points, egg production per female was the most salinity sensitive being 53% lower than the 96-hour LC_{50} (ratio = 0.47).

Table 6.6 Summary of the salinity tolerance of *Chironomus tepperi* and the ratio between sublethal EC₅₀s and the 96-hr LC₅₀ for the species. * Indicates results excluding identified outliers.

<i>Endpoint</i>	Observed optimal EC ($\mu\text{S cm}^{-1}$)	LOEC ($\mu\text{S cm}^{-1}$)	EC₅₀ ($\mu\text{S cm}^{-1}$)	Ratio (sublethal EC₅₀/96-hr LC₅₀)
4 th instar larval 48 hr survival	128	20000	17025 (95% CI 15726-18357)	
4 th instar larval 96 hr survival	5000	20000	14077 (95% CI 12387-15814)	
Egg hatch success (transferred)	138	20000	17381 (95% CI 17058-17705)	1.23
Egg hatch success (laid at EC)	3000	10000	8294 (95% CI 7979-8621)	0.59
Oviposition preference	138	15000-25000	NA	
Full life-cycle test:				
1 st generation egg hatch success	145	3000	7784* (SE 6182-9946)	0.55
1 st generation larval survival	145	>10000	No observed effect	
1 st generation larval growth	3000	7000	7522 (SE 5973-9488)	0.53
Emergence success	145	10000	9784 (95% CI 8237-12659)	0.70
Generation time	145 & 3000	10000	NA	
Egg production per female	145	7000	6658* (SE 4884-6762)	0.47
2 nd generation egg hatch success	145	10000	9072 (95% CI 8728-9468)	0.64
2 nd generation recruitment per female	145	7000	4470 (SE 2124-9260)	0.32
2 nd generation larval survival	3000	>7000	No observed effect	
2 nd generation larval growth	3000	7000	6638 (SE 3921-11571)	0.47
Cumulative life-cycle effects	145	7000	2458* (SE 1777-3360)	0.17

6.4 Discussion

6.4.1 Acute larval salinity tolerance

The short-term salinity tolerance of 4th instar *C. tepperi* did not stabilise during the 96-hour exposure period (as indicated by LC₅₀ values for each 24-hour exposure interval) but decreased with increasing exposure period. The duration of exposure could not be extended as test specimens were beginning to pupate by the end of the exposure period. Eight-day-old larvae were used to ensure all individuals were in the 4th larval instar. Stevens (1998) found *C. tepperi* males (which develop faster than females) to develop into the 4th instar 6.9 days after hatching at 20°C. The 4th instar was found to last 5.3 days at this temperature (Stevens 1998). The onset of pupation during this test 12 days after egg hatching, suggests that *C. tepperi* development time during this study concurs with development times observed by Stevens (1998).

There was a significant reduction in salinity tolerance of *C. tepperi* during the 48-hour to 96-hour exposure period, with a 17% decrease in the LC₅₀ value. Many tolerance testing protocols using midge species use only 48-hour exposure (see ASTM 1997). The results of this test indicate the potential for reliance on only 48 hours of exposure to result in underestimation of the sensitivity of midge species.

Final instar *C. tepperi* larvae displayed a higher salinity tolerance than those recorded for individual Australian Chironomidae species (Kefford *et al.* 2006a) and mixtures of Australian Chironomidae species (Kefford *et al.* 2003a; Dunlop *et al.* 2008). This may suggest *C. tepperi* is a relatively tolerant species. However, Kefford *et al.* (2006a) indicated 72-hour LC₅₀ values of greater than 12800 µS cm⁻¹ for several species, which suggests tolerances for these species may be higher than this salinity. The 10-day survival LC₅₀ of 16200 µS cm⁻¹ for hatch larvae of an unidentified Chironomidae species from the Campaspe River in Victoria (Kefford *et al.* 2007a) was comparable to the tolerance of larval *C. tepperi* observed in this study (i.e. 96-hour LC₅₀ 17025 (95% CI 15726-18357) µS cm⁻¹). Consideration of field distributions of Australian midge species in relation to salinity (Goonan *et al.* 1992, Bailey *et al.* 2002), does suggest that *C. tepperi* may display relatively high tolerance for a freshwater species. The salinity tolerance observed for *C. tepperi* (this study) and other Australian species (Kefford *et al.* 2003a, 2006a, 2007a; Dunlop *et al.* 2008) suggests higher tolerance than observed for Russian Chironomidae species (Berezina 2003).

6.4.2 Egg hatching success

The salinity tolerance of transferred *C. tepperi* eggs observed during this study (EC_{50} 17381 95% CI 17058-17705 $\mu\text{S cm}^{-1}$) was highly comparable to that observed for transferred eggs of the same species by Kefford *et al.* (2004a) (EC_{50} 18000 95% CI 17000-20000 $\mu\text{S cm}^{-1}$). However, as both studies utilised test organisms from cultures originally sourced from Latrobe Universities Genetics Department, results do not reflect the potential for interpopulation variability. Results for transferred eggs were also comparable to the salinity tolerance of unidentified *Chironomus* species eggs collected from the Campaspe River in Victoria and transferred to a range of test salinities (EC_{50} 17700 95% CI 15400-20500 $\mu\text{S cm}^{-1}$) (Kefford *et al.* 2007a).

The hatching success of direct oviposited eggs was much lower than transferred eggs, with an EC_{50} less than half that of transferred eggs. This clearly indicates a critical period of salinity sensitivity during egg oviposition and early development. It is likely that this early sensitivity reflects a developmental stage where water is drawn inside the egg, coming into contact with the embryo. Permeability of the egg may be reduced after this stage reflecting the increased tolerance of eggs exposed at a later developmental stage. A similar phenomenon is observed in fish eggs, however egg permeability is related to external fertilisation, with eggs displaying a much higher salinity tolerance post fertilisation when egg permeability is greatly reduced (Hart *et al.* 1991; Clunie *et al.* 2002). Exclusion of this early critical sensitive phase from testing protocol has great potential for underestimation of the sensitivity of Chironomidae species, not only to salinity but also other toxicants, a point acknowledged by Benoit *et al.* (1997). To avoid this underestimation life-cycle tests with Chironomidae species should begin exposure from oviposition.

While transferred eggs displayed a similar (if not higher) salinity tolerance to that of final instar larvae, the greatly reduced tolerance of direct oviposited eggs indicates the potential for salinity to affect sensitive life stages. This suggests salinity has the potential to affect populations at levels much lower than those affecting the short term survival of older aquatic life stages. Kefford *et al.* (2004a) suggested that the larval life stage of *C. tepperi* was more salinity sensitive than the eggs, however comparison was made with eggs that were not exposed for the critical phase from oviposition.

6.4.3 Oviposition preference

In this experimental design the chosen test salinity levels has the potential to affect the statistical significance of results. With the salinity range used in this test there was a gradual decrease in the number of egg masses oviposited with increasing salinity. There was little difference in oviposition preference between unmodified filtered water and $5000 \mu\text{S cm}^{-1}$. Provision of only two extremely different salinities (e.g. unmodified filtered water and $25000 \mu\text{S cm}^{-1}$) may have resulted in a more clear-cut preference for the lower salinity. None the less, results do indicate a preference for water of a lower salinity for egg oviposition. Salinity preference for egg oviposition has been observed in several species of mosquito (Diptera: Culicidae) (Hinton 1981). Stevens (1995) suggested that female *C. tepperi* are able to identify oviposition sites that suit the ecology of the larvae. Active selection of oviposition sites has also been reported for other *Chironomus* species (Matena 1990 in Stevens 1994).

6.4.4 Full life-cycle

Results of the full life-cycle test with *C. tepperi* indicate significant effects of salinity at levels much lower than those affecting survival of final instar larvae. Many of the observed effects have obvious implications for population viability.

Although differences in pH were evident between treatments in addition to differences in salinity, it is unlikely that the observed affects on *C. tepperi* were due solely to differences in pH, as levels remained within the normal range observed in surface waters (Oliver, 1971). It is possible however, that pH may have acted synergistically with salinity increasing the severity of observed effects. However, as pH increases naturally with increasing salinity (Environment Protection Authority Victoria, unpublished data), this reflects the conditions likely to be encountered with salinity increases in natural ecosystems.

A high degree of variability between replicates was observed during many stages of the full life-cycle test. In many instances outliers were identified, often with little indication of the cause of the variability. Replicate 6 of unmodified filtered water displayed consistently low performance throughout the test compared to other replicates and was excluded from all analyses. The reason for this is unclear however effects were evident from the earliest test stage.

It is unclear why hatching success of first generation eggs was higher at $10000 \mu\text{S cm}^{-1}$ than $7000 \mu\text{S cm}^{-1}$. This was not evident in the second generation or in the independent egg

hatching tests. One explanation may be that the egg masses collected from the 10000 $\mu\text{S cm}^{-1}$ treatment for the test contained an unusually high proportion of viable masses compared to egg masses used for the other treatments.

There were differences in the observed optimal salinity for egg hatching between generations in the full life-cycle test and the independent egg hatch tests. Direct oviposited eggs in the independent test displayed optimal hatching at 3000 $\mu\text{S cm}^{-1}$ while egg hatching at this salinity was significantly lower than in unmodified filtered water for the first generation of the full life-cycle test. There was no significant difference in egg hatching between unmodified filtered water and the 3000 $\mu\text{S cm}^{-1}$ treatment for the second generation.

There was close agreement in the EC_{50} values for hatching of 1st and 2nd generation eggs when results for the 10000 $\mu\text{S cm}^{-1}$ treatment were excluded for the 1st generation. These values also related well to the EC_{50} for direct oviposited eggs in the independent egg hatching test, although the 2nd generation EC_{50} was significantly higher. Reduced egg hatching success has obvious implications for population size, with the number of individuals reduced by 50% at a salinity of approximately 8000 to 9000 $\mu\text{S cm}^{-1}$.

Breaking up of egg masses would appear to have little effect on hatching success. Although the observed optimal hatching success of the unbroken egg masses of the 2nd generation was slightly higher than that of the first generation, it was comparable to that observed for broken egg masses in the independent egg hatching test.

The larval stage of *C. tepperi* was the least salinity sensitive life stage, with no effects on survival evident over the full life-cycle test salinity range. This indicates the potential for test methodologies utilising only larval stage survival to grossly overestimate species tolerance.

While not statistically significant, higher growth of *C. tepperi* larvae was observed at 3000 $\mu\text{S cm}^{-1}$ than in unmodified filtered water ($144.5 \pm 3.7 \mu\text{S cm}^{-1}$). Several other studies have observed higher growth of freshwater macroinvertebrate species at slightly elevated salinities (Kefford and Nugegoda 2005, Kefford *et al.* 2006b, Hassell *et al.* 2006). At salinities above 3000 $\mu\text{S cm}^{-1}$, the growth rate of *C. tepperi* larvae decreased with increasing salinity and the time to emergence increased. The reduction in growth rate with increasing salinity is presumably due to increased energy requirements for osmoregulation and ion balance and

perhaps reduced feeding to avoid ion uptake. Some aquatic insect species have been found to fully compensate for a reduction of growth rate as a result of environmental factors by extension of the development period to enable optimal adult size to be achieved. Hassell *et al.* (2006) observed this for a *Chironomus* species (Diptera: Chironomidae) and Kefford *et al.* (2006b) observed this for the damselfly species *Ischnura heterosticta* (Odonata: Coenagrionidae), both in response to increased salinity. In other insect species extension of development time has been found to only partially compensate for reduced growth rates, resulting in smaller adult size. Clark *et al.* (2004) observed this in the freshwater mosquito species *Aedes aegypti* (Diptera: Culicidae), with increased salinity resulting in increased development time and reduced adult size. As fecundity is often related to female size (Clark *et al.* 2004), this potentially results in reduced reproductive output but minimises the protraction of time to complete a life-cycle, thus allowing for more generations in multivoltine species such as *C. tepperi*. As adult size was not measured during this study, it is unclear if the observed extension of development time fully compensated for the reduction in growth rate with increased salinity. However, the reduction in the number of eggs laid per female with increasing salinity and the observation that adults in the higher salinity treatments did appear smaller (personal observation) suggests that perhaps it did not. In a colonist species such as *C. tepperi*, the production of eggs as quickly as possible is probably more vital to competitive advantage than the absolute number of eggs.

The earlier emergence of male *C. tepperi* adults was also observed by Stevens (1998). That emergence times were only significantly different between sexes in unmodified filtered water during this study was probably due to a reduction in statistical power at the higher salinities as a result of greater variability in time to emergence and lower emergence success rather than a breakdown in this relationship. The trend was certainly evident at each salinity. Protandry with faster male development is a common trait in chironomids from temperate areas (Stevens 1998). Female Chironomidae are larger than males from the final larval instar through pupation and the adult life stage and the longer development time is a result of the extra time required to gain this additional mass (Stevens 1998). The observed increase in time to emergence can not be explained by a change in sex ratio as the higher proportion of males at higher salinities would result in a reduction in mean time to emergence rather than the observed increase.

The mean time to emergence for *C. tepperi* observed in this study in unmodified filtered water (19.5 days) was a few days longer than that observed by Stevens (1998) for the same species

at 20°C (16.1 days). This is most likely due to differences in experimental conditions such as rearing solution (of unknown salinity for Stevens (1998) study), food quality and quantity, and larval density (larvae reared individually in Stevens (1998) study), but may also reflect phenotype differences. This development time is quite short for temperate chironomid species (Stevens 1998).

Altered patterns of emergence have the potential to affect the number of successful mating encounters and the genetic diversity of populations through altered rates of dispersal. As the period between emergence of the different sexes appeared to be relatively unaffected over the experimental salinity range, this maybe of limited concern for *C. tepperi*. However, the increased time to emergence with increasing salinity has potential to reduce to competitive ability of this species, which as a colonist species relies on rapid population establishment. The mean delay in time to emergence was 4.3 days (36%) at 10000 $\mu\text{S cm}^{-1}$. The delay in development time also reduces the number of generations that can be produced within a given time.

The significant reduction in emergence success at 10000 $\mu\text{S cm}^{-1}$ despite larval survival being unaffected up to 13 days post oviposition at this salinity indicates high mortality during the period encompassing pupation and adult emergence. While at other salinities there was no appreciable difference between larval survival and emergence success, at 10000 $\mu\text{S cm}^{-1}$ there was a 50% reduction in survival during this phase. This may reflect reduced salinity tolerance during pupation and/or an inability to accumulate enough energy stores for pupation and metamorphosis to the adult stage at higher salinities due to the increased energy costs of osmoregulation and ion balance. Berezina (1999) investigated the tolerance of a Russian Chironomid species (*Omisus caledonicus*) to changes in salinity, ionic composition and pH. While the pupal phase was found to be less tolerant than larvae, metamorphosis to the adult phase was found to be the most sensitive life stage. Berezina (1999) suggested that the pupal phase was more sensitive than larvae due to the lack of anal papillae (which function in ion exchange) during this stage. Stevens (1998) found mortality of *C. tepperi* raised at different temperatures to be highest during the pupal stage indicating the general sensitive of this developmental stage. This is possibly due to effects on growth manifesting at this stage when accumulated energy is critical to the success of metamorphosis to the adult stage as well as a reduction in physiological tolerance.

Reduced emergence success has obvious implications for reproductive output, with the number individuals surviving to the reproductive stage reduced by 50% at a the EC_{50} value of 9784 (95% CI 8237-12659) $\mu\text{S cm}^{-1}$ during this study. This is highly comparable to the effect of salinity on emergence success of a Victorian *Chironomus* species studied by Hassell *et al.* (2006) for which emergence success was reduced to 50% at approximately 10000 $\mu\text{S cm}^{-1}$.

The protraction of the period between emergence and egg production observed at 10000 $\mu\text{S cm}^{-1}$ and reflected in differences in time for completion of one generation may be a result of longer time requirements for formation and development of eggs.

Despite high variability in sex ratios, results suggest a trend towards a higher proportion of males successfully emerging as adults at higher salinities. This is perhaps a reflection of the lower energy requirements for the development of males. As males are smaller than females they require less energy to attain the minimum requirements for successful metamorphosis to the adult stage. Stevens (1998) found development time for *C. tepperi* to be 10% longer for females than males. It is conceivable therefore that at salinities where high energy requirements for osmoregulation compete with growth, that a higher proportion of males are able to attain the energy required for development to the adult stage.

The reproductive effort of *C. tepperi* was greatly affected by salinity. The obvious trend of a reduced number of eggs in each egg mass with increasing salinity was not statistically significant due to very high variability in the number of eggs per mass within all treatments. However, clearly increased salinity reduced both the number of egg masses oviposited and the number of eggs per mass. Assessment of patterns of emergence and egg mass production suggests that at the lower salinities females produced one large egg mass followed a number of smaller masses. In unmodified filtered water *C. tepperi* females oviposited on average three egg masses each. Martin and Porter (1977 in Stevens 1995) found unfed females of this species to oviposit one to two egg masses with an average of 351 eggs per mass. This is highly comparable to the average number of eggs per mass of 393 ± 33 observed in this study for females in unmodified filtered water. Despite females in the 10000 $\mu\text{S cm}^{-1}$ treatment investing all reproductive effort in an average of only one egg mass, the number of eggs per mass was still lower than for the multiple egg masses produced by females at the lower salinities. The reduction of reproductive effort by 50% at a salinity of approximately 7000 $\mu\text{S cm}^{-1}$ (EC_{50} 6658 95% CI 4884-6762 $\mu\text{S cm}^{-1}$) which is well below salinities that affect survival of larvae has obvious implications for population fitness.

The methodology for assessment of egg hatching success in second generation *C. tepperi*, where the hatching of individual masses was assessed rather than a mixture of eggs from several masses, elucidated that a significant proportion of egg masses in all salinities displayed very low viability (less than 5% hatching success). The proportion of unviable egg masses produced was significantly higher at 10000 $\mu\text{S cm}^{-1}$. This indicates that increased salinity reduced egg hatching success by reducing both the proportion of eggs in viable masses that hatched successfully and increasing the proportion of low viability egg masses produced.

None of the assessed end points for 2nd generation *C. tepperi* indicated increased tolerance to salinity. However, due to the low number of larva available, assessment of effects on growth and survival could not be made at 10000 $\mu\text{S cm}^{-1}$ where effects were most likely to be evident. Extension of this test over a number of consecutive generations would be necessary to effectively investigate the capacity for *C. tepperi* to adapt to higher salinity.

Mean individual weights of 2nd generation larvae were lower than 1st generation larvae as they were weighed at a slightly earlier stage. No differences in growth rate were evident between the generations and the observed optimal salinity for growth was 3000 $\mu\text{S cm}^{-1}$ for both generations. Second generation larval survival was highly comparable to the 1st generation with no effects evident up to 7000 $\mu\text{S cm}^{-1}$.

Many different patterns of response to increasing salinity were displayed by end points examined during the full life-cycle test with *C. tepperi*. Some displayed little difference between unmodified filtered water and the 3000 $\mu\text{S cm}^{-1}$ treatment, others such as larval growth were optimal at 3000 $\mu\text{S cm}^{-1}$ and others suggested an effect between these salinities. Many end points displayed a gradual increase or decrease with increasing salinity (eg. time to emergence and larval recruitment) while others displayed stepped (e.g. number egg masses oviposited per female) or threshold (e.g. egg hatching success) responses. Hassell *et al.* (2006) found the time to emergence, growth and emergence success of a species of *Chironomus* found in Victoria to be optimal at 2500 $\mu\text{S cm}^{-1}$ compared to 650 $\mu\text{S cm}^{-1}$. Time to emergence and growth rate were affected gradually with increasing salinity up to 10000 $\mu\text{S cm}^{-1}$ and then were dramatically affected between 10000 and 15000 $\mu\text{S cm}^{-1}$.

When considered in isolation, egg production per female was the most sensitive endpoint with larval growth the next sensitive. Larval survival was the least salinity sensitive of the examined end points. Even when considered in isolation the effects of salinity on egg hatching success, emergence success and the reproductive effort of *C. tepperi* have obvious implications for population viability. When these effects are taken into account step by step through a full life-cycle, the cumulative effects on the population size of the second generation are astounding. These combined effects displayed a strong negative effect with increasing salinity. All of the effects observed during the full life-cycle test occurred at salinities which results of standard 96-hour larval survival tests would suggest no effects of salinity occur. That the cumulative effects of salinity could potentially reduce the population size of 2nd generation larvae by up to 98% at salinities not affecting larval survival *per se*, indicates the gross inadequacies of short-term lethal tests to predict effects on populations. The magnitude of the cumulative effects of salinity through a full life-cycle on second generation larval recruitment suggest that *C. tepperi* populations at salinities of 10000 $\mu\text{S cm}^{-1}$ may be severely hindered by reduced fitness.

Calculation of the cumulative effects of salinity over one full life-cycle on the recruitment of 2nd generation *C. tepperi* doesn't take into account the additional effects of protracted generation time on the number of generations that can be produced in a given period and the reduced ability to rapidly respond to favourable conditions. These factors would also reduce the competitive ability of this species at elevated salinities.

It is interesting to note that *C. tepperi* has been recorded at field salinities as high as 39800 $\mu\text{S cm}^{-1}$ (Goonan *et al.* 1992), a salinity exceeding even the acute tolerance observed for the species in this study. While field records do not necessarily infer long term persistence of a species at the observed salinity, this may suggest high variability in salinity tolerance between different populations of *C. tepperi*. Research into the salinity tolerance of populations of *C. tepperi* from different geographic regions and with different salinity exposure histories is required.

No other studies have investigated the effects of salinity on freshwater macroinvertebrate species across a full life-cycle of exposure. Research is required to determine if the effects observed in this study occur more broadly for macroinvertebrate species and the relative tolerance of *C. tepperi*.

6.4.5 Relationship between lethal and sublethal salinity tolerance

Acute survival of final instar larvae was the least salinity sensitive of all examined end points for *C. tepperi*. The one exception being the hatching success of transferred eggs, which represents the least likely exposure scenario for natural populations. Rather than a threshold effect where effects are not evident until salinity reaches a certain level, changes in sublethal end points were evident with all changes in salinity. For some end points intermediate salinities were more favourable, while for others negative effects were evident for relatively small increases in salinity. The least sensitive sublethal endpoint examined was adult emergence success. However the 50% effect level for this end point was still 30% lower than the 96-hour LC₅₀ for this species, representing a sublethal to lethal ratio of 0.70. Of the independently assessed life-cycle end points egg production per female was the most sensitive, with a sublethal to lethal ratio of 0.47 (i.e. a 53% reduction in the 50% effect salinity level). This ratio was reduced to 0.32 when the effects of salinity on egg production and egg hatching success were combined to assess 2nd generation recruitment success. The lowest sublethal to lethal ratio observed (0.17) was for the cumulative life-cycle effects of salinity on 2nd generation recruitment success, which indicated a 50% reduction in population number at a salinity 83% lower than the 96-hour LC₅₀ value for this species. As observed optimal values for the investigated sublethal effects to which 50% effect levels are related may not represent true optimal levels sublethal to lethal ratios are likely to be lower than those stated here.

Few studies have investigated both the acute tolerance and sublethal tolerance of freshwater insects. Kefford *et al.* (2007a) found the egg hatching success salinity EC₅₀ for an unidentified Chironomidae species to be higher than the 10-day LC₅₀ for the hatched larvae (EC₅₀:LC₅₀ = 1.09). The high tolerance of eggs transferred to higher salinities after oviposition was also observed in this study, with EC₅₀:LC₅₀ for *C. tepperi* of 1.23. However eggs were exposed in the later stages of development. Investigation of the relationship between acute and sublethal salinity tolerances of other freshwater insect species is required.

7.0 Discussion

7.1 Acute salinity tolerance

7.1.1 Length of the acute lethal response to salinity

The length of the acute lethal response to salinity varied between the investigated species. For none of the species was this period less than the standard 96-hour test exposure period. The shortest period observed for stabilisation of acute salinity related mortality was 96 hours for the worm *L. variegatus*. Acute mortality could not be assessed for longer than 96 hours for final instar *C. tepperi* midge larvae due to the onset of pupation. However, there was no evidence of stabilisation of mortality over 72-hours exposure for this species. The acute tolerance of midge species is often assessed over a 48-hour exposure period rather than 96 hours. The 48-hour salinity LC₅₀ value for final instar *C. tepperi* observed in this study was 21% lower than the 96-hour value, indicating the potential for shorter exposure periods to lead to over estimation of tolerance. For both the snail *P. acuta* and the shrimp *P. australiensis* the acute lethal effects of salinity did not stabilise until after 7 days of exposure. LC₅₀ values for this period of exposure were 10% and 2% less than the 96-hour LC₅₀ values for these species respectively. This difference was statistically significant for *P. acuta* only (indicated by non-overlapping 95% CI).

Kefford *et al.* (2005) investigated the salinity tolerance of five freshwater macroinvertebrate species (4 insect and 1 gastropod species) over both 72-hour and 96-hours of exposure. All of the insect species displayed a decrease in the salinity LC₅₀ value with extension of the exposure period to 96 hours. The 96-hour LC₅₀ values were between 6 and 30% lower, equating to a salinity difference of up to 7000 $\mu\text{S cm}^{-1}$. As tolerance was not assessed over a longer period it is unclear if the acute response to salinity extends for longer than 96 hours for these species. There was no observed decrease in tolerance of the gastropod species between 72 and 96 hours of exposure.

Results of this study and those of Kefford *et al.* (2005) suggest that exposure periods of less than 96 hours are likely to over estimate the acute salinity tolerance of freshwater macroinvertebrate species and that periods longer than 96 hours are required for the assessment of some species. However, shorter exposure periods can provide a reasonable estimate of the relative acute tolerance of a species. In this study differences in LC₅₀ values between exposure periods from 48 hours until stabilisation of mortality were only

significantly different (non-overlapping 95% CI) for *P. acuta*. Wide confidence intervals also resulted in none significant differences in LC₅₀ values between 72 and 96 hours exposure in the study by Kefford *et al.* (2005). This finding would appear to be related to the non-linear relationship between mortality and exposure period, with the majority of mortality occurring during the first 24 to 48 hours of salinity exposure. While investigations of a wider range of species are required, exposure periods of 72 to 96 hours may provide an adequate estimation of the relative acute salinity tolerance of macroinvertebrate species for some applications. This is considering the wide range of variables (eg. temperature, water quality, past salinity exposure, test organism health etc.) that can influence acute tolerance (see section 7.5). However, if an accurate assessment of a species acute salinity tolerance under a specific range of conditions is required, extended exposure periods are recommended to ensure the full extent of acute toxicity is assessed.

7.1.2 Relative acute salinity tolerance

P. australiensis displayed a much higher acute salinity tolerance than the other investigated species, with a 96-hour LC₅₀ value of 39844 (95% CI 37308-43055) $\mu\text{S cm}^{-1}$. The other investigated species displayed acute tolerances within a similar salinity range. *P. acuta* was the most sensitive with a 96-hour LC₅₀ value of 13171 (95% CI 12816-13529) $\mu\text{S cm}^{-1}$ and *L. variegatus* was the most tolerant of the three species with a 96-hour LC₅₀ value of 15255 (95% CI 14720-15809) $\mu\text{S cm}^{-1}$. The tolerance of *C. tepperi* was intermediate to the other two species with a 96-hour LC₅₀ value of 14077 (95% CI 12387-15814) $\mu\text{S cm}^{-1}$, which was not significantly different to the LC₅₀ values of the other species. (See Figure 7.1).

The pattern of acute salinity tolerance displayed by the taxa investigated in this study generally followed that observed in other studies. Crustacea regularly display the highest salinity tolerance of freshwater macroinvertebrate communities (e.g. Berezina 2003; Kefford *et al.* 2003a, 2006a; Dunlop *et al.* 2008). The highest acute salinity tolerances displayed for species collected from the Barwon River were for an Isopod species and a species of freshwater crab, both displayed 72-hour LC₅₀ values in excess of 70000 $\mu\text{S cm}^{-1}$ (Kefford 2003a). Generally non-arthropod taxa (worms, leeches, flatworms, molluscs etc.) are less tolerant than arthropod taxa (insects, crustacea etc.) due to the lack of an exoskeleton. However, within each taxonomic group there are species that display relatively high salinity tolerance and species that are more sensitive. This is particularly true of the Class Insecta, with some species displaying extremely high tolerances and others among the most salinity sensitive of macroinvertebrate species. Tolerance even within one family can vary greatly.

For example tolerance of species within the family Chironomidae ranges from quite sensitive to highly tolerant. Kefford *et al.* (2003a) observed a LC₅₀ of 10000 µS cm⁻¹ for a mixture of chironomid species collected from the Barwon River, while species of the genus *Tanytarsus* regularly occur in hypersaline lakes with salinities as high as ≈375000 µS cm⁻¹ (Bailey *et al.* 2002). Oligochaeta species also display a wide range of salinity tolerances, with some sensitive species and others able to tolerate hypersaline conditions (see Table 3.6). While some gastropod species have adapted to hypersaline environments, within freshwater systems they are often among the most sensitive macroinvertebrate species (e.g. Berezina 2003; Kefford *et al.* 2003a, 2006a; Dunlop *et al.* 2008), with Pulmonates considered particularly sensitive (Hart *et al.* 1991). Given these considerations it is clear that the relative tolerance of the taxa investigated in this study would have been highly dependent on the species selected.

With the exception of *P. australiensis*, which displayed relative high tolerance, the acute salinity tolerance of the species investigated in this study was at the more sensitive end of the range observed for Australian freshwater macroinvertebrate species (Kefford *et al.* 2003a, 2006a; Dunlop *et al.* 2008). The lowest salinity tolerance observed by Kefford *et al.* (2003a) for macroinvertebrate species collected from the Barwon River catchment was for two species of baetid mayfly (Ephemeroptera: Baetidae) with 72-hour LC₅₀ values of 5500 (95% CI 760-9800) µS cm⁻¹ and 6200 (95% CI 3700-7900) µS cm⁻¹. Kefford *et al.* (2003b) observed a 96-hour LC₅₀ value of approximately 4300 µS cm⁻¹ for an Australian species of Hydra (Cnidaria: Hydrozoa).

7.2 Salinity tolerance of sensitive life stages

The salinity tolerance of potentially sensitive life stages was investigated for three of the selected test species. The salinity tolerance of the larval stage of *P. australiensis*, the effect of salinity on the number of *C. tepperi* successfully emerging as adults and the effect of salinity on the egg hatching success of both *C. tepperi* and *P. acuta* was investigated. Different life stages do not occur in the mode of asexual reproduction employed by *L. variegatus*. Therefore, assessment of the tolerances of different life stages was not possible for this test species. Chronic effects of salinity on reproduction/mortality and growth of *L. variegatus* are discussed in the following section (7.3).

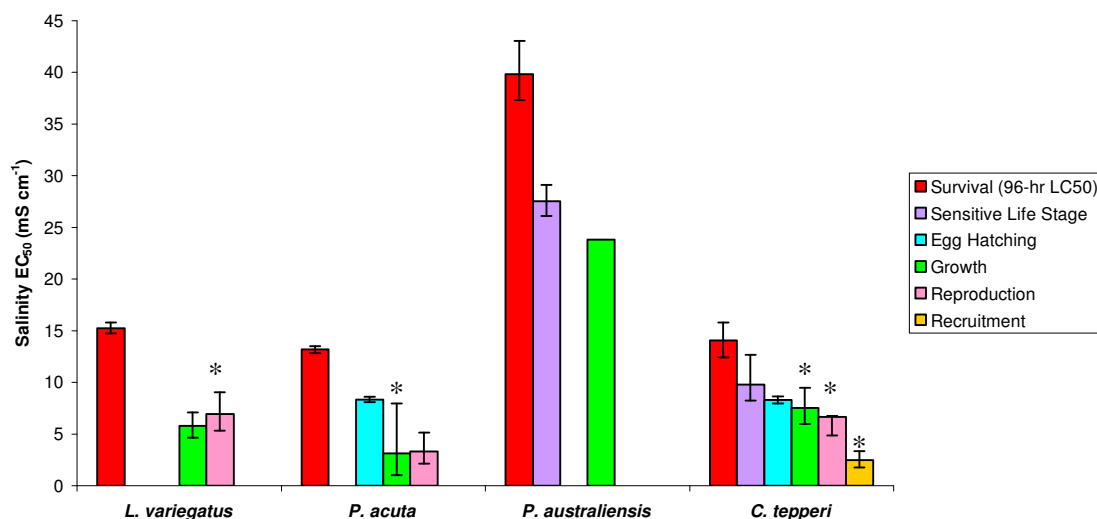


Figure 7.1 96-hr LC₅₀ salinity tolerance values and EC₅₀ values for sensitive life stage and sublethal end points investigated for each of the studied freshwater macroinvertebrate species. Except where indicated with asterisks, error bars represent 95% confidence intervals. Error bars indicated with asterisks represent standard error of the mean.

For each of the investigated species, life stages other than those commonly used for tolerance testing (i.e. older aquatic life stages) were found to be more sensitive to increased salinity (Figure 7.1). The hatching success of eggs of both *P. acuta* and *C. tepperi* was found to be reduced to less than 1% at salinities having only a minimal effect on short-term survival of the older aquatic life stage of the species. The EC₅₀ for emergence success of adult *C. tepperi* was close to 25% lower than the 96-hour LC₅₀ for final instar larvae and *P. australiensis* larvae displayed lower salinity tolerance than the adult stage (Figure 7.1). The effective endpoint of each of these tests was mortality, in that failure to hatch or emerge resulted in death. Although the point in the life-cycle that this mortality occurred differed, in each case this was before sexual maturity and therefore would result in a reduction in the size of the breeding population. Other studies have also found life stages other than the older aquatic stages to be more sensitive (Hubschman 1975; Mills and Geddes 1980; Walsh 1994; Kefford *et al.* 2004a, 2007a). This indicates that tolerance tests conducted with the older aquatic life stages (as is most often the case) are likely to over estimate the tolerance of macroinvertebrate species. Models that utilise the results of these tests without taking sensitive life stages into account may not provide the level of protection that they are designed to offer.

The sensitive life stage end points investigated in this study displayed threshold type responses to increased salinity, with a range over which there was little effect, intermediate

salinities affecting a proportion of individuals and salinities above which all individuals were affected. This is the same response pattern as observed for acute mortality and is likely to be a reflection of the physiology of osmoregulation and the binary nature of the end points (e.g. alive/dead, hatched/unhatched etc.).

Exposure of *C. tepperi* for a full life-cycle revealed egg hatching to be the most salinity sensitive life stage for this species. Egg hatching also appeared to be the most sensitive life stage for *P. acuta*. Although tests investigating tolerance of *P. acuta* hatchlings were unsuccessful, high mortality was not observed at salinities lower than those lethal to the adult stage. Though not directly assessed, the tolerance of *P. australiensis* eggs appeared to be linked to survival of gravid females. At salinities lethal to a proportion of females, high hatching success was observed for the eggs of females that remained alive, while none of the eggs of females that died hatched successfully. Other studies have found egg hatching to be a salt sensitive life stage for freshwater macroinvertebrate species (Kefford *et al.* 2004a, 2007a). However, no other studies have investigated the relative salinity tolerance of all life stages of any one species.

Test methodology is an important consideration in the assessment of salinity tolerance of egg hatching success. Large differences in egg hatching salinity tolerance were observed for *C. tepperi* eggs oviposited at the test salinity versus those that were transferred after oviposition. This indicates that fertilisation or oviposition may be a critical sensitive stage for some macroinvertebrate species. This was not observed for *P. acuta*.

7.3 Sublethal salinity tolerance

Each of the investigated species displayed sublethal effects at salinities significantly lower than those affecting the survival of both the older aquatic life stage and sensitive life stages. The sublethal responses investigated were those affecting growth and reproductive effort. Unlike effects on survival, which displayed threshold responses to increased salinity, sublethal effects displayed more continuous responses to changes in salinity. For most of the investigated variables, any change in salinity resulted in a change in response, even at relatively low salinities. While a variety of responses were observed, curve-linear decreases in response variables were commonly observed above an optimal salinity (e.g. Figures 4.3, 4.11, 6.20).

Due to the observed continuous nature of the responses of sublethal end points to increased salinity (i.e. no threshold below which effects did not occur) and the different shapes of responses, it was necessary to assign a consistent level of effect to enable comparison between different variables and species. As a 50% reduction in growth or reproductive effort can arguably be considered a significant level of effect, this level was selected for comparative purposes. This effect level was also consistent with the effect level considered for lethal end points. NOEC (no observed effect concentrations) values were not considered useful in these circumstances given the observed effect distributions (i.e. non-threshold responses), as these may simply reflect selected salinity concentrations and the level of replication. 50% effect levels were calculated using observed optimal endpoint values. As the observed optimal values are unlikely to represent true optimal levels (as small changes in salinity were found to affect sublethal end points), calculated 50% effects are likely to actually reflect higher levels of effect, resulting in a potential overestimation of tolerance. Investigation of end points in a range of low salinities would elucidate the likely levels of this overestimation.

With the exception of *P. acuta*, the investigated species displayed maximum growth at slightly elevated salinities (Figures 3.5, 5.6 and 6.7). Rather than maximum growth occurring at the lowest salinity, growth was higher at the next salinity in the treatment range. *L. variegatus* also displayed a higher rate of asexual reproduction at 3000 $\mu\text{S cm}^{-1}$ than at the lower salinity of 144 $\mu\text{S cm}^{-1}$, at which it had been cultured. A similar pattern has been observed for emergence success of several freshwater insect species (Hassell *et al.* 2006), growth of a damselfly species (Kefford *et al.* 2006b) and growth of *P. acuta* over a low salinity range (Kefford and Nuggeoda 2005).

While maximum growth of *L. variegatus*, *P. australiensis* and *C. tepperi* was observed at the lowest treatment salinity (i.e. 3000 $\mu\text{S cm}^{-1}$ for *L. variegatus* and *C. tepperi*, and 5000 $\mu\text{S cm}^{-1}$ for *P. australiensis*). The optimal salinity for growth of these species may have occurred anywhere in the range between the lowest test salinity (i.e. unmodified filtered water or river salinity) and the next highest salinity in the treatment range (i.e. 6000 $\mu\text{S cm}^{-1}$ for *L. variegatus*, 10000 $\mu\text{S cm}^{-1}$ for *P. australiensis* and 7000 $\mu\text{S cm}^{-1}$ for *C. tepperi*). This is also true for the asexual reproduction of *L. variegatus*.

The optimal salinity for growth of a species may not be optimal for other end points. For example, while maximum growth of *C. tepperi* larvae was observed at 3000 $\mu\text{S cm}^{-1}$, egg production was reduced at this salinity (see Section 6.3.4). To determine the true optimal

salinity for a species, it is necessary to take into account effects on all end points and the consequences of these effects to overall fitness. This highlights the advantages of full life-cycle tests.

In studies investigating the tolerance of organisms to toxicants, a response pattern displaying stimulation at low doses and inhibition at high doses is termed “Hormesis” (Bailer and Oris 1998). While this is similar to the response of growth to increased salinity observed in this study for *L. variegatus*, *P. australiensis* and *C. tepperi*, where growth was higher at slightly elevated salinities. The mechanism for this response is likely to be different to that described by Hormesis, which is in response to toxic substances. As evident throughout this study, high levels of salinity are toxic to freshwater macroinvertebrates. However, the ions that comprise total salinity (i.e. Na⁺, K⁺, Ca₂⁺, Mg₂⁺, Cl⁻, HCO₃⁻, CO₃²⁻, SO₄ etc.) are essential for cellular function (Hart *et al.* 1991) and therefore required in low doses. The balancing of internal concentrations of these ions through osmoregulation processes requires energy (Potts 1954). Higher growth observed at slightly elevated salinities during this study may have been the result of reduced energy requirements for osmoregulation.

With the exception of *P. australiensis* the effects of salinity on both reproductive effort and growth were investigated for each of the test species. Despite some differences in observed optimal salinities, for each species the sensitivities of these end points were found to be highly comparable, with no significant differences occurring between 50% effect levels (Figure 7.1). While the salinity EC₅₀ for egg production by *P. acuta* estimated from data presented by Kefford and Nugegoda (2005) was lower than the EC₅₀ they observed for growth, the sensitivity of the end points was highly comparable and it is unlikely they differed significantly (see Table 7.1). The correlation between effects on growth and reproduction are likely due to both factors being limited by the energy costs of osmoregulation with increased salinity. Fecundity has also been closely linked with adult size in many invertebrate species.

Effects on growth were observed as not only reduced rates of biomass accumulation but also extensions of development time. The average time required for *C. tepperi* to complete a generation, from laying of initial eggs to laying of eggs by the next generation, increased from 25 days at the lowest salinity (145 µS cm⁻¹) to 34 days at 10000 µS cm⁻¹, an increase of 36%. This would result in four fewer generations in a year (10.7 versus 14.6 generations per year), and may reduce the competitive advantage of this colonist species. Increased development time with increased salinity has also been observed for an unidentified *Chironomus* species

(Hassell *et al.* 2006) and a freshwater species of mosquito (Culicidae: *Aedes aegypti*) (Clark *et al.* 2004). The time required for hatching of *P. acuta* eggs increased from an average of 9 days across a salinity range of 165 to 7000 $\mu\text{S cm}^{-1}$, to 13 days at 10000 $\mu\text{S cm}^{-1}$, an increase of 41%. Kefford *et al.* (2004a) also observed increased egg development time with increased salinity for a species of freshwater limpet (Ancyliidae: *Burnupia stenochorias*) from South Africa. These results indicate that effects of salinity on macroinvertebrate growth can impact a range of life stages and have potential impacts on a species ability to compete and respond to environmental changes. The preferential selection of lower salinities for egg deposition by *C. tepperi* observed in this study (see section 6.3.3) indicates another potential pathway for increased salinity to affect freshwater macroinvertebrates.

The pattern of relative sublethal salinity tolerance of the investigated species followed the same pattern as acute lethal tolerance. *P. acuta* was the most sensitive with growth and egg production reduced by 50% at approximately 3000 $\mu\text{S cm}^{-1}$. *L. variegatus* and *C. tepperi* displayed similar sublethal tolerances, with EC_{50} values for growth and reproduction ranging between approximately 5500 and 7500 $\mu\text{S cm}^{-1}$. *P. australiensis* was the most tolerant, with the growth of juveniles reduced by 50% at approximately 24000 $\mu\text{S cm}^{-1}$, although uninvestigated end points may be more sensitive.

While 50% effect levels were considered for comparative purposes, much higher levels of effect were observed for each of the investigated species at salinities having limited effect on acute survival. At approximately 13000 $\mu\text{S cm}^{-1}$ mortality of *L. variegatus* exceeded asexual reproduction and growth was reduced to zero above approximately 10500 $\mu\text{S cm}^{-1}$, indicating a declining population (Figure 3.9). Egg production by *P. acuta* was reduced by more than 99% at 12000 $\mu\text{S cm}^{-1}$ (in the long-term survival test) and growth was reduced by more than 85% at 10000 $\mu\text{S cm}^{-1}$ (Figure 4.12). Egg production and growth of *C. tepperi* were reduced by approximately 75% at 10000 $\mu\text{S cm}^{-1}$ (Figure 6.21).

High within treatment variability was an issue in determining the effects of salinity on growth for several of the species. These problems may be reduced through increased replication and reducing the initial size range of test organisms. The period of exposure is also likely to be a factor in the magnitude of the observed effects on growth. Further investigation is required to determine if effects are magnified or reduced with extended exposure. Results for *P. acuta*

collected from a site experiencing higher salinities suggest that salinity history may also affect sublethal salinity tolerance (see section 7.5.1).

While some factors influencing sublethal salinity tolerance require further investigation, it is clear that reduced growth, extended generation times and reduced reproduction are likely to have significant impacts on the fitness of populations. The ability of populations experiencing these affects to compete for resources and cope with environmental stressors is likely to be reduced. The level to which this affects the ability to persist at elevated salinities is unclear.

7.4 Full life-cycle salinity tolerance

The short life-cycle of *C. tepperi* allowed not only the salinity tolerance of different life stages and sublethal end points to be investigated individually, but also allowed the cumulative effects of salinity to be assessed over a full life-cycle of exposure. The flow-on cumulative effects of salinity on egg hatching success, adult emergence success, sex ratio and fecundity were evident in the dramatic reduction in recruitment success of the second generation (Figure 6.20). Recruitment displayed an exponential decrease with increasing salinity (Figure 6.20). As a result of exposure for a single life-cycle, recruitment was reduced to just 1% of the observed optimal at 10000 $\mu\text{S cm}^{-1}$, a salinity that had minimal effect on acute survival. The EC_{50} value for recruitment of *C. tepperi* was just 2458 (95% CI 1777-3360) $\mu\text{S cm}^{-1}$, the most sensitive end point for all of the species investigated in this study (Figure 7.1). The combined effects of reduced recruitment and the extension of life-cycle duration (see section 7.3) would be likely to have significant effects on the ability of the species to compete and respond to environmental changes at higher salinities. It is unclear, however, the extent to which the species can adapt to salinity increases. The results of this study did not indicate increased tolerance of the second generation, however, further research into the adaptive abilities of this species is required. It should be noted that *C. tepperi* has been recorded from a lake with a salinity of 39000 $\mu\text{S cm}^{-1}$ (Goonan *et al.* 1992), a salinity exceeding even the acute tolerance of this species observed during this study. No other studies have investigated the effects of increased salinity on a species of freshwater macroinvertebrate across a full life-cycle of exposure. Further research is required to determine if other macroinvertebrate species display the high level of sensitivity observed for *C. tepperi* when exposed for a full life-cycle.

7.5 Relationship between acute, sensitive life stage and sublethal salinity tolerance

To investigate the relationship between the acute salinity tolerance of older aquatic life stage of a species and the tolerance of sensitive life stages and sublethal end points 50% effect levels were considered. This allows consistency of the levels of effects being compared and was necessary in light of the non-threshold and variety of responses observed for sub-lethal end points (see section 7.3). The ratio between the 96-hour LC₅₀ of each of the four investigated species and EC₅₀ values for sensitive life stages and sub-lethal end points are presented in Figure 7.2. Consideration of this ratio allows comparison of the relationship between sensitive life stage and sub-lethal end points to acute tolerance of species with very different absolute tolerances.

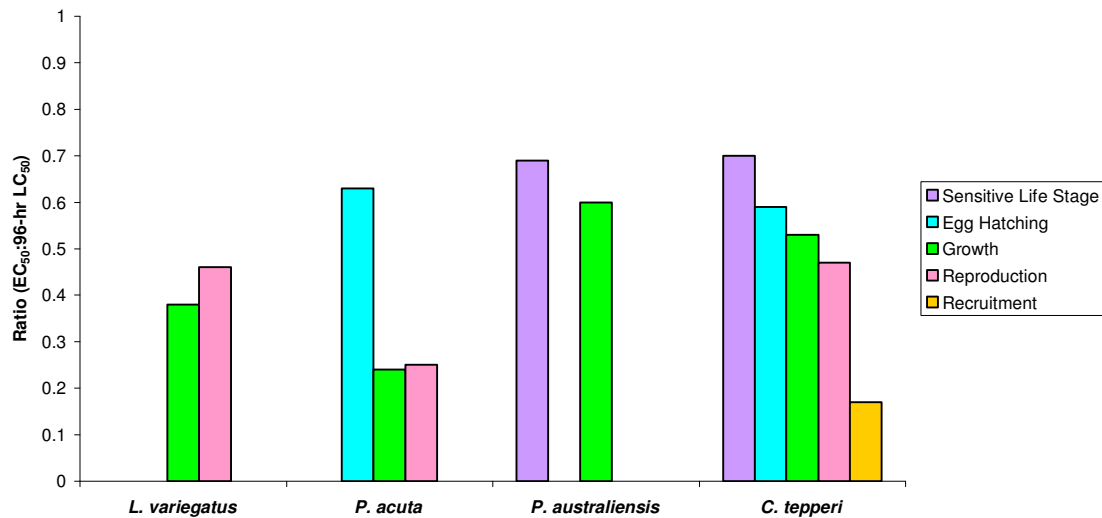


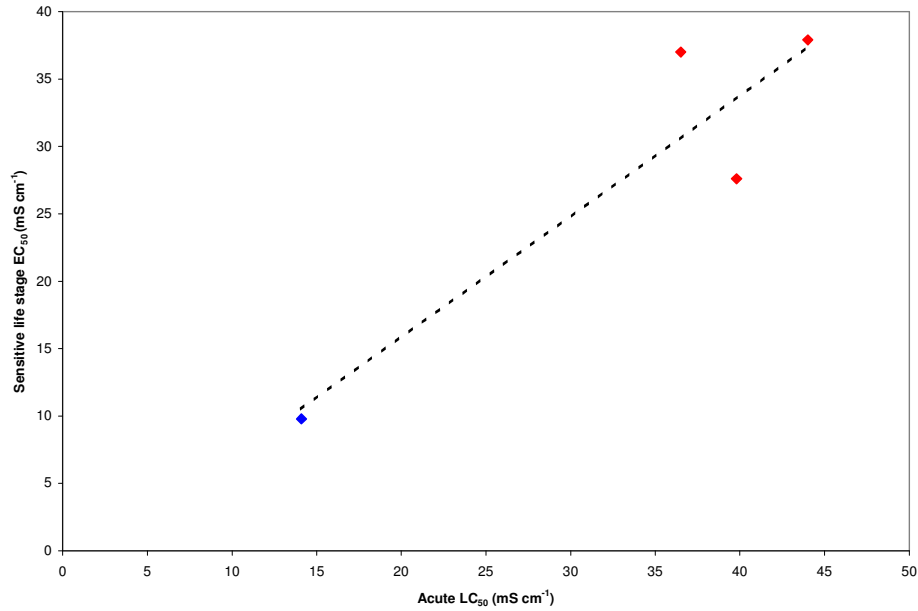
Figure 7.2 The ratio of EC₅₀ values and 96-hr LC₅₀ values for sensitive life stage and sublethal end points for the freshwater macroinvertebrate species investigated in this study.

Relative to acute tolerance, the tolerance of sensitive life stages was the least sensitive of the end points investigated for the test species, with ratios of 0.76 and 0.70 for *P. australiensis* and *C. tepperi* respectively (Figure 7.2). Egg hatching success displayed similar relative tolerances for both *P. acuta* and *C. tepperi* with ratios of 0.63 and 0.59 respectively. The ratios of both growth and reproduction to acute tolerance indicate that, with the exception of recruitment, these end points are more sensitive to increased salinity than the other investigated end points. The similarity of the sensitivity of growth and reproduction end points for each of the investigated species that was evident in Figure 7.1, was again evident in Figure 7.2. Ratios of these end points ranged from 0.60 for growth of *P. australiensis*, to 0.24 and 0.25 for growth and reproduction by *P. acuta* (Figure 7.2). The cumulative effects of

salinity on recruitment of *C. tepperi* after a full life-cycle of exposure was the most sensitive of the investigated end points, with a ratio of EC₅₀ to 96-hour LC₅₀ of 0.17. That is, that the recruitment of second generation *C. tepperi* was reduced by 50% at a salinity 83% lower than the 96-hour LC₅₀ for the species.

There have been very few studies investigating both the acute and sub-lethal salinity tolerances of freshwater macroinvertebrates. Results of this study and the few published studies are summarised in Table 7.1. Ratios of published sub-lethal EC₅₀ and LC₅₀ values were calculated and are also presented in Table 7.1. EC₅₀ values for different sub-lethal and sensitive life stage end points plotted against LC₅₀ values suggest generally linear relationships (Figure 7.3). The small number of data points limits further statistical investigation of these relationships. The combined results of this and other studies does suggest, however, that there is a proportional relationship between the sublethal and sensitive life stage salinity tolerance and the acute salinity tolerance of macroinvertebrate species. This relationship would appear to be true for a wide range of taxa and across a broad range of salinity tolerances (Figure 7.3). It has been suggested that species with low acute salinity tolerance may be relatively more sensitive to sublethal effects than highly tolerant species (Kefford *et al.* 2006a, 2006b). This is not evident in the available tolerance data, with highly tolerant species displaying a similar EC₅₀:LC₅₀ ratio to sensitive species (Figure 7.3). However, the range of species tolerances does not cover the full breadth of acute salinity tolerances observed for freshwater invertebrates and only limited data is available. Therefore a relationship other than a direct proportional one can not be ruled out. Further research is required to investigate these relationships.

a)



b)

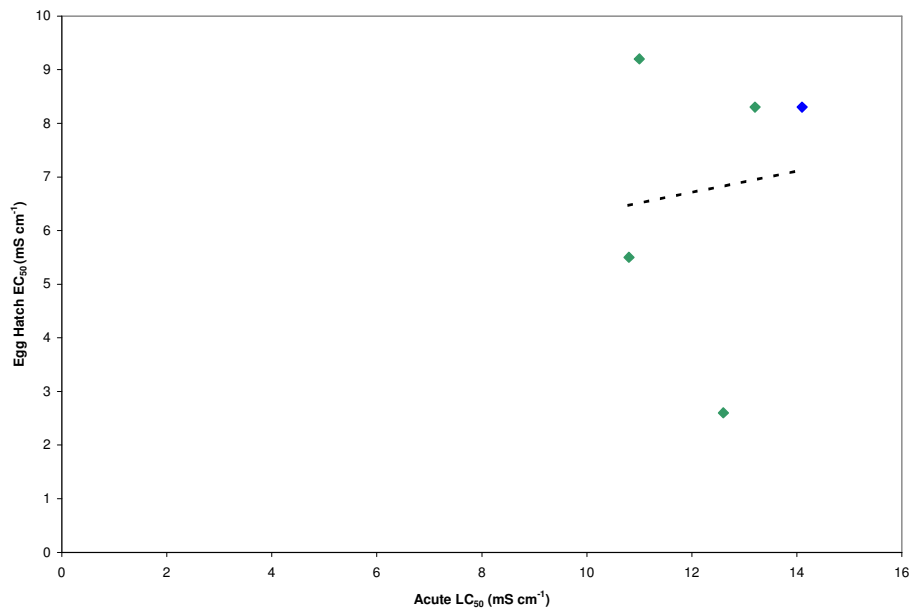
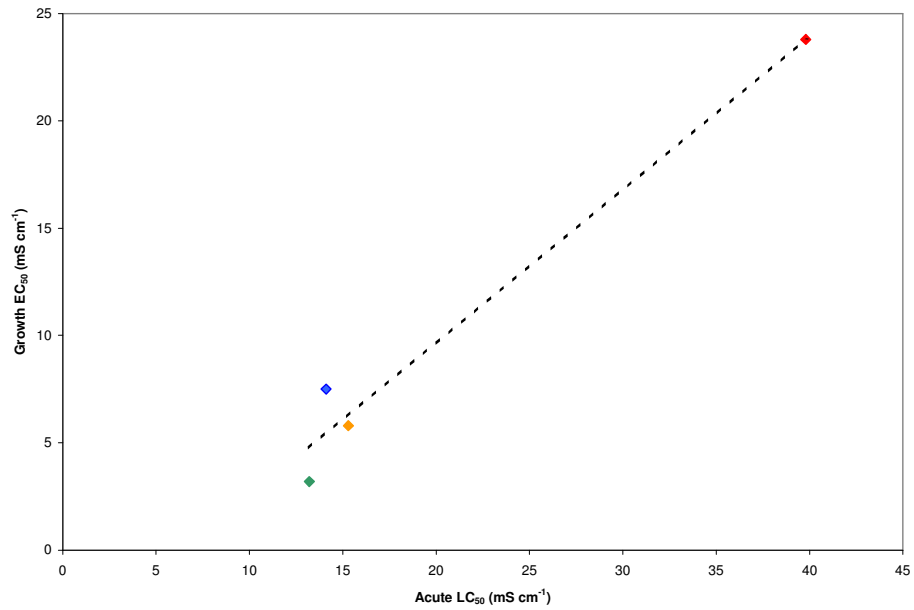


Figure 7.3 The relationship between acute salinity LC₅₀ values and EC₅₀ values (mS cm⁻¹) of different sublethal and sensitive life stage end points for freshwater macroinvertebrate species investigated in this and other studies. a) sensitive life stage, b) egg hatching success, c) growth and d) reproduction. Gastropod species are indicated in green, insects in blue, crustacea in red, oligochaeta species in orange and hydrozoa species in purple. Sources outlined in Table 7.1.

c)



d)

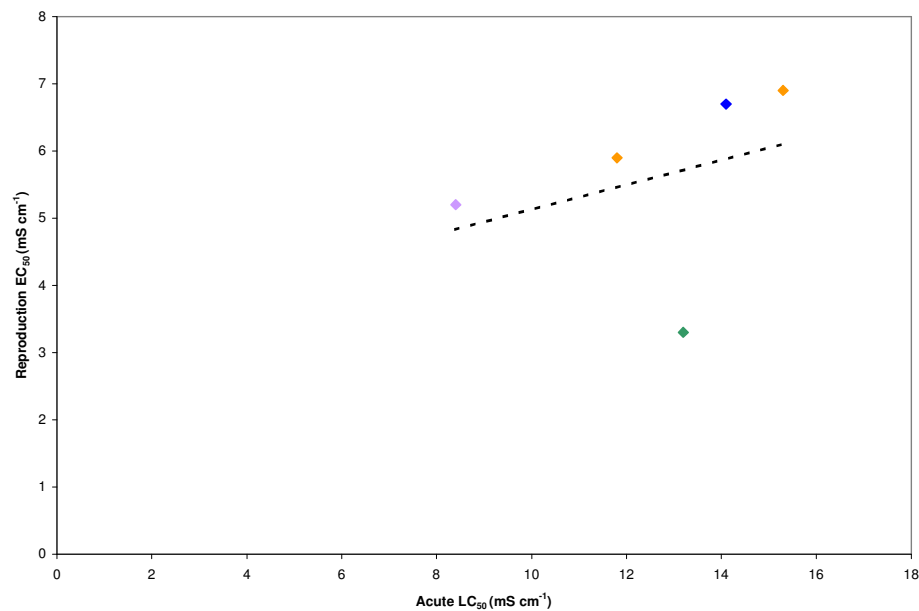


Figure 7.3 continued. The relationship between acute salinity LC₅₀ values and EC₅₀ values (mS cm⁻¹) of different sublethal and sensitive life stage end points for freshwater macroinvertebrate species investigated in this and other studies. a) sensitive life stage, b) egg hatching success, c) growth and d) reproduction. Gastropod species are indicated in green, insects in blue, crustacea in red, oligochaeta species in orange and hydrozoa species in purple. Sources outlined in Table 7.1.

Reproduction by *P. acuta* appears to be quite sensitive relative to the EC₅₀:LC₅₀ ratio of other taxa, with a ratio of only 0.25 (Figure 7.3d.). Ratios for reproduction end points for other macroinvertebrate species ranged from 0.46 for asexual fission by *L. variegatus* and 0.62 for population growth of *Hydra oligactis* (Table 7.1). The relationship between acute tolerance and egg hatching success of gastropod species displayed higher variability than the other investigated end points (Figure 7.3b.), with ratios ranging from 0.21 for *Glyptophysa gibbosa* to 0.84 for *Burnupia stenochorias* (Table 7.1). This may be due to experimental differences, particularly the development stage of eggs at time of exposure and salinity history of the source population.

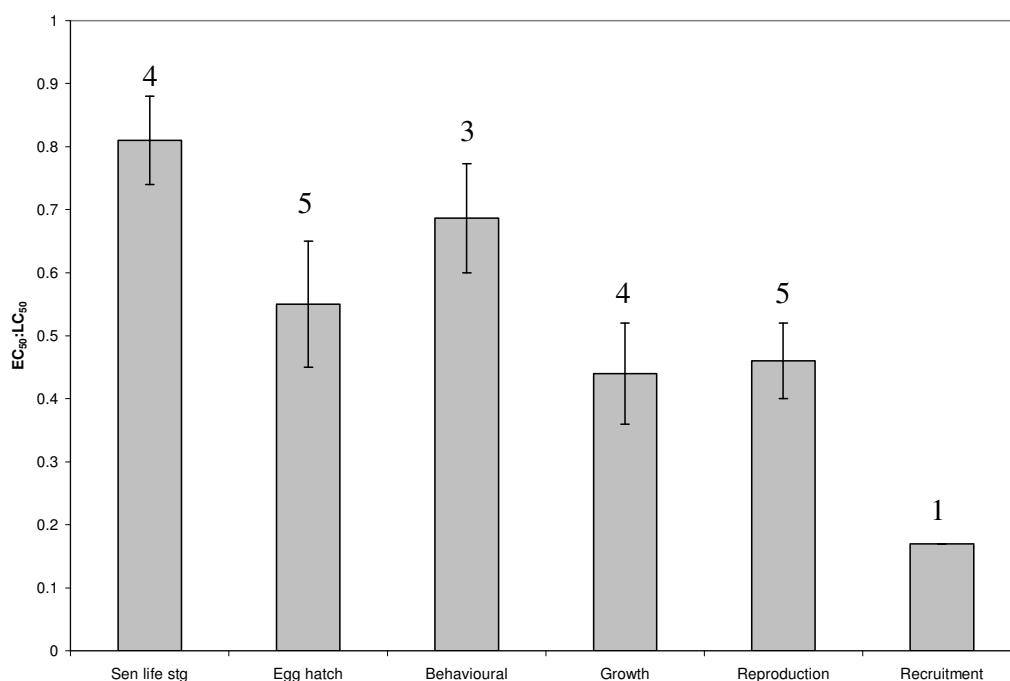


Figure 7.4 Mean values for the ratio of EC₅₀ to LC₅₀ for the salinity tolerance of different sensitive life stages and sublethal end points for freshwater macroinvertebrates investigated in this and other studies. Numbers indicate the number of values used to derive the mean and error bars indicate standard error of the mean. Sources outlined in Table 7.1.

Mean ratios of EC₅₀ values for sensitive life stage and sub-lethal end points to the acute salinity tolerance of macroinvertebrate species from this and other studies are presented in Figure 7.4 (sources outlined in Table 7.1). Egg hatching success was more sensitive relative to acute tolerance than other sensitive life stages, with a mean ratio of $0.55 \pm \text{SE } 0.10$ versus $0.81 \pm \text{SE } 0.07$ for other sensitive life stages (Figure 7.4). While they occur at salinities lower than those affecting acute survival, behavioural responses were not particularly sensitive

indicators of salinity stress (mean ratio $0.69 \pm \text{SE } 0.09$) in comparison to sublethal end points (Figure 7.4). Excluding recruitment, growth and reproduction were the most sensitive of the investigated end points relative to acute tolerance. Mean ratios were $0.44 \pm \text{SE } 0.08$ for growth and $0.46 \pm \text{SE } 0.06$ for reproduction. Data for species additional to those investigated in this study were not available for effects of salinity on macroinvertebrate growth, but data for two additional species were available for effects on reproduction.

The only data available for the cumulative effects of salinity exposure across a full life-cycle were from this study. The recruitment of second generation *C. tepperi* after exposure of a full life-cycle displayed very high salinity sensitivity relative to the acute tolerance of the species, with a ratio of 0.17.

Kefford *et al.* (2007b) investigated the acute and sublethal salinity tolerance of a number of freshwater microinvertebrate species. Ratios of EC_{50} to LC_{50} estimated from the presented data were approximately 0.66 for population growth of *Epiphanes macrourus* (Rotifera: Epiphanidae) and approximately 0.65 for egg hatching success of *Newnhamia fenestra* (Ostrocooda: Notodromadidae). These ratios are higher than mean values observed for macroinvertebrate species but comparable to those observed for some individual species.

Table 7.1 Summary of acute (LC₅₀), sublethal (EC₅₀) and sensitive life stage (EC₅₀) salinity tolerance of freshwater macroinvertebrates. Bold text indicates data from this study.

Taxon	Country	LC ₅₀ (mS cm ⁻¹)	Investigated effect	EC ₅₀ (mS cm ⁻¹)	Ratio EC ₅₀ :LC ₅₀	Sources
Hydrozoa:						
<i>Hydra vulgaris</i> (Hydridae)	Australia	4.3* ^a	Feeding rate (post 48-hrs exposure)	3.7*	0.86	1
<i>Hydra oligactis</i> (Hydridae)	Australia	8.4* ^a	Population growth (96-hrs)	5.2 (SE 4.7-5.6)	0.62	2
Oligochaeta:						
<i>Tubifex tubifex</i> (Tubificidae)	Poland	11.8* ^b	Egg production (12-days)	5.9*	0.50	3
<i>Lumbriculus variegatus</i> (Lumbriculidae)	Australia	15.3 (14.7-15.8)^a	Reproduction/survival (30-days)	6.9 (SE 5.3-9.0)	0.46	4
			Growth (30-days)	5.8 (4.7-7.1)	0.38	4
Gastropoda:						
<i>Burnupia stenochorias</i> (Ancyliidae)	South Africa	11.0 (9.6-13.0) ^b	Egg hatching success	9.2 (8.3-10.0)	0.84	5, 6
<i>Glyptophysa gibbosa</i> (Planorbidae)	Australia	12.6 ^b	Egg hatching success	2.6 (1.8-3.4)	0.21	7, 8
<i>Glyptophysa aliciae</i> (Planorbidae)	Australia	10.8* ^b	Egg hatching success	5.5*	0.51	7, 8
<i>Physa acuta</i> (Physidae)	Australia	14.0 (13.0-15.0) ^b	Egg hatching success	9.5*	0.68	7, 5
	Australia	12.6 ^b	Egg production (14-days)	3.0*	0.24	9, 10
	Australia	14.0 (13.0-15.0) ^b	Adult growth (28-days)	5.0*	0.36	7, 10
	Poland	13.2* ^c	Egg production (10-days)	6.6*	0.50	3
	Australia	13.2 (12.8-13.5)^a	Adult survival (30-days)	10.5 (10.1-10.9)	0.90	4
			Egg hatching success	8.3 (8.1-8.6)	0.63	4
		Egg production	3.3 (2.1-5.1)	0.25	4	
		Adult growth (30-days)	3.2 (SE 1.0-8.0)	0.24	4	

Table 7.1 continued

Taxon	Country	LC ₅₀ (mS cm ⁻¹)	Investigated effect	EC ₅₀ (mS cm ⁻¹)	Ratio EC ₅₀ :LC ₅₀	Sources
Decapoda:						
<i>Cherax destructor</i> (Parastacidae)	Australia	44.0 (42.5-48.7) ^a	Juvenile survival (96-hrs)	37.9 (36.2-39.1)	0.86	11
			Adult threat response (LOEC)	26.5	0.60	11
			Juvenile activity (LOEC)	26.5	0.60	11
<i>Caridina nilotica</i> (Atyidae)	South Africa	36.0-37.0 ^b	Larval survival (48-hrs)	37.0 (33.0-44.0)	1	5
<i>Paratya australiensis</i> (Atyidae)	Australia	38.0 (34.0-42.0) ^b	Larval survival (48-hrs)	33.0 (26.0-44.0)	0.88	7, 5
	Australia	33.7 (32.5-34.7) ^a	Juvenile survival (96-hrs)	31.5 (30.4-32.4)	0.93	12
	Australia	39.8 (37.3-43.1)^a	Larval survival (96-hrs)	39.9 (38.2-41.9)	1	4
			Larval survival (10-days)	27.6 (26.1-29.1)	0.69	4
			Juvenile growth (60-days)	23.8	0.6	4
Diptera:						
<i>Chironomus tepperi</i> (Chironomidae)	Australia	14.1 (12.4-15.8)^a	Egg hatching success	8.3 (8.0-8.6)	0.59	4
			Larval growth (10-12 days)	7.5 (SE 6.0-9.5)	0.53	4
			Adult emergence	9.8 (8.2-12.7)	0.70	4
			Egg production	6.7 (SE 4.9-6.8)	0.47	4
			Recruitment (FLC exposure)	2.5 (SE 1.8-3.4)	0.17	4

Where available 95% confidence intervals are given in parentheses, SE indicates standard error of the mean. LOEC = lowest observed effect concentration, FLC = full life-cycle. ^a = 96-hours exposure, ^b = 72-hours exposure, ^c = 10-days exposure

Sources: 1 = Kefford *et al.* 2003b, 2 = Zaluzniak *et al.* 2006, 3 = Stycznaska-Jurewicz 1972, 4 = this study, 5 = Kefford *et al.* 2004a, 6 = Kefford *et al.* 2005, 7 = Kefford *et al.* 2003a, 8 = Kefford *et al.* 2007a, 9 = Kefford *et al.* 2006a, 10 = Kefford & Nugegoda 2005, 11 = Mills & Geddes 1980, 12 = Walsh 1994.

7.6 Factors affecting salinity tolerance

Many environmental and biotic factors have been shown to affect the acute salinity tolerance of freshwater macroinvertebrates. Factors associated with the salinity exposure regime including rate of increase, duration, periodicity and seasonality have been found to be important (James *et al.* 2003). Acute salinity tolerance is higher when salinity increases gradually (James *et al.* 2003). Marshall and Bailey (2004) found that the same salt load delivered at a continuous rate ($\approx 2206 \mu\text{S cm}^{-1}$) over a five day period had less effect on stream macroinvertebrate abundance than pulsed releases of higher salinity ($\approx 5147 \mu\text{S cm}^{-1}$) interspersed with low salinity over the same period. The ionic composition of the salinity increase has also been found to greatly affect acute tolerance. Ionic proportions most similar to those which the test organisms were naturally exposed (eg similar to seawater in most Australian inland waters) are the most tolerated. Kefford *et al.* (2004b) found sodium chloride to be significantly more toxic than artificial sea salt. Factors that add additional physiological stress to test organisms have been found to reduce the ability of macroinvertebrates to tolerate increased salinity. This includes exposure to temperatures outside the optimal range of a species, reduced water quality (eg increased turbidity, reduced dissolved oxygen, extreme pH levels, etc.) and exposure to toxicants (metals, pesticides, etc.) (James *et al.* 2003). These factors often act synergistically with salinity reducing the tolerance to both stressors. This relationship is less straight forward with toxicants, as chemical reactions often occur with increased salinity that reduce toxicity and bioavailability of the toxicant (Hall and Anderson 1995). Biotic factors such as age, nutritional status, disease and pressures from competition and predation interactions are also likely to affect salinity tolerance (James *et al.* 2003).

It is unclear how these different factors affect the sublethal salinity tolerance of freshwater macroinvertebrate species and if these effects are proportional to effects on acute tolerance. If sublethal tolerance is affected differently by these factors than acute tolerance this could potentially affect the relationship between acute and sublethal tolerance. Research into how these factors affect sublethal salinity tolerance of freshwater macroinvertebrate species is required.

7.6.1 Salinity history

It is thought that past salinity exposure can affect the acute salinity tolerance of freshwater macroinvertebrates (Hart *et al.* 1991; Metzeling *et al.* 1995; James *et al.* 2003). Populations

of a species exposed to different salinity regimes can display different acute tolerances, with those previously exposed to higher salinities displaying higher tolerance. Some studies have observed modest differences in a species acute salinity tolerance between populations with different salinity exposure histories (e.g. Peska 2003, Dunlop *et al.* 2008). However, comparable species tolerances have been observed for populations across broad geographical regions that encompass many different salinity regimes (Kefford *et al.* 2007a; Dunlop *et al.* 2008). This study has highlighted the potential for the sublethal salinity tolerance of freshwater macroinvertebrates to also be affected by past salinity exposure. *P. acuta* from a population with a history of higher salinity exposure displayed a different pattern of egg production with increased salinity than *P. acuta* from a lower salinity population (see Sections 4.3.4.1 and 4.4.6). Seasonal differences in salinity exposure may also have affected sublethal salinity tolerance of *P. acuta*. Egg production was impacted more severely when snails were exposed in mid spring after experiencing lower river salinities than in mid summer when river salinities had been higher (see Section 4.4.6). In both cases prior salinity exposure altered the ratio between the acute salinity tolerance (96-hour LC₅₀) of the species and sublethal tolerance (EC₅₀). The effects of prior salinity exposure on sublethal salinity tolerance of freshwater macroinvertebrates and the relationship between acute and sublethal end points requires further research.

7.7 Conclusions and management implications

The macroinvertebrate species investigated in this study displayed short-term tolerance to salinities much higher than would be encountered in all but the most salt affected freshwater ecosystems. Other studies have also observed very high short-term salinity tolerance of Australian freshwater macroinvertebrates (Kefford *et al.* 2003a, 2006a; Dunlop *et al.* 2008). However, the results of this study indicate the potential for sublethal effects to severely limit the viability of populations at salinities much lower than those affecting short-term survival. This is supported by field observations of changes in macroinvertebrate community structure with relatively small increases in salinity (Metzling 1993; Kefford *et al.* 2006a).

The investigated species displayed both reduced survival of sensitive life stages and significant sublethal effects at salinities lower than those affecting short-term survival of the older aquatic life stages of the species. While different end points displayed different sensitivity, sensitive life stage and sublethal tolerances were generally proportional to the acute tolerance of the species. That is, species displaying high acute tolerance displayed correspondingly high sublethal and sensitive life stage tolerance and vice versa. For each

species, effects on growth and reproduction were the most sensitive of the investigated end points to increases in salinity. Investigations with *C. tepperi* indicate the dramatic impact on the reproductive potential of a species when the effects of salinity on growth, development and reproduction are compounded through a full life-cycle of exposure. The cumulative effects of salinity on recruitment of second generation *C. tepperi* after a full life-cycle of exposure was the most sensitive of all the investigated end points. Investigations with this species identified other pathways for salinity to affect macroinvertebrate species, with effects observed on the sex ratio of emergent adults (with increased salinity favouring males) and preferential selection of lower salinities for egg deposition. Further research is required to determine if other freshwater macroinvertebrate species display similar sensitivity to full life-cycle exposure and whether effects are magnified or diminished with exposure of successive generations.

The sublethal end points investigated displayed non-threshold responses to increased salinity, with changes in salinity above and below an optimal level resulting in a reduction in growth and reproduction. This suggests that any change in salinity may affect the fitness of macroinvertebrate populations. This type of response negates the determination of sublethal salinity thresholds and necessitates consideration of the magnitude of effect. The magnitude of effect on these end points that is likely to significantly impact the survival ability of natural populations requires investigation. The nominal value of 50% effect levels were used in this study for continuity with the level of effect considered for acute tolerance and a perception that this level of effect could potentially significantly reduce population fitness.

To date there has been limited information on the sublethal salinity tolerance of freshwater macroinvertebrates to enable these effects to be taken into consideration in salinity related management decisions. Much research into the relationship between the acute and sublethal salinity tolerance of freshwater macroinvertebrates is still required. However, the general proportional nature of this relationship suggested by data from this and other studies enables some degree of protection against sublethal effects to be factored into management tools utilising acute tolerance data. Using the relationships observed in this study, general inferences about a species likely sublethal salinity tolerance can be made based on the acute tolerance of the species.

Species sensitivity distribution (SSD) models are currently being utilised to assist salinity related management decisions. These models are constructed using acute tolerance data (LC_{50}

values) for species occurring in the aquatic ecosystem being modelled. This information is used to determine the proportion of species that have their LC₅₀ values exceeded at a given salinity (see Kefford *et al.* 2003a, 2003b, 2006a). The information on the sublethal salinity tolerance of freshwater macroinvertebrates derived in the current study can be utilised to incorporate a level of protection (or safety factor) against sub lethal effects into these models.

While there was variability in the relationship between acute and sublethal tolerance for different species, EC₅₀ values for reproduction and growth from all available sources (see Table 7.1) were between 38 and 76% lower than the acute tolerance of the species. The mean ratios between EC₅₀ values and LC₅₀ values for these end points were 0.46 ± 0.06 SE (N = 5) and 0.44 ± 0.08 SE (N = 4) respectively. The EC₅₀ for recruitment of second generation *C. tepperi* after a full life-cycle of salinity exposure was 83% lower than the acute LC₅₀ value for this species. Given these relationships, a safety factor of between 0.1 and 0.4 applied to acute tolerance data could be used to incorporate a level of protection against sublethal effects of salinity. Further research is required to elucidate the relationship between acute and sublethal tolerances and the ramifications of different magnitudes of reductions in growth and reproduction for natural populations. In light of these uncertainties, the level of safety factor applied to acute data may be selected to reflect the importance of protection against sublethal effects in each management scenario.

The observed effects of salinity on sublethal end points such as growth, reproduction and recruitment potential and the relationships between acute and sublethal tolerance observed in this study occurred under controlled laboratory conditions. The four species investigated represent only a tiny fraction of the diversity of macroinvertebrate species occurring in Australian freshwater ecosystems. Further research is required to determine how the findings of this study transfer to natural populations and other macroinvertebrate species. Kefford *et al.* (2006a) investigated the relationship between macroinvertebrate species richness predicted using laboratory derived acute salinity tolerance data and field macroinvertebrate richness. They observed a relationship similar to those between acute and sublethal tolerance observed in this study. This suggests that taking species sublethal tolerances into account using the relationships observed in this study may be applicable to field situations and potentially enable prediction of impacts of salinity on macroinvertebrate communities.

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Appendix A: Salinity LC₅₀ values for investigated freshwater macroinvertebrate species

Table A.1 Salinity LC₅₀ values (EC $\mu\text{S cm}^{-1}$) and 95% confidence intervals for *Lumbriculus variegatus* after different exposure periods in tests with (Test 2) and without (Test 1) food provision.

Exposure period (days)	LC ₅₀ (95% CI) EC $\mu\text{S cm}^{-1}$	
	Test (1) without food	Test (2) with food
1	16102 (15748-16464)	16102 (15748-16464)
2	15567 (15066-16085)	16102 (15748-16464)
3	15358 (14831-15903)	15673 (15189-16171)
4	15255 (14720-15809)	15462 (14946-15996)
5	15255 (14720-15809)	15462 (14946-15996)
6	15255 (14720-15809)	15462 (14946-15996)
7	15255 (14720-15809)	15462 (14946-15996)

Table A.2 Salinity LC₅₀ values (EC $\mu\text{S cm}^{-1}$) and 95% confidence intervals for adult *Physa acuta* after different exposure periods.

Exposure period (days)	LC ₅₀ (95% CI) EC $\mu\text{S cm}^{-1}$
1	14890 (14696-15078)
2	14606 (14410-14795)
3	14296 (14091-14492)
4	13171 (12816-13529)
5	13087 (12734-13441)
6	12136 (11712-12568)
7	11815 (11379-12258)
8	12094 (11663-12533)
9	12346 (11925-12776)
10	12400 (11975-12834)
12	12309 (11886-12739)
13	12096 (11678-12520)
14	11544 (11129-11963)
15	11444 (11024-11868)
16	10915 (10500-11331)
18	10729 (10302-11157)
19	10661 (10234-11089)
20	10739 (10320-11157)
21	10547 (10129-10965)
22	10424 (10011-10836)
23	9806 (9378-10231)
25	9418 (8990-9843)
26	10269 (9856-10682)
27	10369 (9960-10777)
28	11170 (10776-11565)
29	11090 (10685-11497)
30	10539 (10133-10945)

Table A.3 Salinity LC₅₀ values (EC $\mu\text{S cm}^{-1}$) and 95% confidence intervals for adult *Paratya australiensis* after different exposure periods.

Exposure period (days)	LC ₅₀ (95% CI) EC $\mu\text{S cm}^{-1}$
1	41712 (39973-43936)
2	41203 (38925-44102)
3	40172 (37781-43187)
4	39844 (37308-43055)
6	39132 (36322-42711)
7	38957 (36021-42714)
8	38804 (35836-42601)
9	38929 (35795-42993)
10	38835 (35531-43163)
12	37543 (33960-42219)
13	37543 (33960-42219)
16	36218 (32305-41329)

Table A.4 Salinity LC₅₀ values (EC $\mu\text{S cm}^{-1}$) and 95% confidence intervals for larval *Paratya australiensis* after different exposure periods.

Exposure period (days)	LC ₅₀ (95% CI) EC $\mu\text{S cm}^{-1}$
1	65561 (55928-122511)
2	44295 (42670-46035)
3	42426 (40686-44328)
4	39944 (38153-41881)
5	37808 (35860-39974)
6	32826 (31176-34857)
7	33145 (31454-35045)
8	30497 (29048-32173)
9	28865 (27471-30439)
10	27551 (26084-29134)
11	24910 (23317-26483)

Table A.5 Salinity LC₅₀ values (EC $\mu\text{S cm}^{-1}$) and 95% confidence intervals for 4th instar *Chironomus tepperi* larvae after different exposure periods.

Exposure period (days)	LC ₅₀ (95% CI) EC $\mu\text{S cm}^{-1}$
1	-
2	17025 (15726-18357)
3	15271 (13654-16956)
4	14077 (12387-15814)