The Journal of Experimental Biology 209, 4024-4032
Published by The Company of Biologists 2006
doi:10.1242/jeb.02457

Sub-lethal and chronic salinity tolerances of three freshwater insects: *Cloeon* sp. and *Centroptilum* sp. (Ephemeroptera: Baetidae) and *Chironomus* sp. (Diptera: Chironomidae)

Kathryn L. Hassell*, Ben J. Kefford and Dayanthi Nugegoda

Biotechnology and Environmental Biology, School of Applied Sciences, RMIT University, PO Box 71, Bundoora, Victoria, 3083, Australia

*Author for correspondence (e-mail: kathryn.hassell@rmit.edu.au)

Accepted 24 July 2006

Summary

Increased salinity in rivers and streams is a serious environmental concern, and in Australia there is growing information about the acute tolerances to salinity for freshwater macroinvertebrates, but much less information about chronic and sub-lethal tolerances. The effects of increased salinity on the growth and survival of two mayflies, *Cloeon* sp. and *Centroptilum* sp. and one midge *Chironomus* sp. are reported. In both mayfly species survival was variable. Complete mortality was observed in salinities with electrical conductivity of 10 mS cm^{-1} and higher. Salinities causing chronic mortality in mayflies were measured as 21-day LC_{50}, and ranged from 0.90 to 2.7 mS cm^{-1}. Growth rates were not significantly different between treatments. In *Chironomus*, salinity affected the mean number emerging as flying adults as well as the time to emergence. An inverted 'U' shape response was observed for percentage emergence, with the greatest numbers emerging at intermediate salinities (0.65–5.0 mS cm^{-1}). No emergence occurred at salinities of 20 mS cm^{-1} and higher. Time to emergence was delayed by 15–88% with increased salinity, however the size of emerged adults was the same for all treatments. Growth rates were reduced with increased salinity, showing a slow, steady reduction up to 10 mS cm^{-1} then a steep decline between 10 and 15 mS cm^{-1}. The implications of altered growth rates and changes in developmental times are discussed. This study illustrates the variability in responses to increased salinity, and highlights the need to continue studying sub-lethal and chronic exposures in a range of freshwater invertebrates, in order to predict impacts of salinisation on freshwater biodiversity.

Key words: salinity, Ephemeroptera, Chironomidae, stream invertebrate, sub-lethal, toxicity

Introduction

Increasing salinity is a recognised environmental problem world wide, and in Australia approximately 5.7 million hectares of land has been classified as being at risk or affected by dryland salinity (National Land and Water Resources Audit, 2001). Increased salinity in Australian rivers results from historical land clearing and over irrigation, which results in rising saline groundwater making its way to freshwater streams and rivers (Hart et al., 1991; Williams, 1987).

It has been suggested that Australian macroinvertebrate fauna would show adverse effects to increased salinity above 1000 mg l^{-1} [~1.5 mS cm^{-1} (Hart et al., 1991)]. However, the type of exposure and the duration are factors that complicate this statement. For example, it has been reported that the abundance of halosensitive species (including mayflies and gastropods) is reduced at salt concentrations of 1500 mg l^{-1} (~2.2 mS cm^{-1}) following exposure to either a continuous or pulse release of saline water (Marshall and Bailey, 2004). Similarly, changes in macroinvertebrate community structures, from salt-sensitive taxa to salt tolerant taxa, were observed at salinities between 0.8–1.0 mS cm^{-1} in Queensland streams (Horrigan et al., 2005). And in a study of the acute salinity tolerances of a range of macroinvertebrates from the Barwon River in Victoria, a wide range (5.5–76 mS cm^{-1}) of 72 h lethal concentrations (LC_{50}) was observed, which are the concentrations capable of killing 50% of individuals (Kefford et al., 2003).

These examples demonstrate that there is much variability in reported salinity tolerances, and that the values depend on the species and responses that are tested. However, the above examples have all observed short-term responses, and do not provide any insight into the physiological effects of long-term (or chronic) exposure to sub-lethal salinity levels, and since the effects of exposure to environmental contaminants is not only a result of the amount of substance to which an organism is being exposed, but also the amount of time that they are exposed to it (Newcombe and McDonald, 1991), studies of responses to longer-term sub-lethal doses are needed. In the

case of salinity, environmental increases can be slow and gradual over a long period of time, thus highlighting the need for long-term investigations.

Sub-lethal stress may be any response that causes a change in the organism's condition, without mortality. Stress has been defined as a change in biological condition beyond normal state that challenges homeostasis (Barton and Iwama, 1991). Sub-lethal stress responses include changes in growth, development, reproductive fitness, disease resistance, tissue and organ function and cellular processes such as osmoregulation (Barton and Iwama, 1991; Pickering, 1990; Schreck et al., 2001).

It has been traditionally assumed that the effects of increased salinity would display a threshold response, where below a particular level (threshold), no effects would be observed, and above the threshold fitness would be decreased (Hart et al., 1991). In the freshwater cladoceran, *Daphnia magna,* a threshold response, as a decrease in survival, growth and reproduction when salinity levels increased above 6 g l^{-1} (~8.8 mS cm^{-1}), was observed (Smolders et al., 2005). However, in an investigation of sub-lethal salinity tolerance, growth in the freshwater gastropod *Physa acuta* was observed to be lower in low ($\leq$0.05 mS cm^{-1}) and high (>1.0 mS cm^{-1}) salinities than in intermediate salinities (Kefford and Nugegoda, 2005). And in a study of mosquitoes, changes in mass and larval stage duration with increased salinity were reported (Clark et al., 2004). Two species of mosquitoes were investigated, one being the euryhaline *Ochlerotatus taeniorhynchus*, which displayed increases in pupal mass and larval stage duration as salinity increased, and the other was the freshwater *Aedes aegypti,* which displayed a decrease in pupal mass as salinity increased, and a U-shaped pattern of larval duration (being most rapid at intermediate salinities).

This study examined the sub-lethal salinity responses of three species of freshwater invertebrates, in order to assess if the sub-lethal responses to salinity are similar across different species that are known to be salt sensitive in short-term lethal exposures. We chose two baetid mayflies, *Cloeon* sp. and *Centroptilum* sp. (Ephemeroptera: Baetidae) and *Chironomus* sp. midges (Diptera: Chironomidae) because both families have salt-sensitive members with 3-day LC_{50} ranges of 5.5–6.2 mS cm^{-1} and 10 mS cm^{-1}, respectively (Kefford et al., 2003).

Materials and methods

Collection sites

Cloeon sp. and *Chironomus* sp. were collected from the Campaspe River at Kyneton-Heathcote Road (37°23′S; 144°31′E) and *Centroptilum* sp. were collected from King Parrot Creek at Flowerdale (37°23′S; 145°16′E). Both streams are in the southern end of the Murray-Darling Basin in central Victoria, Australia. The electrical conductivities (EC) at these sites were 0.62–0.65 mS cm^{-1} and 0.07 mS cm^{-1}, respectively.

General methods

All specimens were transported from their collection sites and held in river water for 24 h prior to the experiments. Growth was measured in terms of body length (from labrum to end of abdomen) and head width (at the widest point). In the chironomids, the length of the longest wing of emerged adults was also measured (distance from the arculus to the tip) as described (McKie et al., 2004). Tests were conducted in carbon-filtered Melbourne tapwater (wet lab water) and the salt source was Ocean Nature salt (Aquasonic, Wauchope, NSW, Australia), which has the same ionic proportions as seawater and is similar to most Australian inland saline waters (Bayly and Williams, 1973). Experiments were conducted in 500 ml plastic containers provided with aeration.

Mayflies

Mayflies were held individually in aerated containers and fed soft, conditioned leaves from their collection sites. At the beginning of each experiment mayflies were selected such that none had wing buds. Between six and eight salinity treatments were used (0.07–15 mS cm^{-1}), with nine replicates of each. Once per week, water was changed and all uneaten leaves discarded and replenished with newly collected leaves. Preliminary results showed high mortalities in all mayflies held at 20–21°C, therefore experiments reported here were conducted at 15±1°C with a 16 h:8 h light:dark photoperiod. Length and width measurements were taken after 0, 7, 14 and 21 days of exposure. Where emergence occurred, that individual was excluded for calculation of growth rates, since many mayflies undergo limited growth in the final instar. Owing to high mortalities in the wet lab water controls (of the same salinity as river water) in both mayfly experiments, a second *Centroptilum* experiment was carried out with river water as the diluent (instead of wet lab water).

Chironomids

To ensure all individuals were the same age and species, a single *Chironomus* egg mass was collected and held in the laboratory for 24 h prior to being gently broken apart using forceps and a plastic pipette. The individuals were then randomly transferred into different salinity treatments (0.65–25 mS cm^{-1}) with three replicates of each. Each replicate contained 10–15 individuals and was supplied with a 100×220 mm piece of folded, unbleached toilet paper and a small amount of crushed trout pellets (Skretting, Australia). Water was changed weekly, aeration was provided and each container had a mesh covering to allow emerged adults to leave the water surface and be collected. The experiment was conducted at 21±1°C with a 16 h:8 h light:dark photoperiod. The chironomids were observed daily for development of pupae and emergence of flying adults. Any that emerged were carefully removed and immediately frozen (–20°C). To minimise handling stress only the emerged adults were measured.

Statistical analyses

Differences between treatments were evaluated using one-way ANOVA and Tukey honestly significant difference multiple comparisons tests to detect significantly different treatment pairs. Log_{10} transformations were performed where it improved the assumption of normality and homogeneity of variance. Standard logistical regression was used to relate the proportion alive to the salinities tested, and LC_{50}s were calculated from this regression for five time points: 3 days (72 h), 4 days (96 h), 7 days (168 h), 14 days (336 h) and 21 days (504 h). All statistical analyses were performed using SPSS for Windows (version 12.0; SPSS Inc., Chicago, IL USA).

Results

Survival/emergence

The general survival response observed in all insects was U shaped, showing an initial small increase to reach a maximum between 0.20–2.5 mS cm^{-1} depending on the species (Fig. 1). In all cases, survival began to decline at salinities above 2.5 mS cm^{-1}, and in mayflies there was no survival beyond 10 mS cm^{-1} and in chironomids there was no survival beyond 20 mS cm^{-1}.

Survival in all mayfly experiments was variable, and in the highest concentrations tested, no mayflies survived (Fig. 2A–C; Tables 1–4). In *Cloeon*, survival increased at 1.0 and 2.5 mS cm^{-1} to around 50%, then dropped to 33% survival at 5.0 mS cm^{-1} (Fig. 2A). No *Cloeon* survived to 21 days when held at 10 or 15 mS cm^{-1}. All individuals in the 10 mS cm^{-1} treatment were dead after 18 days, and all those in the 15 mS cm^{-1} treatment were dead after 11 days. Mortality was first observed on day 4. LC_{50}s indicate that salinity tolerance in *Cloeon* drops over time and at 21 days is only 10% of the LC_{50} at 3 or 4 days (Table 1). In *Centroptilum* there was an initial increase in survival at a salinity of 0.50 mS cm^{-1} (56%) followed by a decrease at 1.0 mS cm^{-1} (22%) and 2.5 mS cm^{-1} (33%), and no survivors in treatments ⩾5.0 mS cm^{-1} (Fig. 2B). All individuals in the 10 mS cm^{-1} treatment were dead after 8 days, and all those in the 15 mS cm^{-1} treatment were dead after 3 days. Mortality was first observed on day 2. The LC_{50}s for *Centroptilum* dropped over the 21 days of the experiment (Table 1).

However, since survival of both mayfly species was low in all treatments, we concluded that something other than salinity may have been hindering their survival, such as the water used to make up the test solutions. To test this prospect, a second experiment with *Centroptilum* was conducted, using river water as the diluent (instead of wet lab water). Survivorship was generally improved, with around 80–100% survival in treatments up to 0.5 mS cm^{-1} then a steady drop at 1.0 and 2.5 mS cm^{-1}. At the highest concentration tested (5.0 mS cm^{-1}), only 11% survived (Fig. 2C). Mortality was first observed on day 6. As before, the LC_{50}s reduced over the course of the experiment (Table 1).

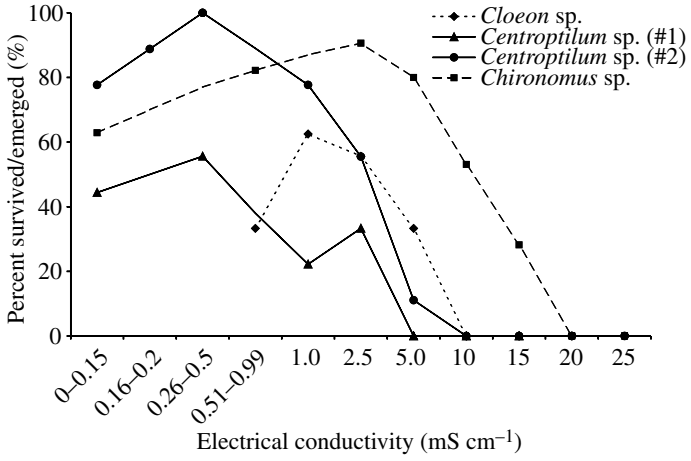

Fig. 1. Mean survival of three insect species exposed to a range of salinities. The mayflies *Cloeon* and *Centroptilum* were exposed for a period of 21 days, while *Chironomus* midges were exposed for their entire larval period. #1 refers to the first *Centroptilum* experiment (using wet lab water) and #2 refers to the second *Centroptilum* experiment (using river water).

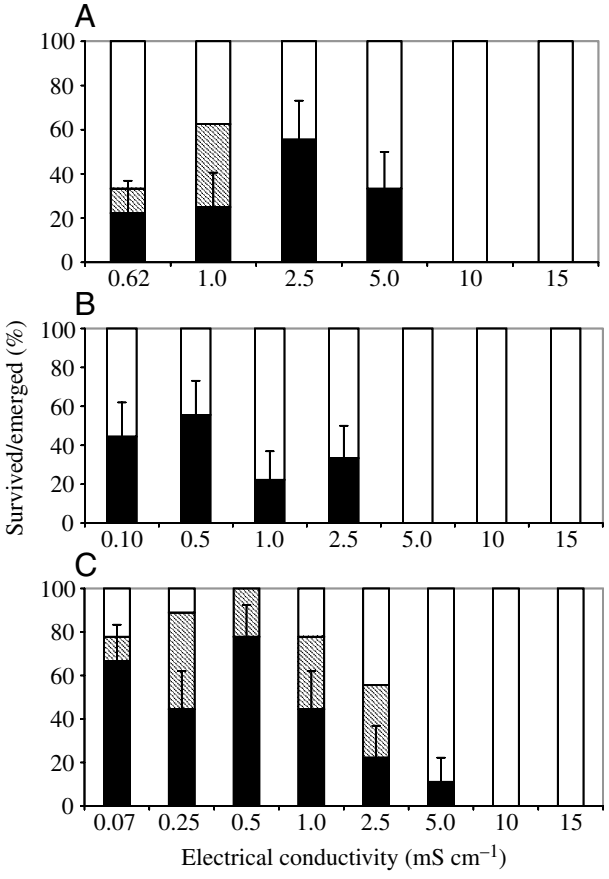

Fig. 2. Mean survival and emergence percentages of mayflies exposed to a range of salinities over 21 days. The black portion of the bars show percentage alive at 21 days and shaded portions show percentage emerged by 21 days. Values are means ± s.e.m. (*N*=9). (A) *Cloeon* (wet lab water); (B) *Centroptilum* (wet lab water); (C) *Centroptilum* (river water).

Table 1. *LC_{50} values of two baetid mayfly species at five different time points*

	LC_{50}				
Genus	3 days (72 h)	4 days (96 h)	7 days (168 h)	14 days (336 h)	21 days (504 h)
Cloeon sp.					
(WLW)	21	21	18.3 (12.4, 69.8)	6.8 (4.6, 10.5)	2.1 (0.0, 4.0)
Centroptilum sp.					
(WLW)	10	10	7.6(5.7, 9.8)	3.0 (1.9, 4.9)	0.89 (0.0, 1.9)
(RW)	–	–	6.4 (4.7, 70.6)	4.2 (3.1, 6.8)	2.7 (1.8, 4.4)

LC_{50}, salinity measured as electrical conductivity (mS cm^{-1}). WLW, values for animals held in wet lab water; RW, values for animals held in river water.

Table 2. *Measurements of Cloeon sp. exposed to a range of salinities in wet lab water*

	Electrical conductivity (mS cm^{-1})						
Measurement	0.62	1.0	2.5	5.0	10	15	ANOVA
Percentage survival	33.3	62.5	55.6	33.3	0	0	
Percent emerged	11.1	33.3	0	0	0	0	
Number of days alive	15.8±1.86^{a}	16.7±2.27^{a}	15.9±2.41^{a}	17.4±1.6^{a}	10.9±1.8^{a,b}	6.8±1.1^{b}	F=4.944, d.f.=5,48, P=0.010
Number of moults	1.3±0.24^{a}	0.9±0.26^{a}	0.9±0.35^{a}	1.1±0.35^{a}	1.1±0.20^{a}	0.6±0.18^{a}	F=0.968, d.f.=5,48, P=0.447
Initial size							
Body length (mm)	4.83±0.23^{a}	5.64±0.14^{a}	5.02±0.25^{a}	5.16±0.26^{a}	5.39±0.15^{a}	5.71±0.30^{a}	F=2.329, d.f.=5,48, P=0.057
Eye width (mm)	0.83±0.04^{a,b}	0.99±0.03^{a}	0.86±0.06^{a,b}	0.89±0.05^{a,b}	0.84±0.02^{a,b}	0.81±0.04^{b}	F=2.501, d.f.=5,48, P=0.043
Final size							
Body length (mm)	6.04±0.17^{a}	6.11±0.39^{a}	5.50±0.32^{a}	5.96±0.39^{a}	–	–	F=0.824, d.f.=3,15, P=0.501
Eye width (mm)	1.05±0.02^{a}	0.91±0.04^{a}	0.91±0.07^{a}	0.98±0.05^{a}	–	–	F=1.546, d.f.=3,15, P=0.244
Growth rates							
Body (mm/day)	0.041±0.06^{a}	0.031±0.012^{a}	0.036±0.010^{a}	0.051±0.017^{a}	–	–	F=0.411, d.f.=3,12, P=0.748
Eyes (mm/day)	0.009±0.001^{a}	0.000±0.005^{a}	0.004±0.003^{a}	0.005±0.005^{a}	–	–	F=0.685, d.f.=3,12, P=0.578
Moulting (moults/day)	0.09±0.02^{a}	0.04±0.01^{a}	0.04±0.02^{a}	0.07±0.02^{a}	0.10±0.02^{a}	0.18±0.11^{a}	F=1.130, d.f.=5,48, P=0.357

Values are means ± s.e.m.; N=9.
Different letters indicate statistically significant differences (P=0.05) between treatments.

Table 3. *Measurements of Centroptilum sp. exposed to a range of salinities in wet lab water*

	Electrical conductivity (mS cm^{-1})							
Measurement	0.10	0.50	1.0	2.5	5.0	10	15	ANOVA
Percentage survival	44.4	55.6	22.2	33.3	0	0	0	·
Percent emerged	0	0	0	0	0	0	0	·
Number of days alive	17.9±1.17^{a}	18.1±1.75^{a,b}	16.1±1.49^{a,b}	14.3±1.81^{a,b}	10.9±1.27^{b}	3.8±0.57^{c}	1.7±0.17^{d}	F=62.756, d.f.=6,55, P=0.000
Number of moults	1.2±0.27^{a}	1.1±0.35^{a}	1.2±0.22^{a}	1.0±0.71^{a}	0.9±0.20^{a}	0.7±0.16^{a}	0	F=10.796, d.f.=5, 47, P=0.558
Initial size								
Body length (mm)	4.00±0.23^{a}	4.22±0.29^{a}	4.31±0.27^{a}	3.70±0.15^{a}	3.83±0.15^{a}	3.97±0.24^{a}	3.65±0.17^{a}	F=1.266, d.f.=6,54, P=0.289
Eye width (mm)	0.62±0.05^{a,b}	0.62±0.04^{a,b}	0.66±0.05^{a}	0.56±0.03^{a,b}	0.56±0.03^{a,b}	0.51±0.04^{a,b}	0.48±0.03^{b}	F=2.735, d.f.=6, 46, P=0.023
Final size								
Body length (mm)	4.59±0.15^{a}	4.44±0.46^{a}	4.55±0.20^{a}	3.77±0.17^{a}	–	–	–	F=1.189, d.f.=3,11, P=0.359
Eye width (mm)	0.63±0.04^{a}	0.65±0.08^{a}	0.66±0.05^{a}	0.57±0.03^{a}	–	–	–	F=0.298, d.f.=3,11, P=0.826
Growth rates								
Body (mm/day)	0.012±0.006^{a}	0.009±0.003^{a}	0.014±0.009^{a}	0.005±0.007^{a}	–	–	–	F=0.336, d.f.=3,11, P=0.800
Eyes (mm/day)	–0.001±0.001^{a}	0.0009±0.002^{a}	0.005±0.005^{a}	0.003±0.002^{a}	–	–	–	F=1.071, d.f.=3,10, P=0.405
Moulting (moults/day)	0.07±0.01^{a}	0.06±0.02^{a}	0.08±0.01^{a}	0.07±0.02^{a}	0.09±0.02^{a}	0.15±0.04^{a}	–	F=2.269, d.f.=5,47, P=0.063

Values are means ± s.e.m.; N=9.
Different letters indicate statistically significant differences (P=0.05) between treatments.

Table 4. *Measurements of Centroptilum sp. exposed to a range of salinities in river water*

Measurement	Electrical conductivity (mS cm^{-1})						ANOVA
	0.07	0.25	0.50	1.0	2.5	5.0	
Percentage survival	77.7	88.8	100	77.7	55.5	11.1	
Percent emerged	11.1	44.4	22.2	33.3	33.3	0	
Number of days alive	18.9±1.4^a	19.8±1.2^a	21.0±0.0^a	20.2±0.5^a	17.8±1.8^a	11.9±2.0^b	F=5.822, d.f.=5,48, P=0.000
Number of moults	2.2±0.36^a	2.2±0.46^a	2.7±0.29^a	2.5±0.33^a	2.1±0.35^a	1.5±0.27^a	F=1.219, d.f.=5,44, P=0.316
Initial size							
Body length (mm)	5.20±0.42^a	4.81±0.31^a	4.94±0.34^a	5.06±0.26^a	4.92±0.38^a	5.57±0.50^a	F=0.522, d.f.=5,48, P=0.758
Eye width (mm)	0.73±0.06^a	0.71±0.04^a	0.76±0.06^a	0.77±0.06^a	0.72±0.06^a	0.72±0.05^a	F=0.230, d.f.=5,41, P=0.947
Final size							
Body length (mm)	5.87±0.32^a	5.88±0.43^a	6.06±0.20^a	6.10±0.20^a	5.25±0.13^a	5.63±0.56^a	F=0.630, d.f.=5, 19, P=0.679
Eye width (mm)	0.86±0.06^a	0.79±0.04^a	0.89±0.04^a	0.82±0.03^a	0.75±0.03^a	0.85±0.13^a	F=0.790, d.f.=5, 19, P=0.570
Growth rates							
Body (mm/day)	0.037±0.017^a	0.049±0.014^a	0.057±0.013^a	0.054±0.020^a	0.071±0.023^a	–0.024 (0.004^a	F=2.124, d.f.=5, 19, P=0.107
Eyes (mm/day)	0.007±0.002	0.006±0.002	0.008±0.001	0.009±0.001	0.012±0.001	0.002*	F=1.32, d.f.=5, 13, P=0.315
Moulting (moults/day)	0.13±0.01^a	0.14±0.03^a	0.13±0.01^a	0.14±0.02^a	0.16±0.01^a	0.12±0.01^a	F=0.866, d.f.=5,31, P=0.515

Values are means ± s.e.m. N=9; *no standard error calculated.
Different letters indicate statistically significant differences (P=0.05) between treatments.

Although we tried to select small mayflies to allow the greatest scope for growth, some *Cloeon* (10%) and *Centroptilum* (25%) did emerge (Fig. 2, Tables 2–4, respectively). No emergence was observed in the second *Centroptilum* experiment in salinities above 2.5 mS cm^{-1}, however, the number of survivors at 5.0 mS cm^{-1} was low and thus may explain why there was no emergence observed at this salinity (Tables 3, 4).

Emergence of flying adult chironomids was significantly affected by salinity and followed an inverted U shape (Fig. 3). No pupation or emergence occurred at 20 or 25 mS cm^{-1}. Time to emergence was also significantly affected by salinity but displayed a different pattern to that of emergence (Fig. 4). Time to emergence increased with increased salinity above 2.5 mS cm^{-1}. However, between 0.15–2.5 mS cm^{-1}, time to emergence was similar. A 15% increase in the time to emergence occurred at 5.0 and 10 mS cm^{-1} relative to 0.15–2.5 mS cm^{-1}, and at 15 mS cm^{-1} a substantial increase of 88% in the time to emergence was observed.

A small number of deformities (abnormal wings or non-hardened exoskeletons) were observed in emerged adult chironomids in the 2.5, 5.0 and 15 mS cm^{-1} treatments, however there didn't appear to be any pattern between treatments (Table 5). The overall male: female ratio of emerged chironomids was 51:49 with no evidence of any difference between salinity treatments (Table 5).

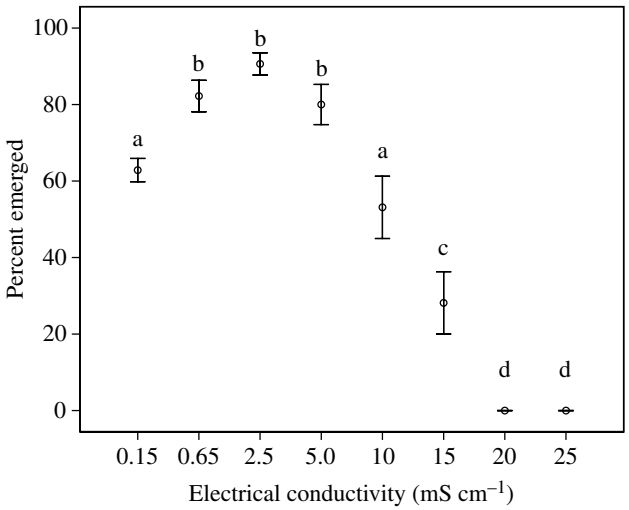

Fig. 3. Percentage of emerged *Chironomus* in eight different salinity treatments. All larvae came from a single egg mass. Values are means ± s.e.m. (N=10–15); different letters indicate statistically significant differences (P=0.05) between treatments.

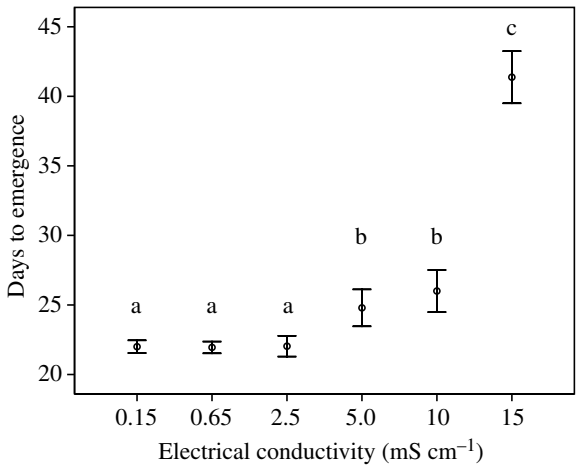

Fig. 4. Time to emergence (days) in *Chironomus* exposed to different salinity treatments. Values are means ± s.e.m. (N=10–15); different letters indicate statistically significant differences (P=0.05) between treatments.

Table 5. *Measurements of Chironomus sp. exposed to a range of salinities in wet lab water*

	Electrical conductivity (mS cm^{-1})								
Measurement	0.15	0.65	2.5	5.0	10	15	20	25	ANOVA
Percent emerged	62.9±1.53^{a}	82.2±2.06^{b}	90.6±1.45^{b}	80.0±2.63^{b}	53.1±4.07^{a}	28.2±4.07^{c}	0	0	F=171.7, d.f.=7,271, P=0.000
Days to emergence	22.0±0.23^{a}	22.0±0.21^{a}	22.0±0.37^{a}	24.8±0.67^{b}	26.0±0.76^{b}	41.4±0.94^{c}	NA	NA	F=175.5, d.f.=5,139, P=0.000
Size at emergence									
Body length (mm)	7.23±0.13^{a}	6.81±0.95^{a}	6.76±0.16^{a}	7.02±0.16^{a}	7.35±0.13^{a}	6.88±0.19^{a}	NA	NA	F=2.642, d.f.=5,125, P=0.062
Wing length (mm)	4.54±0.04^{a}	4.50±0.03^{a}	4.34±0.08^{a}	4.37±0.09^{a}	4.52±0.04^{a}	4.34±0.08^{a}	NA	NA	F=2.225, d.f.=5,130, P=0.056
Head width (mm)	0.87±0.00^{a}	0.85±0.00^{a}	0.84±0.01^{a}	0.85±0.01^{a}	0.87±0.01^{a}	0.84±0.01^{a}	NA	NA	F=2.943, d.f.=5,129, P=0.051
Growth rates									
Body (mm/day)	0.33±0.008^{a}	0.31±0.006^{a}, b	0.31±0.011^{a}, b	0.29±0.009^{b}	0.29±0.012^{b}	0.17±0.009^{c}	NA	NA	F=27.343, d.f.=5,124, P=0.000
Wings (mm/day)	0.21±0.003^{a}	0.21±0.003^{a}	0.20±0.005^{a}	0.18±0.006^{b}	0.17±0.005b	0.11±0.004^{c}	NA	NA	F=51.427, d.f.=5,128, P=0.000
Head (mm/day)	0.04±0.001^{a}	0.04±0.000^{a}	0.04±0.001^{a}	0.04±0.001^{b}	0.03±0.001^{b}	0.02±0.001^{c}	NA	NA	F=66.921, d.f.=5,127, P=0.000
Wet mass (mg)	1.94±0.21^{a}	2.07±0.11^{a}	1.86±0.26^{a}	2.11±0.57^{a}	2.07±0.08^{a}	1.58±081^{a}	NA	NA	F=0.477, d.f.=5,8, P=0.785
Dry mass (mg)	0.92±0.09^{a}	0.97±0.02^{a}	0.85±0.11^{a}	0.73±0.19^{a}	1.03±0.03^{a}	0.75±0.29^{a}	NA	NA	F=0.664, d.f.=5,8, P=0.662
Percent water	52.3±1.30^{a}	52.7±1.92^{a}	53.7±1.79^{a}	55.5±11.6^{a}	50.3±0.80^{a}	48.0±8.45^{a}	NA	NA	F=0.521, d.f.=5,9, P=0.755
Percentage deformed (N)	0	0	11.9 (1)	20.8 (2)	0	12.5 (1)	NA	NA	
Sex ratio (M:F)	68: 32	32: 68	41: 59	61: 39	56: 44	46: 54	NA	NA	

Values are means ± s.e.m.; N=10–15.
Different letters indicate statistically significant differences (P=0.05) between treatments. NA, not applicable.

Chironomids of the same treatment were grouped together for weight determination and no apparent differences were observed in dry mass or wet mass across treatments (Table 5).

Growth

Mayflies

Growth rates in *Cloeon* and *Centroptilum* grown in wet lab water were highly variable, and not significantly different between treatments in terms of body length or head width (Tables 2–4). In the second *Centroptilum* experiment, the highest mean growth rates (0.071 mm day^{-1}) were observed at 2.5 mS cm^{-1} and below this salinity, body length growth rates ranged from 0.037–0.054 mm day^{-1} (Fig. 5). At 5.0 mS cm^{-1} the mean growth rate was zero or negative. No statistical differences were found between any of the treatments. However, the sample size, especially at 5.0 mS cm^{-1} was low (three individuals) due to high mortality and it is thus unwise to rule out the possibility of a type II error (Fig. 2C).

Chironomids

The growth rates of *Chironomus* were determined by the relationship between size at emergence and the number of days to emergence. There were no differences between any

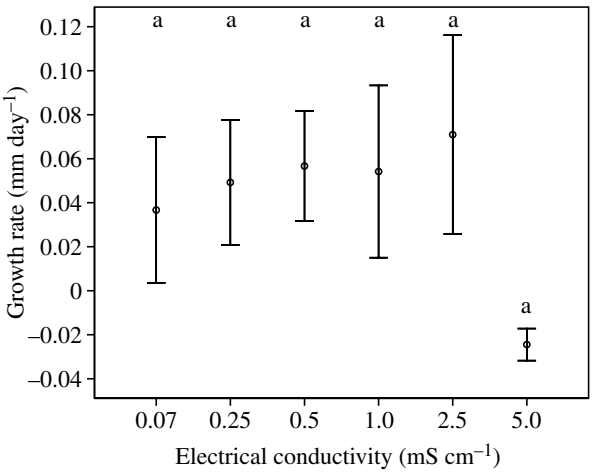

Fig. 5. Mean growth rate (measured as increase in body length; mm), in *Centroptilum* exposed to different salinity treatments over 21 days. Values are means ± s.e.m. (N=9); there was no statistically significant differences (P=0.05) between treatments (a).

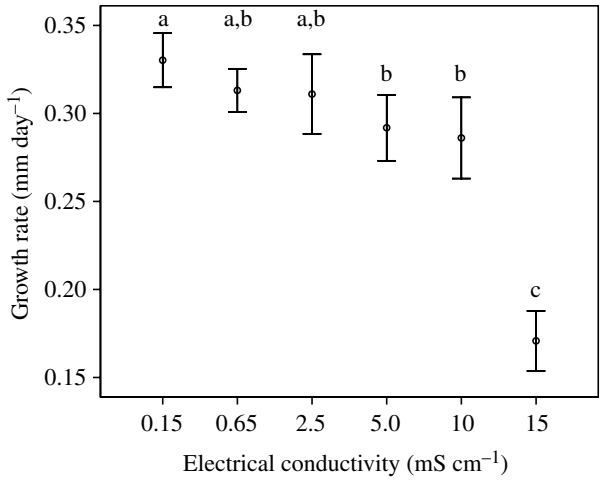

Fig. 6. Mean growth rate (measured as increase in body length; mm), in *Chironomus* exposed to different salinity treatments from egg to emergence. Values are means ± s.e.m. (N=10–15); different letters indicate statistically significant differences (P=0.05) between treatments.

treatments for the size at emergence (body length, head width or wing length), however there were significant differences in body length growth rates from different salinity treatments. Growth rates were slightly reduced in the 5 and 10 mS cm^{-1} treatments relative to the 0.15–2.5 mS cm^{-1} treatments, and greatly reduced in the 15 mS cm^{-1} treatment (Fig. 6). Wing length and head width measurements displayed similar trends (Table 5).

Discussion

Survival in mayflies was variable but definitely affected by increased salinities ($\geqslant$10 mS cm^{-1} for *Cloeon* and $\geqslant$5.0 mS cm^{-1} for *Centroptilum*). The growth rates of mayflies did not display any statistically significant differences across treatments, however in the highest salinity treatment, where there were survivors at 21 days, the growth rate was lower than the other treatments for which there were survivors at 21 days. Growth is an energy-dependent function, and the reduction in growth observed in mayflies as salinity increased may be evidence of a shift in energy partitioning towards maintaining osmoregulatory functions.

For example when the beetle *Tenebrio molitor* was exposed to different levels of oxygen, changes in growth rates, moulting frequency and survival were observed (Greenberg and Ar, 1996). In hypoxic environments (10% oxygen) low growth rates, increased moulting and increased mortality occurred. These authors concluded that the lower growth rates in hypoxic environments could have been due to water loss associated with frequent opening of the spiracles and increased moulting, using energy that would have otherwise been invested in growth (Greenberg and Ar, 1996). It is therefore possible to conclude that mayflies similarly partition energy away from growth when exposed to environmental stressors.

The 21 day LC_{50} for *Centroptilum* in river water (2.7 mS cm^{-1}) was approximately half that of the 3 days LC_{50} (5.5–6.2 mS cm^{-1}) previously reported (Kefford et al., 2003). A similar finding has been reported for the acute and chronic salinity tolerances in the salt tolerant damselfly *Ishnura heterosticta* (Kefford et al., 2006). They found that the chronic salinity concentration over a 21 day period was between 20–30 mS cm^{-1}, whereas the acute, 3 day LC_{50} was around 50 mS cm^{-1}. It is curious that two taxa with very different salt tolerances have similar ratios of acute to chronic tolerances (ACR ~2). This may give some indication of the level of safety required when predicting chronic salinity tolerances from acute salinity tolerance data. Further studies of this nature on other species are required to confirm this ratio.

This study did not follow all mayflies right through to eclosion, which may have provided evidence of smaller adults emerging at increased salinity levels due to lowered growth rates in the nymphs prior to the initiation of metamorphosis. When *Ephemerella* mayflies were collected from water of different temperatures, those with low larval masses tended to metamorphose at a similar time to those with high larval masses, resulting in smaller adults. It was hypothesised that once the process of metamorphosis had been started (*via* hormonal regulation), no postponement was able to allow for much additional larval tissue growth, hence those that received the signal to initiate metamorphosis at a smaller size tended to emerge as smaller adults (Sweeney and Vannote, 1981). From the low growth rates yet similar level of emergence across salinities in the second *Centroptilum* experiment we can speculate that a similar phenomenon has occurred in our study. However, without further experimentation this cannot be confirmed.

It would also seem that the tested baetid mayflies are from very sensitive taxa that do not perform particularly well in laboratory experiments, since the survival rates were low in all salinities and in the controls. This is not usually the case with other species that we have tested (Kefford and Nugegoda, 2005; Kefford et al., 2006). The improved performance of *Centroptilum* mayflies in the second experiment where river water was used to make up the saline solutions suggests that the wet lab water could be one cause of the lower survival rates, and this factor should be considered in future experiments where mayflies and other similarly sensitive species are studied.

Emergence in chironomids was greatest in intermediate salinities, and decreased above or below that, producing an inverted U-shaped survival response. Chironomids held in high salinities (>2.5 mS cm^{-1}) had lowered growth rates and extended larval periods, yet the size of emerged adults was not significantly different between salinity treatments. Likewise, although there were differences in growth rates, time to emergence and survival in the damselfly *I. heterosticta*, the size of final instar and emerged adults were similar across all treatments (Kefford et al., 2006). These studies indicate that the process of development is protracted with increased salinity, which is in contrast to the current results on mayflies where development time was not affected by increased salinity.

Mayflies have very short adult life spans that generally last for only a few days (Carey, 2002). Adult mayflies do not feed and the process of oviposition results in the female's abdomen bursting, subsequently resulting in death (Carey, 2002). Owing to their short adult existence, mayflies have developed life histories that are synchronous, ensuring emergence is timed with other individuals to allow for successful reproduction (Newbold et al., 1994). Perhaps a delay in pupation and subsequent emergence of chironomids or damselflies is a less important factor than for mayflies, since they have longer adult life spans (Ruppert and Barnes, 1994) and the requirement for synchronous emergence to ensure successful reproduction is less critical.

An asymptotic relationship was observed between temperature and pupal duration in a study of some Australian chironomids, and at higher temperatures development time was faster, yet wing length was reduced (McKie et al., 2004). In our study, larval development time was prolonged with increased salinity and the number of individuals emerging decreased, but their size and weight was similar between salinity treatments. So, whereas temperature initiated an increase in the rate of

development and a decrease in the growth rate, we have observed salinity to cause a delay in development time but not growth. If we assume that the size of the emerged adults is a major factor influencing successful reproduction, then increased salinity may have little overall effect on reproduction, as it may just affect the frequency of reproductive events rather than the success of those events.

Although we generally presume that a reduction in growth is undesirable, in some situations it may actually benefit an organism. For example, it may be beneficial for a chironomid to delay pupation if adverse salinity conditions exist, since salinity tolerance in chironomids is much lower in pupae than larvae, because the pupae have no means of osmotic regulation (Berezina, 2003). Yet this delay would only be a temporary solution because the stress would need to be alleviated to still allow for successful emergence.

Similar to that hypothesised for lowered growth in mayflies, the increased times to emergence in chironomids may be due to increased energy requirements for osmoregulation as the salinity increased. Likewise in low-salinity environments ionic stress would be apparent and require energy to hold on to ions (rather than having them lost to the environment), which helps explain the U-shaped survival curve that was observed.

Inverted U-shaped growth patterns in response to salinity have been observed in freshwater snails (Jacobsen and Forbes, 1997; Kefford and Nugegoda, 2005) and mosquitoes (Clark et al., 2004; McGinnis and Brust, 1983). However, in the salt tolerant damselfly *I. heterosticta*, growth followed a step (or step then ramp) function, with low salinities having no effect on growth, intermediate salinities resulting in maximum growth, and at very high salinities reduced growth and eventually mortality (Kefford et al., 2006). For the present study we calculated growth rates in chironomids based on size at emergence and time to emergence, and observed a non-linear decrease in response to increased salinity. Again this illustrates that not all growth patterns in response to increased salinity are the same, therefore further studies of a variety of species is still needed.

This study focussed on the individual-level traits of growth and survival, but it has been suggested that environmental assessment should focus on changes at the population level (Admiraal et al., 2000; Forbes and Calow, 1999). For studies of salinity tolerance this could be a desirable way of assessing impacts, since there is much variation in salinity tolerances in different freshwater species (Hart et al., 1991; Kefford et al., 2003; Metzeling et al., 1995), making it very difficult to assign threshold levels of impact.

No consistency was found in which chosen traits (i.e. growth, survival, fecundity) were most sensitive to toxicant stress (Forbes and Calow, 1999), and hence these authors concluded that in order to get a proper indication of impact, organisms should be studied at the population level, in particular the population growth rate. Since the change in population growth rate incorporates all life stages and all life history traits, it may provide a better measure of effect. Similarly, it has been suggested that shifts in population life cycles and growth dynamics provide better indications of heavy metal stress in chironomids than simply observing one generation, and this also allows for determination of induced tolerance across generations (Postma and Davids, 1995). However, measuring effects at the population level can be much harder to demonstrate scientifically because it relies on continual monitoring data, which is often not available (Admiraal et al., 2000). In addition it is only possible to study populations when species are common, and common species may not reflect responses of all species.

This study has demonstrated that intensive investigation of different species is important when assessing the effects on freshwater biodiversity from increasing salinity, because subtle differences exist in species' physiological responses to salinity stress, and understanding the physiology of an organism's response to stress helps in the implementation of environmental management procedures to reduce the impact (Schreck et al., 2001). Safety factors applied to salinisation of freshwaters should be recalculated as results of more such studies become available.

We conclude that increased salinity appears to interfere with normal growth processes in mayflies, resulting in smaller and presumably less fecund adults, whereas in chironomids developmental processes are prolonged, resulting in a longer life cycle that has potential to alter life cycle dynamics and the frequency of reproductive events. This illustrates the need for further investigation of sub-lethal responses to salinity stress for a wide range of freshwater invertebrates to establish likely outcomes of increased salinity in Australian waterways.

We are grateful for funding from Land and Water Australia (LWA), the Murray Darling Basin Commission (MDBC) under the National River Contaminant Program (LWA Project RMI 12) and the Queensland Department of Natural Resources and Mines. The project benefited from a steering committee consisting of Satish Choy, Brendan Edgar, Richard Marchant, Leon Metzeling, Daryl Neilsen, Carolyn Palmer and Phil Papas.

References

Admiraal, W., Barranguet, C., van Beusekom, S. A. M., Bleeker, E. A. J., van den Ende, F. P., van der Geest, H. G., Groenendijk, D., Ivorra, N., Kraak, M. H. S. and Stuijfzand, S. C. (2000). Linking ecological and ecotoxicological techniques to support river rehabilitation. *Chemosphere* **41**, 289-295.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1**, 3-26.

Bayly, I. A. E. and Williams, W. D. (1973). *Inland Waters and their Ecology*. Camberwell: Longman Australia.

Berezina, N. A. (2003). Tolerance of freshwater invertebrates to changes in water salinity. *Russ. J. Ecol.* **34**, 261-266.

Carey, J. R. (2002). Longevity minimalists: life table studies of two species of northern Michigan adult mayflies. *Exp. Gerontol.* **37**, 567-570.

Clark, T. M., Flis, B. J. and Remold, S. K. (2004). Differences in the effects of salinity on larval growth and developmental programs of a freshwater and a euryhaline mosquito species (Insecta: Diptera, Culicidae). *J. Exp. Biol.* **207**, 2289-2295.

Forbes, V. E. and Calow, P. (1999). Is the per capita rate of increase a good measure of population-level effects in ecotoxicology? *Environ. Toxicol. Chem.* **18**, 1544-1556.

Greenberg, S. and Ar, A. (1996). Effects of chronic hypoxia, normoxia and hyperoxia on larval development in the beetle *Tenebrio molitor*. *J. Insect Physiol.* **42**, 991-996.

Hart, B. T., Bailey, P., Edwards, R., Hortle, K., James, K., McMahon, A., Meredith, C. and Swadling, K. (1991). A review of the salt sensitivity of the Australian freshwater biota. *Hydrobiologia* **210**, 105-144.

Horrigan, N., Choy, S., Marshall, J. and Recknagel, F. (2005). Response of stream macroinvertebrates to changes in salinity and the development of a salinity index. *Mar. Freshw. Res.* **56**, 825-833.

Jacobsen, R. and Forbes, V. E. (1997). Clonal variation in life-history traits and feeding rates in the gastropod, *Potamopygrus antipodarum*: performance across a salinity gradient. *Funct. Ecol.* **11**, 260-267.

Kefford, B. J. and Nugegoda, D. (2005). No evidence for a critical salinity threshold for growth and reproduction in the freshwater snail *Physa acuta*. *Environ. Pollut.* **134**, 377-383.

Kefford, B. J., Papas, P. and Nugegoda, D. (2003). Relative salinity tolerance of macroinvertebrates from the Barwon River, Victoria, Australia. *Mar. Freshw. Res.* **54**, 755-765.

Kefford, B. J., Zalizniak, L. and Nugegoda, D. (2006). Growth of the damselfly *Ishnura heterosticta* is better in saline water than freshwater. *Environ. Pollut.* **141**, 409-419.

Marshall, N. A. and Bailey, P. C. E. (2004). Impact of secondary salinisation on freshwater ecosystems: effects of contrasting, experimental, short-term releases of saline wastewater on macroinvertebrates in a lowland stream. *Mar. Freshw. Res.* **55**, 509-523.

McGinnis, K. M. and Brust, R. A. (1983). Effect of different sea salt concentrations and temperatures on larval development of *Aedes togio* (Diptera: Culicidae) from British Columbia. *Environ. Entomol.* **12**, 1406-1411.

McKie, B. G., Cranston, P. S. and Pearson, R. G. (2004). Gondwanan mesotherms and cosmopolitan eurytherms: effects of temperature on the development and survival of Australian Chironomidae (Diptera) from tropical and temperate populations. *Mar. Freshw. Res.* **55**, 759-768.

Metzeling, L., Doeg, T. and O'Connor, W. (1995). The impact of salinization and sedimentation on aquatic biota. In *Conserving Biodiversity: Threats and Solutions* (ed. R. A. Bradstock, T. D. Auld, D. A. Keith, R. T. Kingsford, D. Lunney and D. P. Sivertsen), pp. 126-136. Chipping Norton: Surrey Beatty & Sons.

National Land and Water Resources Audit (2001). Australian dryland salinity assessment 2000. Extent, impacts, processes, monitoring and management options. Canberra: Land and Water Australia.

Newbold, J. D., Sweeney, B. W. and Vannote, R. L. (1994). A model for seasonal synchrony in stream mayflies. *J. N. Am. Benthol. Soc.* **13**, 3-18.

Newcombe, C. P. and MacDonald, D. D. (1991). Effects of suspended sediments on aquatic ecosystems. *N. Am. J. Fish. Manage.* **11**, 72-82.

Pickering, A. D. (1990). Stress and the suppression of somatic growth in teleost fish. In *Progress in Comparative Endocrinology* (*Progress in Clinical and Biological Research, Vol. 342*) (ed. A. Epple, C. G. Scanes and M. H. Stetson), pp. 473-479. New York: Wiley-Liss.

Postma, J. F. and Davids, C. (1995). Tolerance induction and life cycle changes in cadmium-exposed *Chironomus riparius* (Diptera) during consecutive generations. *Ecotoxicol. Environ. Saf.* **30**, 195-202.

Ruppert, E. E. and Barnes, R. D. (1994). *Invertebrate Zoology* (6th edn). Orlando, FL: Saunders College Publishing.

Schreck, C. B., Contreras-Sanchez, W. and Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality and progeny. *Aquaculture* **197**, 3-24.

Smolders, R., Baillieul, M. and Blust, R. (2005). Relationship between the energy status of *Daphnia magna* and its sensitivity to environmental stress. *Aquat. Toxicol.* **73**, 155-170.

Sweeney, B. W. and Vannote, R. L. (1981). *Ephemerella* mayflies of white clay creek: bioenergetic and ecological relationships among six coexisting species. *Ecology* **62**, 1353-1369.

Williams, W. D. (1987). Salinization of rivers and streams: an important environmental hazard. *Ambio* **16**, 180-185.