Using Chironomidae to assess water and sediment quality

Submitted by
Kallie Rose Townsend

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Department of Zoology
Faculty of Science
The University of Melbourne

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Abstract

Biological assessments with Chironomidae (chironomids) are often used to provide information on the causes and effects of aquatic pollution. However, these applications are constrained by several factors, some of which were investigated in this thesis.

Laboratory toxicity test results are used to determine toxicity in the field, but these are ecologically irrelevant and often fail to account for the influence of complicating factors in the field. Two such factors are food limitation, potentially caused by an indirect effect of pollution, and the exposure of previous generations to stressors. The effect of food limitation was investigated in a multigenerational laboratory experiment with *Chironomus tepperi*. Responses to food limitation were similar to toxicity, with reduced survival, reproduction and increased development times as food became limited. The offspring of food limited parents were also affected. There was evidence that the quality of these offspring were compromised, even when offspring were raised under standard food conditions. Males and females responded differently, with greater developmental delays seen in female offspring.

There is also a scarcity of data regarding the effects of various pollutants on different chironomid species, particularly for Australian chironomids, under ecologically relevant conditions. In field-based microcosm experiments the effects of 17α-ethinylestradiol (EE2), tebufenozide and diuron were investigated. High nominal concentrations (10 µg L⁻¹) of EE2 consistently induced a novel antennal deformity in *Procladius* species larvae, but no effects on assemblages, abundances or development rate were observed except for an increased number of taxa at lower concentrations. Similarly, aside from increasing the number of taxa at the lowest nominal concentration of 0.004 mg L⁻¹, diuron had no effect on assemblages or species. Tebufenozide had no effect on these parameters.

Another constraint is an inadequate understanding and insufficient use of alternative endpoints, such as sex ratio skewing and adult responses *in situ*. Experimental studies showed that sex ratio skewing was stressor specific, occurring after exposure to
tebufenozide and diuron but not EE2 or food limitation. It was also species specific; tebufenozide and diuron caused skewing in different species. There was evidence that skewing was consistent, with the feminised sex ratios in *Procladius villosimanus* caused by diuron similar to that in *P. paludicola* in a previous study. In addition to sex ratios, other adult endpoints such as abundance and diversity are also useful for measuring impairment, although these are rarely including in biomonitoring studies. To measure adult responses *in situ* a method was developed using yellow sticky traps. The method was successfully able to detect differences between sites based on the composition and sex ratios of the chironomid fauna captured. Some of these biological parameters could be related to zinc pollution in sediments.

The results of these studies demonstrate the value of considering factors aside from toxicity, continued pollutant testing and the use of alternative endpoints in bioassessments with chironomids. Multigenerational testing and measuring sex ratios should be routinely included in studies with chironomids and, along with adult biomonitoring, represent useful additions to current approaches that need further investigation.
Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the preface,
ii. due acknowledgement has been made in the text to all other material used,
iii. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Kallie Rose Townsend

15th January 2013
Preface

The experimental results of this thesis are presented in five self-contained chapters (Chapters 2 to 6).

Chapter 5, A method for biomonitoring with adult Chironomidae, has been submitted to *Environmental Monitoring and Assessment* and is under review. This paper is co-authored with V. J. Pettigrove and A. A. Hoffmann. However, the contents are my own work and co-authorship represents a supervisory role of the other authors, in addition to providing scientific advice and site selection, assistance with statistical analyses, and reviewing the manuscript before submission.

Chapter 4 is presented as the substantially unchanged paper submitted as: Townsend, K. R., Pettigrove, V. J. and Hoffmann, A. A. (2012). Food limitation in *Chironomus tepperi*: Effects on survival, sex ratios and development across two generations. *Ecotoxicology and Environmental Safety* **84**, 1 – 8. The contents of this paper are my own work and co-authorship represents a supervisory role, in addition to advice for experimental design and statistical analyses, and revision of the paper prior to submission.
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This thesis is the result of the hard work, sacrifices and support of many people throughout my candidature. While people who contributed to specific parts of an experiment are acknowledged in those chapters, in this space I want to express my thanks to those who have helped me get this far.

Firstly, I thank my supervisors Ary Hoffmann and Vin Pettigrove for giving me inspiration, instruction and correction when I needed it. Without them this thesis would not exist (alternatively, it would be filled with very lengthy paragraphs and poor statistics). I also want to acknowledge the effort of people from CAPIM and CESAR who gave me advice, helped me with field work and made me laugh. I especially thank Sara Hoskin for being there while I went through the trauma of Starvation Creek, Bryant Gagliardi (I owe him a lot for making him dig up the dam bank in the 40 °C heat), Daniel MacMahon who was dragged out on a lot of field work expeditions, Dave Sharley for encouraging me to get this finished, two anonymous reviewers who gave me helpful comments on this thesis, and to Emily Thomson and Cindy Halliwell. Thanks also to Nancy Endersby and others at Bio21, the Department of Zoology and the University of Melbourne who have been involved in various parts of this process.

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Chapter 1: Introduction

Introduction

The introduction to this thesis consists of four sections followed by an outline of the thesis. The first section introduces freshwater ecosystems and highlights their importance for human activity. The second section follows on from this by describing the status of the world’s freshwater ecosystems and describes the major threats to these with specific reference to pollution. The third section is a discussion of the approaches used for identifying the pollution of aquatic ecosystems. Chemical and biological assessments are evaluated, and types of biological assessments are further elaborated on. The fourth section describes the use of macroinvertebrates for biological monitoring, with particular emphasis on the Chironomidae. Finally an outline of the chapters in this thesis is presented.

Freshwater ecosystems

Water is the most valuable resource and is essential for all known life forms. The total volume of water on Earth is estimated to be 1.4 billion km$^3$. Of this 2.5 % is freshwater and only 0.3 % of freshwater is available in surface waters (United Nations Environment Programme, 2008). Fresh surface waters include streams, lakes, floodplains, peatlands, marshes, wetlands, ponds, cave waters and small bodies such as phytotelm and soil cavities (Revenga and Kura, 2003). Fresh surface waters are important ecosystems and carry a disproportionately large number of taxa for their volume, providing habitats for over 6 % of all described species (Dudgeon et al., 2006) and one third of all known vertebrate species (Dudgeon et al., 2006). Around 40 % of all fish species, for example, are found in freshwater ecosystems, and new species are continually being discovered (Lundberg et al., 2000).

Human history is closely intertwined with fresh waters. Freshwater ecosystems were utilised as sources of food well before agricultural practices were established and major civilisations began in close association with surface waters. Human activities
rely on the services provided by aquatic ecosystems, which the Millenium Ecosystem Assessment (Millenium Ecosystem Assessment, 2005) has grouped into four main categories:

- **Supporting services** (e.g. nutrient cycling, soil formation, pollination and primary production).
- **Provisioning services** (e.g. provision of water, food, energy, transportation and materials).
- **Regulating services** (e.g. regulating floods and hydrology, groundwater recharge and discharge, climate, water purification, waste disposal, pollution control and detoxification).
- **Cultural services** (e.g. aesthetic, spiritual, recreational and educational services).

Costanza *et al.* (1997) estimated the services provided by wetlands, lakes and rivers to equate to about US$9,810 billion per year. However, ecosystem valuing is the subject of much contention, and the reality of how valuable ecosystem services are goes unheeded until ecosystems are degraded to the point that services are impaired (Daily *et al.*, 2000) or people lack access to these services.

Australia is the driest inhabited continent and Australians depend on fresh surface waters, with approximately 95% of the water used extracted from surface waters (Hatton *et al.*, 2011). The three largest uses are agriculture (49.6%), water supply including sewerage and drainage services (17%) and households (12.5%). Other major uses include manufacturing, mining, energy production, forestry and fisheries. The variability of the Australian climate, water resource development (harvesting and flow regulation), historical and current land use practices, and invasive species are causing serious degradation of Australia’s aquatic ecosystems and the services they provide.

**Threats to freshwater ecosystems**

Aquatic ecosystem services are closely linked to their biological integrity, but our close association with aquatic ecosystems places them at risk. Aquatic ecosystems are threatened by human activities more than other ecosystems. The Living Planet Index
(LPI), which is calculated from the average population changes of 1,686 vertebrate species relative to populations in 1970, declined by nearly 30% in 2005. Freshwater ecosystems fared the worst with declines of around 35%, whereas marine ecosystems experienced 14% declines (Hails et al., 2008). Aquatic species are at the greatest risk of extinction (Revenga et al., 2005). In North America the extinction rates of freshwater species are predicted to be five times greater than for their terrestrial counterparts (Ricciardi and Rasmussen, 1999). Loss of biodiversity can affect the functionality of aquatic ecosystems (reviewed by Covich et al., 2004), which then affects ecosystem services. For instance, in the USA eutrophication of freshwater systems costs an estimated US$2.2 billion annually by affecting water front property sales, drinking water, recreation and conservation programs (Dodds et al., 2008).

Dudgeon et al. (2006) described five broad, interacting threats to aquatic ecosystem biodiversity:

Overexploitation of services or species can be detrimental to the whole ecosystem. For example, in 2008 90 million tonnes of fish were caught from wild stocks, 28% of which were overexploited and 3% depleted (Food and Agriculture Organization, 2010). Ten million tonnes of freshwater fish were apparently supplied. Freshwater fish stocks have been depleted by systematic commercial fishing practices and recreational fishing (Allan et al., 2005). Overfishing, bycatch, altering community composition, and habitat degradation through fishing practices affect whole aquatic ecosystems (Kura et al., 2004). Another example is the overexploitation of freshwater, and in many regions water stock depletion is faster than replenishment, or human needs exceed water availability. Currently 700 million people from 43 countries live under “water-stress conditions”, which will increase to three billion people by 2025 (Watkins, 2006). In Australia, irrigation is important for sustaining agriculture in arid and semi-arid areas, and in 2009/2010 1.84 million hectares of irrigated land was worth $11.5 billion in production (Australian Bureau of Statistics, 2011). Over half of Australia’s irrigation occurs in the Murray-Darling Basin (MDB), where excessive water extractions have reduced flood frequency and current water licensing favours license holders over environmental flow requirements (Hatton et al., 2011).
Habitat degradation, of both aquatic and surrounding terrestrial habitats, has negative consequences for aquatic communities and is widespread. For example, over 50 million hectares of wetlands were drained in midwestern America since the 1850s (McCorvie and Lant, 1993) and globally 50% of wetland area has been lost in the last century (Hails et al., 2008). Habitat degradation can occur at the scale of the catchment. For instance, Australian freshwater ecosystems are threatened by increased sedimentation from catchment land clearing and riparian vegetation removal (DeRose et al., 2003). Most of this is deposited on floodplains, riverbeds and in reservoirs, and it homogenises the riverbeds, smoothers aquatic organisms and forms barriers to migration. Habitat degradation can also be local, such as removing snags and rocks from waterways, with consequences for many native fishes (Norris et al., 2001), or the removal of riparian vegetation, which threatens the integrity of aquatic ecosystems (NSW Department of Primary Industries, 2005).

Flow modification is also a threat, with waterways frequently regulated by structures for storage and controlled water release, modified by straightening or surrounded by structures such as levees to mitigate floods, and replaced with concrete channels or pipes to improve water conveyance. Globally nearly 60% of 292 large river systems are regulated (Nilsson et al., 2005) and four of 12 major drainage basins in Australia are in poor condition due to substantially changed flows (Hatton et al., 2011). Regulation inundates riparian habitats upstream of dams (Nilsson and Berggren, 2000), increases sedimentation and reduces water temperatures and flows. Downstream riparian vegetation is deprived of water and erosion is increased (Nilsson and Berggren, 2000) while the movement of aquatic biota is impeded. Straightening streams reduces streambed heterogeneity and habitat structures (Ligon et al., 1995).

Exotic species threaten aquatic ecosystems by competing with and preying on native species. Nile perch Lates niloticus, for example, contributed to the extinction or threatened status of around 200 endemic cichlid fish species after being introduced to Lake Victoria, Africa (Witte et al., 1992). Invasive species can also alter the abiotic conditions of the waterway, such as reduced flows and sunlight infiltration, and increased evapotranspiration caused by dense growths of water hyacinth (Eichhornia crassipes) (NSW Department of Primary Industries, 2010). Readily established exotic species often thrive in disturbed ecosystems, such as in regulated rivers of the MDB.
where European carp *Cyprinus carpio* dominate fish communities (Gehrke *et al.*, 1995).

*Aquatic pollution* is ubiquitous and is the threat focussed on in this thesis. Even areas with no history of contamination can be polluted (e.g. Braune *et al.*, 1999). Every day two million tonnes of human waste are released into waterways, and developing countries dispose of 70 % of their industrial waste into waterways untreated (United Nations Water, 2011). Aquatic pollution is a human health concern. In developing nations one in five people lack access to clean water while half have inadequate sanitation (Watkins, 2006). Water is often contaminated by faecal matter and unsafe water causes approximately 1.8 million deaths, mostly children, from diarrhoea each year (World Health Organization, 2012). Developed nations are not immune to the problems of pollution and deteriorating water quality is recognised as the most important challenge facing society today. In a 2010 survey of environmental concerns, 50 % of Americans were greatly worried about the pollution of drinking water and 46 % were worried about water body pollution. People were more concerned about these issues than about air pollution (38 %), tropical rainforest loss (33 %), species extinctions (31 %) and global warming (28 %) (Gleick, 2011).

Pollution can enter waterways through point sources or diffuse (nonpoint) sources, and types of pollutants range from coarse debris and litter to organic matter, suspended solids, sediments and chemicals. Pollution impacts aquatic ecosystems by affecting aquatic taxa, which in turn changes the community composition of those ecosystems (e.g. Patrick, 1949), and this ultimately alters the ecosystem and its services. For example, nutrient enrichment contributed to a 1,000 km long cyanobacterial bloom in the Darling River, contaminating drinking water and killing livestock and wildlife (Hallegraeff, 1992). Some pollutants can enter food chains by bioaccumulating in the tissues of aquatic organisms and biomagnifying at higher trophic levels. Mercury concentrations, for instance, were higher in two fish species than in lower trophic taxa in Ozark stream ecosystems, USA, presumably coming from mercury within the crayfish these fish preyed upon (Schmitt *et al.*, 2011). Some pollutants persist in the environment over long periods of time. DDT use, for example, has been banned since 1983 in China but DDT and its degradation products are still detectable in river sediments (Wang *et al.*, 2007).
Current and past land use practices, in conjunction with Australia’s unique climate and landscape, have polluted Australian freshwater ecosystems. Nutrients have been found in waterways associated with agricultural production (e.g. Markich and Brown, 1998; Mitchell et al., 2005; Bainbridge et al., 2009), sewage and wastewater effluent (e.g. Markich and Brown, 1998), urbanisation (Markich and Brown, 1998) and increased sedimentation (DeRose et al., 2003). All waterways in metropolitan areas and most areas under intensive agriculture exceed guideline values for nutrients (Hatton et al., 2011), and nutrient enrichment has been responsible for toxic algal blooms in Australian waterways (e.g. Hallegraeff, 1992; Hodgkin and Hamilton, 1993). Pesticides have been detected in Australian waterways associated with urban areas (e.g. Waugh and Padovan, 2004; Schäfer et al., 2011) and in rural and agricultural waters (e.g. Muschal, 2001a, 2001b; Rose and Kibria, 2005, 2006; Tran et al., 2007; Schäfer et al., 2011). Metal contamination has been associated with mining (e.g. Harrison et al., 2002; Taylor, 2007), agriculture (Bennet-Chambers et al., 1999), urbanisation (e.g. Gale et al., 2006; Markich and Brown, 1998) and industrial activity (Gale et al., 2006). Hydrocarbons have been found in urban and industrial catchments (Brown and Maher, 1992; Carew et al., 2007). Low concentration environmental contaminants have also been identified, such as environmental estrogens (e.g. Mispagel et al., 2009; Ying et al., 2009), pharmaceuticals and personal care products (e.g. Watkinson et al., 2007; Fisher and Scott, 2008; Watkinson et al., 2009).

Advances in technology have enabled the detection of a greater number of contaminants at lower concentrations than previously. However, as waterways are often contaminated by numerous pollutants, it is not feasible to screen for every single contaminant that could possibly be present. In addition, many pollutants have little to no ecotoxicological data available, and existing data are generally restricted to single species toxicity tests derived from exotic species. For most chemicals the effects on Australian biota are unknown. Also, the hydrology, geology and climate of Australia greatly differs from those of countries where the environmental fate of many contaminants have been determined; chemicals may behave differently under Australian conditions. To summarise, there is a paucity of data regarding the toxicity and environmental fate of many pollutants in the Australian environment.
Assessing aquatic ecosystem pollution

In terms of assessing aquatic pollution, a range of tools have been developed to identify what pollutants are present, predict if impairment to aquatic biota is likely, and to determine if impairment has occurred as a result of contamination. The type of assessment chosen by a manager will often depend on the suspected cause of impairment, the questions being asked and the resources available for assessment. These assessments can be quite specific in what they address and each has its advantages and disadvantages. For the convenience of further discussion these are broadly categorised into two groups: chemical assessments and biological assessments.

Chemical assessments

Chemical assessments involve the direct measurement of chemicals in the water, pore water, sediment and organism tissues of aquatic ecosystems. Chemical analyses are relatively inexpensive, fast and require less effort and expertise in sample collection than biological assessments (Maher et al., 1999). The early identification of contaminants in an ecosystem can allow preventative and remedial actions to take place before visible ecological damage has occurred. Through the strategic monitoring of waterways, chemical analyses can help identify sources of pollution (e.g. Martin, 2004; Anderson et al., 2007; Acquavita et al., 2010).

However, chemical analyses are not a direct measure of ecosystem health. The presence of a chemical does not mean degradation has occurred and not all forms of a chemical are bioavailable. In consequence many studies now examine different fractions of a chemical to determine bioavailability (e.g. Davide et al., 2003; Ebrahimpour and Mushrifah, 2008) and guideline values have been developed to link ecological effects with chemical concentrations (e.g. ANZECC/ARMCANZ, 2000; MacDonald et al., 2000; Canadian Council of Ministers of the Environment, 2002). However, guideline values are usually derived from single species acute toxicity tests under standardised laboratory conditions, which may fail to explain ecological effects (Norris and Thoms, 1999) and the responses of other taxa to the chemical. The chronic, sublethal, population and community effects of many chemicals are generally unknown. Guidelines are often inadvertently misused as the sole determinant of
ecological degradation, whereas they should be used to identify sites where specific follow-up investigations are needed (Maher et al., 1999).

Another problem is that many chemicals may be present in an environmental sample and it is too expensive, time-consuming and practically impossible to determine the presence and concentration of every one of these. *A priori* predictions about what chemicals are likely to be present or of most concern can be useful, but these can still result in the chemical causes of degradation going undetected (e.g. Townsend et al., 2009). Chemical assessments are of limited use as early warning indicators of degradation except where the chemicals monitored are in fact the cause (or correlate with the cause) of actual degradation (Cairns et al., 1993). At best, chemistry by itself can only indicate that degradation is “potentially occurring” (Maher et al., 1999).

**Biological assessments**

Previously chemical assessments of aquatic ecosystems were preferred over biological assessments, which were considered expensive, time consuming, difficult to standardise and required a level of expertise that was difficult to access (Karr et al., 1986; Maher et al., 1999). Yet biological analyses were regarded as crucial for understanding the state of aquatic ecosystems (Krantzberg, 1992; Hart and Fonseca, 1996; Norris and Thoms, 1999). When considered alone, chemical assessments could suggest a healthy ecosystem was impaired, or conversely identify an ecosystem as healthy when in fact degradation was occurring.

Ultimately the biota are affected when changes to chemical parameters occur, and biological assessments seek to measure this. Biological assessments are used to determine if degradation has occurred, identify the potential sources and causes of degradation, and predict whether degradation is likely under different scenarios. Cairns *et al.* (1993) described two approaches for evaluating ecosystem degradation. *Top down approaches*, or *biological monitoring* (*biomonitoring*), are where direct changes in communities or ecosystems are measured and causes then diagnosed. *Bottom up approaches* (*experimental approaches*) are typically conducted under standardised, simple laboratory conditions to model the effects and changes of stressors, which are then extrapolated back to the field. In the following discussion I
have added a third category, *field-based experimental approaches*, which take a middle ground between these two approaches. Each approach has advantages and disadvantages, and there is potential for much overlap between the methodologies used to identify impairment and the causes and mechanisms of impairment.

*Field based approaches (biomonitoring)*

Field based approaches involve the measurement of biological parameters *in situ* to determine ecosystem status or the presence of stressors, usually in conjunction with physical or chemical analyses. Different levels of organisation, from whole ecosystems to suborganismal endpoints, as well as multiple species, can be monitored. Suitable biomonitoring species can be studied *in situ* to indicate the presence and magnitude of impairment by their presence, abundance, morphology, physiology and behaviour, and sentinel or bioaccumulator species can be used to measure the bioavailability of contaminants through accumulation in their tissues (Beeby, 2002). Groups commonly used for biomonitoring include macroinvertebrates (e.g. Chessman, 1995; Connolly *et al.*, 2004), fish (e.g. Harris and Silveira, 1999; Karr, 1999) and algae (e.g. Barinova *et al.*, 2011).

Biomonitoring is not a recent idea. In the early 20th century the Saprobien system was developed where aquatic organisms were used to identify the state of an aquatic ecosystem (Kolkwitz and Marsson, 1909). Today a variety of analytical methods are used in biomonitoring. Diversity indices, for example, are a function of species richness and evenness that allows comparison between sites, although these are heavily criticised (Hurlbert, 1971; Karr *et al.*, 1986). Multivariate approaches use data from reference sites to predict the fauna at impacted sites, and deviations from the predicted fauna are used to form a score of impairment which is easy to interpret, comparable between sites (Norris, 1995), and useful for identifying degradation and evaluating remediation strategies (Karr, 1999). These predictive models form standard approaches for biomonitoring in many countries, including the River Invertebrate Prediction And Classification System (RIVPACS) in the United Kingdom (Wright, 1995), the BEEnthic Assessment of SedimenT (BEAST) in Canada (Reynoldson *et al.*, 1995), and the Australia River Assessment Scheme (AusRivAS) (e.g. Smith *et al.*, 1999) and Stream Invertebrate Grade Number – Average Level (SIGNAL) (Chessman, 1995) in Australia. However, they are only sensitive to large differences in
degradation (Boulton, 1999; Smith et al., 1999), lack significance testing and assume all environmental variables driving biotic changes have been measured (Norris, 1995).

In general, effects measured at the scale of ecosystems, communities and populations suggest that significant degradation has already occurred, which limits their usefulness as early warning indicators (Krantzberg, 1992). Early responses to stress, such as biomolecular changes or sublethal responses in individual animals, potentially solve this problem and are widely used in biomonitoring. They can be highly specific for certain stressors, such as acetylcholinesterase which is inhibited by organophosphates (Ricciardi et al., 2006). These parameters can include molecular biomarkers (reviewed in Livingstone, 1993; e.g. Vindimian et al., 1991), deformities (e.g. Warwick, 1985) and histology (e.g. Gibbs, 2009) among other things. However data for the repercussions that sub-lethal effects have on higher levels of organisation are lacking for many endpoints.

Ecosystems are complex and organisms are often exposed to multiple stressors, only some of which are anthropogenic. Isolating stressors and determining which are causing impairment is challenging. Interactions between stressors can change organism responses to any given stressor. Also field organisms may have complex exposure histories, which affect how they respond to stress. Experimentally it has been shown that exposure to a stressor can lead to local adaptation (e.g. Knapen et al., 2004; Barhndorff et al., 2006), conferral of a survival advantage to offspring under continued stress (e.g. Harshman et al., 2001) or increasing sensitivity to stressors as exposed individuals produce poor quality offspring (Janssens de Bisthoven et al., 2001). These effects should be expected in the field and make it difficult to compare biological responses between sites.

Analytical and statistical problems exist with biomonitoring studies. Inferential statistics are often applied to data sets where the “treatments” (e.g. sites) are not replicated or samples within sites are treated as replicates (pseudoreplication) (Hurlbert, 1984). Pseudoreplication can be overcome by treating similar sites as replicates, but how sites are deemed similar could be contentious. The use of quantitative methods, such as Surber sampling, can overcome many statistical problems associated with rapid bioassessment techniques, and are found to be more
accurate for measuring community richness and composition (da Silva et al., 2005). However, these are more time consuming and expensive. Another problem is with reference sites and the reference condition. Experimental approaches require a control and many biomonitoring studies designate some reference sites to a “control group”. Predictive multivariate analyses also require numerous reference sites (Reynoldson et al., 1997). Ideally reference sites should be similar in biotic and abiotic characteristics to test sites, but for highly disturbed areas reference sites may not be available. Also, the terms “reference site” and “reference condition” are often used ambiguously and assigned different meanings (Stoddard et al., 2006), and approaches can reach different conclusions about which sites are reference sites and which are impacted (Reynoldson et al., 1997).

*Experimental laboratory approaches*

Controlled experimental methods address many of the problems encountered in biomonitoring and are based on traditional toxicology. These methods are mostly used for chemical toxicity testing, where the toxicity of environmental samples is determined. The chemicals causing toxicity and the concentrations at which toxicity is observed are also identified. The premise of the test is that a test organism is exposed to a chemical or environmental sample (e.g. sediment, water, effluent) over a defined period of time, its response is recorded and then compared to controls. The endpoints measured are similar to those used in field studies and range from the levels of sub-organism to population. Advantages of laboratory experiments include replication with controls so inferential statistical analyses can be conducted, treatments can be manipulated and controlled so only variables of interest are tested, the experiments are reproducible and allow for the measurement of endpoints that are difficult to measure in the field (e.g. reproduction, behaviour, developmental stage specific responses), the exposure history of an organism or population is known so confounding multigenerational effects are prevented, the taxonomy of the test organism is known, and the experiments have greater scope for identifying the mechanisms by which a stressor affects an organism.

The model organisms used are selected because they are readily available, have a well known biology, are suitable for answering questions of interest, have an appropriate size, survive under laboratory conditions, have short generation times, and are fecund
(Ankeny and Leonelli, 2011). Model organisms represent the responses of a larger
group of organisms to a stressor (Ankeny and Leonelli, 2011), but in that purpose it is
assumed their responses are similar to those of other taxa and perhaps even whole
communities or ecosystems. However, this is unlikely and responses can differ
between species of the same genus (e.g. Watts and Pascoe, 2000) or strains and
populations of the same species (e.g. Oda et al., 2006). Thus organism responses from
the laboratory will not represent organism responses in the field. Testing multiple taxa
with different levels of relatedness (multispecies test systems) partly resolves this (e.g.
Sánchez and Tarazona, 2002; Jaser et al., 2003; Beckage et al., 2004). Although
massively simplified, multispecies tests give a broader understanding of how
taxonomic groups differ in their responses and can reflect ecological interactions
between taxa. Another problem is that laboratory populations are susceptible to
inbreeding, which can affect the sensitivity of organisms to tested stressors (e.g.
Nowak et al., 2007).

Laboratory exposures are simplified and do not reflect field conditions, limiting how
observed responses can be extrapolated to the field. To increase the relevance of
laboratory assays studies can investigate the effects of multiple stressors (e.g. Hooper
et al., 2003; Flaherty and Dodson, 2005) and whole environmental samples such as
effluents can be used (e.g. Jobling et al., 2003; Porter and Janz, 2003). Most testing
previously focussed on acute toxicity and neglected the effects of chronic exposure,
life-stage specific responses and behavioural responses to stressor (Krantzberg, 1992).
Acute tests such as Lethal Concentration tests are criticised for lacking ecological
relevance and limited usefulness as early warning indicators or predictors (Krantzberg,
1992). Alternative tests, such as full life-cycle and chronic toxicity testing, overcome
these difficulties. Experiments can also be conducted over multiple generations to
replicate field scenarios where populations have more complicated exposure histories
(e.g. Janssens de Bisthoven et al., 2001; Clubbs and Brooks, 2007; Dussault et al.,
2008).

Field-based experimental approaches
Field-based experimental approaches combine aspects of biomonitoring and
laboratory methods to experimentally examine the ecological effects of impairment.
Theoretically results from these tests are more ecologically relevant than laboratory
tests and easier to interpret than field surveys (Belanger, 1997). These experiments may use artificial ponds, streams, lakes and wetlands, and microcosms, mesocosms and macrocosms. Natural lakes and ponds can also be experimentally manipulated. Giesy and Odum (1980) defined microcosms as experimental units that are a bounded (either artificially or naturally) subset “of naturally occurring environments which are replicable”, allowing for experimental manipulation and statistical testing. This definition can also be loosely applied to the other field-based experimental methods. Test systems can be gnotobiotic (species of the system are defined and added by the experimenter) or derived (assemblages are sourced from natural populations and may be collected or colonise the system) (Giesy and Allred, 1985). Gnotobiotic systems are useful for examining, testing and modelling processes and interactions between biota and stressors. They are easy to replicate and repeat, but are more costly, require more maintenance, take longer to establish and are less ecologically relevant than derived systems (Giesy and Allred, 1985). Derived systems, though, tend to be more difficult to interpret and are less repeatable.

Field-based experiments can simulate natural processes such as flows simulated by artificial outdoor streams (Belanger, 1997). They can show which stressors cause impairment under ecologically relevant conditions and which biological responses would be useful to measure in situ (e.g. Pestana et al., 2009). They are useful for determining the behaviour and fate of chemicals (e.g. Sundaram, 1997; Thompson et al., 2004; Peters et al., 2007) and through simple experimental manipulations (such as transplanting environmental samples or taxa) they can investigate local adaptation of populations to stressors (e.g. Bahrndorff et al., 2006).

Biomonitoring studies are usually limited to correlating stressors with biological responses. Field experiments have the advantage of establishing causal links between impairment and particular stressors (Boudou and Ribeyre, 1997; Pettigrove and Hoffmann, 2005) and factors that are potentially confounding to researchers can be controlled (Giesy and Allred, 1985). The microcosm approach by Pettigrove and Hoffmann (2005), for instance, allowed the effects of sediment quality to be isolated from other co-occurring factors in the field.
Laboratory experiments often fail to account for interactions between organisms and the biotic and abiotic components of their habitats (Giesy and Odum, 1980) whereas field-based experiments can investigate the direct and indirect effects of stressors, such as trophic interactions (Fleeger et al., 2003). Field-based experiments can be ecologically complex in terms of species diversity and food web complexity. For example, stream microcosms had structures comparable to natural streams (Brown et al., 2011). Multiple species are tested, usually over several trophic levels and from different functional groups. As such, field-based experiments can incorporate numerous taxa with greatly differing sensitivities to stressors that would not be available in the laboratory (Pettigrove and Hoffmann, 2005).

However field-based experiments are simpler than natural ecosystems and many field conditions cannot be adequately reproduced (Giesy and Allred, 1985). The species and responses represented in field-based experiments can differ to those in field surveys. For example, physico-chemistry, taxon presence and sensitivity were similar between field-based microcosms and field sites, but some taxa were not present in both studies and other taxa exhibited different sensitivities under field and experimental conditions (Carew et al., 2007). The microcosms probably did not accurately reflect all of the stressors animals from field sites were facing. Field-based experimental systems are often restricted in the types of organisms are present, especially when organisms are selected a priori (Giesy and Odum, 1980) or colonisation by certain taxa is inhibited. Natural assemblages and interactions will not be accurately represented. Some microcosms, for example, only allow colonisation by aerial macroinvertebrates (Pettigrove and Hoffmann, 2005). Factors that normally influence natural communities may deliberately be excluded from the test system as well (Giesy and Odum, 1980). Compared to laboratory studies and biomonitoring, field-based experiments are generally more time consuming and expensive. They also require greater taxonomic and ecological expertise, and the results produced are usually more complex and difficult to interpret. Thus, field-based experiments are not considered to be suitable for the rapid screening of a large number of stressors.
Biological assessments with macroinvertebrates

Macroinvertebrates are commonly used in biological assessments for a number of reasons, some of which are cited in Rosenberg and Resh (1993) and Chessman (1995). They are ubiquitous and diverse in freshwater environments, functionally important, form a significant part of the diet of other organisms, taxa vary in their sensitivity to different stressors, many taxa are sedentary with short life cycles and therefore reflect recent impacts at field site, and their small size makes them suitable for examination, capturing, storage and transport. In addition, the use of macroinvertebrates avoids the ethical constraints associated with vertebrate species, and their size makes them easy to culture in the laboratory at sufficiently high numbers for experimentation.

The Chironomidae

The Chironomidae are a species diverse family of Diptera that are frequently included in biological assessments of aquatic ecosystems. They are widely distributed and inhabit a range of different environments. Although most species inhabit freshwater, some chironomids are terrestrial and several species are marine (reviewed in Oliver, 1971). Chironomid species occupy numerous ecological niches and differ in their behaviours, habitat use and feeding preferences (Oliver, 1971). Chironomids are ecologically important because of their functional roles, such as nutrient cycling and maintaining prey populations (e.g. Hansen et al., 1998), and they form a significant part of the diets of predators such as fish, birds and other organisms (e.g. King and Wrubleski, 1998; Dreyer et al., 2012).

Chironomids are included in bioassessments because of their ecological significance and ability to indicate impairment. Their small size, diversity, relatively well known biology, short life cycles and numerous responses make chironomids suitable organisms for bioassessment. Biomonitoring with chironomids has a long history dating back to the early 20th Century where Chironomus plumosus was included in the Saprobien system to indicate organic pollution (Kolkwitz and Marsson, 1909). Today many biomonitoring approaches use chironomids to indicate ecological impairment, and predictive models such as AusRivAS and SIGNAL assign the family, as well as its subfamilies, scores that denote pollution tolerance (e.g. Chessman, 1995; Barmuta
Biomonitoring with macroinvertebrates often relies on coarse taxonomic resolution such as this, with the benefit of saving time, money and requiring less expertise. However, the assumption that all taxa within a family exhibit similar sensitivities to a stressor is incorrect (Lenat and Resh, 2001), even within the Chironomidae (e.g. Carew et al., 2011). Although more difficult and time consuming, genus and species level biomonitoring give a more sensitive, sometimes stressor-specific indication of impairment (e.g. Carew et al., 2007).

Chironomids have many endpoints that are readily measured in field, field-based experimental and laboratory approaches, providing informative links between the methods. For example, head capsule deformities in larvae have been measured in field populations linked to pollution (e.g. Madden et al., 1992; Williams et al., 2001; Bhattacharyay et al., 2005), observed in field-based experiments (e.g. Townsend et al., 2009) and have been induced in laboratory experiments (e.g. Vermeulen et al., 2000; Meregalli et al., 2001). Several species, usually Chironomus species, can be reared in the laboratory and are standard test organisms for aquatic toxicity (e.g. US EPA, 2002; OECD, 2004a, 2004b).

There are problems specifically related to using chironomids in addition to those already discussed for specific types of biological assessments. There is taxonomic uncertainty due to the presence of cryptic species, old or incomplete identification keys, and missing links between the different developmental stages of species. Molecular techniques have alleviated this problem by determining and confirming the identification of species and revealing cryptic taxa (e.g. Carew et al., 2003; Sharley et al., 2004; Carew et al., 2011). However, molecular and morphological identification of chironomids to species or genus are still expensive, time consuming and require greater expertise compared to subfamily level identifications. Also, the responses of chironomids to stressors will not represent the responses of other taxa (e.g. Dussault et al., 2008). Another related problem, particularly for laboratory tests, is that the test organism is usually from the genus Chironomus, and its responses to stressors differ to other Chironomidae (Pinder, 1986). The use of other genera in the laboratory could rectify this but to date the establishment of new species in the laboratory has been difficult.
The greatest problem with using chironomids is the lack of information regarding their biology, ecology and responses to stressors, especially in field surveys and field-based experiments. Interpreting observed effects and relating this to ecosystem impairment is difficult without this information. Data are lacking for species-specific responses to different stressors; these data could be powerful tools for identifying specific types of environmental stress. There are also many useful chironomid endpoints that could potentially be used but have yet to be developed, especially for adult chironomids. One of these is sex ratio skewing. In other taxa skewed sex ratios can indicate specific types of environmental stress and the mechanisms by which effects are occurring. One of the best examples comes from studies on endocrine disruption, where feminisation of fish populations has been linked to the presence of estrogenic compounds (e.g. Länge et al., 2001; Vajda et al., 2008). Although only a few studies have examined sex ratio skewing in chironomids, it appears that skewed sex ratios are stressor specific (e.g. Rakotondravelo et al., 2006; Townsend et al., 2009) and species specific (Ayres et al. unpublished). There are several mechanisms by which skewing may occur, including sex reversal (e.g. Olmstead et al., 2009; Larsen and Baatrup, 2010), the effects of parental condition, sex allocation and different reproductive fitness of the sexes (Hamilton, 1967; Trivers and Willard, 1973; Charnov, 1982), and differing susceptibilities to stressors between the sexes (e.g. Moran and Hurd, 1994; Ramasamy and Murugan, 2002).

Knowing sex determination and developmental pathways is important in understanding the mechanisms by which skewing may occur. Sex determination in insects occurs by a variety of mechanisms, some of which are reviewed in Sánchez (2008). Sex determination varies even within the Diptera, although it is generally genetically determined and involves a primary signal with maternal products that regulate the activity of a particular gene. This in turn activates a double switch gene to determine either male or female development (reviewed in Shearman, 2002). Sex determination in chironomids is not well understood, although in Chironomus sex appears to be determined by the presence of male dominant genes that switch the default female pathway to male, and these male dominant genes possibly act at different points in the sex determination pathway for different species (Martin and Lee, 2000). Insect hormones are also involved in sexual development. Juvenile hormone regulates vitellogenesis, oocyte development and vitellogenin uptake by
oocytes in females and accessory gland development in males. It also inhibits oogenesis and spermatogenesis, whereas ecdysone regulates these processes (reviewed in Pinder et al., 1999). Although the specific details of sex determination and differentiation are not established for chironomids, the measurement of sex ratio skewing is still a useful indicator of stress. However, chironomid sex ratios have largely been ignored and other adult endpoints have been notably excluded from field surveys. These endpoints represent the end of the life cycle and organisms that have successfully survived in an environment, therefore providing a better measure of field conditions (Bouchard and Ferrington, 2011). For this reason understanding and evaluating adult endpoints in biomonitoring warrants further investigation.

In addition, data are lacking that link the results of laboratory and field studies; often it is assumed that toxicity observed in the laboratory must also be occurring in the field to a similar degree. Efforts should be made to make laboratory studies with chironomids more ecologically relevant, such as conducting multigenerational studies to understand the effects of previous population exposures to stress (e.g. Janssens de Bisthoven et al., 2001). The results of laboratory experiments should also be validated in the field, and laboratory studies should be used to further enhance our understanding of the biology of chironomids.
Outline of study
Biological assessments with the Chironomidae have potential for answering questions about ecological impairment, but current data are insufficient and incomplete. This limits how useful chironomids are for indicating impairment and how their responses should be interpreted. Most notably, connections between laboratory and field studies need to be established. Alternative endpoints, testing methods and exposure to stressors other than chemicals need to be explored in the laboratory to make these more ecologically relevant. Endpoints with adult chironomids should also be investigated further, especially in field surveys, because these are informative but less commonly used or understood than larval endpoints. This thesis aims to investigate these areas where data are needed through the use of field, field-based experimental and laboratory approaches.

Chapter 2: The effects of an environmental estrogen, 17α-ethinylestradiol, on Chironomidae
The synthetic estrogen 17α-ethinylestradiol (EE2) is a common low-concentration contaminant in aquatic ecosystems with well-documented endocrine-disruptive effects in aquatic vertebrates. There are few data for effects in aquatic macroinvertebrates and most are limited to single species laboratory comparisons. Data for effects on macroinvertebrate assemblages and Australian fauna are lacking. To investigate the effects of EE2 on Australian Chironomidae, two field-based microcosm experiments were conducted. In addition to effects on assemblages, the effects on populations, development rate and sex ratios of common species were examined. The larvae of two species of Procladius were also examined for head capsule deformities.

Chapter 3: The effects of tebufenozide and diuron on Chironomidae
There is evidence that chemicals can have sex specific effects in Chironomidae. Responses are potentially chemical and species specific, which could provide a powerful tool for assessing the presence and impacts of specific chemicals in the field. Two pesticides that have caused sex specific effects previously are tebufenozide and diuron, although the former was limited to a single-species laboratory study. To assess and confirm the effects of these chemicals on Australian chironomids, a field-based
microcosm experiment was conducted and effects on chironomid assemblages, populations and sex ratios were examined.

Chapter 4: Food limitation in Chironomus tepperi: effects on survival, sex ratios and development across two generations
Toxicity testing can be limited in adequately representing the responses of organisms in situ. Two areas where this is the case is in terms of food availability and previous population exposure. Altered food availability can exert effects similar to toxicity on organisms and can affect the way organisms respond to other stressors. Food availability is likely to differ between field sites, and can be affected by changes to population density, however it is rarely accounted for in field studies and not investigated in laboratory tests. In addition, field populations of organisms can have complicated exposure histories to stressors, which can ultimately affect offspring quality or how offspring respond to stress. To investigate the effects of food limitation over two generations a laboratory experiment was conducted using the Australian test chironomid, Chironomus tepperi. Traditional toxicity endpoints (survival, development and reproduction) were measured in addition to sex specific endpoints (sex ratio and sex-specific development rate).

Chapter 5: A method for biomonitoring with adult Chironomidae
Adult endpoints are highly informative because they represent an organism that has successfully survived surrounding conditions. These endpoints are widely used in field-based experiments and laboratory tests with chironomids. However their use in biomonitoring is limited. There are several methods available for capturing adult chironomids in the field for ecological surveys and nuisance chironomid control. These methods are reviewed with the objective of determining which could be used for biomonitoring. The method chosen was yellow sticky traps, which have previously been used in biomonitoring of terrestrial arthropods and ecological studies of chironomids and other wetland insects. The method was trialled in three pilot surveys to determine if chironomids could be captured on the sticky traps, if chironomids were easily identified on the traps, if trap placement in a wetland affected chironomid capture, what sampling period was appropriate for gaining an accurate representation of the chironomid fauna at a site, and if the captured fauna
differed between different sites. Endpoints such as abundance, species richness and diversity, population size and sex ratios were investigated.

**Chapter 6: Biomonitoring with adult Chironomidae using yellow sticky traps in the Greater Melbourne Area, Australia**

The results of the three pilot surveys in Chapter 5 showed that yellow sticky traps were suitable for capturing adult chironomids. To further demonstrate their biomonitoring potential traps were deployed at 24 sites across the Greater Melbourne Area for a period of five weeks. These sites were selected based on recent chemical analyses of sediments, with sites representing varying types and magnitudes of sediment pollution. Differences in the chironomid fauna at sites were determined, and correlations were made between biological parameters and sediment chemistry.

**Chapter 7: Conclusions**

The main findings from the experimental chapters are summarised and recommendations are made for future related research.
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Chapter 2: The effects of an environmental estrogen, $17\alpha$-ethinylestradiol, on Chironomidae

Abstract
Environmental estrogens such as $17\alpha$-ethinylestradiol (EE2) are common low-concentration contaminants of aquatic ecosystems, with well documented reproductive and developmental effects in aquatic vertebrates. There are few data for the effects of EE2 on the aquatic Diptera Chironomidae, and these are largely limited to the genus *Chironomus*. Little is known of the effects of EE2 on aquatic macroinvertebrate assemblages, and no published data currently exist for Australian macroinvertebrates. To understand the effects of EE2 on Australian chironomids two field-based microcosm experiments were conducted, the first using five concentrations of EE2 and the second testing a high concentration (10 $\mu$g L$^{-1}$) of EE2. Chironomid assemblages, populations and development rate were not affected by EE2 in either experiment. No effects on survival or sex ratios were observed, supporting the results of other studies in *Chironomus*. However, an antennal deformity was observed in two species of *Procladius* at high nominal concentrations of EE2. The antennal deformity involved constrictions or “buckling” of the first antennal segment and has not previously been described in the literature.
Introduction

Endocrine disrupting compounds (EDCs) represent a group of chemical contaminants that first gained considerable attention in the 1990s (Holmes et al., 1997; Ankley et al., 1998; deFur et al., 1999) and have caused concern over the last two decades for several reasons. Firstly, they are capable of exerting effects on organisms at very low concentrations (e.g. Duft et al., 2003; Roepke et al., 2005; Hogan et al., 2006). This is partly due to their highly specific modes of action, which involves disrupting some part of endocrine pathways. Secondly, the number of compounds being added to this group is increasing; compounds that are structurally dissimilar can exert similar effects on the endocrine system. Thirdly, effects on the endocrine system are often difficult to detect with traditional endpoints (e.g. lethality), requiring the use of finer resolution endpoints to determine if impairment has occurred (e.g. Angus et al., 2005). Fourthly, the process of linking observed endocrine disruption to individual fitness and population effects has been slow, although more evidence is emerging that EDCs are detrimental to populations (e.g. Kidd et al., 2007). Finally, many EDCs are widespread in the environment and persist over decades. The EDC tributyltin, for example, has a half-life of over 360 days in aerobic freshwater sediments and a half-life of decades in anaerobic sediments (Dowson et al., 1996).

Environmental estrogens are a group of EDCs that mimic estrogen. Chemicals belonging to this group are structurally diverse, ranging from natural and synthetic estrogens (e.g. 17β-estradiol and 17α-ethinylestradiol) to industrial chemicals and plastics (e.g. bisphenol-A), pesticides (e.g. endosulfan) and phytoestrogens. Environmental estrogens affect human health and cause adverse effects in wildlife (reviewed in Crisp et al., 1998). The synthetic estrogen 17α-ethinylestradiol (EE2) has been implicated in aquatic ecosystem impairment. It is more estrogenic than natural estrogen and is commonly used in female contraceptives and hormone replacement therapy. It is frequently detected in wastewaters and surface waters at low but ecologically significant concentrations in the range of < 1 ng L⁻¹ to 10 ng L⁻¹ (Cargouët et al., 2004; Hintemann et al., 2006; Zuo et al., 2006) but has also been detected at concentrations as high as 15 ng L⁻¹ in river water in Taipei, Taiwan (Chen et al., 2007) and 101.9 ng L⁻¹ in the Douro River estuary, Spain (Ribeiro et al., 2009). In Australia it has been detected in wastewater treatment plant effluents (Leusch et al., 1996).
2006; Tan et al., 2007; Allinson et al., 2010) and is suspected of impacting aquatic organisms in waters receiving effluents (Ying et al., 2008). The effects of EE2 on aquatic vertebrates are well documented and include vitellogenin production (Örn et al., 2006) and demasculinisation (Scholz and Gutzeit, 2000; Angus et al., 2005; Parrott and Blunt, 2005) in males, disrupting female reproduction and development (Scholz and Gutzeit, 2000), feminising sex ratios (Parrott and Blunt, 2005; Hogan et al., 2008) and causing reproductive failure (Nash et al., 2004).

The Chironomidae, a species diverse Dipteran family, are frequently used in biological assessments of aquatic ecosystem integrity (e.g. Chessman, 1995; Wright, 1995; Hawkins et al., 2000) and laboratory toxicity testing (e.g. OECD, 2004a, 2004b). However, few studies have examined the effects of environmental estrogens on chironomids and most studies are restricted to single species laboratory tests with the genus Chironomus. Two of these studies showed conflicting results. Meregalli and Ollevier (2001) reported that EE2 concentrations up to 100 µg L$^{-1}$ had no effect on the frequency of deformities after early instars were exposed for 9 days. In contrast, mouthpart deformities were observed in fourth instar larvae exposed to EE2 at concentrations as low as 10 ng L$^{-1}$ throughout their entire larval life stage (Watts et al., 2003). Data for other species under ecologically relevant conditions are lacking. Field-based experimental approaches, such as the microcosms used by Pettigrove and Hoffmann (2005), are useful for providing such data because they allow multiple species to be tested under conditions similar to those of the field, while using replication, controls and reducing the environmental complexity associated with field surveys.

In vertebrates, estrogen-responsive endpoints are used to detect the presence of estrogenic compounds in effluents and the aquatic environment (e.g. Flammarion et al., 2000; Pettersson et al., 2006). Sex ratio skewing is one such endpoint, with fish populations feminised after exposure to estrogenic compounds (e.g. Länge et al., 2001; Parrott and Blunt, 2005; Vajda et al., 2008). Stressors can induce sex ratio skewing in chironomids, and this appears to be species and stressor specific (Rakotondravelo et al., 2006; Townsend et al., 2009; Ayres et al., unpublished). Sex ratio skewing in chironomids is a potentially sensitive, stressor-specific endpoint that has rarely been
studied, and the effects of estrogenic compounds on chironomid sex ratios have not been reported.

To assess the effects of EE2 on Australian Chironomidae two-field based microcosm experiments were conducted. The first experiment was conducted using five EE2 concentrations to determine if effects in chironomids occur at ecologically relevant concentrations. The second experiment was conducted testing a single high dose of EE2 to confirm the results of the first experiment. Assemblage, population (abundance and sex ratios) and sublethal endpoints (development rate and larval deformities) were assessed. It was hypothesised that sublethal endpoints in Chironomidae would be more sensitive to EE2 exposure than assemblage or population endpoints, and that species would differ in their sensitivities to EE2. From these data it would be determined whether chironomids were suitable for use in other studies to detect and assess the ecological impacts of EE2.
Materials and methods

Experimental design
Two field-based microcosm experiments were conducted to assess the effects of 17α-ethinylestradiol (EE2) on Australian Chironomidae. The first experiment was conducted at a private, disused farm dam at Cottles Bridge, 30 km north east of Melbourne, Australia (37°38’ S, 145°13’E) from the 19th of November, 2008, until the 25th of February, 2009. The site was surrounded by native vegetation and situated in a small catchment free from recent agricultural activity. The experiment consisted of five EE2 treatments (10 ng L⁻¹, 100 ng L⁻¹, 1 µg L⁻¹, 10 µg L⁻¹ (a) and 10 µg L⁻¹ (b)), a control and a solvent control. The 10 µg L⁻¹ (a) treatment was administered at the beginning of the experiment, while the 10 µg L⁻¹ (b) treatment was administered at a later date (16th December 2008). The control treatment was discarded from the analysis because of cross contamination. The second experiment was conducted to validate the results of the first. It was run at Starvation Creek, over 70 km north east of Melbourne (37°43’ S, 145°48’ E) from the 4th of September, 2009, until the 18th of May, 2010. The site was located in a forested catchment with no public access. In this experiment a high estrogen concentration (10 µg L⁻¹) was compared to the control and solvent control treatments.

Each microcosm was an experimental replicate consisting of a 20 L clear polypropylene tank (Starmaid Australia) containing 500 mL of reference sediment and wetland water filtered through a 64 µm nybolt mesh net. Reference sediment was taken from the site where the experiment was conducted. Fine surface deposits of sediment were collected and filtered through a 64 µm nybolt mesh net to standardise particle size and remove coarse debris and indwelling macroinvertebrates (Simpson et al. 2005). In the first experiment 15 L of filtered farm dam water was added to each microcosm, whereas in the second experiment only 10 L of filtered creek water was added to reduce the risk of containers flooding. The microcosms of the first experiment were arranged around the littoral zone of the farm dam, while in the second experiment they were arranged on rafts close to the creek bank to protect the rafts from flash flooding in the creek. In both experiments the microcosms were arranged in a random block design.
EE2 (solid compound, > 98 %, Sigma-Aldrich Pty Ltd, Australia) was dissolved in 100 % ethanol (Merck, Australia) to make a stock solution of 100 mg EE2 L$^{-1}$. The stock was diluted with ethanol to create nominal EE2 concentrations of 10 µg L$^{-1}$, 1 µg L$^{-1}$, 100 ng L$^{-1}$ and 10 ng L$^{-1}$. The solvent control was 2 mL of 100 % ethanol. A solvent control treatment consisted of 2 mL of 100 % ethanol administered to each replicate. In all microcosms the addition of the treatment solution was < 0.5 % of the total volume. Treatment solutions were added to their respective replicates via glass syringes. Each treatment in the first experiment consisted of six replicate microcosms. Two non-experimental replicates were also set up to be sacrificed during the experiment for chemical analyses. In the second experiment the EE2 treatment and solvent control consisted of 10 replicates while 16 replicates were used for the control. In addition, two replicates of each treatment were arranged nearby with the intention of sacrificing these for chemical analyses, but these were destroyed in a flood event.

In both experiments microcosms were covered by coarse netting (hole size 2 cm) to prevent large predatory macroinvertebrates (e.g. dragonflies) from colonising the experiment and to prevent disturbance by vertebrates. The premise of the microcosm approach is that adult aerial macroinvertebrates emerge from a waterway, mate and lay their eggs in the microcosms. The eggs hatch and the larvae develop exposed to any contaminants within the microcosm (Pettigrove and Hoffmann, 2005). When adult Chironomidae began emerging from the microcosms (Day 33 in the first experiment, Day 103 in the second experiment) the microcosms were covered by stockings (Kayser, Victoria) to prevent adults from escaping. Emerging adults were collected twice weekly in the first experiment and weekly in the second experiment using mechanical aspirators (Hausherr’s Machine Words, Toms River, NJ USA) until adult emergence declined and the experiments were terminated. Adults were stored in 100 % ethanol at 4 °C. During the first experiment warm and dry weather (maximum daily temperatures from November to February ranged from 16.6 °C to 46.8 °C) throughout the experiment caused evaporation from the microcosms and these were refilled with wetland water. As the water level of the dam fell, microcosms were shifted to ensure they were partially submerged in the water to regulate microcosm temperature. During December rain events caused the water level in the dam to rise suddenly and on two occasions flooded the microcosms. The microcosm experiments were terminated on Day 91 of the first experiment and Day 256 of the second...
experiment. Larvae remaining in the microcosms were collected and stored in 100 %
ethanol at 4 °C.

Adult and larval chironomids from the microcosms were identified to species level
using the larval key by Cranston (2000) and the adult keys by Freeman (1961) and
Glover (1973). Adults and larvae of Procladius were present in both experiments. A
combined molecular and morphological study on Australian Procladius has found that
P. villosimanus is distinct whereas individuals morphologically belonging to P.
apaludicola may represent a mix of lineages (Carew et al., 2011).

Deformity analyses
Procladius larvae were present in both microcosm experiments and were examined
for head capsule deformities according to Warwick (1989). Animals were dissected
and head capsules were mounted in Hoyers fixative. Mounted specimens were left to
clear for up to a week and then examined for deformities under a compound
microscope. In the first experiment all Procladius paludicola larvae were mounted
and larval instar was calculated from the width of the ligula. In the second experiment
a maximum of 30 predominately fourth instar Procladius villosimanus larvae were
mounted per microcosm. Structures examined for deformities in Procladius larvae
included deformities of the ligula (extra teeth, missing teeth, misshapen teeth, stunted
teeth), paraligulae (asymmetry, bifurcations of spines, stunted size, fusion to the
ligula), mandibles (misshapen, missing teeth), paralabial combs (reduced tooth size
and number) and antennae (misshapen, stunted, absent or additional antennal
segments).

Segment 1 of Procladius paludicola antennae was frequently observed to be
misshapen (Figure 2.6). To quantify the abnormality measurements were taken of the
first antennal segment in experiment 1. The measurements taken were of the first
segment’s inner length (il), outer length (ol), apical width (al) and basal width (bl)
(Figure 2.1). For each antenna the average length (il and ol) and average width (al and
bl) were plotted against each other. Figure 2.2 shows that antennal width was similar
between instars, regardless of abnormality, but the buckling in abnormal individuals
caused a shortening of the length of the antennal segment. The abnormality was therefore a quantifiable deformity.

**Chemical analyses**
In the first experiment non-experimental replicates of each treatment and control group were sacrificed for analyses on two occasions (16th December 2008 and 24th February 2009) with 2 L of water and 20 g of sediment taken from each replicate. A sample of the initial 100 mg EE2 L⁻¹ ethanol stock solution was also taken. Water samples were spiked with 500 µL of concentrated sulphuric acid and all samples were stored at 4 °C in amber glass bottles. Analyses of EE2 concentrations in sediments and water samples from the first experiment were conducted using Solid Phase Extraction onto Oasis HLB 6cc (500mg) cartridges followed by Liquid-Chromatography-Mass Spectrometry/Mass Spectrometry. Analyses were conducted by CSIRO Land and Water (Urrbrae, South Australia).

For the controls, an average reading of 48.6 (SEM = 9.3) ng L⁻¹ was detected in water. This value did not differ significantly to values obtained from any of the treatments at day 97 suggesting that all EE2 had been lost by this time. The control treatment also did not differ from EE2 concentrations detected for any of the treatments at day 27 with the exception of the 10 µg L⁻¹ (b) treatment which had a value of 703.6 (SEM = 74.3) ng L⁻¹. All other readings had an average of < 50 ng L⁻¹. These results point to a rapid loss of EE2 from the microcosms. The 10 µg L⁻¹ (b) treatment had only been added within 24 hours prior to the analysis being undertaken and yet 92.96 % of the EE2 had been lost from this treatment. For the sediment analysis, all samples were at or below the detection limit of 1 µg kg⁻¹.

In the first experiment water quality parameters for electrical conductivity (µS cm⁻¹), temperature (°C) and pH were measured using a field water quality meter (TPS WP-81, Australia) on the 24th of February, 2009. All water quality data were normally distributed (Kolmogorov-Smirnov test, P > 0.05) except for conductivity, which became normal after log-transformation (P = 0.14). The average conductivity ranged from 546.2 (SEM = 19.2) to 629.7 (SEM = 69.5) µS cm⁻¹, the average temperature ranged from 22.2 (SEM = 1.1) to 23.0 (SEM = 0.6) °C and the average pH ranged
from 7.46 (SEM = 0.27) to 8.02 (SEM = 0.12) across the treatments. None of these significantly differed between treatments \( F_{5,27} = 0.12, P = 0.99; F_{5,27} = 1.19, P = 0.34; F_{5,27} = 0.81, P = 0.55 \) for temperature, pH and log-transformed conductivity respectively). In the second experiment water quality was measured in situ on the 14th May 2010 for conductivity, pH and temperature. Data were normally distributed \( (P > 0.05) \) and treatment had no significant effect on temperature \( F_{2,32} = 0.20, P > 0.05 \), conductivity \( F_{2,32} = 0.042, P > 0.05 \) or pH \( F_{2,32} = 0.48, P > 0.05 \). In this experiment the average conductivity ranged from 33.8 (SEM = 3.9) to 35.8 (SEM = 6.4) \( \mu S \ cm^{-1} \), the average temperature ranged from 9.5 (SEM = 0.2) to 9.7 (SEM = 0.2) °C and the average pH ranged from 6.0 (SEM = 0.1) to 6.1 (SEM = 0.1) across the treatments.

Chemical analyses of the water and sediment for EE2 were not conducted due to destruction of the non-experimental replicates in the second experiment.

**Statistical analyses**

Multi-Response Permutation Procedures (MRPP) were used to examine species composition and Indicator Species Analyses were used to examine associations between species and EE2 treatment in PC ORD version 5 (McCune and Mefford, 2006). All other analyses were conducted in PASW Statistics version 18 (SPSS Chicago, IL). Data were tested for normality using Kolmogorov-Smirnov tests, and data that were not normal were log-transformed prior to further analysis. Only adults and fourth instar larvae were analysed further because these represent animals that have successfully survived in a treatment. ANOVAs were used to test for the effects of block and EE2 treatment on the overall abundance of macroinvertebrates, number of species and the abundances of common taxa. EE2 treatment was either treated categorically or as a log-transformed nominal concentration (with the 10 \( \mu g \ L^{-1} \) (b) treatment excluded). Block effects were not significant and did not contribute to the interpretation of the data, so statistics for these are not included. Common species were selected for further analyses if they comprised over 1 % of the total abundance and occurred in more than 10 % of all microcosms. Binary logistic regressions were performed for the presence/absence of common taxa using a forward-Wald method. Significant groupings were determined with post hoc Tukey-B tests.
Sex ratios were analysed for common taxa that had over 90% adult emergence to avoid discrepancies due to differences in male and female emergence rates. Sex ratios were expressed as the proportion female for each microcosm with 10 or more adults present, and were analysed using ANOVAs after arcsine square root transformation. The effects of EE2 on development rate were assessed by calculating adult to larvae ratios for common taxa present in the first experiment as both adults and larvae, and only for microcosms with more than ten animals. Ratios were expressed as the proportion of adults over the total abundance and were arcsine square root transformed prior to analysis with ANOVAs. The first day and median day of adult emergence of common taxa were also determined for each microcosm and analysed using ANOVAs.

In both experiments Likelihood Ratio Statistics ($G$ tests) were conducted to determine if the frequency of antennal deformities significantly differed between instars and EE2 treatment. Contingency table analyses were used to determine the variability of deformity frequency across replicates within individual treatments, and to determine if either treatment or instar (first experiment only) had an effect on deformity frequency. In the first experiment hierarchical log-linear analysis was used to compare the frequency of deformities among treatments and instars. These analyses were not conducted in the second experiment because only fourth instar larvae were examined for deformities. Probability values were corrected with the Bonferroni procedure because multiple tests were conducted using the same organisms from each treatment.
Results

Experiment 1
Assemblage and population level effects

A total of 7,431 individuals from 32 macroinvertebrate taxa colonised the 33 microcosms. Over 96.5% of the total abundance was composed of individuals from the family Chironomidae (Table 2.1). An MRPP test of common taxa that occurred in more than 10% of microcosms showed species composition did not significantly differ across microcosms ($A = 0.0003, P = 0.46$). Indicator Species Analysis also found no significant associations between any common taxa and EE2 treatment except for *Cladopelma curtivalva*, which had a strong association with the 100 ng L$^{-1}$ EE2 treatment ($P = 0.02$).

The data for the average number of taxa were normally distributed (Kolmogorov-Smirnov test, $P = 0.28$). The average number of taxa significantly differed between treatments when considered categorically ($F_{5,27} = 2.74, P = 0.04$) with more taxa occurring in the three lower EE2 treatments (Figure 2.3a). The later addition of the 10 µg L$^{-1}$ (b) EE2 treatment did not result in a significantly different number of taxa than the earlier 10 µg L$^{-1}$ (a) EE2 treatment ($t = 1.03, P > 0.05, d.f. = 9$). When treatment was considered as a log-transformed nominal concentration there was no significant effect of treatment on the number of taxa ($F_{4,23} = 2.69, P = 0.06$).

The average abundance per treatment was normally distributed when log-transformed (Kolmogorov-Smirnov test, $P = 0.128$). The average abundance was higher in the 100 ng L$^{-1}$ treatment (Figure 2.3b) and EE2 treatment had a significant effect on abundance when considered categorically ($F_{5,27} = 2.70, P = 0.042$) and as a log transformed nominal concentration ($F_{4,23} = 2.83, P = 0.048$).

Common taxa that occurred in ten or more microcosms with abundances that comprised over 1% of the total abundance are given in Table 2.1 and were analysed separately. Treatment with EE2 did not significantly determine the presence of common taxa (Table 2.2).
Paratanytarsus grimmii was the most abundant species and was present in over half of the microcosms (Figure 2.4a). Its abundance was not normal, even after log transformation (Kolmogorov-Smirnov, $P < 0.05$). Treatment did not affect the abundance of P. grimmii ($\chi^2 = 3.98$, $P = 0.41$, d.f. = 4). Cladotanytarsus bilinearis had a relatively high frequency of occurrence in all treatments. Its abundance was normally distributed ($P = 0.20$). Treatment had no significant effect on its abundance when considered categorically ($F_{5,27} = 0.64$, $P = 0.67$) or when the nominal concentration was log-transformed ($F_{4,23} = 0.25$, $P = 0.91$) although fewer animals occurred in the 10 $\mu$g L$^{-1}$ (b) treatment (Figure 2.4b).

Procladius paludicola occurred in over 50% of all microcosms for every treatment (Figure 2.4c). Its abundance was normally distributed ($P = 0.081$) and was not significantly affected by categorical treatment ($F_{5,27} = 0.91$, $P = 0.49$) or log-transformed EE2 concentration ($F_{4,23} = 1.18$, $P = 0.35$). Kiefferulus martini exhibited a patchier distribution across microcosms than the preceding species (Figure 2.4d) and its abundance was not normally distributed, even after log transformation ($P < 0.05$). The abundance of K. martini was not affected by EE2 treatment ($\chi^2 = 6.83$, $P = 0.23$, d.f. = 5).

Other taxa were less common in the microcosms and had more patchy distributions, which were not normally distributed even after log-transformation ($P < 0.05$). The abundance of Cladopelma curtivalva was not significantly affected by EE2 treatment ($\chi^2 = 9.23$, $P = 0.1$, d.f. = 5) and only exceeded 10 individuals in the 100 ng L$^{-1}$ EE2 treatment. Dicrotendipes conjunctus occurred in less than 40% of microcosms and was not significantly affected by EE2 ($\chi^2 = 2.67$, $P = 0.75$, d.f. = 5). Similarly, Coelopynia pruinosa occurred in less than 40% of microcosms for any treatment, and was not affected by EE2 treatment ($\chi^2 = 5.32$, $P = 0.38$, d.f. = 5). Only larvae of this taxon were present. Tanytarsus fuscithorax was present in less than 50% of microcosms for any treatment and was not affected by EE2 treatment ($\chi^2 = 8.21$, $P = 0.15$, d.f. = 5). Polypedilum nubifer had very low abundances of less than five animals in three of the microcosms it was present in. However, in one microcosm of the 100 ng L$^{-1}$ treatment it had an abundance of 165 animals. It was not significantly affected by EE2 treatment ($\chi^2 = 6.72$, $P = 0.24$, d.f. = 5). Larsia albiceps had a low frequency of occurrence in all treatments except in the 10 $\mu$g L$^{-1}$ (a) treatment where it was
present in 80% of replicates. It was not significantly affected by EE2 ($\chi^2 = 9.94, P = 0.093, d.f. = 5$). *Polypedilum leei* was present in less than 60% of the microcosms for any treatment and its average abundance never exceeded seven animals. It was not significantly affected by EE2 ($\chi^2 = 6.38, P = 0.27, d.f. = 5$).

**Sex ratios**

Sex ratios for *C. bilinearis* were not significantly skewed by EE2 treatment when treatment was considered categorically ($F_{5,17} = 2.26, P = 0.095$) or as log-transformed nominal concentrations ($F_{4,16} = 2.63, P = 0.073$), although there was a male bias in all treatments and solvent control except for the 10 ng L$^{-1}$ EE2 treatment (Table 2.3). Treatment caused a male bias in *K. martini* compared to the solvent control (Table 2.3), but this was not significant when considered categorically ($F_{4,6} = 0.85, P = 0.54$) or as log-transformed nominal concentrations ($F_{4,6} = 0.85, P = 0.54$). *Dicrotendipes conjunctus* sex ratios were female biased in all EE2 treatments except in the 10 µg L$^{-1}$ (b) treatment (Table 2.3) but could not be analysed for an effect of treatment due to insufficient animals. *Cladopelma curtivalva* sex ratios were male biased in all treatments and the solvent control (Table 2.3), with no significant effect of treatment when treatment was considered categorically ($F_{2,1} = 0.21, P = 0.84$) or as a log-transformed nominal concentration ($F_{2,1} = 0.22, P = 0.84$). No adults of *T. fuscithorax* were present in the solvent control and 10 ng L$^{-1}$ treatment. In the other treatments the sex ratio became male biased with increasing EE2 concentration (Table 2.3) but was not significantly affected by categorical treatment ($F_{2,3} = 0.85, P = 0.51$) or log-transformed nominal EE2 concentration ($F_{1,3} = 0.38, P = 0.58$). There was no effect of EE2 on the sex ratio of *L. albiceps* when treatment was considered categorically ($F_{1,1} = 0.32, P = 0.67$) or as log-transformed nominal concentrations ($F_{1,1} = 0.32, P = 0.67$) although sex ratios were variable (Table 2.3). *Polypedilum leei* and *P. nubifer* occurred in insufficient numbers for analyses to be conducted.

**Development**

Common taxa were analysed for effects on development except for *Paratanytarsus grimmii*, a parthenogenic species capable of reproducing within the microcosms, and *Coelopyinia pruinosa*, which were only present as larvae. The adult to larvae ratio of *C. bilinearis* was not affected by either categorical EE2 treatment and log-transformed nominal EE2 concentrations ($F_{5,17} = 1.22, P = 0.34$ and $F_{4,16} = 1.38, P = 0.29$ for...
categorical and log-transformed nominal treatments respectively). Similarly, *P. paludicola* adult to larvae ratios were not affected by categorical treatment ($F_{5,11} = 2.23, P = 0.13$) or log-transformed nominal treatment ($F_{4,9} = 2.61, P = 0.11$). The adult to larvae ratio of *K. martini* was not significantly affected categorical treatment ($F_{4,6} = 0.89, P = 0.53$) or log-transformed nominal EE2 concentration ($F_{4,6} = 0.89, P = 0.53$). *Cladopelma curtivalva* could not be analysed due to its patchy distribution and low abundance across microcosms.

The effects of EE2 on development were also investigated in terms of the rate of adult emergence between treatments. The first day of emergence for *Cladotanytarsus bilinearis* was similar across treatments (Table 2.4) and there was no effect of categorical treatment ($F_{5,27} = 0.45, P = 0.81$) or log-transformed concentration ($F_{4,23} = 0.49, P = 0.75$). The median day of emergence for *C. bilinearis* was also similar between treatments (Table 2.5) with no effect of treatment considered categorically ($F_{5,27} = 0.29, P = 0.91$) or nominally ($F_{4,23} = 0.23, P = 0.92$). *Procladius paludicola* began emerging from the 1 ng L$^{-1}$ and 10 µg L$^{-1}$ EE2 treatments earlier than other treatments (Table 2.4) but this was not significantly different between treatments when considered categorically ($F_{5,19} = 0.58, P = 0.72$) or as log-transformed concentrations ($F_{4,17} = 0.46, P = 0.76$). The median day of emergence was similar across treatments ($F_{5,20} = 1.02, P = 0.43$ for categorical treatment; $F_{4,18} = 1.03, P = 0.44$ for log-transformed nominal treatment) (Table 2.5). The first day of emergence of *K. martini* was delayed in the 10 µg L$^{-1}$ EE2 treatment (Table 2.4). However, first day of emergence did not significantly vary between treatment considered categorically ($F_{5,10} = 2.42, P = 0.11$) or log-transformed nominal treatment ($F_{4,10} = 1.34, P = 0.32$). The median day of emergence was earlier in the 10 ng L$^{-1}$ and 10 µg L$^{-1}$ EE2 treatments than others (Table 2.5). However, this was not significantly different when considered categorically ($F_{5,10} = 1.36, P = 0.32$) or as log-transformed nominal treatment ($F_{4,10} = 1.03, P = 0.44$).

While *C. curtivalva* began emerging from the solvent control later than other treatments (Table 2.4), the first day of emergence did not significantly differ between EE2 treatments considered categorically ($F_{5,12} = 0.80, P = 0.57$) or as log-transformed nominal concentrations ($F_{4,12} = 0.98, P = 0.48$). Median emergence day was later in the solvent control but was not significantly affected by categorical treatment ($F_{5,12} = 0.58, P = 0.72$).
1.18, \( P = 0.38 \) or nominal EE2 concentration \( (F_{4,12} = 1.41, \ P = 0.29) \) (Table 2.5). Table 2.4 shows that for \( D. \ conjunctus \) the first day of emergence varied a lot across treatments, but was not significantly affected by EE2 treatment \( (F_{4,2} = 3.62, \ P = 0.23 \) for categorical EE2 treatment and \( F_{3,1} = 1.01, \ P = 0.61 \) for log-transformed nominal concentration). The day of median emergence was much earlier in the 10 \( \mu \text{g L}^{-1} \) (b) treatment (Table 2.5) but this difference was not significant when EE2 was considered categorically \( (F_{4,2} = 2.75, \ P = 0.28) \) or as nominal EE2 concentrations \( (F_{3,1} = 0.26, \ P = 0.85) \).

**Larval deformities**

In the first experiment 1,057 \( P. \ paludicola \) individuals were collected and 216 of these were larvae. It was present in over 50 \% of all microcosms for every treatment. The frequency of antennal deformities did not significantly differ between instars \( (G^2 = 4.30, \ P = 0.12, \ d.f. = 2) \). There was a strongly significant effect of EE2 treatment on the frequency of antennal deformities \( (G^2 = 20.07, \ P = 0.001, \ d.f. = 5) \). Antennal deformities occurred more frequently at higher concentrations of EE2, particularly in both 10 \( \mu \text{g L}^{-1} \) treatments. For \( P. \ paludicola \) deformities of the paralabial combs, ligula and mandibles were uncommon and comprised 11 \% of all deformities present (Table 2.6; Figure 2.5). Bifurcations and asymmetry of the paraligulae were more common, making up 33.8 \% of all deformities, and in the 10 \( \mu \text{g L}^{-1} \) (a) EE2 treatment one animal had a paraligula fused to the ligula. Over 54 \% of all deformities were the novel antennal deformity. Contingency table analyses showed that replicates did not significantly differ in deformity frequency within each treatment (Pearson \( \chi^2 = 0.19, \ P = 0.86, \ d.f. = 1 \) for 10ng L\(^{-1}\); \( \chi^2 = 1.73, \ P = 0.65, \ d.f. = 3 \) for 100 ng L\(^{-1}\); \( \chi^2 = 2.43, \ P = 0.31, \ d.f. = 1 \) for 10 \( \mu \text{g L}^{-1} \) (b)) except in the 10 \( \mu \text{g L}^{-1} \) (a) treatment, where replicates significantly differed in deformity frequency \( (\chi^2 = 11.70, \ P = 0.002, \ d.f. = 2) \). In replicate 5 of this treatment the proportion of deformed individuals was lower than for other replicates, however this made little difference to further analyses. Analyses were not conducted for the solvent control where there was only one case or for the 1 \( \mu \text{g L}^{-1} \) treatment because there was no variation between replicates (i.e. no individuals were deformed). Treatment with EE2 had a significant effect on deformity frequency \( (\chi^2 = 28.49, \ P < 0.001, \ d.f. = 5) \) but deformity frequency was not affected by instar \( (\chi^2 = 3.51, \ P = 0.18, \ d.f. = 2) \). The analyses were then conducted by grouping similar treatments, so comparisons were made between treatments with low deformity concentration.
frequency (i.e. solvent control, 10 ng L\(^{-1}\), 100 ng L\(^{-1}\) and 1 µg L\(^{-1}\)) and high deformity frequency (10 µg L\(^{-1}\) (a) and 10 µg L\(^{-1}\) (b)). Again EE2 was found to have a significant effect on deformity frequency (\(\chi^2 = 27.18, P < 0.001, d.f. = 1\)).

Hierarchical log-linear analysis led to a model with two-way interactions with instars varying across treatments (\(\chi^2 = 37.25, P < 0.001, d.f. = 10\)) and the frequency of deformities varying across treatments (\(\chi^2 = 32.31, P < 0.001, d.f. = 5\)) (Figure 2.5) but no interaction between instars and deformity frequency (\(\chi^2 = 0.95, P = 0.62, d.f. = 2\)). When treatments with no deformities (i.e. the solvent control and the 1 µg L\(^{-1}\) treatment) were excluded from the analysis, significant interactions were found with the frequency of deformities varying across larval instars (\(\chi^2 = 28.44, P < 0.001, d.f. = 6\)) and across EE2 treatment (\(\chi^2 = 27.35, P < 0.001, d.f. = 3\)), but with no significant effect of instar on deformity frequency (\(\chi^2 = 0.96, P = 0.62, d.f. = 2\)).

**Experiment 2**

*Assemblage and population level effects*

In the second microcosm experiment over 7,154 animals from 18 taxonomic groups were collected. The most abundant taxonomic group was the Chironomidae, which comprised 95.15 % of the total abundance, and were dominated by five taxa: *Procladius villosimanus, Paramerina levidensis, Chironomus* species, *Cladopelma curtivalva* and *Tanytarsus inextentus* (Table 2.7). Other taxonomic groups that were much less abundant included larvae from the Trichopteran family Hydroptilidae, larvae from the Coleopteran family Dytiscidae, and Dipterans from Culicidae and Ceratopogonidae. Species composition did not significantly vary across the EE2 treatments (MRPP; \(A = 0.006, P = 0.30\)). Indicator species analysis revealed that *P. levidensis* had a weak positive association with the 10 µg L\(^{-1}\) EE2 treatment group (\(P = 0.04\)). *Cladopelma curtivalva* were not analysed further because animals only occurred in one control replicate. The presence of common taxa was not affected by treatment (Table 2.8).

The average number of taxa was normally distributed (Kolmogorov-Smirnov, \(P = 0.28\)). EE2 treatment had a significant effect on the number of taxa (\(F_{2,32} = 4.73, P = 0.02\)) with a greater number of taxa occurring in the EE2 treatment than the control or
solvent control (Figure 2.7a). Data for the total abundance were also normally distributed (Kolmogorov-Smirnov, $P = 0.16$) but treatment had no effect ($F_{2,32} = 0.59$, $P = 0.56$) (Figure 2.7b).

Data for *Chironomus* species were normally distributed ($P = 0.47$). *Chironomus* spp. occurred in over 90% of microcosms for each treatment and were almost twice as abundant in the solvent control than in the control and EE2 treatment, however treatment had no effect on its abundance ($F_{2,32} = 2.18$, $P = 0.13$). *Procladius villosimanus* was the second most abundant species with 1,341 adults and fourth instar larvae present in the microcosms. It had a high frequency of occurrence across all treatments and data were normally distributed ($P = 0.71$). While average abundance seemed to decline in the solvent control and the EE2 treatment compared to the control, treatment also had no effect on its abundance ($F_{2,32} = 0.86$, $P = 0.43$).

Data for *Paramerina levidensis* were normally distributed after log-transformation ($P = 0.14$) and it was present in 65% to 70% of microcosms for all treatments. The average abundance was less than 18 animals for all treatments, and EE2 treatment did not significantly affect its abundance ($F_{2,32} = 0.022$, $P = 0.98$). *Tanytarsus inextentus* was less common and in less than 60% of microcosms for all treatments. Data for this species were not normally distributed after log-transformation ($P < 0.001$). EE2 treatment had no effect on its abundance (Kruskal-Wallis tests; $\chi^2 = 1.68$, $P = 0.43$, d.f. = 2 for treatment).

**Sex ratios**

The sex ratios of *Chironomus* spp. were similar across treatments with 47.5% female for the control, 45.5% female for the solvent control and 48.6% female for the EE2 treatment. Treatment had no effect on sex ratios ($F_{2,23} = 0.74$, $P = 0.49$ for treatment). *Procladius villosimanus* sex ratios were also similar across treatments and ranged between 35 to 40% female. EE2 treatment did not affect *P. villosimanus* sex ratios ($F_{2,17} = 0.80$, $P = 0.47$). The proportion female of *P. levidensis* were between 51 to 57% in the control and solvent control, while a female bias was seen in the EE2 treatment with 65.1% of adults female. Treatment with EE2 did not significantly affect its sex ratio ($F_{2,14} = 0.58$, $P = 0.58$). *Tanytarsus inextentus* sex ratios were also not affected by EE2 treatment ($F_{1,2} = 0.24$, $P = 0.67$), although the solvent control was
male biased with only 38.5% females whereas the control and EE2 treatment had over 50% females.

*Larval deformities*

In the second experiment *P. villosimanus* had a total abundance of 3,348 individuals, and 2,732 of these were adults. It occurred across all treatments. Again, the most common deformities in *Procladius* larvae were of the first antennal segment, followed by deformation of the paraligulae (Table 2.9). EE2 treatment had a significant effect on deformity frequency when deformities were expressed as the number of individuals deformed ($G^2 = 80.47, P < 0.001, d.f. = 2$). Contingency table analysis showed that the deformity frequency did not significantly differ between replicates within a treatment after correction for multiple comparisons (3) ($\chi^2 = 27.44, P = 0.015, d.f. = 13$ for control; $\chi^2 = 2.43, P = 0.73, d.f. = 4$ for solvent control; $\chi^2 = 14.07, P = 0.025, d.f. = 6$ for EE2). Treatment had a significant effect on the frequency of deformities ($\chi^2 = 29.94, P < 0.001, d.f. = 2$). When the control and solvent control were grouped together and compared to EE2 the effect of group was significant ($\chi^2 = 28.59, P < 0.001, d.f. = 1$), demonstrating that EE2 treatment caused an increase in the frequency of deformities (Figure 2.8a).

Some individuals expressed more than one deformity, while others had damaged characters that could not be assessed. To account for these, deformities were also analysed as the number of all characters assessed that exhibited deformation. Treatment had a significant effect on the number of deformed characters ($G^2 = 28.28, P < 0.001, d.f. = 2$). When deformed characters were analysed by contingency table analysis deformity frequency was not found to significantly differ between replicates within treatments after correction for multiple comparisons (3) ($\chi^2 = 31.97, P = 0.19, d.f. = 26$ for control; $\chi^2 = 4.49, P = 0.81, d.f. = 8$ for solvent control; $\chi^2 = 21.61, P = 0.042, d.f. = 12$ for EE2). Treatment significantly affected the number of characters deformed ($\chi^2 = 31.07, P < 0.001, d.f. = 4$) and when the control and solvent control were considered as a single group, EE2 treatment caused a significant increase in the number of characters deformed ($\chi^2 = 29.17, P < 0.001, d.f. = 2$) (Figure 2.8b).

Antennal deformities were the most common deformity (Table 2.9) so these were also analysed separately. Treatment significantly affected the frequency of antennal
deformities ($G^2 = 59.33, P < 0.001, d.f. = 2$). Antennal deformities did not significantly differ within treatments ($\chi^2 = 23.20, P = 0.091, d.f. = 13$ for control; $\chi^2 = 4.81, P = 0.37, d.f. = 4$ for solvent control) except in the EE2 treatment ($\chi^2 = 20.67, P = 0.002, d.f. = 6$). Two replicates within this treatment had a low number of antennal deformities; however there were fewer fourth instar larvae in these replicates and further analysis of the results were not affected by this variation. Treatment had a significant effect on the number of antennal deformities present ($\chi^2 = 28.51, P < 0.001, d.f. = 2$). More antennal deformities were present in larvae from the EE2 treatment than either the control or solvent control (Figure 2.8c). This difference was shown to be significant when the control and solvent control were combined and compared to the EE2 treatment ($\chi^2 = 28.42, P < 0.001, d.f. = 1$).
Discussion
Chronic exposure to 17α-ethinylestradiol increased the number of taxa present in the microcosms but had no significant effects on assemblage composition or populations of Australian Chironomidae. Development rate and sex ratios of common species were also unaffected. High nominal concentrations of EE2 induced head capsule deformities in the larvae of Procladius paludicola and P. villosimanus. The most common deformity was “buckling” of the first antennal segment, which has not been described in the literature before. However, deformity frequency was greatest at high concentrations of EE2 and thus is unlikely to occur at ecologically relevant concentrations.

Environmental fate of EE2
Compared to natural estrogens, EE2 is more persistent in the environment and resistant to biodegradation (Cargouët et al., 2004). However, under aerobic conditions EE2 rapidly undergoes microbial degradation (Colucci and Topp, 2001; Ying and Kookana, 2005; Czajka and Londry, 2006). In aerobic soils EE2 can degrade within seven days (Ying and Kookana, 2005), while the presence of suspended solids in aquatic environments rapidly increases EE2 removal by increasing the surface area for bacterial attachment (Liu et al., 2009). Photolysis is another important degradation pathway. In seawater under natural irradiation EE2 has a halflife of 1.5 days (Zuo et al., 2006) and in river waters the half-life is 2.3 hours (Lin and Reinhard, 2005). Microalgae enhance the photodegradation of EE2; in one study the presence of microalgae effectively doubled the rate of degradation (Ge et al., 2009).

EE2 rapidly degraded in the first experiment and photolysis was presumably the dominant degradation pathway. The weather conditions were generally hot and dry, with maximum daily temperatures ranging from 26.6 °C to 46.8 °C and below average rainfall (Bureau of Meteorology, 2012). Microcosms were exposed to full sunlight for much of the day (K. T. personal observation) and water loss from the microcosms was excessive. EE2 is hydrophobic, with a high log K_{oc} of 3.8, and readily adsorbs to sediments and organic carbon (e.g. Lai et al., 2000; Ying et al., 2003). It also adsorbs to substances such as iron oxide (Lai et al., 2000), stainless
steel, polycarbonate plastic and to a lesser extent glass, Teflon and PVC (Walker and Easton, 2010). EE2 was not detected in the sediments in the current study, although it may have adsorbed to the polypropylene tanks and sediments at concentrations below the detection limits.

**Effects of EE2 on aquatic macroinvertebrates**

Pollutants in wastewater effluents can impact the aquatic communities of receiving waters. For example, in the Central Texas Plains ecoregion (USA) fish communities from sites receiving effluent were dominated by more tolerant species while several sensitive species and top predators were absent (Porter and Janz, 2003). Increased plasma vitellogenin in males of one species, *Lepomis megalotis*, provided evidence that estrogenic substances in the effluent were affecting the fish. Effluents have also been shown to affect aquatic macroinvertebrate communities (e.g. Coimbra et al., 1996) but studies exploring the impacts of environmental estrogens on macroinvertebrate communities are lacking. While chironomid assemblages were not affected by EE2, more species were present in the lower EE2 treatments of the first experiment and the only EE2 treatment of the second experiment. This could be due to an indirect effect of EE2 via microbial responses. The natural estrogen 17β-estradiol increased the microbial biomass and altered the structure of microbial communities in soils, including increasing the dominance of bacteria with aerobic respiration (Chun et al., 2006). Micro-organism diversity in activated and digested sewage sludge decreased in the presence of EE2, although at concentrations over 1000 times greater than the highest used in the current study (Wang et al., 2013). While EE2 was possibly affecting microbiota in the microcosms, the concentrations of EE2 available were two to three orders of magnitude lower than those used by Wang et al. (2013), so subsequent effects on chironomids were likely to be minimal. While other effects of EE2 on chironomid assemblages were not observed in the current study, effects on assemblages could still occur *in situ* by affecting the predators and prey of chironomids. Fish populations, for example, are susceptible to EE2, and Kidd et al. (2007) showed that addition of EE2 to a lake caused *Pimephales promelas* populations to crash because of reproductive failure over a period of several years.
Reproductive and multigenerational endpoints were not measured in this study, and it is conceivable that if EE2 affected reproduction, chironomid populations could change over several generations. However, other studies have shown estrogenic chemicals do not affect chironomid reproduction. Bisphenol-A and EE2 did not have a dose dependent effect on the number of egg ropes produced by female *Chironomus riparius*, and adult emergence was not affected over two generations (Watts et al., 2001). *Chironomus tentans* reproduction was only affected at very high EE2 concentrations of 3.1 mg L\(^{-1}\) when no adults emerged (Dussault et al., 2008).

Reproductive effects in some other invertebrates are negligible or do not occur at ecologically relevant concentrations. In *Hydra vulgaris* sperm activity and oocyte production was reduced at 500 µg L\(^{-1}\) of EE2 (Pascoe et al., 2002). 17β-estradiol, EE2 and medroxyprogesterone had little effect on the survival or reproduction of *Ceriodaphnia dubia* at concentrations up to 5 mg L\(^{-1}\) (Jujosky et al., 2008). Molluscs are more sensitive to estrogenic compounds, with *Potamopyrgus antipodarum* reproduction stimulated at lower EE2 concentrations and inhibited at 100 ng L\(^{-1}\), similar to the fathead minnow *Pimephales promelas* (Jobling et al., 2003).

In the first experiment EE2 appeared to skew the sex ratios of three species, although this was highly variable within a treatment and not dose dependent. Reduced variability in the second experiment showed that EE2 did not skew sex ratios, concurring with other studies that found no effect of EE2 on sex ratios in *Chironomus riparius* (Watts et al., 2001) or *Chironomus tentans* (Dussault et al., 2008). In contrast, sex-specific endpoints in vertebrates are highly sensitive to environmental estrogen exposure (e.g. Scholz and Gutzeit, 2000; Parrott and Blunt, 2005; Hogan et al., 2008). This is likely due to differences in sexual development, which, in insects, is not regulated by androgens and estrogens but by juvenile hormones and ecdysteroids. Vertebrate-like steroids such as estrogens have been detected in several insect orders including Diptera (e.g. Mechoulam et al., 1984) and an estrogen-related gene that responds to an estrogenic compound (di(2-ethylhexyl)phthalate) has been characterised in *Chironomus riparius* (Park and Kwak, 2010). The presence and role of estrogens and estrogen-related genes in insects are still poorly understood, although in the silkmoth *Bombyx mori* estrogen is produced endogenously, has a functional role in the posterior silk glands (Keshan and Ray, 2001) and has physiological effects in the fat bodies of female larvae (Roy et al., 2007). The chironomids in the current
The growth and development of an organism are often susceptible to a variety of stressors, and this is especially true of chemicals that may directly interfere with developmental pathways. Previous studies have shown that *Chironomus riparius* exposed to 1 ng L\(^{-1}\) of EE2 emerged earlier than controls (Watts *et al*., 2001). At higher EE2 concentrations delayed emergence was only observed in females, while bisphenol-A caused females to emerge earlier than controls, while males in later generations experienced delayed emergence. Similarly, Dussault *et al*. (2008) found that only females had delayed emergences at 0.14 mg L\(^{-1}\), but this was attributed to experimental variation rather than EE2 treatment. This differs from the results of the current study where the emergence of several chironomid species was not affected by EE2 exposure.

**Effects of EE2 on deformity frequency in *Procladius* species**
High concentrations of EE2 caused an increased incidence of deformities in *P. paludicola* larvae in the first experiment and in *P. villosimanus* larvae in the second experiment. “Buckling” of the first antennal segment was the most common type of deformity in both species. Antennal deformities of *Procladius* species have been comprehensively described in the past (Warwick, 1989; 1991) but the antennal deformity observed in the current study was not described in those or other studies where deformities in *Procladius* were noted (e.g. Pettigrove, 1989; Dermott, 1991; Townsend *et al*., 2009). Similar deformities in other chironomids are also not described in the literature (e.g. Warwick, 1985; Janssens de Bisthoven *et al*., 1998b; Bhattacharyay *et al*., 2005).

Chironomid deformities have been linked to instar, with certain types of deformities more prevalent in specific instars, and some deformities that are present in early instars repaired at moulting (Servia *et al*., 2002). Deformities induced by EE2 were not affected by instar; in particular the antennal deformity was present in all instars and was possibly transferred from one instar to the next, as has been observed with some other deformities (Vermuelen *et al*., 2000b; Servia *et al*., 2002). Larval instars
can differ in their sensitivities to toxicants (e.g. Nebeker et al., 1984; Williams et al., 1986), but there was no evidence of this in regards to deformity induction by EE2. Deformity incidence was similar at high estrogen exposures, regardless of whether larvae were exposed from an early age (in the early 10 μg L⁻¹(a) treatment) or whether exposure occurred later, as in the 10 μg L⁻¹(b) treatment.

Despite over 30 years of research into the use of chironomid deformities for bioindication, little is known about the mechanisms by which deformities occur. Links have been made between subcellar activities and deformity incidence, such as increased numbers of active nucleoli in larvae with deformities (Meregalli et al., 2002). This indicated increased rRNA synthesis and by consequence increased protein synthesis, probably to improve larval tolerance to the toxicant. However, these links are correlative and fail to demonstrate the mechanism by which deformations are occurring. Deformation of the antennal segment may have been due to pressure applied to the coverslip during mounting. However, all head capsules from each treatment were mounted the same way and the resultant “buckling” was more prevalent in high EE2 treatments, indicating that EE2 was having some effect on the antennae. It suggests possible weakening of the exoskeleton.

Deformities do not develop as larvae age but occur during moulting, which indicates a physiological disturbance at moulting is the cause (Janssens de Bisthoven et al., 1992). Moulting is a crucial time in exoskeleton development in insects and is an endocrine controlled process involving complicated hormonal and neurohormonal regulation. The link between deformities and moulting could indicate endocrine disruption in chironomids. Chironomid deformities caused by known vertebrate EDCs, such as 4-nonylphenol (Meregalli et al. 2001), bisphenol A and EE2 (Watts et al., 2003) support this, with the two latter chemicals also affecting moulting in C. riparius. The induction of deformities in Procladius species could therefore be indicative of endocrine disruption or physiological disturbance by EE2. Disruptions to normal processes leading up to and during moulting, such as local disruptions in the epidermis, inhibition of epidermal cell proliferation, changes to actin filament patterning, induction of irregular cell growth, alterations to sclerotisation and impairment of repair systems have all been suggested as possible mechanisms by
which toxicants may cause deformities at moulting (Servia et al., 2002). Each of these mechanisms could also potentially weaken the exoskeleton.

Interference of normal chitin formation by EE2 could have caused the antennal deformity. Interference with chitin has caused morphological deformities in other insects, such as in adults of the lepidopteran *Solanum tuberosum* L. that survived exposure to the chitin synthesis inhibitor lufenuron (Edomwande et al., 2000). Alternatively EE2 could interfere with the sclerotisation of the antennae, leading to a weakened exoskeleton. In insects bursicon is released from neurosecretory cells in the ventral ganglia after a new exoskeleton has been synthesised. It sclerotises the exoskeleton, but normally only parts of the exoskeleton are sclerotised; the pattern of sclerotisation is both taxon and life stage specific. For example, in *Drosophila melanogaster* bursicon is involved in adult cuticle hardening and wing expansion (Baker and Truman, 2002) and pupal cuticle hardening (Loveall and Deitcher, 2010) but not the sclerotisation of the entire larval cuticle. In chironomid larvae the head capsule is sclerotised. Any interference with sclerotisation could result in a weakened exoskeleton that would deform more easily under pressure. However, if this were the case there would also be a higher incidence of breakages and deformities in other structures of the head capsule but these were not observed.

The consequences of deformation on the survival and fitness of individuals is not fully understood, with studies presenting differing results. One study found no difference in larval length, dry weight or imaginal disc development between normal and deformed larvae (Janssens de Bisthoven et al., 1992). Other studies have shown that deformed larvae, compared to normal larvae, have reduced emergence (Gerhardt and Janssens de Bisthoven, 1995; Janssens de Bisthoven et al., 1998a), reduced locomotion and increased ventilation (Gerhardt and Janssens de Bisthoven, 1995), decreased weight, increased energy content, lower survival and slower emergence (Janssens de Bisthoven et al., 1998a). However, these effects were not consistent between sites, with no differences observed between deformed and normal larvae at some sites. Population or toxicant differences between sites may have caused the different responses.
The effects of deformities on populations and communities are also unknown. Reduced chironomid abundances due to deformity-caused mortalities could potentially exert trophic effects on predator and prey species of chironomids. Deformed larvae may also have higher body burdens of some contaminants than normal larvae (e.g. Dickman et al., 1992; Janssens de Bisthoven et al., 1992), which could constitute a significant risk to chironomid predators including fish, birds and other invertebrates. Increased body burdens of EE2 have been measured in chironomid larvae (Dussault et al., 2009) and thus could potentially exert effects on higher trophic organisms that prey on affected chironomids.

**Conclusions**
Aside from increasing the number of chironomid species present, 17α-ethinylestradiol had no effects on assemblages, populations, sex ratios or the development rate of several Australian chironomid species. A novel antennal deformity was observed in two species from the genus *Procladius*, demonstrating a possible species-specific and chemical specific endpoint, but significant responses were only observed at high nominal concentrations of EE2. Chironomidae are unlikely to be directly impaired by exposure to EE2 at ecologically relevant concentrations, and would not be useful for monitoring ecological stress due to environmental contamination by the synthetic estrogen.
Acknowledgements
I would like to thank CSIRO Land and Water, South Australia for the chemical analyses, Maria Rajendran for allowing us to use her dam at Cottles Bridge, and Melbourne Water for access to Starvation Creek. Bryant Gagliardi, Victor Kabay, Matthew O’Brien, Steve Marshall, Katy Jeppe, Rebecca Brown, Sara Hoskin and Daniel MacMahon assisted with fieldwork. I also thank Ary Hoffmann and Vincent Pettigrove for their help with experimental design, statistical analyses and reviewing this chapter. Funding for this research was provided by Melbourne Water Corporation and the Australian Research Council through their Fellowship scheme.

References


Figures

Figure 2.1. Measurements taken of a *Procladius paludicola* first antennal segment. al = apical width, il = inner length, ol = outer length, bl = basal length.
Figure 2.2. Comparison between the average length and average width of the first antennal segment of *Procladius paludicola*. White points represent normal antennae and black points represent “buckled” antennae. Diamonds represent second instar larvae. Circles represent third instar larvae. Triangles represent fourth instar larvae.
Figure 2.3. (a) Number of taxa per EE2 treatment (average ± SEM) and (b) log-transformed average abundance per EE2 treatment (average ± SEM).
Figure 2.4. The average abundance of (a) *Paratanytarsus grimmii*, (b) *Cladotanytarsus bilinearis*, (c) *Procladius paludicola* and (d) *Kiefferulus martini* across the 17α-ethinylestradiol (EE2) treatments. Columns represent the average number of individuals for each EE2 treatment (log-transformed ± SEM). Diamonds represent the proportion of microcosms colonised per treatment.
Figure 2.5. Total deformity frequency in larvae of *Procladius paludicola* in the first microcosm experiment. Error bars are the upper 95% and lower 95% confidence intervals.
**Figure 2.6.** Deformities of the first antennal segment in *Procladius paludicola* larvae with (a) a normal antenna and (b)-(d) showing varying degrees of “buckling”. The arrow points to the deformity.
Figure 2.7. (a) The number of taxa and (b) the abundance of adult and larval macroinvertebrates (average ± S.E.).
Figure 2.8. The frequency of deformities in Procladius villosimanus fourth instar larvae given as (a) the proportion of individuals with deformities, (b) the proportion of deformed characters out of all characters assessed and (c) the proportion of individuals with antennal deformities in the second microcosm experiment. Error bars are the upper and lower 95% confidence intervals.
**Tables**

**Table 2.1.** List of common taxa from the Chironomidae.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Taxon</th>
<th>Number of individuals</th>
<th>% total abundance</th>
<th>Number of adults</th>
<th>Number of microcosms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Paratanytarsus grimmii</em></td>
<td>2905</td>
<td>39.09</td>
<td>953</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td><em>Cladotanytarsus bilinearis</em></td>
<td>1174</td>
<td>15.80</td>
<td>1166</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td><em>Procladius paludicola</em></td>
<td>1057</td>
<td>14.22</td>
<td>841</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td><em>Kiefferulus martini</em></td>
<td>557</td>
<td>7.50</td>
<td>485</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td><em>Cladopelma curtivalva</em></td>
<td>311</td>
<td>4.19</td>
<td>303</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td><em>Coelopynia pruinosa</em></td>
<td>248</td>
<td>3.34</td>
<td>0</td>
<td>7</td>
</tr>
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<td>7</td>
<td><em>Dicrotendipes conjunctus</em></td>
<td>193</td>
<td>2.60</td>
<td>185</td>
<td>7</td>
</tr>
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<td>8</td>
<td><em>Tanytarsus fuscithorax</em></td>
<td>178</td>
<td>2.40</td>
<td>178</td>
<td>8</td>
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<tr>
<td>9</td>
<td><em>Polypedilum nubifer</em></td>
<td>171</td>
<td>2.30</td>
<td>170</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td><em>Larsia albiceps</em></td>
<td>133</td>
<td>1.80</td>
<td>129</td>
<td>14</td>
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<tr>
<td>11</td>
<td><em>Polypedilum leei</em></td>
<td>100</td>
<td>1.35</td>
<td>98</td>
<td>12</td>
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</table>
**Table 2.2.** The effect of $17\alpha$-ethinylestradiol treatment on the presence of a species in a microcosm. Treatment is considered categorically (Categorical) or as log-transformed nominal EE2 concentration with the exclusion of data for the solvent control and $10\mu g \text{ L}^{-1}$ (b) treatments (Log-transformed). Degrees of freedom for categorical treatment = 5. Degrees of freedom for log-transformed treatment = 1. $P = 0.0025$ after correction for multiple comparisons (20).

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment (Categorical)</th>
<th>Treatment (Log-transformed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald</td>
<td>$P$</td>
</tr>
<tr>
<td><em>Paratanytarsus grimmii</em></td>
<td>5.30</td>
<td>0.38</td>
</tr>
<tr>
<td><em>Cladotanytarsus bilinearis</em></td>
<td>5.78</td>
<td>0.33</td>
</tr>
<tr>
<td><em>Procladius paludicola</em></td>
<td>3.28</td>
<td>0.66</td>
</tr>
<tr>
<td><em>Kiefferulus martini</em></td>
<td>6.71</td>
<td>0.24</td>
</tr>
<tr>
<td><em>Cladopelma curtivalva</em></td>
<td>10.52</td>
<td>0.06</td>
</tr>
<tr>
<td><em>Dictotendipes conjunctus</em></td>
<td>3.08</td>
<td>0.69</td>
</tr>
<tr>
<td><em>Coelopynia pruinosa</em></td>
<td>5.68</td>
<td>0.34</td>
</tr>
<tr>
<td><em>Tanytarsus fuscithorax</em></td>
<td>7.78</td>
<td>0.17</td>
</tr>
<tr>
<td><em>Polypedilum nubifer</em></td>
<td>5.15</td>
<td>0.40</td>
</tr>
<tr>
<td><em>Larsia albiceps</em></td>
<td>9.12</td>
<td>0.10</td>
</tr>
<tr>
<td><em>Polypedilum leei</em></td>
<td>5.78</td>
<td>0.33</td>
</tr>
<tr>
<td>Treatment</td>
<td>Species</td>
<td>SC</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>Cladotanytarsus bilinearis</td>
<td>0.38 (0.07 - 0.08)</td>
</tr>
<tr>
<td></td>
<td>Kiefferulus martini</td>
<td>0.80 (0.21 - 0.16)</td>
</tr>
<tr>
<td></td>
<td>Dicrotendipes conjunctus</td>
<td>- (0.00 - 0.00)</td>
</tr>
<tr>
<td></td>
<td>Cladopelma curtivalva</td>
<td>0.41 (0.17 - 0.20)</td>
</tr>
<tr>
<td></td>
<td>Tanytarsus fuscithorax</td>
<td>- (0.11 - 0.11)</td>
</tr>
<tr>
<td></td>
<td>Larsia albiceps</td>
<td>- (0.24 - 0.56)</td>
</tr>
</tbody>
</table>
Table 2.4. The first day of adult emergence (average ± S.E.) of common Chironomidae species from microcosms treated with 17α-ethinylestradiol.

<table>
<thead>
<tr>
<th>Species</th>
<th>17α-ethinylestradiol treatment</th>
<th>Solvent control</th>
<th>10 ng L⁻¹</th>
<th>100 ng L⁻¹</th>
<th>1 µg L⁻¹ (a)</th>
<th>10 µg L⁻¹</th>
<th>10 µg L⁻¹ (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladotanytarsus bilinearis</td>
<td></td>
<td>43.40 ±</td>
<td>43.40 ±</td>
<td>44.33 ±</td>
<td>43.20 ±</td>
<td>47.17 ±</td>
<td>43.75 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.60</td>
<td>0.98</td>
<td>1.23</td>
<td>2.20</td>
<td>4.64</td>
<td>2.75</td>
</tr>
<tr>
<td>Kiefferulus martini</td>
<td></td>
<td>66.00 ±</td>
<td>47.50 ±</td>
<td>57.00 ±</td>
<td>60.67 ±</td>
<td>55.50 ±</td>
<td>80.00 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00</td>
<td>4.27</td>
<td>4.16</td>
<td>1.89</td>
<td>4.92</td>
<td>0.00</td>
</tr>
<tr>
<td>Cladopelma curtivalva</td>
<td></td>
<td>62.67 ±</td>
<td>45.00 ±</td>
<td>47.67 ±</td>
<td>59.00 ±</td>
<td>51.00 ±</td>
<td>55.00 ±</td>
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<td></td>
<td></td>
<td>9.13</td>
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<td>5.90</td>
<td>0.00</td>
<td>5.83</td>
<td>0.00</td>
</tr>
<tr>
<td>Procladius paludicola</td>
<td></td>
<td>60.50 ±</td>
<td>55.33 ±</td>
<td>69.50 ±</td>
<td>63.60 ±</td>
<td>58.67 ±</td>
<td>53.33 ±</td>
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<tr>
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<td></td>
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<td>7.45</td>
<td>8.62</td>
<td>4.77</td>
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<td>2.96</td>
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<tr>
<td>Dicrotendipes conjunctus</td>
<td></td>
<td>80.00 ±</td>
<td>59.00 ±</td>
<td>81.50 ±</td>
<td>73.00 ±</td>
<td>66.00 ±</td>
<td>46.50 ±</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0.00</td>
<td>8.50</td>
<td>0.00</td>
<td>0.00</td>
<td>5.50</td>
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</tbody>
</table>
Table 2.5. The median day of adult emergence (average ± S.E.) of common Chironomidae species from microcosms treated with 17α-ethinylestradiol.

<table>
<thead>
<tr>
<th>Species</th>
<th>Solvent control</th>
<th>10 ng L-1</th>
<th>100 ng L-1</th>
<th>1 µg L-1</th>
<th>10 µg L-1 (a)</th>
<th>10 µg L-1 (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cladotanytarsus bilinearis</em></td>
<td>2.18</td>
<td>1.89</td>
<td>3.12</td>
<td>2.76</td>
<td>4.29</td>
<td>8.63</td>
</tr>
<tr>
<td><em>Kiefferulus martini</em></td>
<td>70.00 +</td>
<td>55.50 +</td>
<td>68.67 +</td>
<td>66.33 +</td>
<td>62.75 +</td>
<td>40.00 +</td>
</tr>
<tr>
<td><em>Cladopelma curtivalva</em></td>
<td>8.08</td>
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<td>5.79</td>
<td>0.00</td>
<td>4.82</td>
<td>0.00</td>
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<tr>
<td><em>Procladius paludicola</em></td>
<td>70.50 +</td>
<td>63.67 +</td>
<td>77.25 +</td>
<td>72.60 +</td>
<td>75.50 +</td>
<td>72.00 +</td>
</tr>
<tr>
<td><em>Dicrotendipes conjunctus</em></td>
<td>0.00</td>
<td>80.00 +</td>
<td>70.00 +</td>
<td>81.50 +</td>
<td>73.00 +</td>
<td>48.00 +</td>
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<td></td>
<td>0.00</td>
<td>0.00</td>
<td>8.50</td>
<td>0.00</td>
<td>7.00</td>
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Table 2.6. The number and type of head capsule deformities in *Procladius paludicola* larvae treated with 17α-ethinylestradiol in the first microcosm experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Instar</th>
<th>n</th>
<th>Number deformed</th>
<th>Antennae</th>
<th>Ligula</th>
<th>Paraligulae</th>
<th>Paralabial combs</th>
<th>Mandibles</th>
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<tbody>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
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</tr>
<tr>
<td>10 ng L(^{-1})</td>
<td>2</td>
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<td>0</td>
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<td>0</td>
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<tr>
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</tr>
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<td>100 ng L(^{-1})</td>
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<td>2</td>
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<td>1 µg L(^{-1})</td>
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<td>(a)</td>
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<td>1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.7. List of common Chironomidae taxa from the microcosms.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Taxon</th>
<th>Number of individuals</th>
<th>% total abundance</th>
<th>Number of adults</th>
<th>Number of microcosms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Procladius villosimanus</em></td>
<td>3348</td>
<td>46.8</td>
<td>616</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td><em>Chironomus</em> species</td>
<td>1779</td>
<td>24.87</td>
<td>1761</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td><em>Paramerina levidensis</em></td>
<td>535</td>
<td>7.48</td>
<td>426</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td><em>Tanytarsus inextentus</em></td>
<td>289</td>
<td>4.04</td>
<td>216</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td><em>Cladopelma curtivalva</em></td>
<td>61</td>
<td>0.85</td>
<td>22</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2.8. The effect of 17α-ethinylestradiol treatment on the presence of a species in a microcosm. Degrees of freedom = 2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Wald</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chironomus</em> spp.</td>
<td>0.90</td>
<td>0.64</td>
</tr>
<tr>
<td><em>Procladius villosimanus</em></td>
<td>2.97</td>
<td>0.23</td>
</tr>
<tr>
<td><em>Paramerina levidensis</em></td>
<td>0.03</td>
<td>0.99</td>
</tr>
<tr>
<td><em>Tanytarsus inextentus</em></td>
<td>1.37</td>
<td>0.50</td>
</tr>
</tbody>
</table>
Table 2.9. Deformities in the head capsules of fourth instar *Procladius villosimanus* larvae exposed to 17α-ethinylestradiol in the second microcosm experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Number deformed</th>
<th>Antennae</th>
<th>Ligula</th>
<th>Paraligulae</th>
<th>Paralabial combs</th>
<th>Mandibles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>267</td>
<td>38</td>
<td>19</td>
<td>7</td>
<td>15</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Solvent control</td>
<td>84</td>
<td>17</td>
<td>5</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 µg L⁻¹ EE2</td>
<td>137</td>
<td>52</td>
<td>33</td>
<td>4</td>
<td>18</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Chapter 3: The effects of tebufenozide and diuron on Chironomidae

Abstract
A field-based microcosm experiment was used to expose Australian Chironomidae to two pesticides, tebufenozide and diuron, that have previously been shown to have sex specific effects in Chironomidae. Chironomid assemblages were not strongly affected by either pesticide. *Chironomus* species abundance was affected by tebufenozide, while the abundance of other taxa were unaffected by either pesticide. A more sensitive endpoint was sex ratio skewing; both compounds caused sex ratio skewing in several chironomid species, indicating this endpoint may be sensitive to low concentrations of contaminants. Responses were species-specific and chemical dependant. *Procladius villosimanus* showed a particularly strong female bias in exposure to diuron, confirming previous results in another *Procladius* species and highlighting the potential use of feminised *Procladius* sex ratios to indicate diuron contamination in the field. While the mechanisms of action for both pesticides were not investigated, sex ratio skewing may indicate endocrine disruption in Chironomidae.
Introduction
Aquatic ecosystems are contaminated by a vast number of different chemicals, many of which are present at very low concentrations with unknown effects on aquatic organisms. The challenges for environmental managers are to identify which contaminants are present and determine if biological impairment is occurring. Biomonitoring approaches can be used to determine if impairment has occurred and indicate the potential causes of impairment. For example, by using both rapid bioassessment and field-based microcosm approaches, O’Brien et al. (2010) demonstrated that deteriorating ecosystem health in the Yarra River, Victoria, was due to habitat disturbance rather than sediment quality.

In 2007 sediments from the River Murray (New South Wales, Australia) and several of its tributaries caused biological impairment in a microcosm experiment (Townsend et al., 2009). Changes in abundances, species composition and deformity incidence in Chironomidae indicated that sediments from several sites were polluted. Sex ratios of Tanytarsus fuscithorax in sediments from two sites were significantly skewed, with one sediment causing feminisation and the other masculinisation of the population. The different skews suggested that sex ratio skewing was not a general stress response but was stressor-specific, although causes of stress were never elucidated. Other studies have also shown that sex ratio skewing in chironomids is chemical specific (Rakotondravelo et al., 2006) and also species specific (Ayres et al., unpublished). It is therefore a potentially useful endpoint for identifying specific stressors in biomonitoring and ecotoxicology. However, data on sex ratio skewing in chironomids are lacking.

Sex specific effects have been noted in chironomids exposed to the pesticides tebufenozide and diuron. Tebufenozide is an insect growth regulator that mimics the insect hormone 20-hydroxyecdysone (20HE) by inducing moulting. Tebufenozide is highly specific against lepidopteran pests; once ingested larvae cease feeding and undergo a precocious, incomplete and fatal moult. It causes the forced formation of a new cuticle and procuticle, along with structural changes to the prothoracic gland and foregut tissues (Smagghe et al., 1997). Two products containing tebufenozide are registered in Australia for use against Lepidoptera on pome fruit, citrus, grapevines,
avocados, kiwi fruits, longans, lychees, macadamias and eucalypts (APVMA, 2010). Tebufenozide has been sporadically detected in waterways internationally (e.g. Rhône River, France- Comoretto et al., 2007) but no data concerning tebufenozide contamination of Australian ecosystems exist in the peer-reviewed literature. It causes little toxicity in other insects such as Hymenoptera (e.g. Suh et al., 2000) and Hemiptera (e.g. Studebaker and Kring, 2003) but can affect non-target insects including mosquitoes (Beckage et al., 2004; Song et al., 1997). In the chironomid *Chironomus riparius* tebufenozide caused higher mortality in males than females at pupation, indicating sex specific effects in chironomids may be linked to endocrine disruption (Hahn et al., 2001).

The phenylurea herbicide diuron is a general use herbicide that acts by inhibiting photosystem II in plant photosynthesis. Significant responses in aryl hydrocarbon receptor (AhR) yeast assays provide some evidence of endocrine disrupting effects of diuron (Noguerol et al., 2006). Diuron caused antiestrogenicity in another yeast screen assay, reduced testosterone levels and ovulation in adult African clawed frog *Xenopus laevis* females (Orton et al., 2009), reduced growth and increased deformity incidence in the embryos and tadpoles of several frog species (Schuytema and Nebeker, 1998) and increased urothelial carcinogenesis in rats (da Rocha et al., 2010). Diuron is toxic to invertebrates. In the sea urchin (*Paracentrotus lividus*) larval development was completely arrested at 7.5 mg L\(^{-1}\), sea urchin sperm lost its fertilisation ability after diuron exposure, and when fertilisation did occur offspring were malformed (Manzo et al., 2006). Larvae of *Chironomus tentans* experienced delayed moulting between first and second instars in diuron concentrations of 1.9 mg L\(^{-1}\) (Nebeker and Schuytema, 1998).

Diuron is currently registered in Australia in 117 products as an herbicide and algaecide for use in various agricultural, urban, domestic, industrial and commercial situations (APVMA, 2010). Its use raises concerns about environmental contamination and impacts. In Queensland diuron has been frequently detected in coastal streams, (Mitchell et al., 2005), nearshore areas (Shaw et al., 2010) and offshore in river plumes (Bainbridge et al., 2009). It has been implicated in coral bleaching (Negri et al., 2005), mangrove dieback (Bell and Duke, 2005; Duke et al., 2005) and seagrass toxicity (Haynes et al., 2000; McMahon et al., 2005). In New
South Wales diuron was detected at concentrations of 30 – 40 µg kg$^{-1}$ in sediments near a marina in the lower Hawkesbury-Nepean River (Matthai et al., 2009). It was also detected in water at concentrations up to 12 µg L$^{-1}$ from northern inland rivers, where it is used as a defoliant in cotton (Muschal and Warne, 2003). Data for the toxicity of diuron to Australian macroinvertebrates are lacking, although data from Ayres et al. (unpublished) show adverse effects should be expected. They found that diuron reduced the taxonomic diversity and abundance of Australian chironomid assemblages, and two chironomid species had sex ratios that were female biased following exposure to diuron. One species, Procladius paludicola, exhibited complete population feminisation at the highest diuron concentration of 40 mg L$^{-1}$.

The aim of current study was to investigate the effects of tebufenozide and diuron on Australian Chironomidae assemblages, populations and sex ratios. Tebufenozide was tested to determine what effects a known insect endocrine disrupter would have on Australian Chironomidae, and hence if it could be used as a positive control in other experiments testing potential endocrine disrupters. Diuron was tested to confirm the results of Ayres et al. (unpublished) on a different population of Australian Chironomidae.
Materials and methods

Chemical preparation and analyses
Stock solutions of 100 mg L\(^{-1}\) of diuron dissolved in 10 % acetone and 90 % methanol, and 100 mg L\(^{-1}\) of tebufenozide (Mimic, Dow Agrosciences) dissolved in methanol were provided by Gavin Rose (Department of Primary Industries, Victoria, Australia). From the diuron stock solution the nominal concentrations of 0.4 mg L\(^{-1}\), 0.04 mg L\(^{-1}\) and 0.004 mg L\(^{-1}\) in 10 L of stream water per microcosm were achieved by adding 40 mL, 4 mL and 0.4 mL of stock solution to the microcosms of each treatment respectively. The tebufenozide treatments of 100 µg L\(^{-1}\), 10 µg L\(^{-1}\) and 1 µg L\(^{-1}\) were made by adding 10 mL, 1 mL or 0.1 mL of tebufenozide stock solution to 10 L of filtered stream water for each treatment respectively. Treatment concentrations were derived from concentrations used by Ayres et al. (unpublished) for diuron and Hahn et al. (2001) for tebufenozide. In all microcosms the addition of the treatment solution was less than 0.5 % of the total volume. A solvent control for diuron (40 mL of 10% acetone and 90 % methanol) and for tebufenozide (10 mL of 100 % methanol) (Ajax Finechem, Australia) were also prepared as treatments. Two replicate microcosms for each treatment were intended for destructive sampling for chemical analyses of water and sediments, but these were damaged during a flood event. Instead water samples were taken from one block on Day 54 of the experiment and were analysed for diuron by a consulting laboratory (ACS Laboratories, Victoria Australia). Water quality was measured twice for conductivity, pH and temperature using a water quality meter (TPS WP-81, Australia) but the first set of measurements taken on the 15\(^{th}\) of September 2009 (Day 11) were lost. Water quality was also measured towards the end of the experiment on Day 245.

Experimental design
The field-based microcosm experiment was run at Starvation Creek, 37.76 °S and 145.85 °E, from the 4\(^{th}\) September 2009 until the 18\(^{th}\) May 2010. Starvation Creek was selected because it is unpolluted and situated in a forested catchment with no public access. Each treatment consisted of 5 replicate microcosms comprising a 20 L polyethylene container (Starmaid International, Melbourne) with 10 L of filtered stream water and 500 mL of Starvation Creek sediment. The sediment was filtered
through a 64 μm mesh net to standardise particle size and remove debris and sediment-dwelling fauna. The control treatment consisted of 16 replicates. The microcosms were arranged in a random block design on rafts, which were positioned along the littoral zone of the creek. Rafts were used to overcome difficulties with fluctuating water levels, which can be quite rapid and large in Starvation Creek, and to ensure that each microcosm was partially submerged in the water to regulate temperature.

To each replicate the appropriate treatment was administered using glass syringes. The microcosms were covered in coarse netting to prevent large macroinvertebrates and debris from entering the microcosms. The microcosms were left for colonisation by flying macroinvertebrates. Heavy rainfall on several occasions caused the microcosms to fill and the sacrificial replicates were destroyed by flooding. On the 22nd of October the microcosms were covered with stockings (Kayser, Melbourne) to prevent emerging adults from escaping. The microcosms were frequently checked for adult macroinvertebrate emergence. On the 15th of December adult emergence from the microcosms was noted, and adult collections began on the 21st of December. Adult collections involved removing flying adults from the microcosms using a mechanical aspirator (Hausherr’s Machine Works, Toms River, NJ, USA) and storing the adults in 100 % ethanol (MERCK, Australia) at 4 °C until identification. Collections were conducted weekly except on several occasions when the creek was in flood and rafts could not be accessed. Several microcosms were lost during the experiment due to vandalism, colonisation by tadpoles and destruction by fish, so any data collected from these was not included in the final analysis. The experiment was terminated on Day 256 of the experiment and remaining larvae and adults were collected for identification.

**Macroinvertebrate identification**

Adult insects from the family Chironomidae were identified using keys by Freeman (1961) for the subfamilies Orthocladiinae, Chironominae and Tanypodinae, and Glover (1973) for tribe Tanytarsini within the subfamily Chironominae. Chironomid adults were identified to species, except for *Chironomus* which was identified to genus. Chironomid larvae were identified to genus or species level using a key by
Cranston (2000). Chironomid adults and larvae were identified by dissecting and mounting specimens in Hoyers medium and examining these under a compound microscope. Other taxa were identified using a field guide (Gooderham & Tsyrlin, 2002) and Trichoptera were identified using the key by Dean et al. (2004).

**Statistical analyses**

Differences in taxonomic composition between treatments were tested using Multi-Response Permutation Procedures (MRPP) and Indicator Species Analysis in PC ORD version 5 (McCune and Mefford, 2006). All other analyses were conducted in PASW Statistics version 19 (SPSS Chicago, IL). Physico-chemical data for water temperature, conductivity and pH were tested for normality using Kolmogorov-Smirnov tests and differences in the physico-chemical parameters for treatments were determined using ANOVAs. The overall abundance of animals in a treatment was calculated using data for all animals, whereas the abundances of individual taxa were determined using only adults, pupae and fourth instar larvae. The effect of tebufenozide and diuron treatment on the number of taxa, overall abundance and abundances of individual taxa were determined using non-parametric Kruskal-Wallis tests because the data contained many zero values. If a significant result was obtained treatments were compared against the control using Mann-Whitney U-tests to determine which groups were different. Sex ratios were given as the proportion of the total adult abundance that was female. The effects of treatment on sex ratios for common taxa were analysed in two ways. In the first, the number of males and females per treatment were pooled across replicates and analysed by contingency table analysis. In the second, sex ratios were calculated for each replicate within a treatment, and replicates with less than two individuals were excluded. These data were then analysed by ANOVA after arcsine square root transformation, and significant groupings were determined using post hoc Tukey-B tests. For all analyses of species richness, abundance and sex ratios data for the controls never significantly differed from the solvent control (Mann-Whitney U test; \( P > 0.10 \)); therefore the solvent control was excluded from analysis.
Results

Physico-chemical analyses
The temperature, conductivity and pH of the microcosms were normally distributed (Kolmogorov-Smirnov, $P > 0.05$), and did not significantly vary between treatments for tebufenozide ($F_{4,30} = 0.31, P = 0.87$; $F_{4,30} = 1.51, P = 0.23$; $F_{4,30} = 0.98, P = 0.43$ for temperature, conductivity and pH respectively) or diuron ($F_{4,27} = 0.33, P = 0.85$ for temperature and $F_{4,27} = 0.68, P = 0.62$ for pH). Conductivity was slightly higher in the 0.004 mg L$^{-1}$ diuron treatment ($F_{4,27} = 3.05, P = 0.034$). Water temperatures were in the range of 9 to 10 °C, conductivity was between 25 to 60 µS cm$^{-1}$ and pH ranged between 5.9 to 6.5. The actual differences in these measurements were not considered great enough to have an effect on macroinvertebrates or the tested chemicals.

Tebufenozide
Macroinvertebrate responses
Over 6,554 individuals from eight macroinvertebrate taxa colonised the 35 microcosms in this experiment. The fauna were dominated by five chironomid taxa, which made up 99 % of the animals collected. Other taxa present included Ceratopogonidae with 42 individuals and 20 animals from the Trichopteran family Hydroptilidae. Tebufenozide treatment did not affect the number of taxa present in the microcosms ($\chi^2 = 1.39, P = 0.70, d.f. = 3$) (Figure 3.1a). Although fewer animals were present at the highest nominal concentration of tebufenozide (Figure 3.1b) the overall abundance of animals in the microcosms was not significantly affected by tebufenozide treatment ($\chi^2 = 3.48, P = 0.32, d.f. = 3$). The composition of taxa did not vary between the microcosms (MRPP; $A = 0.02, P = 0.16$) and no species were significantly associated with any tebufenozide treatment after correction for multiple comparisons (Indicator Species Analysis, $P > 0.0125$).

Procladius villosimanus was the most abundant species, with 1,702 adults and fourth instar larvae present across most of the 35 microcosms (Figure 3.2a). Abundance was not affected by tebufenozide treatment ($\chi^2 = 2.71, P = 0.43, d.f. = 3$). Chironomus spp. were the second most abundant taxonomic group with 1,658 adults and fourth instar larvae collected from the microcosms. This taxon also had a relatively high frequency
of occurrence (Figure 3.2b). Treatment had a significant effect on the abundance of \textit{Chironomus} spp. ($\chi^2 = 11.73, P = 0.008, d.f. = 3$) with abundance in the 100 µg L$^{-1}$ tebufenozide treatment significantly lower than the control (Mann-Whitney U test; $P = 0.015$).

The third most abundant species was \textit{Paramerina levidensis} with 314 individuals. Treatment had no effect on \textit{P. levidensis} abundances ($\chi^2 = 1.71, P = 0.64, d.f. = 3$) (Figure 3.2c). \textit{Tanytarsus inextentus} adult and fourth instars amounted to 304 individuals and were patchily distributed across treatments (Figure 3.2d). Treatment did not affect the abundance of \textit{T. inextentus} ($\chi^2 = 1.36, P = 0.72, d.f. = 3$).

\textbf{Sex ratios of common taxa}

Tebufenozide treatment did not have a significant effect on \textit{Procladius villosimanus} sex ratios when data were pooled across replicates ($G^2 = 9.08, P = 0.06, d.f. = 4$) (Figure 3.3a) or analysed by microcosm ($F_{3,17} = 0.21, P = 0.89$). \textit{Chironomus} spp. sex ratios were significantly affected by treatment ($G^2 = 11.72, P = 0.02, d.f. = 4$), with the proportion of males slightly higher in 10 µg L$^{-1}$ and 100 µg L$^{-1}$ treatments (Figure 3.3b). However, analysis by microcosm failed to find a significant difference in sex ratios ($F_{3,20} = 1.06, P = 0.39$).

The sex ratio of \textit{Paramerina levidensis} was also significantly different across tebufenozide treatments when data were pooled ($G^2 = 13.13, P = 0.01, d.f. = 4$). The lowest tebufenozide concentration had a strong male bias, which became increasingly feminised with increasing tebufenozide concentration (Figure 3.3c). However, within a treatment there was a lot of variation in sex ratios, and when microcosms were analysed individually treatment had no significant effect on sex ratios after correction for multiple comparisons ($F_{3,13} = 3.87, P = 0.035$). Treatment had no significant effect on the sex ratios of \textit{Tanytarsus inextentus} when data were pooled ($G^2 = 5.25, P = 0.26, d.f. = 4$) or analysed by microcosm ($F_{3,4} = 1.733, P = 0.30$) (Figure 3.3d).
**Diuron**

*Chemical analyses*

Measured diuron concentrations in water were lower than nominal concentrations on Day 54 of the experiment (Table 3.1), and this may have been a result of dilution due to several rainfall events. In these events the 20 L microcosms, which were initially only filled with 10 L of water, overflowed.

*Macroinvertebrate responses*

The microcosm fauna was composed of ten taxa and 6,766 individuals; 98.42% of these were Chironomidae. Other common taxa included Ceratopogonidae, with 14 individuals, and Hydroptilidae (Trichoptera), with 85 individuals. Species composition did not significantly differ between the microcosms (Multi-Response Permutation Procedure; $A = -0.027$, $P = 0.83$) and Indicator Species Analysis did not show any taxa associating with the diuron treatments. Diuron treatment significantly affected the average number of taxa ($\chi^2 = 11.6$, $P = 0.009$, $d.f. = 3$). The 0.004 mg L$^{-1}$ diuron treatment had significantly more taxa than the control (Mann-Whitney U test; $P = 0.001$) (Figure 3.4a). The abundance of animals across the microcosms were not affected by treatment with diuron ($\chi^2 = 0.44$, $P = 0.93$, $d.f. = 3$) (Figure 3.4b).

*Procladius villosimanus* was the most abundant taxon with 4,127 fourth instar larvae and adults. The abundance of *P. villosimanus* was not affected by diuron treatment ($\chi^2 = 2.86$, $P = 0.41$, $d.f. = 3$) (Figure 3.5a). *Chironomus* spp. were the second most abundant taxon with 1,659 individuals. Diuron treatment had no effect on its abundance ($\chi^2 = 1.93$, $P = 0.59$, $d.f. = 3$), although fewer animals were present in the lowest diuron treatment (Figure 3.5b). *Paramerina levidensis* had 432 individuals. Treatment did not affect its abundance ($\chi^2 = 3.67$, $P = 0.30$, $d.f. = 3$), although its abundance was low in the 0.04 mg L$^{-1}$ treatment (Figure 3.5c). *Tanytarsus inextentus* only had 239 individuals, and treatment had no effect on its abundance ($\chi^2 = 2.44$, $P = 0.49$, $d.f. = 3$) (Figure 3.5d).

*Sex ratios*

*Procladius villosimanus* sex ratios were significantly affected by diuron when data for replicates were pooled ($G^2 = 70.98$, $P < 0.001$, $d.f. = 4$). All diuron treatments had a higher proportion of females than the control, and the population at the 0.004 mg L$^{-1}$
treatment was nearly completely feminised (Figure 3.6a). Increasing diuron treatment showed decreasing feminisation. Analysis by microcosms showed sex ratio was significantly affected by diuron \((F_{3,5} = 5.71, P = 0.008)\) and post hoc Tukey-B test showed that the 0.004 mg L\(^{-1}\) treatment had a significantly higher proportion female than the control and the 0.4 mg L\(^{-1}\) treatment.

*Chironomus* spp. sex ratios did not significantly differ across diuron treatments when data were pooled \((G^2 = 5.69, P = 0.22, d.f. = 4)\) or analysed by individual microcosm \((F_{3,19} = 2.63, P = 0.08)\) (Figure 3.6b). *Paramerina levidensis* sex ratios did not significantly differ across treatments \((G^2 = 6.57, P = 0.16, d.f. = 4)\) although there was a female bias evident in the two lowest diuron treatments (Figure 3.6c). When sex ratios were analysed by microcosm treatment had no effect \((F_{3,11} = 1.998, P = 0.17)\). The sex ratios of *Tanytarsus inextentus* did not significantly differ across diuron treatments when data were pooled \((G^2 = 5.72, P = 0.22)\) or considered by microcosm \((F_{3,4} = 5.18, P = 0.073)\) (Figure 3.6d).
Discussion
Tebufenozide and diuron had an impact on Chironomidae over prolonged exposure at presumably low concentrations. While species composition and abundance were unaffected by either pesticide, sex ratios were affected and the responses were species specific. The results suggest that skewed sex ratios in different taxa may be chemical specific and could potentially be used as positive controls for future field-based experiments (tebufenozide) and in biomonitoring (diuron).

The environmental fate and assemblage effects of tebufenozide and diuron
Tebufenozide had little effect on the composition of macroinvertebrate assemblages or chironomid populations, which may be partly due to the length of the experiment and the dilution of tebufenozide by frequent heavy rainfall. Tebufenozide readily adsorbs onto surface sediments and has a half-life of 64 days in the top layer of sediment under constant light at 15 °C (Sundaram, 1997a). Under fluctuating light tebufenozide persists for longer and accumulates at concentrations up to 25 times greater than concentrations applied to water. Tebufenozide is also stable in acidic and neutral buffers (Sundaram, 1994). However, tebufenozide rapidly undergoes photodegradation (half-life = 83 hours in sunlight) and microbial degradation (Sundaram, 1994). In sterilised water tebufenozide has a half-life of 734 days compared to only 181 days in unsterilised water. Tebufenozide can also be lost through algal uptake (Sundaram, 1997b). While cool water temperatures (less than 10 °C), fluctuating natural light and slightly acidic water may have allowed tebufenozide to persist in the sediments of the current study, photodegradation, microbial breakdown and uptake from algae observed growing in the microcosms would have significantly contributed to tebufenozide losses throughout the experiment.

While tebufenozide concentrations were presumably low and few significant impacts on chironomid assemblages or populations were observed, the effects of tebufenozide on these endpoints cannot be completely ruled out. An interesting pattern was observed for Chironomus species, where its abundance was lowest at the highest nominal concentration of tebufenozide and highest at the lowest tebufenozide treatment. Tebufenozide can stimulate algal growth, presumably because algae utilise...
the breakdown products of tebufenozide as nutrients (Sundaram, 1997b), although at high concentrations it can inhibit phytoplankton growth (de Barreda Ferraz, et al. 2004). By promoting the growth of Chironomus prey items, such as algae and phytoplankton, at low concentrations and inhibiting growth at high concentrations tebufenozide may have indirectly affected Chironomus species. Tebufenozide can cause chronic toxicity and competitive release in zooplankton communities (Kreutzweiser and Thomas, 1995), which would also indirectly impact chironomids. The reduced abundance of Chironomus spp. at high nominal concentrations could also be an effect of direct toxicity. Concentrations of tebufenozide greater than 1 µg L\(^{-1}\) caused mortality and delayed development in Chironomus plumosus, and an increased incidence of mouthpart deformities were also observed (Kwak and Lee, 2005).

By Day 54 of the experiment, diuron concentrations were considerably lower than the nominal concentrations; the lowest concentration was almost 10 times lower in water samples than the nominal concentration of 0.004 mg L\(^{-1}\). A number of factors would have contributed to this, including dilution from rainfall and degradation. In water diuron has a half-life of 90 days and is considered stable to hydrolysis (Okamura, 2002) except at high and low pH (Salvestrini et al., 2002). Diuron is not rapidly degraded by photolysis (Okamura, 2002), so neither hydrolysis nor photolysis would have caused significant degradation. Partitioning to the solid phase from water is generally low with no effect of pH or salinity (Voulvoulis et al., 2002). Diuron has a strong adsorption affinity to organic particles in the soil (K\(_{oc}\) = 485) and has a half-life in soils of up to 100 days (Alva and Singh, 1990). It can persist in soils even between yearly applications (Field et al., 2003). However, diuron can rapidly dissipate in environmental samples due to microbial degradation. Many microorganisms that degrade diuron have been isolated (e.g. Castillo et al., 2006) and some strains of fungi and bacteria may also degrade breakdown products of diuron (Tixier et al., 2001). Pesce et al. (2010) showed that diuron in sediments had a half-life of only 1 – 3 weeks due to microbial degradation. In the current study it is expected that biodegradation would have caused considerable loss of diuron.

Diuron undergoes transformation into several products, most of which are toxic, and in some cases are more toxic than the parent compound (Tixier et al., 2001). Environmental samples containing diuron often contain its degradation products. For
example, DCPMU, a breakdown product produced when diuron undergoes biological demethylation, was commonly detected in river water samples containing high levels of diuron (Field et al., 2003). The principle product of hydrolysis (Salvestrini et al., 2002) and biodegradation, 3,4-dichloroaniline (3,4-DCA), is considered to be more toxic than diuron (reviewed in Giacomazzi and Cochet, 2004). At low concentrations 3,4-DCA caused deformities and increased Hsp70 content (a measure of proteotoxicity) in zebrafish Danio rerio larvae, and at higher concentrations impaired locomotor activity and increased mortality (Scheil et al., 2009). While degradation products of diuron were not measured or considered in the current study, it is assumed these products were present and were contributing to any observed effects.

Diuron had little effect on the composition of chironomid assemblages or abundances across the microcosms. Very few studies have examined the effects of diuron in chironomids and the general conclusion is that diuron is not toxic to chironomids. One study reported that effects in Chironomus tentans only occurred at high concentrations (Nebeker and Schuytema, 1998). The results of the current study and Ayres et al. (unpublished) challenge this and suggest low concentrations of diuron can affect chironomids. The 0.04 mg L\(^{-1}\) treatment had fewer taxa than the control, which could be the result of indirect effects via changes to microbial communities. Diuron and some of its degradation products are toxic phytoplankton (Gatidou et al., 2007), and phytoplankton in biofilms exposed to diuron have reduced photosynthetic efficiency, while bacteria in biofilms are less abundant, with indirect trophic effects evident (Ricart et al., 2009). The reduced abundance of Paramerina levidensis in the 0.04 mg L\(^{-1}\) treatment could indicate adverse effects of diuron on its prey species, including the larvae of other chironomids that feed on microbiota. Changes to microbiota can also affect water chemistry (e.g. Perschbacher and Ludwig, 2004), which may then affect chironomids. Further evidence for potential trophic effects comes from the increased number of taxa in the lowest diuron treatment. This coincided with reduced abundances of Procladius villosimanus, possibly releasing other species from predation and competition. Two prey taxa of P. villosimanus, Chironomus spp. and Tanytarsus inextensus, had slightly increased abundances in the lowest diuron treatment and may have benefited from fewer P. villosimanus.
The effects of tebufenozide and diuron on chironomid sex ratios

Tebufenozide affected the sex ratios of chironomids, although taxa responded differently. While Hahn et al. (2001) found male Chironomus riparius pupae suffered higher mortality than females when exposed to tebufenozide, Chironomus spp. from the current study exhibited a different result, with a male bias occurring at the 10 µg L\(^{-1}\) and 100 µg L\(^{-1}\) nominal concentrations. The slight female bias in the 1 µg L\(^{-1}\) treatment where abundance was greatest, and male bias in the 10 µg L\(^{-1}\) and 100 µg L\(^{-1}\) treatments where adult abundance was lower, indicate that female mortality contributed to the observed sex ratios. Similarly, the most male biased treatment for tebufenozide also corresponded with a lower abundance for Paramerina levidensis, once again indicating female mortality. In contrast, Procladius villosimanus showed a female bias in the 10 µg L\(^{-1}\) and 100 µg L\(^{-1}\) treatments, although this bias was not significant.

Tebufenozide probably affected sex ratios through differences between the sexes in the susceptibility and responses of ecdysteroid pathways to stressors. This has been observed with other chemicals. For example, exposure of C. riparius to bis(tri-n-tributyltin) oxide resulted in slower genital imaginal disc development in females (Hahn and Schulz, 2002). Exposed females also had reduced ecdysteroid synthesis, while exposed males had increased ecdysteroid synthesis. As a consequence males developed faster than controls while females developed slower. By mimicking 20HE tebufenozide is potentially affecting a different part of the ecdysone pathway than bis(tri-n-tributyltin), but the result is similar in that males and females are responding differently and either male development is enhanced or unaffected, and female development is being disrupted.

The most conclusive evidence of an effect of diuron on Chironomidae also came from the sex ratio analysis. Increased Procladius villosimanus feminisation in all diuron treatments confirmed the results of Ayres et al. (unpublished), where diuron caused significant sex ratio skewing in Procladius paludicola. In that study, feminisation increased with increasing diuron concentration and no males were present at the 4 mg L\(^{-1}\) concentration. Chemical-induced skewing of sex ratios in vertebrate populations
indicates endocrine disruption, where a stressor alters the ability of one sex to survive (sex-specific mortality) or causes phenotypic changes in one sex (e.g. Kidd et al., 2007; Hogan et al., 2008).

Sex ratio skewing is not well understood in invertebrates, and while it may indicate endocrine disruption, it cannot be called such unless the mechanisms by which skewing occurs are identified. In some cases sex ratio skewing may be the result of changes to environmental conditions that favour the survival of one sex, or increase the mortality of another. For instance, juvenile female Chinese mantids *Tenodera sinensis* are more susceptible to starvation and mortality when food is limited, resulting in a male biased sex ratio (Moran and Hurd, 1994). For many chironomid species female larvae are larger, require greater energy reserves (Atchley, 1971; Servia et al., 2006; Benbow, 2008) and would therefore be more susceptible to starvation. Conversely, in other invertebrates there are examples of larger animals having a competitive advantage when resources are limited (e.g. Goulden et al., 1982). If female *P. villosimanus* were larger they might have lower survival in treatments where food is scarce, or alternatively have higher survival due to a competitive advantage. However, neither of these explain why the sex ratio was most female biased in the lowest tebufenozide treatment, because *P. villosimanus* abundance was low but prey species (i.e. other chironomids) were more abundant. Furthermore, all diuron treatments had reduced *P. villosimanus* abundances but female biased sex ratios. An indirect of diuron via food limitation does not appear to be responsible for the skew; rather it appears that male mortality is occurring in treatments with diuron.

The mechanism of diuron-induced sex ratio skewing can only be speculated at. In vertebrates, the sex-specific effects of diuron relate to its action as both an antiestrogen and an antiandrogen (Orton et al., 2009). Although androgenic glands, androgenic hormones, and a role for these in male differentiation exist in some crustaceans (e.g. Nagamine et al., 1980), there is no evidence of endogenous androgens, androgen receptors or androgen producing tissues in insects; the one case of androgen-controlled sex determination in glowworms was later refuted (Maas and Dorn, 2005). Similarly, while estrogens and other sex steroids have been detected in insects (e.g. Mechoulam et al., 1984) the role of these estrogens is unknown, but a role of estrogen in sex determination is unlikely. Further evidence that diuron is
unlikely to be acting via estrogenic pathways comes from the experiment described in Chapter 2 of this thesis, where exposure to the synthetic estrogen 17α-ethinylestradiol had no sex-specific effects in Chironomidae.

Instead, diuron may be interfering with ecdysone receptors in invertebrates. Moulting is controlled by 20HE in many arthropods. Exposure to diuron increased the frequency of moulting in the isopod *Asellus aquaticus*, indicating an interference with ecdysone receptors (Weltje and Oehlmann, 2006). Hahn *et al.* (2001) suggested that tebufenozide increased male mortality in *C. riparius* pupae by altering the availability of ecdysone in exposed animals. Ecdysone also has a role in sexual development, and ecdysteroid concentrations differ between the sexes prior to ecdysis, which indicates that ecdysteroids are involved in sexual differentiation for some species (e.g. in *Orgyia postica*- Gu *et al.*, 1992). If the ecdysone required for male development is greater, any interference with ecdysone would thus have a greater impact on males. Thus, sex ratio skewing in *P. villosimanus* may be due to sex specific mortality from diuron interfering with ecdysone pathways. Interestingly, the female bias caused by tebufenozide in the study by Hahn *et al.* (2001) is similar to the results seen for diuron in the current study but not for tebufenozide, where a male bias was more common.

The sex ratios of different species skewed in response to different contaminants, providing further evidence that sex ratio skewing is not a general response but stressor-specific. For example, *P. villosimanus* sex ratios were skewed in exposure to diuron but not to tebufenozide. At present the use of sex ratio skewing is limited to demonstrating stress in chironomids (e.g. Townsend *et al.*, 2009) and more data are needed before it can be utilised as a tool for monitoring the presence and effects of specific contaminants in the environment. The consistent result between the current study and Ayres *et al.* (unpublished) with *Procladius* species suggest that *Procladius* sex ratios may therefore be a useful indicator of diuron contamination in field samples, and field surveys are needed to confirm this. In addition, future work should also include determining if *Procladius* spp. sex ratios are skewed by other compounds, particularly other phenylurea herbicides.

Aside from biomonitoring purposes, sex ratio skewing may also be an important endpoint for measuring the population health of chironomids after chemical exposure.
In other taxa, skewed sex ratios can cause population declines and localised extinctions. A well documented example comes from widespread population declines in marine gastropods exposed to the pollutant tributyltin (TBT). Reproductive output from exposed populations decreased as sex ratios became male biased due to female mortality (Bryan et al., 1986). The operational sex ratio of these populations also declined because even when females were present imposex caused by TBT often resulted in female sterility. Similarly, extreme male biased sex ratios in the lepidopteran Thyridopteryx ephemeraeformis contributed to the extinction of local populations as there was reduced reproduction and therefore very little local recruitment of animals into the next generation (Horn and Sheppard, 1979). Skewed sex ratios in different chironomid groups could have adverse population effects over time, with consequences for other taxa, abiotic factors and even the functionality of the affected aquatic ecosystems.

**Conclusions**

Tebufenozide and diuron affected different chironomid taxa. The concentrations of each pesticide were presumably low for much of the experiment, and this is perhaps reflected in the way chironomid assemblages and abundances were not greatly impacted by either chemical. However, sex ratio skewing proved to be a sensitive endpoint, with several taxa exhibiting skewed sex ratios in response to tebufenozide, and one species, Procladius villosimanus, showing feminisation in response to diuron. The feminisation of P. villosimanus in response to diuron is consistent with previous results in another Procladius species, and therefore sex ratios for Procladius may be useful in monitoring environmental contamination by diuron.
Acknowledgements
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References


http://www.pesticideinfo.org


Figure 3.1. The (a) number of taxa and (b) abundance of macroinvertebrates occurring in the tebufenozide treated microcosms (average ± S.E.).
Figure 3.2. The average abundance of (a) *Procladius villosimanus*, (b) *Chironomus* species, (c) *Paramerina levidensis* and (d) *Tanytarsus inextentus* adults and fourth instars. Columns are average ± S.E. and diamonds are the proportion of microcosms per treatment the taxon was present in. * denotes a significant difference compared to the control (*P* < 0.05).
Figure 3.3. The sex ratios of (a) Procladius villosimanus, (b) Chironomus species, (c) Paramerina levidensis and (d) Tanytarsus inextentus adults given as the proportion female. Errors bars are the upper and lower 95% confidence intervals.
**Figure 3.4.** The (a) number of taxa and (b) abundance of macroinvertebrates present in the microcosms (average ± S.E.). * indicates a significant difference compared to the control ($P < 0.01$).
Figure 3.5. The abundance of (a) Procladius villosimanus, (b) Chironomus species, (c) Paramerina levidensis and (d) Tanytarsus inextentus adults and fourth instar larvae. Columns are the average abundance ± S.E. and diamonds are the proportion of microcosms per treatment the taxon were present in.
Figure 3.6. Sex ratios of (a) Procladius villosimanus, (b) Chironomus species, (c) Paramerina levidensis and (d) Tanytarsus inextentus adults. Letters denote significant groupings by post hoc Tukey-B test with the exclusion of the solvent control. Error bars are the upper and lower 95% confidence intervals.
### Tables

**Table 3.1.** Measured concentrations of diuron in water samples taken from Replicate Block 4 on day 54 of the experiment. Detection limit = 0.0001 mg L\(^{-1}\).

<table>
<thead>
<tr>
<th>Nominal diuron treatment</th>
<th>Measured diuron concentration (mg L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Solvent control</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>0.004 mg L(^{-1})</td>
<td>0.0006</td>
</tr>
<tr>
<td>0.04 mg L(^{-1})</td>
<td>0.0035</td>
</tr>
<tr>
<td>0.4 mg L(^{-1})</td>
<td>No sample</td>
</tr>
</tbody>
</table>
Chapter 4: Food limitation in *Chironomus tepperi*: effects on survival, sex ratios and development across two generations

Abstract
Species from the Dipteran family Chironomidae are widely used in laboratory tests and field studies to identify toxicity in freshwater environments. However, toxicity assessments can be influenced by food availability, which can alter endpoints in assays including viability, sex ratios and development time. The aim of this study was to determine if food limitation affected the endpoints used in toxicity tests with the Australian model organism, *Chironomus tepperi*, including responses in offspring. First instar larvae were subjected to food treatments with larval density controlled, and offspring were either raised under the same food conditions as their parents or under standard conditions. In lower food density treatments adults in the F₀ generation experienced delayed emergence and females produced fewer egg masses. F₀ diet affected the performance of F₁ under continued exposure and there was evidence that the quality of the offspring was compromised. Although sex ratios were not skewed, males and females responded differently to food limitation, especially in the F₁ generation where female development was more delayed. These results demonstrate that endpoints used in toxicity evaluation in *C. tepperi* also respond to food availability, highlighting the need to control for food in both laboratory and field toxicity studies. Multiple generations should ideally be exposed to stressors under laboratory conditions to elucidate likely long term effects in the field.
Introduction

In aquatic ecotoxicology invertebrate taxa are commonly used to indicate biological impairment from contaminant exposure. The Chironomidae represents one species diverse family within the Diptera that is frequently used in field surveys to assess aquatic ecosystem health (e.g. Hawkins et al., 2000; EPA Victoria, 2003), in field-based experimental approaches to isolate the effects of factors such as sediment pollution from hydrology and habitat (e.g. O’Brien et al., 2010), and in laboratory toxicity testing (e.g. Rakotondravelo et al., 2006). In these types of studies any biological responses are usually assumed to be the result of the stressor/s of interest. However the responses measured may be confounded by other factors including nutrition.

Changes to resource availability can potentially exert effects on chironomids that resemble those due to toxicity, affecting viability and delaying development. In natural systems and field-based experiments, food availability can vary considerably between sites and environmental samples collected. This heterogeneity can result in differences in the availability of food items for different chironomid species, which may consume detritus, algae and organic matter, other macroinvertebrates and even wood (Oliver, 1971). For example, the abundance of algae can be directly influenced by pollutants such as nutrients and herbicides, leading to indirect effects on algal grazers including chironomids. The effect of these changes and ability to mimic macroinvertebrate toxicity can also depend on the density of the chironomid populations (e.g. Postma et al., 1994).

Mortality, adult emergence, development and reproduction are commonly used endpoints in Chironomus tests. In other taxa these endpoints have been shown to be susceptible to food limitation (e.g. Anger and Dawirs, 1981; Juliano, 1986). In C. riparius high larval densities reduced survival, adult emergence, development rate, fecundity and adult body weight as a result of decreased resource availability (Hooper et al., 2003). Because food limitation can affect the sexes differently, there is also the potential to influence sex ratio. For example, female Chinese mantids Tenodera sinensis (Saussure) were more susceptible to starvation than males because they were larger and had greater food requirements (Moran and Hurd, 1994). Female larvae and
pupae of several chironomid species also tend to be larger and have greater energy reserves (Atchley, 1971; Servia et al., 2006; Benbow, 2008). Therefore chironomids may be susceptible to sex specific effects of food limitation, with sex ratios skewed in populations in the same way that chemical stressors affect sex specific endpoints in chironomids (e.g. Hahn et al., 2001; Rakotondravelo et al., 2006).

Both toxicity and nutrition effects may extend across generations by influencing resources that parents allocate towards offspring. For instance, females of two fish species that provision their young through yolk content produced larger offspring with greater energy reserves when they were fed a low food ration (Reznick et al., 1996). In chironomids, multigenerational effects occur following contaminant exposure, and in some cases the magnitude of effects appear greater in later generations (Janssens de Bisthoven et al., 2001). However, later generations can also show increased tolerance towards contaminants, implying that previously exposed generations confer an adaptive advantage to their offspring (Vogt et al., 2007). There is little information on the potential multigenerational effects of food limitation in chironomids, even though this could confound the detection of toxicity in the field where multiple generations are exposed.

Although the effects of nutrition have been demonstrated to affect the endpoints used in toxicity assays and the sensitivity of test species to toxicants (e.g. Liess et al., 2001; Beketov and Liess, 2005), there is limited data available on the effects of nutrition in Chironomus. The effects of food limitation have not been assessed in the Australian chironomid Chironomus tepperi, which is used in field surveys (e.g. Carew et al., 2007) and toxicity studies (e.g. Stevens, 1992). The aims of the current study are to provide data on the effects of food limitation on C. tepperi and to test the importance of controlling food availability in Chironomus toxicity tests. The experiments were conducted over two generations to determine if parental condition affected the quality and performance of offspring. I hypothesised that under limited food availability adult emergence would be delayed, and that this effect would be greater in female chironomids, which are likely to have greater developmental energy requirements because of their larger size and egg provisioning. I also hypothesised that the offspring of food-limited parents would be of poor quality even when raised using recommended feeding levels.
Methods
The experimental protocol was adapted from the guidelines recommended by the OECD (OECD, 2004a, 2004b). *Chironomus tepperi* were obtained from three different laboratory cultures originating from the University of Melbourne, Latrobe University and CSIRO Land and Water, South Australia. Inbreeding depression is a problem with laboratory experiments, so to increase the genetic variability of the laboratory population and mask potential inbreeding depression the cultures were crossed. First instar larvae for the experiment were obtained by collecting several egg ropes from the mixed laboratory population. First instar larvae from the egg ropes were collected within 24 hours of hatching and were added to the test vessels (below).

Each food treatment was represented by three replicate test vessels. Each vessel consisted of a 600 mL Pyrex beaker with an artificial substrate and approximately 400 mL of Martin’s solution, a reconstituted water solution that is a suitable medium for rearing *Chironomus tepperi* (Martin, 2009). The reconstituted water had a pH of 6.43, dissolved oxygen content of 92.0 % saturation, conductivity of 237 µS cm⁻¹, general hardness of 53.7 ppm, carbonate hardness of 17.9 ppm and ammonia of 0 ppm at 22 °C. The artificial substrate was two squares of toilet tissue (Coles Soft & Strong Toilet Tissue, Coles Australia) previously rinsed in ethanol, dried and shredded. Toilet tissue was selected rather than other substrates like sediments to reduce food availability from alternative sources. Test vessels were rinsed in 10 % nitric acid then distilled water before use. After the addition of substrate and reconstituted water, the food treatment was added to each vessel followed by 20 first instar larvae. Each vessel was securely covered by a piece of translucent white nylon cloth. After an initial 24 hours the test vessels were aerated. Food treatments consisted of finely ground TetraMin Tropical Flakes (Tetra, Germany). The treatments were designated as “standard” (upper limit of 1 mg larva⁻¹ day⁻¹ recommended by the OECD (2004a, 2004b) for *C. riparius* larvae), “2.0” (2 mg larva⁻¹ day⁻¹, twice the standard amount), “0.50” (the lower limit recommended by the OECD guidelines of 0.5 mg larva⁻¹ day⁻¹), “0.25” (0.25 mg larva⁻¹ day⁻¹) and “0.10” (0.1 mg larva⁻¹ day⁻¹). Early instar larvae were fed half the experimental quantity, as recommended in the OECD guidelines. Larvae were counted twice weekly by gently searching through the substrate with a
clear plastic pipette. Food was added to each test vessel twice weekly. The amount of food added was calculated based on the number of larvae remaining in the test vessel to avoid increased food availability with decreasing larval density. The experiment was run at 20 °C under 16 hours light: 8 hours dark in a temperature controlled cabinet. Water and substrate were renewed weekly.

Emerging adults were collected daily using an aspirator and their sex was noted. Adults from all replicates of a treatment were pooled into one breeding tank (clear Perspex, 24 cm depth x 30 cm width x 30 cm height, courtesy of J. Martin) with a tray of reconstituted water at the bottom for reproduction. Adult *Chironomus tepperi* live for three to five days with males emerging one to two days earlier than females. F₁ treatments were set up within five days of first emergence from a treatment. This meant that stressed treatments with delayed emergence were set up a few days later than other treatments. Egg masses were removed from the tray daily, placed in petri dishes with reconstituted water under experimental conditions and checked daily for hatching.

A multigenerational experiment was conducted using these egg masses. Newly hatched larvae from several egg masses of a treatment were mixed together before the experiment to decrease relatedness of the larvae. Larvae were then selected and were either raised under the same food conditions as their parents or were raised under standard conditions. The experiment was conducted following the same experimental procedure described above. F₁ adults were also allowed to breed, and the number and hatching success of egg masses noted.

Water quality was measured prior to water and substrate renewal using a field water quality meter (TPS WP-81, Australia). Water quality was similar among all treatments with temperatures between 19 °C and 20 °C, pH between 5.7 to 6.7, and conductivity between 210 and 274 μS cm⁻¹. Dissolved oxygen was not measured due to a faulty probe; however it was not expected to significantly differ between treatments because in the treatment where it was most likely to decrease (i.e. 2.0 food treatment) other water quality parameters were unaffected, there was no evidence of excessive decomposition or microbial growth, and *C. tepperi* larvae from this
treatment were large, red, “healthy” and similar in appearance to larvae from the standard and 0.5 food treatments (K.T. personal observation).

**Statistical analyses**

All tests were conducted in PASW 18 (SPSS Chicago, IL) and parametric tests were used because data were normally distributed (Kolmogorov-Smirnov tests, P > 0.05). Analysis of variance (ANOVAs) were used to test for effects of food treatment on survival (adult emergence), sex ratios (proportion female of adults), overall development (time until adult emergence) and sex specific development in the parental chironomids (F₀) and offspring (F₁) under the different food regimes. Sex ratios were arcsine transformed prior to analysis. *Post hoc* Tukey’s B tests were run to identify homogenous groupings. Independent t tests were used to identify if exposure to continued food deprivation or exposure to standard conditions affected F₁ responses. Data for the number of egg masses laid and the hatching rate of egg masses were presented but were not statistically analysed because breeding animals for each treatment were pooled across replicates to reduce inbreeding, removing replication.
Results

Survival
Survival to adulthood (successful adult emergence) was not significantly affected by food treatment in the F₀ generation ($F_{4,9} = 1.17, P = 0.39$), although survival was lower and more variable in the 0.10 food treatment (Figure 4.1a). The offspring of these adults were affected by food treatment. In the case of F₁ raised in standard feeding conditions, the food treatment of parents had a significant effect on survival ($F_{3,8} = 10.87, P = 0.003$), with animals of the 0.50 ration parents having higher survival than animals with parents from other treatment groups (Figure 4.1b). When larvae were raised under the same conditions as their parents, a significant effect of food treatment on survival was also observed ($F_{3,7} = 19.32, P = 0.001$). Animals in the 2.0 and 0.50 food treatments had higher survival than the standard and 0.25 ration fed treatments (Figure 4.1c). Comparisons were made between F₁ from the same parents but reared under different feeding regimes. Survival was not significantly different for F₁ from the 0.50 or 0.25 parents ($t_{4} = 0.25, P = 0.82$ for 0.50 F₁ and $t_{4} = 0.32, P = 0.82$ for 0.25 F₁). F₁ from 2.0 food treatment parents had greater adult emergence when raised under the same conditions as their parents than when reared under standard conditions ($t_{3} = -5.81, P = 0.01$). Overall, F₁ survival benefitted when parents were exposed to an intermediate level of nutrition or when both F₀ and F₁ generations had excess nutrition.

Sex ratios
Food treatment did not significantly affect the sex ratios of the parental generation ($F_{4,9} = 0.48, P = 0.75$), F₁ raised under standard conditions ($F_{3,8} = 1.90, P = 0.21$) or F₁ raised under the same food treatment as their parents ($F_{3,7} = 3.20, P = 0.09$), although for both F₁ treatments there was a trend of increasing female bias with decreasing food (Figure 4.2b, 4.2c). Similarly sex ratios did not significantly differ between F₁ under standard conditions and F₁ under continued exposure ($t_{3} = 0.71, P = 0.53; t_{4} = 0.39, P = 0.72; t_{4} = 0.74, P = 0.50$ for 2.0, 0.50 and 0.25 respectively). These results suggest only minor or no effects of nutrition on sex ratios.
Development rate

Food treatment had a significant effect on the first day of emergence in F₀ chironomids ($F_{4,9} = 285.52, P < 0.001$), with the 0.25 treatment adults emerging significantly later than standard, 2.0 and 0.50 adults, and the 0.10 treated adults emerging significantly later than all other treatments (Tukey’s B test) (Figure 4.3a). Food treatment did not significantly affect the first day of emergence for F₁ raised under standard conditions ($F_{3,8} = 4.00, P = 0.052$) or under continued exposure ($F_{3,7} = 2.79, P = 0.12$) (Figure 4.3b, 4.3c). No significant differences were observed between F₁ raised under continued exposure and standard food conditions ($t_{1.1} = -0.54, P = 0.68$ where variances were unequal; $t_{4} = 0.45, P = 0.68; t_{4} = -0.71, P = 0.52$ for 2.0, 0.50 and 0.25 respectively).

The median day of emergence for F₀ chironomids was significantly affected by food treatment ($F_{4,9} = 7.44, P = 0.006$), with the 0.10 treatment having a significantly delayed median emergence date compared to all other groups except the 0.25 treatment (Figure 4.3a). F₁ of treated parents that were fed the standard diet did not significantly differ in the median day of emergence ($F_{3,8} = 0.67, P = 0.60$) (Figure 4.3b) whereas F₁ exposed to the same food treatment as parents were significantly affected by food treatment ($F_{3,7} = 15.70, P = 0.002$), with all treatments delayed compared to the standard food treatment (Figure 4.3c). Comparisons between F₁ from the same parents but under different food regimes revealed no effect of treatment on the median day of emergence ($t_{1.23} = -3.16, P = 0.16$ where unequal variances were assumed; $t_{4} = -2.5, P = 0.067; t_{4} = -1.60, P = 0.18$ for 2.0, 0.5 and 0.25 treatments respectively).

Development rate in female C. tepperi

The first day of emergence significantly differed between food treatments in F₀ animals ($F_{4,9} = 12.80, P = 0.001$), with animals fed 0.10 ration emerging later than all other treatment groups (Table 4.1). The food treatment of parents had no significant effect on first day of emergence for F₁ raised under standard conditions ($F_{3,8} = 0.97, P = 0.06$) or continued exposure to food treatment ($F_{3,7} = 2.80, P = 0.11$). However, differences were apparent between standard and continued exposure treated F₁ females from the same F₀ treatments. Female F₁ from the 2.0 treated parents emerged significantly earlier when they were raised under standard conditions compared to
continued exposure to 2.0 food ($t_3 = 6.20, P = 0.008$). Similarly $0.25$ $F_1$ raised under standard conditions also began emerging earlier than those raised under 0.25 conditions ($t_4 = 4.00, P = 0.016$). $F_1$ of the 0.50 treatment did not significantly differ in the first day of emergence regardless of standard or continued exposure ($t_2 = 2.50, P = 0.13$).

Median day of emergence for female chironomids was also significantly affected by food treatment in the $F_0$ generation ($F_{4,9} = 23.30, P < 0.001$). A Tukey’s B test identified the 0.25 group and the 0.10 group as being delayed more than the other groups (Table 4.1). $F_1$ raised under standard conditions did not significantly differ for the median day of emergence ($F_{3,8} = 0.26, P = 0.85$) whereas exposure to the same conditions as parents had a significant effect on emergence ($F_{3,7} = 8.09, P = 0.011$), with the 2.0 and 0.25 treatments emerging significantly later than the standard treatment. $F_1$ raised under standard conditions had an earlier median day of emergence than those under continued exposure for the 2.0 treatment ($t_3 = -5.38, P = 0.013$) and the 0.5 treatment ($t_4 = -3.54, P = 0.024$) and there was a marginally non-significant difference with the 0.25 treatment ($t_4 = -2.75, P = 0.051$). Females of both generations experienced delays in the first and median day of emergence when food ration was reduced.

*Development rate in male C. tepperi*

The first day of emergence for male chironomids significantly differed with food treatment in the $F_0$ generation ($F_{4,9} = 285.52, P < 0.001$) with the 0.25 group emerging later than all groups except the 0.10 treatment, which had the latest emergence of all treatments (Table 4.1). $F_1$ under both exposure scenarios did not significantly differ in emergence ($F_{3,8} = 0.86, P = 0.50$ for $F_1$ raised under standard conditions and $F_{3,7} = 0.99, P = 0.45$ for $F_1$ raised under continued exposure). When $F_1$ from the same $F_0$ exposure were compared, their current exposure (i.e. continued exposure or standard exposure) had no significant effect on their emergence ($t_3 = -0.93, P = 0.42; t_4 = -0.63, P = 0.56; t_4 = 0.15, P = 0.89$ for 2.0, 0.50 and 0.25 respectively).

The median day of emergence was significantly affected by food treatment for $F_0$ males ($F_{4,9} = 20.74, P < 0.001$) with the median day of emergence for the 0.10 treatment significantly later than those for other treatment groups (Table 4.1). For $F_1$
of these animals raised under standard conditions, F0 exposure had no effect ($F_{3,8} = 1.14, P = 0.39$) whereas for F1 raised under continued food exposure conditions treatment did have a significant effect ($F_{3,7} = 13.01, P = 0.003$). Tukey’s B tests showed that the median emergence of standard fed males was earlier than for other treatments (Table 4.1). Comparisons between F1 raised under different conditions but from the same parental diet group showed no differences in median emergence day ($t_3 = -1.46, P = 0.24; t_4 = -1.89, P = 0.13; t_4 = -1.60, P = 0.18$ for 2.0, 0.50 and 0.25 treatments respectively). Male F1 development rates were less sensitive to food limitation than those of female F1.

**Fecundity and egg mass development**

Fecundity, represented by the average number of egg masses laid per female, appeared to be affected by food treatment in the F0 generation (Figure 4.4), where a slight decline in the number of egg masses laid was observed with decreasing food availability in comparison to the standard and 2.0 food treatments. When larvae from exposed adults were then raised under standard conditions, this effect seemed more pronounced except for the 2.0 treatment female F1, which laid fewer egg masses than their parents. This trend was also observed in F1 raised under the same food treatment as their parents.

In general egg masses did not appear to differ in the number of days to hatch, regardless of the food treatment in either generation (Figure 4.5). An exception is in the 0.25 treatment F1. Egg masses from these F1 hatched up to a day earlier when F1 were raised under standard conditions than when 0.25 F1 were reared under continued food deprivation. All egg masses laid by the F0 generation hatched, but one egg mass from the standard F1 and both 0.25 F1 groups failed to hatch.
Discussion

Chironomus tepperi were affected by food limitation, and endpoints commonly used in Chironomus toxicity tests were significantly impacted. Parental diet affected the response of F₁ under continued food limitation and also the quality of F₁ reared under standard conditions. Food limitation affected the development of both sexes, although effects were especially pronounced for female fecundity. These results have implications for laboratory and field studies where multiple generations may be exposed to a stressor, and where food availability may vary between sites or treatments due to differences in population density or the indirect effects of pollutants.

Effects on ecotoxicological endpoints

Survival until adult emergence was not affected in the F₀ generation by food limitation in the current study because food rations were selected to avoid high mortality. Adult survival in Chironomidae is a frequently-used endpoint, whether it be measured as successful development until adult emergence in toxicity studies (e.g. Schweitzer et al., 2010) or as the abundance of adult chironomids collected in field experiments (e.g. Sharley et al., 2008). Previous studies with chironomids have shown that adult emergence is affected by the food available for larvae. For example, more second generation C. riparius larvae survived under higher food rations when larval density increased (Hooper et al., 2003). Changes to larval density due to mortality or adult emergence can increase food availability for remaining larvae, which may alter the apparent toxicity of chemical stressors. For example, C. riparius emergence decreased at low densities in the presence of the growth inhibitor lufenuron; at higher densities lufenuron no longer had a significant effect on emergence because mortality from lufenuron exposure was offset by increased resources for survivors (Hooper et al., 2005). Nevertheless, the results of the current study suggest that nutrition levels can be selected to minimize any likely confounding effects on survival.

Sex ratio skewing may be affected by food limitation through the increased susceptibility of one sex to starvation. There is evidence from the current study and the literature that the sexes of larval chironomids differ in their morphology and development. Female chironomids can be substantially larger than male chironomids.
at the immature stage (Benbow, 2008) and this includes some Australian *Chironomus* species like *C. tepperi* (Atchley, 1971). Studies in other taxa have shown that the larger sex tends to be more susceptible to starvation, such as in Chinese mantids, *Tenodera sinensis* (Moran and Hurd, 1994). Therefore although nutrition might not directly influence sex ratio, it may exert indirect effects through altered resource availability when pollutants are present.

Food limitation did cause sex-specific developmental delays in *C. tepperi* at lower food treatments in both F₀ and F₁ generations, similar to what has been observed in *C. riparius* (Postma et al., 1994; Hooper et al., 2003). While both males and females experienced delays, their responses were different, which is likely to reflect levels of resources required by the sexes. Female *C. tepperi* emerge later than males (Stevens, 1998; K. T. personal observation) and in the related *C. riparius* female larvae must reach a certain size before emergence (Stanko-Mishic et al., 1999; Hooper et al., 2003), which suggests they have higher resource requirements and longer exposure times to stressors. This may explain the relatively greater developmental delays in females, particularly for the comparison between the standard and lowest food treatment in the F₀ generation.

We found that females from lower food rations laid fewer egg masses, most likely reflecting lower energy reserves. Food availability, habitat and chemical stressors affect female reproduction in midges. For example, high density, food limited populations of *C. riparius* experienced delayed reproduction and produced relatively fewer offspring than control populations (Hooper et al., 2003; Servia et al., 2006). Similarly, *T. torrenticola* female larvae from high quality habitats contained more eggs and more germ tissue than smaller larvae from other habitats (Benbow, 2008). Cadmium exposure resulted in *C. riparius* producing fewer egg masses and fewer eggs (Postma et al., 1994). Reproductive output may therefore be influenced by a variety of factors and represent an unclear endpoint for toxicological studies unless other factors are controlled.

As food availability affected chironomid responses, laboratory studies need to control the amount of food available per individual to avoid misinterpreting food limitation as toxicity. In addition, food availability should be consistent across experiments and
recommended guidelines should be developed and followed. When new model organisms are being tested, an optimal feeding regime should be specifically tailored to the organism. For example, our model organism, *C. tepperi*, responded better to the 0.50 ration than to the standard ration recommended by the OECD guidelines (2004a, 2004b), with greater survival observed in the 0.50 ration. Food and related factors, such as nutrient enrichment or organic carbon content, can cause toxicity directly and also affect the toxicity of other substances. Food can have toxic effects when in excess as well as deficit; we found delayed emergence when offspring were exposed to excess food conditions. Food can cause toxicity by altering the physico-chemical conditions of the test system, such as by reducing the dissolved oxygen concentration in water (Ankley *et al.*, 1993) which can delay the emergence of chironomids (e.g. Connolly *et al.*, 2004) and also change the toxicity or bioavailability of a chemical. For instance, food increased the acute toxicity of cadmium to *C. riparius* because cadmium adsorbed to food and was ingested by the larvae (Pascoe *et al.*, 1990).

Food limitation can increase sensitivity to a stressor, as demonstrated by Postma *et al.* (1994) where mortality increased by 30% in cadmium exposed larvae when food was restricted. Alternatively, food limitation and stressors can interact in such a way that organisms appear to benefit. For example, fecundity was greater when *C. riparius* was exposed to cadmium and food limitation than when exposed to food limitation alone (Postma *et al.*, 1994). Mortality from chlorpyrifos released surviving *C. tentans* larvae from competition, resulting in larvae developing faster and weighing more than controls (Rakotondravely *et al.*, 2006). In studies where the effects of food or density are not of interest, treatments where significant mortality have occurred should be avoided for the testing of endpoints such as growth and reproduction, which are likely to be affected by changes to population size (US EPA, 2002). Nutrition is also likely to be a problem in detecting toxicity effects in field surveys and field-based experiments; here nutrition levels may directly or indirectly influence communities by acting through trophic effects (e.g. Langdon *et al.*, 2006) as well as by potentially masking toxicity caused by pollutants through the benefits nutrient enrichment provides to opportunistic taxa (e.g. Pettigrove *et al.*, 2007).
Multigenerational effects

Animals used in laboratory tests often come from a population with no previous exposure to a contaminant, whereas in the field animals often have complex exposure histories. As a result, cross-generation effects and evolutionary adaptation need to be considered when interpreting pollution responses. For some contaminants, the effect of exposures in later generations can be larger than in the $F_0$ generation. For example, 17α-ethinylestradiol affected adult emergence and sex ratios more in the second generation than in the first generation of *C. riparius* (Watts *et al.*, 2001). Mortality in *C. riparius* increased over nine generations of exposure to 156.9 nM of cadmium, eventually resulting in population extinction (Postma and Davids, 1995).

Multigenerational experiments can be useful in highlighting potential issues when interpreting contamination effects in field environments.

Multigenerational effects can be positive or negative; there was evidence in our experiments that parental diet conferred an advantage to offspring reared on the same diet. For example, offspring that were fed the 2.0 ration had much greater survival than 2.0 $F_1$ raised under standard conditions. A similar pattern has been observed in the lepidopteran *Pieris rapae*, with mothers producing offspring that developed best under the same (extreme high quality or extreme low quality) food conditions, although these offspring also did well on the optimal diet (Rotem *et al.*, 2003).

Previous exposure to pollution can increase the survival of offspring under similar conditions. Bahrndorff *et al.* (2006) found that *C. februarius* survived relatively better in polluted sediments if their parents were from a polluted site. This difference may reflect adaptive shifts in response to pollution or cross generation effects, particularly through maternal factors contributed to eggs by mothers (e.g. Munkittrick and Dixon, 1988; Mousseau and Fox, 1998; Hercus and Hoffmann, 2000). Populations given suboptimal nutrition or starvation can develop resistance to starvation through selection, which can also affect their resistance to other stressors. *Drosophila melanogaster* population lines, for instance, that were subjected to starvation or suboptimal diet (lemon), had greater starvation and desiccation resistance than control populations (Harshman *et al.*, 2001). Parents in poor condition may also allocate more resources to offspring to increase offspring fitness. For example, female flycatchers *Ficedula albicollis* that were in poor condition increased the androstenedione concentrations in their egg yolk, which correlated with larger
hatchling size and increased recruitment over the next two years for their offspring (Hegyi et al., 2011). Nutrient-deprived *Drosophila melanogaster* produced faster developing offspring (Vijendravarma et al., 2009). The increase in survival we observed in F₁ groups from 0.50 parents may therefore be the result of increased provisioning and other benefits gained from parental investment or selection.

Parents that are nutritionally stressed often produce offspring with a lower fitness; for instance female yellow dung flies that are nutritionally deprived produce smaller egg volumes, resulting in reduced adult emergence (Jann and Ward, 1999). In our experiments, the F₁ generation of 0.25 treated adults produced fewer egg masses than other treatments, regardless of food conditions. Even when reared under standard conditions, 0.25 treated offspring had the lowest adult emergence of all treatments, suggesting that offspring quality was compromised by parental condition. However, low nutrition in the parental generation does not always lead to reduced fitness (Grech et al., 2007) and any effects will need to be investigated on a case-by-case basis.

**Conclusions**

Food limitation affected several endpoints that are frequently used in ecotoxicological studies with *Chironomus* species. Although mortality and sex ratios were unaffected, development rate was delayed under food limitation. Both sexes were affected, although the magnitude of delays and reduced fecundity in food limited females suggest higher nutritional developmental requirements in female *C. tepperi*. These results also suggest that females may be more sensitive to other stressors and highlight the need to control for food-related effects in experiments generally. The effects of food limitation extended across generations. There was evidence that the feeding rations of F₀ chironomids affected F₁ quality and sensitivity to food limitation. The results provide evidence that food limitation can affect ecotoxicological endpoints in *C. tepperi*, and they may be useful in the interpretation of field data where food limitation is likely to occur.
Acknowledgements
I would like to thank Jon Martin from the University of Melbourne, Anupama Kumar from CSIRO and Latrobe University for the provision of the stock cultures of *Chironomus tepperi*. Jon Martin is also thanked for providing breeding and culture tanks for *C. tepperi*. Sara Long provided advice on conducting the laboratory tests, and my supervisors Ary Hoffmann and Vin Pettigrove gave valuable comments regarding this chapter. Thanks also to the comments of two anonymous reviewers who reviewed this chapter for publication. Funding for this research was provided by Melbourne Water Corporation, the Victorian Department of Business and Innovation through support of the Centre for Aquatic Pollution Identification and Management, and the Australian Research Council through their Fellowship scheme.

References


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Figure 4.1. Survival of *Chironomus tepperi* to successful adult emergence for (a) larvae exposed to different amounts of food, (b) offspring of the parental larvae from a given food treatment that were raised under standard food conditions and (c) offspring of the parental larvae from a given treatment that were raised under identical food conditions as the parents (average ± standard error). Letters indicate significant grouping by Tukey’s B test ($P < 0.05$).
Figure 4.2. Sex ratios of adult *Chironomus tepperi* given as the proportion female for (a) animals exposed to different amounts of food, (b) offspring of the parental animals from a given food treatment that were raised under standard food conditions and (c) offspring of the parental animals from a given treatment that were raised under identical food conditions as the parents (average ± 95% confidence intervals).
Figure 4.3. Emergence of adult *Chironomus tepperi* for (a) animals exposed to different amounts of food, (b) offspring of the parental animals from a given food treatment that were raised under standard food conditions and (c) offspring of the parental animals from a given treatment that were raised under identical food conditions as the parents. = standard, = excess, = 0.5 standard, = 0.25 standard, = 0.10 standard.
Figure 4.4. The number of egg masses laid per female. Error bars are upper and lower 95% confidence intervals. Adult males and females from each treatment were pooled across replicates, so these data are not statistically replicated. The number of females varied per treatment.
Figure 4.5. The average number of days it took for egg masses from the different treatments to hatch. Adult males and females from all replicates of a treatment were pooled for breeding purposes, so these data are not statistically replicated. The error bars given are upper and lower 95% confidence intervals, except for in (b) 0.25 control treatment, where n =2. The number of egg masses laid varied per treatment.
### Tables

**Table 4.1.** The first and median day of emergence for *Chironomus tepperi* adults under different food treatments (average ± standard error).

<table>
<thead>
<tr>
<th>Generation/conditions</th>
<th>Treatment</th>
<th>First day of emergence</th>
<th>Median day of emergence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td><strong>F₀</strong></td>
<td>Standard</td>
<td>14.7 ± 1.7</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Excess</td>
<td>14.0 ± 0.6</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>½ standard</td>
<td>14.0 ± 0.0</td>
<td>13.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>¼ standard</td>
<td>17.3 ± 0.3</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1/10 standard</td>
<td>26.5 ± 3.5</td>
<td>22.5 ± 0.5</td>
</tr>
<tr>
<td><strong>F₁ in standard</strong></td>
<td>Standard</td>
<td>14.7 ± 0.3</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Excess</td>
<td>14.3 ± 0.3</td>
<td>13.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>½ standard</td>
<td>14.0 ± 0.0</td>
<td>13.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>¼ standard</td>
<td>13.3 ± 0.3</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td><strong>F₁ in continued exposure</strong></td>
<td>Standard</td>
<td>14.7 ± 0.3</td>
<td>11.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Excess</td>
<td>17.0 ± 0.0</td>
<td>14.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>½ standard</td>
<td>15.7 ± 0.7</td>
<td>14.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>¼ standard</td>
<td>16.0 ± 0.6</td>
<td>13.7 ± 1.7</td>
</tr>
</tbody>
</table>
Chapter 5: A method for biomonitoring with adult Chironomidae (Diptera)

Abstract
Chironomidae larvae are often used in aquatic biomonitoring, whereas adult chironomids are not normally used. Several methods are available for capturing adult chironomids in situ but these are usually only applied in ecological studies or pest management. Yellow sticky traps have been successfully used for ecological studies with chironomids and with other insects in terrestrial biomonitoring. The current study explores whether the sticky trap method can be used to successfully sample adult chironomids in a biomonitoring study. Sticky traps were deployed in three separate surveys of waterways in Victoria, Australia, and successfully captured multiple chironomid species that could be easily identified. A sampling period of five weeks was necessary to capture 90% of taxa, however shorter sampling periods gave data that were sufficient to distinguish between sites. The sticky traps were able to measure endpoints potentially indicative of ecological impairment including changes in sex ratio. Assemblages of chironomids captured in traps were relatively stable across time. The above features indicate that the method is suitable for further investigation as a biomonitoring tool with a number of advantages over traditional larval collection methods.
Introduction
The Chironomidae are frequently used in biomonitoring programs to indicate ecological impairment and to identify the causes, magnitude and extent of stress (e.g. Chessman, 1995; Wright, 1995; Smith et al., 1999). Larvae, and occasionally pupal exuviae, are used in biomonitoring approaches. Adult endpoints have largely been ignored, despite their demonstrated usefulness in field-based experimental approaches (e.g. Pettigrove and Hoffmann, 2005a; Sharley et al., 2008a) and laboratory toxicity testing (e.g. Watts et al., 2001; Hahn et al., 2001; Rakotondravelo et al., 2006). The presence of a larva does not necessarily represent an organism that will survive its present conditions, especially given that chironomid life stages exhibit different sensitivities to stressors. The insect growth regulator, tebufenozide, for example, only elicited significant toxic responses in Chironomus riparius pupae, not larvae (Hahn et al., 2001). Bouchard and Ferrington (2011) advocated the use of an endpoint that represents the end of the life cycle to better characterise the health of a waterway; they selected pupal exuviae to measure the successful emergence of adult chironomids. The presence of an adult chironomid represents an organism that has successfully survived in its environment. Therefore biomonitoring with adults is a potentially useful approach that warrants further investigation and development.

Numerous techniques have been developed for capturing adult Chironomidae in situ for ecological and biological investigations (e.g. Tokeshi, 1991; Verneaux and Aleya, 1999) and controlling nuisance chironomid species (e.g. Ali et al., 1986; Ali et al., 2008). These techniques include adult sweep netting (Takamura, 1999; Verneaux and Aleya, 1999), collection of pupal exuviae (Hardwick et al., 1995; Verneaux and Aleya, 1999; Raunio et al., 2007), emergence traps (Lynch et al., 2002; Lundström et al., 2010), light traps (Ali et al., 1986; Ali et al., 2008) and sticky intercept traps (King and Wrubleski, 1998; Lynch et al., 2002). For a method to be suitable for biomonitoring purposes it must be repeatable, easily standardised, robust enough to endure variable field conditions, deployable for sufficient time periods to accurately represent chironomid assemblages, allow multiple sites to be sampled simultaneously, and capture a sufficient number of animals for abundance and sex ratios to be reliably estimated. Most of the techniques listed above have characteristics that make them unsuitable or at least challenging for use in biomonitoring. For example, it is difficult
to standardise the effort used in sweep netting, while light traps and emergence tents are expensive, require substantial maintenance and may need supervision under some circumstances (e.g. adverse weather conditions or risk of public interference). These factors can reduce the number of sites that can be sampled with such techniques.

Yellow sticky traps have been successfully used to capture insects in terrestrial biomonitoring (e.g. Thomson et al., 2004; Sharley et al., 2008b) and in aquatic ecological studies on Chironomidae (King and Wrubleski, 1998; Lynch et al., 2002) but not in aquatic biomonitoring. Sticky traps are cheap and require little ongoing maintenance. Numerous traps can be deployed within a site to provide good spatial coverage, and many sites can be simultaneously sampled. Sticky traps are fairly robust, able to withstand adverse weather conditions and can be left in the field over long periods of time. However, the traps can catch a lot of non-target species and caught specimens can be damaged, making identifications difficult.

Three surveys were conducted to test the feasibility of using sticky traps as a biomonitoring method for adult chironomids. The first survey aimed to confirm if sticky traps were suitable for capturing chironomids, if chironomids could easily be identified and if sticky trap placement in a wetland affected animal capture. The second survey investigated how sampling period affected assemblages, populations and sex ratios. Finally, the third survey tested if the sticky traps could differentiate Chironomidae assemblages between sites.
Materials and methods

Experimental design
Yellow sticky traps (Agrisense) are commercially available plastic cards (240 mm X 100 mm) with adhesive on both sides and are used to intercept insects. In all three surveys 15 yellow sticky traps were deployed at each field site and hung from vegetation, star posts, fences or other suitable structures using tie wire. Sites differed in the presence, suitability and accessibility of structures so the placement of the sticky traps varied with site. When sticky traps were replaced in a survey, the replacement traps would be placed in the same positions as those previously deployed. Retrieved traps were stored in clear low density polyethylene press-seal bags (180 mm X 330 mm) (Grayson Packaging) at 4 °C. Captured adult Chironomidae were examined using a dissecting microscope and identified to species level with the keys by Freeman (1961) for subfamilies Tanypodinae, Orthocladiinae, Podonominae and Chironominae, Glover (1973) for Chironominae tribe Tanytarsini and Martin (2011) for Chironomus species. The Orthocladiinae key by Hergstrom (1974) was also consulted although it has not been peer-reviewed.

Survey 1
The first survey was conducted at Glynns Wetland, Warrandyte Victoria (37°43’ S, 145°12’ E). Glynns Wetland has been used as a reference site in previous experiments with Chironomidae (Pettigrove and Hoffmann, 2005a, 2005b; Sharley et al., 2008a) because it has a diverse macroinvertebrate fauna, is not polluted and is not susceptible to public interference. The experiment was conducted from the 22nd September until the 8th October, 2010. Fifteen sticky traps were deployed at the wetland and detailed notes were taken of their location in relation to height above water (30 cm to 120 cm), distance to bank (approximately 0 cm to 500 cm), and if the trap was attached to a stake or vegetation, positioned in riparian vegetation (yes/no), amongst emergent aquatic vegetation (yes/no) or over open water (yes/no). The sticky traps were left at the site for 17 days.

Survey 2
The second survey was conducted to investigate how chironomid assemblages and populations changed over time to determine appropriate sampling periods, and to
determine if sticky traps could be used to measure chironomid sex ratios. The survey was conducted at Trin Warren Tam-boore wetland, Royal Park, Parkville Victoria (37°47' S 144°56' E). This site was selected because of its close proximity to the University of Melbourne Parkville campus, making repeat collections feasible. The experiment was conducted from the 17th January until the 12th March, 2011. Fifteen sticky traps were deployed at the site and these were replaced every two days. An initial adult sweep was also undertaken to help determine species presence. Wire cages were constructed (hole size 0.9 cm) and sticky traps were hung in these to prevent birds from interacting with traps.

To test for pollution levels at this site, surface sediments were collected from the wetland on the 17th January 2011 and filtered through a 63 µm nylon net for chemical analyses by ALS Laboratory Group. Trace metals were not present at concentrations expected to affect aquatic biota (ANZECC/ARMCANZ, 2000) with the exception of nickel and zinc, which had concentrations of 61 mg kg\(^{-1}\) and 525 mg kg\(^{-1}\) respectively. These concentrations exceeded the ISQG high values, above which ecological impairment can be expected. TPH did not exceed values at which ecological impairment is likely to occur (Pettigrove and Hoffmann, 2005b). Total Kjeldahl nitrogen was 3,790 mg kg\(^{-1}\), total phosphorus was 839 mg kg\(^{-1}\) and total organic carbon was 4.51 %.

The diversity of chironomids were described by the Shannon-Wiener Index of Diversity, given as

\[
H' = -\sum_{i=1}^{S} p_i \ln p_i
\]

where \(H'\) = the Shannon-Wiener Diversity Index, \(p_i\) = the proportion of the total population made up by the \(i\)th species for all species \(S\).

Survey 3
The third survey was conducted to determine if differences in chironomid communities could be detected between sites using sticky traps. Eight regional sites in
Victoria (Table 5.1) were selected for the study based on their inclusion in previous monitoring programs by the Centre for Aquatic Pollution Identification and Management (University of Melbourne) and the Department of Primary Industries (Victoria), with chemical and biological data available for these sites. The experiment was conducted from the 25th October until the 10th November 2011. At each site 15 sticky traps were deployed. On the 3rd November the sites were revisited and the sticky traps were replaced with new traps. The experiment was terminated early at Site 4, Site 7 and Site 8 because of flash flooding and at Site 5 because of strong winds.

In addition, sweep netting was conducted at each site throughout the study for the purpose of assisting in chironomid identifications on the sticky traps. This involved the capture of aerial and resting adult chironomids, which were preserved in 100% ethanol for identification. The technique used and time devoted to sweep netting at each site was not consistent because the aim was to collect as many different taxa as possible to aid in identifications, not for statistical comparisons between sweep net and sticky trap data.

**Statistical analyses**

All statistical analyses were conducted with SPSS 20 (IBM Chicago, IL). In the first survey, Spearman correlations were computed between chironomid numbers/diversity and continuous variables, and Kruskal Wallis tests were undertaken to compare chironomid numbers/diversity to discontinuous wetland features.

In the second survey contingency table analysis was performed to determine if week affected the abundance of chironomids captured on the sticky traps. Data for all sticky traps were pooled within each sampling period (2 – 3 days). Local weather data were obtained for the study period (Bureau of Meteorology, 2012). Total rainfall was calculated for the days over which each sampling event occurred, while the temperature of each sampling period was calculated by averaging the maximum daily temperatures for the days over which each sampling period occurred. Spearman rank correlations were computed between abundance and meteorological data to test for associations between weather conditions and chironomid numbers. Correlations were
also computed to test if sex ratio (expressed as the proportion of females, and only computed for samples of 10 or more animals) varied with abundance for common taxa. When < 10 animals were caught, numbers from different sampling intervals were randomly selected and pooled to generate samples > 10. Mean abundance was then used in correlating sex ratio to abundance.

In the third survey, the number of taxa, overall abundance and the abundance of common taxa were calculated for individual sticky traps and these were analysed for an effect of week or site by univariate ANOVAs when data were normally distributed based on Kolmogorov-Smirnov tests. The abundances of common taxa were log-transformed prior to analysis. Site and week were treated as random factors. The proportion of females (as a measure of sex ratio) was arcsine square root transformed prior to analysis. If data were not normally distributed, the effects of site and week were analysed with Kruskal-Wallis tests. Sex ratio was correlated with abundance for common taxa.
Results

Survey 1
A total of 1,461 Chironomidae belonging to 26 taxa were caught in this survey. Five taxa comprised more than 94% of total abundance (Table 5.2). Captured chironomids were generally in good condition, despite several rain events. Species with distinct markings were easily identified on the traps. For others, particularly several taxa within the Orthocladiinae and species with few male samples, species could not be confidently identified although the number of species present could be ascertained. The number of taxa present on each of the 15 sticky traps was not affected by the sticky trap’s height above water (Spearman correlation, \(\rho = -0.42, P = 0.12, N = 15\)), distance to the littoral zone (\(\rho = 0.36, P = 0.19, N = 15\)), attachment to a stake or vegetation (\(\chi^2 = 0.37, P = 0.54, d.f. = 1\)), proximity to emergent aquatic vegetation (\(\chi^2 = 0.05, P = 0.82, d.f. = 1\)), proximity to riparian vegetation (\(\chi^2 = 0.37, P = 0.54, d.f. = 1\)) or proximity to open water (\(\chi^2 = 0.14, P = 0.71, d.f. = 1\)).

The number of animals present on each sticky trap was not affected by trap height above water (\(\rho = -0.07, P = 0.81, N = 15\)), proximity to the littoral zone (\(\rho = -0.08, P = 0.78, N = 15\)), attachment to stake or vegetation (\(\chi^2 = 2.52, P = 0.11, d.f. = 1\)), position relative to emergent aquatic vegetation (\(\chi^2 = 0.52, P = 0.47, d.f. = 1\)) or position relative to open water (\(\chi^2 = 1.09, P = 0.30, d.f. = 1\)). Traps positioned within riparian vegetation had more animals than those that were not, but this was only weakly significant (\(\chi^2 = 5.33, P = 0.02, d.f. = 1\)). These data therefore suggest that the distribution of Chironomidae on sticky traps is not especially dependent on trap placement within a wetland.

Survey 2
In the second survey 2,528 individuals belonging to 23 taxa were caught on the traps over 56 days. An adult sweep was conducted initially but this resulted in very few animals (37) belonging to only five taxa. The abundance of chironomids caught throughout the sampling period varied through time (Figure 5.1a) although the pattern of variation was largely driven by the most common species, *Limnophyes vestitus*. Exclusion of *L. vestitus* from the results showed a decrease in total abundance from
Day 4 to Day 10 (Figure 5.1b). Shannon-Wiener diversity appeared to be similar throughout the experiment except for reduced diversity on Day 8 when *L. vestitus* dominated (Figure 5.1c). Of the 23 taxa caught on the sticky traps, eight contributed to over 97% of the total abundance (Table 5.3) and these were considered further.

The contribution of each of the common taxa to the composition of the chironomid community was relatively constant over time when *L. vestitus* was excluded (compare Figure 5.2b to 5.2a). There were no significant changes in the numbers of the common taxa across weeks (contingency test, $\chi^2 = 165.67$, $P = 0.38$, d.f. = 161). Total abundance (when excluding *L. vestitus*) did not significantly correlate with temperature ($\rho = -0.08$, $P = 0.70$, $N = 28$) or rainfall ($\rho = 0.11$, $P = 0.59$, $N = 28$) throughout the study period.

There was a steady increase in the cumulative number of taxa caught throughout the experiment (Figure 5.3). By Day 35 over 90% of the taxa present in the experiment had been caught, and there was no further increase in taxon number collected beyond Day 42.

The sex ratios of common taxa were considered except for the two parthenogenetic species *L. vestitus* and *P. grimmii*, and for *Polypedilum* species and *P. pluriserialis* which had few samples > 10 individuals. Because sex ratio may be affected by swarming, we examined the relationship between abundance and sex ratio for several species by computing correlations. For *T. fuscithorax*, > 10 animals were collected for each sampling period except on two occasions and these were combined. For three relatively abundant samples, sex ratios were strongly biased towards females (Figure 5.4a). However, overall there was no significant correlation between abundance and sex ratio for this species regardless of whether the entire data set was considered (Spearman rank correlation; $\rho = 0.25$, $P = 0.21$, $N = 27$) or when the three most abundant samples were excluded ($\rho = -0.015$, $P = 0.95$, $N = 24$).

*Kiefferulus* “tinctus” samples consisted of < 10 animals on 20 occasions, and data from these samples were randomly combined into five groups to investigate the correlation with abundance. Although sex ratio varied in the smaller samples, females tended to comprise around half the sample regardless of abundance (Figure 5.4b) and
there was no significant correlation between these variables ($\rho = 0.17, P = 0.59, N = 13$). The abundance of *Chironomus* species was low and data were randomly assigned to six groups for the subsequent analysis. The sex ratio became increasingly feminised as abundance increased (Figure 5.4c), and this was significant ($\rho = -0.16, P = 0.71, N = 8$). *Cricotopus albitarsis* samples were combined into three groups except on the one occasion where its abundance was greater than 10. There was no significant correlation between its abundance and sex ratio ($\rho = -0.63, P = 0.34, N = 4$) (Figure 5.4d).

**Survey 3**
Thirty taxa and 34,826 individual chironomids were caught on the sticky traps, while 30 taxa and 8,097 individuals were collected with aerial sweep netting. At several sites, fewer taxa were caught by sweep netting than on the sticky traps (Table 5.4). The exceptions were at Site 5 where strong winds destroyed most of the sticky traps and animals were observed flying close to the ground (personal observation), and at Site 7 where sticky traps were retrieved after only three days due to a flash flooding event. Site 1, Site 2, Site 3 and Site 6 were sampled with sticky traps twice and were analysed further. Identification of animals on the sticky traps was facilitated through the availability of specimens in good condition from sweep netting. Data for abundance and number of taxa were pooled by week given that the previous surveys showed only small changes in species composition across a week.

The number of taxa present at each site was not normally distributed even after log-transformation (Kolmogorov-Smirnov test; $P < 0.001, N = 108$). The number of taxa differed significantly between sites (Kruskal-Wallis test; $\chi^2 = 26.82, P < 0.001, d.f. = 3$) (Figure 5.5a) but week had no significant effect ($\chi^2 = 2.10, P = 0.15, d.f. = 1$). The total abundance of all chironomid taxa was normally distributed after log-transformation ($P = 0.64, N = 108$). Overall abundance differed significantly between sites ($F_{3,3} = 86.95, P = 0.002$), and this factor accounted for 69.19% of the variance in abundance (Figure 5.5b). Week accounted for < 1% of the variance and had no significant effect on abundance ($F_{1,3} = 0.031, P = 0.87$).
Four common taxa that were present at two or more sites and contributed to over 71% of the abundance of animals caught on sticky traps were examined to determine the relative contributions of site and week to variation in abundance. *Limnophyes vestitus* was the most abundant species with 19,921 individuals. Its abundance was significantly affected by site ($F_{3,3} = 38.27, P = 0.007$), which explained 94.9% of the variance, but not by week ($F_{1,3} = 1.34, P = 0.45$; variance < 1%). It was more common at Site 1 than at other sites (Figure 5.6a). *Austrocladius terjugus* was the second most abundant species with 2,772 individuals. Its abundance was greatest at Site 3 (Figure 5.6b) and was significantly affected by site ($F_{3,3} = 19.27, P = 0.018$), which explained 89.1% of the variance. Week had no significant effect on its abundance ($F_{1,3} = 0.50, P = 0.53$, variance = 1.2%).

*Chironomus tepperi* was present at all four sites with 1,388 individuals collected. Its abundance did not significantly differ between sites ($F_{3,3} = 8.22, P = 0.06$), although site did account for 77.34% of the variance and abundance was higher at Site 2 (Figure 5.6c). Its abundance was not affected by weeks ($F_{1,3} = 0.77, P = 0.45$, variance = 1.24%). There were 648 individuals of *Paratrichocladius pluriserialis*, which was only present at two of the sites (Figure 5.6d). At these sites its abundance was not significantly affected by site ($F_{1,1} = 1.91, P = 0.40$) or week ($F_{1,1} = 0.001, P = 0.98$), and these factors accounted for 23.24% and 25.64% of the variance in abundance respectively.

The sex ratios of common taxa (excluding parthenogenetic *L. vestitus*) were compared between site/week samples. *Chironomus tepperi* sex ratios were highly skewed in different directions at different sites (Figure 5.7a). When sites and weeks were included as independent samples, sex ratio became increasingly male biased as the abundance of *C. tepperi* increased at the trap level (Figure 5.7b), but this correlation was not significant ($\rho = -0.48, P = 0.006, N = 32$). The sex ratio of *A. terjugus* also differed between sites (Figure 5.7c) and there was a strong negative correlation between abundance and the proportion of females at the level of individual sticky traps ($\rho = -0.56, P < 0.001, N = 53$) (Figure 5.7d). At the two sites where *P. pluriserialis* occurred its sex ratios were similar (63.5% and 62.1% of animals were female at Site 2 and Site 3 respectively), and no significant correlation existed between sex ratio and abundance ($\rho = -0.05, P > 0.05, N = 42$).
Discussion

Yellow sticky traps were an effective method for capturing adult Chironomidae \textit{in situ} and produced results that could be analysed to determine the chironomid community composition of a waterway. Diversity, abundance and sex ratios were quantifiable using sticky traps and these measurements varied among sites. The successful measurement of these endpoints with adults \textit{in situ} highlights the potential for biomonitoring with adult Chironomidae.

Species with distinctive morphological features, such as the white frosted wing bases of \textit{Paralimnophyes albibasis}, could be easily identified on the sticky traps. Others that required identification under a compound microscope were more difficult because animals could not be removed from the sticky traps without damaging characters crucial for identification. These animals could still be grouped based on morphological similarities but not identified to species. Having sweep net samples of intact insects available resolved this problem. Netted animals were in good condition for mounting, easy to identify and could be linked to samples on sticky traps.

Survey 2 showed that faunal composition of a site changed over time and also that sampling periods of several weeks were required to gain an accurate representation of fauna present at a site. Sampling with sticky traps over a period of five weeks was necessary to acquire 90\% of the species present at Trin Warren Tam-boore wetland; this period is similar to that of Bouchard and Ferrington (2011) who found sampling for six to eight weeks was necessary to accurately measure chironomid communities from pupal exuviae.

Chironomid community composition can largely be determined by the natural features of the habitat (Oliver, 1971) and ecological impairment such as pollution (e.g. Rae, 1989; Carew \textit{et al.}, 2007) or habitat degradation (O’Brien \textit{et al.}, 2010). In addition, changes to assemblages, populations and sex ratios can also indicate specific causes of impairment. For example, species richness, diversity and evenness of chironomid larvae were much lower at polluted sites than unpolluted sites (Janssens de Bisthoven and Gerhardt, 2003); \textit{Riethia stictoptera} larvae were found to be more sensitive to zinc pollution than other species (Pettigrove and Hoffmann, 2005a); the sex ratios of
Procladius species became heavily feminised after exposure to diuron (Ayres, unpublished; Townsend, unpublished). Biomonitoring approaches measure differences in community composition and other endpoints to predict and identify ecosystem impairment at a site (e.g. Chessman, 1995; Janssens de Bisthoven and Gerhardt, 2003; Carew et al., 2007). In Survey 3 the sticky traps were able to measure differences in chironomid assemblages, populations and sex ratios between sites, demonstrating their potential for indicating ecological impairment.

A problem with adult capture methods is discrepancies due to sampling bias, which may occur due the biology of different species. For example, species exhibit different emergence phenologies (Yamagishi and Fukuhara, 1971; Boerger, 1981; Ferrington et al., 1993) and within a species the sexes often emerge at different times (e.g. Titmus, 1979; Stevens, 1998). Chironomid assemblages can be seasonal and vary substantially throughout the year (e.g. Oliver, 1971; Bouchard and Ferrington, 2011), so to avoid measuring seasonal rather than site differences it is important that field sites are surveyed at the same time. In addition, weather conditions such as temperature also affect chironomid emergence (Boerger, 1981; Bouchard and Ferrington, 2009) and behaviours (Syrjämäki, 1968; Kon, 1984; Kon, 1989). However, rainfall and temperature did not appear to affect chironomid abundance in the current study.

Sampling biases may also occur due to behaviour and microhabitat utilisation differences of species and sexes. For example, some species have swarming behaviours that involve a “quasi-stationary flight over a landmark, often undertaken by many insects together, and during which mating takes place” (Downes, 1969). Swarming behaviours and the landmarks used for swarming vary between species and swarms are often composed of one sex, usually males (e.g. Downes, 1969; Kon et al., 1986; Kon, 1989; Hahn and Reinhardt, 2006). The proximity of a sticky trap to a swarm marker could result in the capture of a disproportionate number of animals from one species or sex. At Glynns Wetland in Survey 1 the position of the sticky traps did not greatly affect the numbers or diversity of chironomids caught; however in the third survey there was some evidence that extreme male biases at sites with high abundances, such as Site 2 for C. tepperi and Site 3 for A. terjugus. While no data exist in the literature for Austrocladius species, the sex ratios of C. tepperi in the laboratory are typically around 50 – 60 % female (Townsend et al., 2012). The
interception of a male swarm could account for such large numbers of males, although it has been suggested that *C. tepperi* is capable of reproducing without swarming (Martin and Porter, 1977) or does not swarm at all (Martin, 1974). Male biases were also seen in sweep net captures at Lake Cooper and also Site 5, Lake Buloke, where large numbers of predominantly male *C. tepperi* were caught (D. MacMahon, personal communication). These animals were not swarming but flying close to the ground or water surface. While the cause of the male bias could not be determined, the consistency of sticky trap results with those of the sweep netting would suggest that the observed relationship between abundance and sex ratio was real rather than an artefact of the sticky trap method.

Another problem inherent in many adult capture methods is that samples could contain animals that did not emerge from the site of interest (Verneaux and Aleya, 1999). However this problem is not limited to adult capture techniques. It may also occur with pupal exuviae and larval collections. Water currents and winds can affect the distribution of pupal exuviae so that collected specimens come from “an undefined area of stream” (Wilson and McGill, 1977). The distribution of larvae can be affected by drift (Ladle and Ladle, 1992; Ramírez and Pringle, 1998), stream disturbance, seasonal shifts in plant and sediment distribution, biological stressors (e.g. predation, competition, disease, parasitism), physical stressors and chemical stressors (Ladle and Ladle, 1992). Understanding the biology of the species involved can help minimise this issue when monitoring with all life stages. This is particularly true for understanding adult chironomid dispersal and migration, which appears to be species specific. For example, some *Chironomus* species, such as *C. flaviplumus* and *C. yoshimatsui*, do not disperse far from their place of emergence (Kon, 1989) and others like *C. anthracinus* migrate a short distance from the water body they emerged from until they sexually mature and migrate back to that water body (Tokeshi and Reinhardt, 1996). Other species, such as the midge *C. tepperi* found in the current study, are known to be an early colonising species of recently inundated waterways (Maher and Carpenter, 1984) with females preferentially avoid ovipositing in waters containing conspecific larvae (Stevens *et al.*, 2003). This species could be expected to migrate away from the waterway of emergence and adult data should therefore be used with caution as adults may be aggregating at sites from which they did not emerge. Assumptions can also be validated through the further development of the
sticky trap method, comparison of adult taxa with larval and pupal taxa present at a site, and by determining if adult responses at a field site indicate ecological impairment similar to larval responses.

**Conclusions**
Adult chironomids were successfully caught on the yellow sticky traps. Informative endpoints that are frequently used in biomonitoring and experimental approaches, such as community composition, diversity, abundance and sex ratios, could be measured using the sticky traps. The traps were relatively robust and required little ongoing maintenance or supervision. To gain an adequate representation of the fauna at a site, sampling needed to extend across a period of more than five weeks, although sampling for two weeks was still sufficient to show distinct differences in the chironomid fauna between sites. Differences such as these could be used to indicate the health of waterways and with further development yellow sticky traps could potentially be used for routine biomonitoring.
Acknowledgements
We wish to thank Linda Thomson for advice regarding the use of yellow sticky traps, David Sharley for his comments on this manuscript, Parks Victoria for the use of Glynns Wetland, Melbourne Water for the use of Trin Warren Tam-boor Wetland, and to Julian Woolhouse from the City of Melbourne and the Friends of Royal Park for keeping a watchful eye on Survey 2. We are especially grateful to Daniel MacMahon for his assistance with field work, and to Sara Long, Claudette Kellar, Rebecca Brown, Lisa Golding, Michele Schiffer, Steve Marshall and Stephen Tan for assisting with collections from Royal Park. Funding for this research was provided by Melbourne Water Corporation, the Victorian Department of Business and Innovation through support of the Centre for Aquatic Pollution Identification and Management, and the Australian Research Council through their Fellowship scheme.

References


Figure 5.1. The (a) total abundance of chironomids, (b) abundance when *Limnophyes vestitus* was excluded, and (c) Shannon-Wiener Index of Diversity $H'$ of chironomids caught on the sticky traps for each day the survey was carried out.
Figure 5.2. The contribution each of the common taxa to total abundance (a) including all common taxa and (b) excluding *Limnophyes vestitus* at different sampling times. □ = *Limnophyes vestitus*. §§ = *Tanytarsus fuscithorax*. □ = *Paratanytarsus grimmii*. □ = *Kiefferulus “tinctus”*. §§ = *Chironomus species*. §§ = *Cricotopus albitarsis*. §§ = *Polypedilum species*. §§ = *Paratrichocladius pluriserialis*. 
Figure 5.3. The cumulative number of taxa (% of total) caught on the sticky traps throughout the duration of the survey.
Figure 5.4. The sex ratio (proportion female) of (a) Tanytarsus fuscithorax, (b) Kiefferulus “tinctus”, (c) Chironomus species and (d) Cricotopus albitarsis samples plotted against the number of adults collected in samples.
Figure 5.5. The (a) number of taxa and (b) abundance of Chironomidae caught on the yellow sticky traps at four regional sites in Victoria, Australia. Open columns = week 1, filled columns = week 2.
Figure 5.6. The abundance of the common chironomid taxa (a) *Limnophyes vestitus*, (b) *Austrocladius terjugus*, (c) *Chironomus tepperi* and (d) *Paratrichocladius pluriserialis*. White columns = abundance in Week 1. Black columns = abundance in Week 2.
Figure 5.7. (a) *Chironomus tepperi* sex ratios (proportion female) at four rural sites, (b) *C. tepperi* sex ratios expressed in relation to abundance, (c) *Austrocladius terjugus* sex ratios at four rural sites, and (d) *A. terjugus* sex ratios expressed in relation to abundance. White columns = week 1. Black Columns = week 2. Black squares = Site 1, white squares = Site 2, black triangles = Site 3 and asterisks = Site 6, with each data point representing a single sticky trap.
### Tables

**Table 5.1.** Description of the rural biomonitoring sites in Survey 3.

<table>
<thead>
<tr>
<th>Site</th>
<th>Location</th>
<th>Waterway</th>
<th>Location</th>
<th>Land use type</th>
<th>Land use activities</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Tatura</td>
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<td>36.43°S</td>
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<td></td>
<td></td>
<td>Depression</td>
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<td>Dairy</td>
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<td>Viticulture</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Wanalta Creek</td>
<td>Wanalta Creek</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>144.51°E</td>
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Table 5.2. Common Chironomidae present on sticky traps at Glynns Wetland.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Species</th>
<th>Subfamily</th>
<th>Abundance</th>
<th>% total abundance</th>
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<td>“Orthoclad 1”</td>
<td>Orthocladiinae</td>
<td>1,171</td>
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<td>“Orthoclad 2”</td>
<td>Orthocladiinae</td>
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<td>Paralimnophyes albibasis</td>
<td>Orthocladiinae</td>
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<td>Tanytarsus inextentus</td>
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<td>36</td>
<td>2.46</td>
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Table 5.3. Common Chironomidae on the yellow sticky traps at Trin Warren Tamboore wetland.

<table>
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<tr>
<th>Rank</th>
<th>Taxon</th>
<th>Subfamily</th>
<th>Abundance</th>
<th>% total abundance</th>
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<td><em>Cricotopus albitarsus</em></td>
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<td>1.42</td>
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<td>8</td>
<td><em>Paratrichocladius pluriserialis</em></td>
<td>Orthocladiinae</td>
<td>36</td>
<td>1.42</td>
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Table 5.4. The number of taxa caught at each regional site with the two collection methods. Sticky trap data for Sites 4, 5, 7 and 8 are limited to shorter sampling events due to inclement weather.

<table>
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<tr>
<th>Site</th>
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Chapter 6: Biomonitoring with adult Chironomidae using yellow sticky traps in the Greater Melbourne Area, Australia

Abstract
Adult Chironomidae are useful for indicating toxicity in field-based surveys and laboratory experiments as they vary in their sensitivity to different types of toxins, but it can be difficult to sample these at field sites. Yellow sticky traps were demonstrated in the previous chapter to be suitable for capturing adult Chironomidae in the field. In the current study, yellow sticky traps were deployed at 24 sites with varying magnitudes and types of sediment pollution in the Greater Melbourne Area. The sticky traps provided measures of site differences in community composition, diversity, abundance and sex ratios of common chironomid species. In an exploratory analysis, measures of two contaminants in sediment – zinc (a surrogate for trace metals) and Total Kjeldahl Nitrogen (TKN) – were correlated with the chironomid data. Taxon abundance, sex ratio and biodiversity were not correlated with TKN, but for zinc there were positive correlations with the abundance and sex ratios of several species, suggesting the feasibility of using sticky traps for monitoring the health of waterways and wetlands. Several species of Chironomidae caught on the sticky traps were suspected of having terrestrial or semi-aquatic larval life stages, and these taxa might be useful for monitoring other aspects of the environment. As the biology and ecology of different chironomid species become better characterised, it should be possible to link Chironomidae caught on sticky traps to environmental contamination.
Introduction

Biological monitoring (biomonitoring) is a top down approach for assessing ecological condition by directly measuring the biological components of an ecosystem to identify impairment (Cairns et al., 1993). Biomonitoring is more costly than chemical assessments and laboratory toxicity testing in terms of money, time, effort and expertise. However, chemistry and toxicity assessments can only predict if impairment has occurred. Laboratory assays also lack ecological relevance in terms of test organisms and test conditions. Biomonitoring overcomes these disadvantages by allowing the inclusion of taxa that are difficult to maintain in the laboratory and by measuring responses that are the end result of interactions between an organism, stressors, and other components of its environment.

Unfortunately ecological complexity in biomonitoring can make it difficult to determine which stressors are responsible for observed responses. Often only correlations can be made between stressors and responses. However, concordance has been found between the results of field surveys and experiments, which suggest these correlations can indicate impairment. For example, many Chironomidae species caught in a field survey exhibited similar tolerances to zinc pollution as those collected in field-based microcosm experiments (Carew et al., 2007). Biomonitoring can also point to the involvement of specific stressors. For example, imposex, the growth of male genitalia in female neogastropods, appears to be a specific response to tributyltin (TBT), a persistent organic pesticide, and is subsequently being used to monitor TBT contamination in marine environments (Axiak et al., 2003).

The Dipteran family Chironomidae (chironomids) have a long history of use in biomonitoring. In the early 20th Century Chironomus plumosus was included in the Saprobien system to indicate pollution, organic enrichment and oxygen levels (Kolkwitz and Marsson, 1909). Chironomids are still widely used in biomonitoring approaches, such as the predictive modelling of AusRivAS (Smith et al., 1999), SIGNAL (Chessman, 1995), BEAST (Reynoldson et al., 1995) and RIVPACS (Wright, 1995). While many of these approaches rely on family and subfamily level identifications, species identifications can give more accurate conclusions about ecosystem state because taxa within families can differ in their sensitivities, habitat
preferences and biology (Lenat and Resh, 2001). Within the Chironomidae it has been shown that species can exhibit different sensitivities even within a genus (e.g. Watts and Pascoe, 2000; Carew et al., 2007; Carew et al., 2011).

Chironomids are also used in field-based experimental approaches (e.g. Pettigrove and Hoffmann, 2005; Sharley et al., 2008a; O’Brien et al., 2010) and laboratory toxicity assays (e.g. Benoit et al., 1997; Hahn et al., 2001; Dussault et al., 2008). Adult endpoints are used in these approaches and are highly informative. Adult emergence in the laboratory or adult abundance in a field experiment measure the effects of a stressor over the whole life of an animal. The sex ratios of adult chironomids have also been experimentally demonstrated to skew in response to specific stressors, such as female biases in Procladius paludicola exposed to diuron (Ayres et al., unpublished) and males biases in Chironomus tentans exposed to DDT (Rakotondravelo et al., 2006). Delays in the emergence of adult chironomids also indicate stress (e.g. Wilson et al., 2000; Watts et al., 2001).

Endpoints that represent the end of the life cycle can be considered better indicators of ecosystem condition because they “correspond to an organism that successfully utilized the habitat” (Bouchard and Ferrington, 2011). While adult Chironomidae can indicate ecological impairment at a site (e.g. Ruse et al., 2000) they are not commonly used in biomonitoring, with larvae used instead. This can be problematical for several reasons. Identifying larvae to lower taxonomic levels is difficult (Bouchard and Ferrington, 2008). Taxonomic keys for larvae are often incomplete or lack links with later life stages. Also, the presence of a larva does not guarantee the successful emergence of an adult because chironomid life stages can have different sensitivities to stressors. For example, the toxicity of tebufenozide to Chironomus riparius was only evident at the pupal moult (Hahn et al., 2001). In this example, if toxicity was only assessed in larvae, tebufenozide may have been wrongly identified as non-toxic to C. riparius. To account for chronic and life stage specific effects full life cycle testing has been developed in the laboratory (Benoit et al., 1997), but biomonitoring has largely disregarded this approach.

There are numerous methods available for capturing adult chironomids in situ for reasons such as pest control (Ali et al., 1986; Ali et al., 2008) and ecological and
biological research (Tokeshi, 1991; Verneaux and Aleya, 1999). In order for a method to be suitable for biomonitoring it needs to easily standardised, repeatable, robust enough to endure variable field conditions, involve deployment over a sufficient time period that chironomid assemblages are well-represented, allow multiple sites to be sampled simultaneously, and allow the capture of a sufficient number of animals for abundance and sex ratios to be reliably estimated. In Chapter 5 of this thesis the yellow sticky trap method was chosen for development and testing in pilot studies. The technique has successfully been applied in terrestrial biomonitoring of beneficial and pest invertebrate species in agricultural settings (e.g. Thomson et al., 2004; Sharley et al., 2008b). Chironomidae and other aquatic insects have also been caught on sticky traps in ecological studies (King and Wrubleski, 1998; Lynch et al., 2002).

Yellow sticky traps have many characteristics that make them suitable for biomonitoring. Sampling effort is easily standardised and repeatable. The traps are relatively cheap and easy to replace. Sampling can occur continuously over the course of weeks, giving good temporal coverage of chironomid assemblages, and the distribution of traps around a field site can ensure good spatial coverage. The technique requires little maintenance or supervision over the course of the experiment. As very little sampling effort, maintenance or supervision is required, numerous field sites can be sampled simultaneously with this method. The results of three pilot studies showed that sticky traps were suitable for collecting adult chironomids over a period of several weeks, with traps replaced weekly, and that significant differences in chironomid assemblages could be observed between different sites (Chapter 5). The aims of the current study were to further test the suitability of using yellow sticky traps for aquatic biomonitoring. For this purpose, yellow sticky traps were deployed at 24 sites in the Greater Melbourne Area. Site selection was based on current land use and previous chemical contamination data. Comparisons could therefore be made between biological responses and site chemistry to determine if biological responses were indicative of chemical contamination.
Methods

Yellow sticky traps (Agrisense) were used to intercept adult Chironomidae. These were commercially available plastic cards (240 mm X 100 mm) with adhesive on both sides. The sticky traps were deployed at 24 sites around the Greater Melbourne Area, Victoria, from the 17th May to the 1st July 2011 (Table 6.1). The sites were selected based upon recent chemical assessment, with some sites reported as clean while others had varying degrees and types of sediment pollution. At each field site 15 yellow sticky traps were deployed, which involved hanging these from vegetation, star posts, fences, bridges or other suitable structures using tie wire. The placement of the sticky traps varied between sites due to differences in the presence, availability and accessibility of structures suitable for hanging the traps. Wire cages to house the traps were constructed (9 mm mesh) to prevent interference by birds. The traps were left at each field site for a week before being collected and replaced with new traps. Collected sticky traps were stored in clear low density polyethylene press-seal bags (180 mm X 330 mm) (Grayson Packaging), which allowed the sticky traps to be stored without adhering to each other. Collected traps were stored at 4 °C.

There was insufficient time to conduct adult sweeps at each site for assisting in adult identifications. Instead, another approach was used where chironomid specimens were cut from the sticky traps and soaked in a citric acid based solvent (Orange Power; < 5 % citric acid) for up to three weeks to dissolve the glue on the sticky trap. Later, an improved method was developed (I. Valenzuela, personal communication). Chironomids were cut from sticky traps and soaked in a solution containing 40 – 80 % aliphatic hydrocarbon solvent and 8 – 20 % D-limonene (De-Solv-It Solution, RCR International, Australia) in a hot water bath (60 °C ) for 10 minutes. After this period animals were completely freed from the adhesive, rinsed in 100 % ethanol and stored for later identification. This method also allows DNA to be successfully extracted from animals to assist in later identification.

**Chironomidae identification**

Chironomidae that were removed from the sticky traps were dissected and mounted in Hoyers mounting medium on glass slides for examination under a compound microscope. Chironomidae were identified to species level where possible using the
keys by Freeman (1961) for subfamilies Tanypodinae, Orthocladiinae, Podonominae and Chironominae, Glover (1973) for Chironominae tribe Tanytarsini, Martin (2011) for *Chironomus* species and Hergstrom (1974) for Orthocladiinae. Identifications from Hergstrom (1974) have not been published in the peer reviewed literature so these were compared to Freeman (1961).

**Richness, diversity and evenness indices**

Diversity indices were calculated for each site and each week. Margalef’s Index of Diversity (equation 1) was used as a measure of species richness and calculated as:

\[
d = \frac{(S - 1)}{\ln N}
\]

where \(d\) = Margalef’s Index of Diversity, \(S\) = number of species and \(N\) = number of individuals. Diversity was measured in two ways. The first was with the Shannon-Wiener Index of Diversity (equation 2), which is given as:

\[
H' = -\sum_{i=1}^{S} p_i \ln p_i
\]

where \(H'\) is the Shannon-Wiener Index of Diversity, \(p_i\) is the proportion of individuals belonging to the \(i\)th species for all species \(S\). Simpson’s Diversity Index was also calculated for each site using equation 3:

\[
D = 1 - \sum_{i=1}^{S} p_i^2
\]

where \(D\) = Simpson’s Index of Diversity and \(p_i\) is the proportion of individuals belonging to the \(i\)th species for all species \(S\). In addition, Pielou’s Index of Evenness \((J')\) was calculated using equation 4:

\[
J' = \frac{H'}{\ln (S)}
\]

where \(H'\) is the Shannon-Wiener Index of Diversity and \(S\) is the total number of species.
Sediment chemistry

Sediment chemistry for the sites were derived from data collected during monitoring programs by the Centre for Aquatic Pollution Identification and Management, the Centre for Environmental Stress and Adaptation Research, Melbourne Water Corporation and the Department of Primary Industries Victoria. Where multiple datasets for a site existed only the most recent data were used. Data collected earlier than 2002 were excluded. All sediment chemistry data were derived from the fine fraction (< 63 µm) of surface sediments. The chemicals analysed often differed between sites depending on the aims of each monitoring program. Most sites had analyses conducted for total Kjeldahl nitrogen (TKN) and zinc (Zn), so these measures were linked to taxon data. Zn concentrations were positively correlated with other trace metals in this study ($\rho > 0.63$, $P < 0.05$, $N = 19 – 22$), with the exception of mercury, so Zn contamination was used as a surrogate for trace metal contamination.

Statistical analyses

The program PC Ord version 5 (McCune and Mefford, 1999) was used for examining aquatic chironomid community structure. Multi-response permutation procedures (MRPP) were conducted to determine if community structure differed between sites and weeks. Biological data were pooled across weeks and used in non-metric multidimensional analyses (NMS), which were conducted with relative Sørensen’s distance measures in the “slow and thorough” mode to identify significant axes. Relative Sørensen’s distance measure was used because the abundance of taxa differed sharply. Rare species that were only present at one site were excluded from the analysis. SPSS version 20 (IBM Chicago, IL) was used for all other analyses.

Spearman Rank correlations were computed between the sediment chemistry parameters and the richness and diversity indices. Differences in the chironomid fauna between sites and weeks were examined. Data were initially tested for normality using Kolmogorov-Smirnov tests. Data that were normally distributed were analysed using ANOVAs while those that were not normally distributed were analysed using Kruskal-Wallis tests. These were performed to determine if the number of taxa, overall abundance, diversity, abundance of common taxa and sex ratios differed.
between weeks and sites. Common taxa were selected for further analysis providing they met several criteria:

1. They must occur in no fewer than 10 % of samples (that is, the number of sites multiplied by the number of weeks sampled).

2. They must be relatively abundant and comprise greater than 1 % of the total abundance of all chironomid taxa.

Sex ratios were expressed as the proportion female and were arcsine square-root transformed prior to analysis; sex ratios were only computed for samples that had \( \geq 10 \) individuals. Analyses were corrected for multiple comparisons using Bonferroni corrections.

In general, week had little effect on the biological parameters (see below) so data were pooled across weeks for each site. Spearman Rank correlations were computed between the abundances of common taxa and the significant NMS axis scores to determine which species were contributing to specific axes. Correlations were also computed between the two measures of sediment pollution, zinc (log-transformed) and TKN, and biological measures including the significant NMS axis scores. Zinc concentrations were log-transformed because sites differed greatly in Zn contamination. Finally the association between taxon abundance and sex ratio of common taxa was examined to test if samples with a high proportion of one sex (particularly males) might occur when there were large aggregations of swarming individuals.

Several common species were identified that have unknown larval habitats or are terrestrial. Although these were excluded from analyses of community structure, the species-specific analyses described above were also used to explore the relationships between sediment measures on the abundance and sex ratios of these species.
Results
During the survey 63,326 individuals belonging to approximately 99 chironomid taxa were collected on the yellow sticky traps. Of these, 43,680 individuals from 55 species were known to have aquatic larval stages, and ten of these were considered common enough for further analysis (Table 6.2). One exception was *Austrocladius trichiatus*, which had an abundance of 0.91% of all taxa but was considered for analysis to provide a contrast with other *Austrocladius* species. Chironomid community structure differed significantly between sites ($A = 0.49$, $P < 0.001$). Two measures of species richness were originally calculated for each site (number of taxa and Margalef’s index of diversity). These were significantly correlated (Spearman Rank Correlation; $\rho = 0.89$, $P < 0.001$, $N = 22$) so only the number of taxa was used in subsequent analyses. Species diversity was measured using the Shannon-Wiener index ($H'$) and Simpson’s index of diversity ($1 - D$), and species evenness was also calculated for each site using Pielou’s index of evenness ($J'$). These were also significantly correlated with each other ($\rho > 0.93$, $P < 0.002$, $N = 22$) so $H'$ was used to represent diversity in subsequent analyses.

Site had a significant effect on the total abundance of aquatic taxa ($F_{23,92} = 29.14$, $P < 0.001$), the number of aquatic taxa ($F_{23,92} = 10.11$, $P < 0.001$), the Shannon-Wiener index of diversity $H'$ ($F_{23,92} = 10.01$, $P < 0.001$) and the abundances of all common taxa (Kruskal-Wallis test; $\chi^2 = 80.63 - 105.35$, $P < 0.001$, d.f. = 23). Week had a significant effect on the number of taxa ($F_{4,92} = 3.17$, $P = 0.017$) and diversity $H'$ ($F_{4,92} = 6.75$, $P < 0.001$) but not on other biological parameters.

Community structure
Non-metric multidimensional scaling showed chironomid communities were structured along three axes (with $P$ values from 0.004 to 0.04). These axes were correlated with biological parameters. Axis 1 had a strong positive correlation with diversity $H'$ ($\rho = 0.74$, $P < 0.001$, $N = 22$) and the abundance of *Austrocladius terjugus* ($\rho = 0.51$, $P = 0.016$, $N = 22$). Axis 2 was positively correlated with the abundance of *Polypedilum vespertinus* ($\rho = 0.59$, $P = 0.004$, $N = 22$) and negatively correlated with the abundance of *Austrocladius trichiatus* ($\rho = -0.70$, $P < 0.001$, $N =$
Axis 3 had a weak positive correlation with the overall abundance of aquatic taxa ($\rho = 0.53, P = 0.012, N = 22$) and the number of aquatic taxa ($\rho = 0.43, P = 0.048, N = 22$). It also strongly correlated with the abundances of *Paratanytarsus grimmii* ($\rho = 0.64, P = 0.001, N = 22$) and *Paralimnophyes pullulus* ($\rho = 0.82, P < 0.001, N = 22$ for *P. pullulus*).

**Environmental associations**

To determine what environmental variables were driving community structure, Spearman rank correlations were made between biological parameters and sediment chemistry data. Of the significant NMS axes, only Axis 1 had a significant negative correlation with Zn concentration ($\rho = -0.61, P = 0.003, N = 22$). This axis did not correlate with TKN and neither Axis 2 nor Axis 3 correlated with either sediment chemistry variable.

Zinc concentration negatively correlated with the number of aquatic taxa present at a site ($\rho = -0.48, P = 0.024, N = 22$) and the abundance of *A. terjugus* ($\rho = -0.75, P < 0.001, N = 22$) (Figure 6.1a). The response of *A. terjugus* differed to that of other *Austrocladius* species, which were not correlated with this measure, and the contribution of *A. terjugus* to the total abundance of *Austrocladius* spp. declined as Zn concentrations increased (Figure 6.1b). No *A. terjugus* were present at sites with Zn concentrations greater than 1,790 mg kg$^{-1}$. Neither Zn nor TKN significantly correlated with any of the other biological parameters tested.

**Sex ratios**

*Limnophyes vestitus* and *Paratanytarsus grimmii* sex ratios were not analysed because they are parthenogenetic. The sex ratios (female proportions) of the other common aquatic taxa were analysed in two ways. In the first, which focused on differences among sites, sex ratios were calculated for every site in each week (but only when ten or more animals of a species were present). This analysis showed that sex ratio did not significantly vary between weeks for any species.

The sex ratios of four species were significantly different between sites ($F_{11,29} = 18.65, P < 0.001$ for *P. pullulus*; $F_{4,6} = 10.31, P < 0.001$ for *P. vespertinus*; $F_{10,26} = 3.29, P <$
0.001 for *A. terjugus*; $F_{4,9} = 31.77, P < 0.001$ for *P. variegatus*). The sex ratios for other species were not significantly different between sites after correction for multiple comparisons.

The second analysis focussed on associations with sediment variables. Data were pooled across weeks for each site, and sex ratios were calculated when ten or more animals were present at a site before being correlated with Zn and TKN. The sex ratios of most species did not significantly correlate with Zn or TKN except for *A. numerosus* and *A. trichiatus*. *Austrocladius numerosus* sex ratios showed a weak positive correlation with Zn ($\rho = 0.90, P = 0.037, N = 5$), reflecting female biased sex ratios at high Zn concentrations, although this was no longer significant after correction for multiple comparisons. *Austrocladius trichiatus* sex ratios showed a positive correlation with Zn ($\rho = 1.00$), but this was based on only 4 samples.

In general there was no relationship between abundance and sex ratio. *Limnophyes pelurgis* and *P. variegatus* showed feminised sex ratios regardless of abundance (Figure 6.2d and 6.2e respectively), while for *A. terjugus* there was no clear pattern (Figure 6.2c). For *Paralimnophes pullulus* and *P. crassipennis* high abundances tended to have a male bias (Figure 6.2a and 6.2b respectively), but there were only a few data points and the correlation between these variables was not significant.

**Other Chironomidae**

Of the Chironomidae caught on the sticky traps, eight species with 2,695 individuals were terrestrial, while the life history and larval habitat of 16,951 Orthocladiinae belonging to 36 taxa were unknown. Of these, four were considered common (Table 6.3), and the abundance of each differed significantly between sites ($\chi^2 = 101.17 – 108.74, P < 0.001, \text{ d.f.} = 23$). The abundance of these species did not significantly correlate with Zn or TKN ($\rho < +0.33 P > 0.05, N = 16 - 22$), although a male bias was evident when the abundance of *P. latifurca* increased (Figure 6.3).

Sex ratios were examined for two species, *P. latifurca* and the terrestrial chironomid *S. aterrima*. The sex ratio of *P. latifurca* did not significantly differ between weeks ($F_{4,43} = 0.90, P = 0.47$) but did differ between sites ($F_{15,43} = 10.08, P < 0.001$). For this
species, sex ratio was not significantly correlated with Zn ($\rho = 0.38, P = 0.19, N = 14$) or TKN ($\rho = 0.31, P = 0.30, N = 13$) but was female biased (Figure 6.4a), and there was no significant correlation between abundance and the proportion female ($\rho = -0.14, P = 0.63, N = 14$). *Smittia aterrima* sex ratios also differed significantly between sites ($F_{12,31} = 3.14, P = 0.005$) but not weeks ($F_{4,31} = 0.72, P = 0.58$). Its sex ratios did not significantly correlate with TKN ($\rho = 0.36, P = 0.22, N = 13$) but weakly correlated with Zn ($\rho = 0.60, P = 0.02, N = 14$). Similar to *P. latifurca*, the sex ratio of *S. aterrima* was generally female biased (Figure 6.4b); there was no significant correlation between sex ratio and abundance ($\rho = 0.28, P = 0.38, N = 14$) although in the most abundant samples females predominated.
Discussion

The sticky trap method provided evidence for differences between sites in the abundance, richness, diversity and sex ratios of aquatic Chironomidae caught on the yellow sticky traps. Moreover, in the exploratory analysis there was evidence that some variables measured from the sticky trap catches may be correlated to Zn concentrations and perhaps trace metal pollution more generally. There was also concordance between this study and the pilot studies in Chapter 5, with significant differences between the fauna captured at sites but little effect of week on faunal composition.

Zinc was used as a surrogate measure of sediment pollution because it was highly correlated with other trace metals. However, other factors may also be important, particularly as previous work in the Greater Melbourne Area has shown Zn concentrations to be closely associated with anthropogenic activities, especially catchment urbanisation as measured by increased impervious surface area (Pettigrove, 2006). Urbanisation, which often results in increased pollutant loads in waterways, is also associated with riparian and aquatic habitat loss, altered stream hydrology and changed geomorphology, all of which affect aquatic biota (Paul and Meyer, 2001). The effects of other groups of chemical pollutants also need to be assessed. For example, synthetic pyrethroids such as permethrin and bifenthrin are known to be toxic to chironomids at low concentrations (Maul et al., 2008), and have been detected in waterways within the study area.

Chironomid communities differed substantially between sites, but this variation may be associated with a number of environmental variables. Based on larval surveys from the same region, Carew et al. (2007) found that chironomid assemblages were unable to adequately discriminate between polluted and unpolluted sites, although they found that some individual species were sensitive to environmental variables. Indices of ecological impairment based on subfamily identifications assume all species within a subfamily have similar sensitivities and tolerances. However, this assumption is not always accurate, as even species within a genus can show markedly different responses to stressors (Lenat and Resh, 2001; Carew et al., 2011). This was demonstrated here, where A. terjugus abundance strongly declined at sites with higher
Zn concentrations (trace metal concentrations), while no relationship existed between Zn and two congeneric species, *A. trichiatus* and *A. numerosus*. *Austrocladius* species have not been collected in previous larval surveys, so it is not possible to compare our results to those obtained previously. However *A. terjugus* was relatively more sensitive to Zn in this study than other species, such as *P. grimmi*, which has previously been found to have an intermediate level of tolerance of pollution (Carew *et al.*, 2007). The sticky trap method may therefore be able to collect other species that could act as sensitive pollution indicators.

Although sex ratio skewing is not well understood in chironomids, previous experiments have shown that skewing is not a general stress response but is specific to certain chemicals. For example, sex ratios of *Chironomus tentans* were male biased after exposure to DDT but not chlorpyrifos or atrazine (Rakotondravelo *et al.*, 2006), while sediments from sites along the River Murray system, Australia, caused adverse effects in *Tanytarsus fuscithorax*, including sex ratio skewing at two sites and in different directions (Townsend *et al.*, 2009). Responses are also taxon specific. Diuron caused the feminisation of sex ratios in *Procladius paludicola* (Ayres *et al.*, unpublished) and *P. villosimanus* but not in other genera (Chapter 3). Thus, being both stressor- and species-specific, sex ratio skewing may represent a potentially powerful tool for indicating the presence of particular stressors in an ecosystem. In the current study, sex ratios of different chironomid species varied between sites but were only weakly related to Zn concentrations. It remains to be seen if sex ratio can be linked to specific chemicals and particularly pesticides, which are more likely to affect sex ratios than trace metals.

A challenge in using sex ratio changes as a measure of ecological impairment is the possibility that it may also be affected by the relative abundance of chironomids. Many Diptera, including several species of chironomids, exhibit swarming behaviours during which flying insects aggregate together, usually over a landmark, for the purposes of courtship and mating (Downes, 1969). Chironomid swarms are mostly composed of males from one species (e.g. Downes, 1969; Kon *et al.*, 1986; Kon, 1989; Hahn and Reinhardt, 2006). Swarm interception by a sticky trap could result in male biased sex ratios. A similar phenomenon was observed in the pilot studies, albeit with different taxa. Female swarming behaviours have also been noted in Diptera such as
several species of Empididae (Svensson, 1997; Svensson and Petersson, 2000) and female aggregations in other Diptera may also be associated with ovisposition (e.g. Judd and Borden, 1992; McCall et al., 1994; Wertheim et al., 2002). Evidence of female swarming or aggregations have not been recorded in the literature for chironomid species.

A limitation in using sticky traps is that captured animals may not have emerged from the waterway of interest. This problem is not unique to sticky traps; pupal exuviae are also collected from an undefined area, especially in lotic environments where currents can carry exuviae downstream (Wilson and McGill, 1977). Understanding the ecology of the species caught on the traps can give confidence about whether animals emerged from a site. Species differ in their use of adult habitats and migration between habitats (Delettre et al., 1992). Broad generalisations about habitat use and behaviour, even within a genus, can be misleading. For example, two Chironomus spp. in Japan used habitats close to their place of emergence for resting and swarming (Kon, 1984; 1989). Although the species were sympatric, their use of habitats did not overlap in the 1989 study because they emerged from water bodies 100 m apart.

Further evidence that a captured adult emerged from a site can be derived from comparisons between the larvae and adults, and comparing characteristics of a site to sites where larvae of the species have been found. Larval data for 15 of the sites used in the current study were collected in 2003 and 2004 as part of a larger study (Carew et al., 2007), however comparisons were not made with the current study because data were collected seven years apart, and at a different time of year. Chironomid assemblages vary substantially across seasons and even years (e.g. Oliver, 1971; Carew et al., 2007; Bouchard and Ferrington, 2011).

**Monitoring other larval habitats**
Chironomid larvae are not restricted to freshwater environments; many Orthocladiinae have terrestrial life stages, and this group was caught on the sticky traps. Some species of Orthocladiinae that were captured do not have described larval habitats, such as Pseudosmittia latifurca. Terrestrial and semi-aquatic chironomids live in temporary habitats that may become unsuitable due to desiccation stress. These
species have developed a range of mechanisms including recolonisation, diapausing lifestages, cocooning and larval migration to persist in these environments (reviewed in Frouz et al., 2003).

One terrestrial species, *Smittia aterrima*, differed in its abundance between sites. This species has global distribution and has been recorded in Europe (Laurence, 1954; Elberling and Olesen, 1999; Gardarsson et al., 2004; Delettre, 2005), Atlantic islands (Raposeiro et al., 2009), Asia (Chaudhuri and Bhattacharyay, 1989; Tanaka et al., 2003) and Australia (Freeman, 1961), and occupies a range of disturbed terrestrial habitats including fields (Jones, 1976; Delettre, 2005), rice paddies (Tanaka et al., 2003), alpine areas (Elberling and Olesen, 1999), tall heathlands (Delettre, 1994) and vending machines in cities (Hirabayashi et al., 2001). Adults are more common over open areas, with females preferentially ovipositing in open vegetation (Frouz, 1997) while larvae are saprophagic (Jones, 1976). Animals are most abundant in autumn, spring and milder winters (Laurence, 1954; Tanaka et al., 2003; Hirabayashi et al., 2001) and emergence is favoured by cool damp weather (Jones, 1976). It is not clear if site variation in this species is related to these ecological factors or environmental contamination.

Another orthoclad detected here, *Limnophyes vestitus*, has larvae known to inhabit very shallow waters where it feeds on algae and detritus. In laboratory cultures with water depths greater than 5 mm, pupae of this species die (Edward and Colless, 1968). This species has not been reported in previous larval surveys of the Greater Melbourne Area, possibly because its preferred habitats of temporary ponds, puddles or recently inundated areas are not typically included in aquatic surveys. Temporary waterways are ecologically important, highly threatened by anthropogenic activities and not well understood or appreciated (Schwartz and Jenkins, 2000). Recently inundated areas, such as floodplains, are also ecologically important (e.g. Tronstad et al., 2007) and have a role in exchanging nutrients, organic matter and sediments with permanent waterbodies (e.g. Tockner et al., 1999). Harmful and persistent substances can be deposited in shallow or temporary waterways (e.g. Middelkoop, 2000; Xiao et al., 2011), potentially causing toxicity to organisms using these habitats. Although the abundance and sex ratios of *L. vestitus* did not correlate with measures of pollution in the current study, its prevalence and the fact it can be cultured in the laboratory
(Edward and Colless, 1968) make it another species that is potentially suitable for ecotoxicological studies.

**Conclusions**

Yellow sticky traps were used to test for differences in the chironomid fauna between sites. Variables such as species richness, diversity, abundance and sex ratios have previously been used in biomonitoring with larvae or experimental studies with adults to demonstrate impairment due to a stressor. All of these variables can clearly be measured with sticky traps, demonstrating the potential of using this approach for biomonitoring. Although the current surveys were not designed to test for pollution effects, some measurements were correlated with Zn pollution, and in particular the abundance of a species not previously collected in high numbers through larval surveys was potentially negatively affected by pollution. The sticky trap method is also useful in detecting several taxa known to inhabit terrestrial or shallow water environments.
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I would like to thank my supervisors Ary Hoffmann and Vincent Pettigrove for advice on experimental design, site selection, data analyses and for revising this chapter. Thanks also to Cameron Amos and Daniel MacMahon for assisting with fieldwork, and to Bryant Gagliardi, Rebecca Brown, David Sharley and Simon Sharp for contributing to field work. David also helped with revising this chapter. I am grateful to the terrestrial biomonitoring group at the Centre for Environmental Stress Adaptation and Research (CESAR) for information on using sticky traps and removing animals from them, and especially to Isabel Valenzuela for developing an improved method of dissolving the adhesive. Thank you to the past and present staff of CESAR and the Victorian Centre for Aquatic Pollution Identification and Management for the sediment chemistry data collected over the years. This research was funded by the Melbourne Water Corporation, the Victorian Department of Business and Innovation through support of the Centre for Aquatic Pollution Identification and Management, and the Australian Research Council through their Fellowship scheme.

References


Figure 6.1. Sediment zinc concentrations (log-transformed mg kg\(^{-1}\)) versus (a) the log-transformed abundance of *Austrocladius terjugus* and (b) the proportion of *A. terjugus* of all *Austrocladius* spp. caught from 22 waterways in the Greater Melbourne Area.
Figure 6.2. The proportion female versus the abundance of (a) *Paralimnophyes pullulus*, (b) *Pseudosmittia crassipennis*, (c) *Austrocladius terjugus*, (d) *Limnophyes pelurgis* and (e) *Parakiefferiella variegatus*. 
Figure 6.3. Sediment zinc concentrations (log-transformed mg kg$^{-1}$) versus the log-transformed abundance of *Pseudosmittia latifurca* caught from 16 waterways in the Greater Melbourne Area.
Figure 6.4. Abundance versus the sex ratio (proportion female) of (a) *Pseudosmittia latifurca* and (b) *Smittia aterrima*. 
### Tables

**Table 6.1.** Location of sticky trap survey sites, Melbourne, Victoria, Australia.

<table>
<thead>
<tr>
<th>Site</th>
<th>Name</th>
<th>Location</th>
<th>Suburb</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Jacksons Creek</td>
<td>Riddells Creek Road</td>
<td>Riddells Creek</td>
<td>37.49° S</td>
<td>144.68° E</td>
</tr>
<tr>
<td>2</td>
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<td>Annandale Road</td>
<td>Melbourne Airport</td>
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<td>144.84° E</td>
</tr>
<tr>
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<td>Tullamarine</td>
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</tr>
<tr>
<td>4</td>
<td>Maribyrnong River</td>
<td>Flora Street</td>
<td>Keilor</td>
<td>37.72° S</td>
<td>144.84° E</td>
</tr>
<tr>
<td>5</td>
<td>Trin Warren Tamboore Wetland</td>
<td>Royal Park</td>
<td>Parkville</td>
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<tr>
<td>6</td>
<td>Edgars Creek Wetland</td>
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<td>Reservoir</td>
<td>37.71° S</td>
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</tr>
<tr>
<td>7</td>
<td>Mount Cooper Wetland</td>
<td>Bundoora Park</td>
<td>Bundoora</td>
<td>37.71° S</td>
<td>145.05° E</td>
</tr>
<tr>
<td>8</td>
<td>Greswell Reserve wetland</td>
<td>Main Drive</td>
<td>Bundoora</td>
<td>37.71° S</td>
<td>145.06° E</td>
</tr>
<tr>
<td>9</td>
<td>Glynn's Wetland</td>
<td>Glynns Road</td>
<td>North Warrandyte</td>
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<tr>
<td>10</td>
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<td>Macintyre Lane</td>
<td>Yering</td>
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<td>11</td>
<td>Brushy Creek</td>
<td>Black Springs Road</td>
<td>Chirnside park</td>
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<td>145.29° E</td>
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<tr>
<td>12</td>
<td>Croydon wetland</td>
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<tr>
<td>13</td>
<td>Bungalook Creek Sediment Trap</td>
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<td>Bayswater North</td>
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<td>14</td>
<td>Liverpool Road Retarding Basin</td>
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<td>The Basin</td>
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</tr>
<tr>
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<td>McMahons Creek</td>
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<tr>
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<td>Altona</td>
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<td>Newport</td>
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<td>Highett</td>
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<td>145.03° E</td>
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<td>Craigmore Avenue</td>
<td>Mentone</td>
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<td>145.09° E</td>
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<td>Lynbrook Estate Wetland</td>
<td>Banjo Patterson Park</td>
<td>Lynbrook</td>
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<td>145.25° E</td>
</tr>
<tr>
<td>22</td>
<td>Eumemmerring Creek</td>
<td>Frog Hollow Reserve</td>
<td>Endeavour Hills</td>
<td>37.99° S</td>
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<td>Belgrave Lake</td>
<td>Belgrave Lake Park</td>
<td>Belgrave</td>
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<td>Ferny Creek Retarding Basin</td>
<td>Gilmour Park</td>
<td>Upper Ferntree Gully</td>
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<td>Rank</td>
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<td>Subfamily</td>
<td>Abundance</td>
<td>No. of sites</td>
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<tr>
<td>------</td>
<td>--------------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>--------------</td>
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<td>3</td>
<td><em>Polypedilum vespertinus</em></td>
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<td>5</td>
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<td><em>Paratanytarsus grimmii</em></td>
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<td><em>Parakiefferiella variegatus</em></td>
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<td>9</td>
<td><em>Austrocladius numerosus</em></td>
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<td>10</td>
<td><em>Austrocladius trichiatus</em></td>
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</table>
Table 6.3. Common Orthocladiinae caught on the sticky traps with unknown or terrestrial larval habitats.

<table>
<thead>
<tr>
<th>Species</th>
<th>Habitat</th>
<th>Abundance</th>
<th>No. of sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudosmittia latifurca</em></td>
<td>Unknown</td>
<td>11,606</td>
<td>17</td>
</tr>
<tr>
<td><em>Sparsicladius graminis</em></td>
<td>Unknown</td>
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<td><em>Smittia aterrima</em></td>
<td>Terrestrial</td>
<td>2,007</td>
<td>15</td>
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<tr>
<td><em>Kiefferophyes arthrothrix</em></td>
<td>Unknown</td>
<td>742</td>
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Chapter 7: General conclusions

It is recognised that the protection of valuable water resources requires maintaining the ecological integrity of aquatic ecosystems. Biological assessments have been crucial in this regard, as they measure and predict ecological impairment, whereas chemical assessments can only predict that degradation may occur (Krantzberg, 1992). The Chironomidae (chironomids) are frequently used as bioindicators of ecological health, although there are important limitations regarding their use. These include, but are not limited to, the oversimplification of laboratory tests, an insufficient understanding of how factors other than direct toxicity can affect responses, scarcity of data and conflicting results regarding the effects of specific stressors, and the neglect of adult endpoints in biomonitoring. This thesis presents research that addresses some of these limitations. In the following discussion the main outcomes of the thesis will be discussed with implications and recommendations for further research.

Laboratory tests are important for testing the toxicity of environmental samples and pollutants, but in most cases lack ecological relevance (Beketov and Liess, 2012). The oversimplification of these tests can give misleading results because the exposure of an organism to a stressor in the field is more complicated than simply direct toxicity. Two factors that can affect biological responses are trophic effects (Fleeger et al. 2003) and complicated exposure histories. These were examined in Chapter 4. *Chironomus tepperi* were exposed to food limitation to simulate the indirect effects a pollutant may have on prey species of chironomids. Reduced nutrition induced effects that resembled toxicity, including reduced survival, delayed emergence and decreased reproduction. The experiment was conducted over two generations to examine multigenerational effects. Offspring of food limited parents were of poor quality, even when offspring were raised under standard food conditions. These results highlight an important problem in ecotoxicology and biomonitoring, where the responses of an organism are attributed to direct toxicity without acknowledging that other factors may be contributing to responses. Previous work has shown ways in which food availability can affect chironomids (e.g. Hooper *et al.* 2003) and contaminants can
exert effects over several generations (e.g. Janssens de Bisthoven et al., 2001; Bahrndorff et al. 2006; Vogt et al., 2007), but these are still not widely included in testing. In other taxa it has been demonstrated that food availability can affect sensitivity to a stressor (e.g. Liess et al., 2001; Beketov and Liess, 2005) and stressors can exert effects over multiple generations (e.g. Brennan et al., 2006; Oliveira-Filho et al., 2009). The effects of food availability and multigenerational effects should be routinely considered in bioassessments with chironomids, but these are rarely considered.

Sex ratio skewing is another area that is not routinely included in bioassessments, and one of the main purposes of this thesis was to investigate sex ratio skewing in chironomids as a measure of ecological impairment. Skewed sex ratios have been used in other organisms to indicate the presence of specific stressors and the mechanisms by which stress is occurring (e.g. Länge et al., 2001; Vajda et al., 2008). In sexually dimorphic species, such as chironomids, sex ratios are easy to measure, and the results of a few studies have shown that sex ratio skewing in chironomids is stressor specific (Rakotondravelo et al., 2006; Townsend et al., 2009) and species specific (Ayres et al., unpublished). Therefore, chironomid sex ratios are potentially useful endpoints for indicating specific types of stress, and yet little data exist for these.

Sex ratios were measured in all experiments, and evidence was found that supports the notion that skews are stressor-specific and likely to be induced by certain chemicals, with skews induced by diuron and tebufenozide (Chapter 3), but not 17α-ethinylestradiol (EE2) (Chapter 2) or food limitation (Chapter 4). In Chapter 3 it was evident that skews were also species-specific, with tebufenozide and diuron causing skewed sex ratios in different species. Diuron feminised the sex ratios of Procladius villosimanus, which concurred with the results of Ayres et al., (unpublished) who also observed extreme female biased sex ratios in the congeneric P. paludicola after diuron exposure. Female biased sex ratios in Procladius spp. could be used to indicate diuron contamination of environmental samples. Further work is needed to determine if Procladius sex ratios are skewed by other contaminants, particularly other phentylurea herbicides, and to validate the result using diuron contaminated environmental samples. Sex ratio skewing was also measured in adults captured in the
field (Chapters 5 and 6), indicating the presence of different sources of ecological
stress at field sites. Sex ratio skewing was shown to be a potentially specific and
sensitive measure of pollution effects. While more data are required regarding other
contaminants, the mechanisms of skewing and implications for populations, these
results provide a case for routinely measuring sex ratios in ecotoxicology.

Chemicals can have adverse effects on organisms that may not result in mortality, so
endpoints that measure these effects can be more sensitive indicators of impairment
than lethality. One such endpoint is head capsule deformities in chironomid larvae.
These have been observed in the field (e.g. Madden et al., 1992; Williams et al., 2001;
Bhattacharyay et al., 2005) and laboratory (e.g. Vermeulen et al., 2000; Meregalli et
al., 2001), and their presence has been associated with different chemical pollutants.
Different types of deformities have been linked to the presence of specific chemicals
(Janssens de Bisthoven et al., 1998). In Chapter 2 of this thesis, high nominal
concentrations of EE2 induced a novel antennal deformity in Procladius paludicola
and P. villosimanus larvae, whereas effects on other biological endpoints were not
observed. Although the deformity occurred at concentrations unlikely to be
encountered in the environment, it reinforces the idea that some sublethal effects can
be used to indicate specific types of contamination. Further investigations into this
particular deformity have little relevance for biomonitoring, even though it would be
interesting to investigate if other estrogenic compounds or environmental samples
elicit the same effect, and if similar responses can be observed in other species.

Chironomid development rate is another useful, sublethal endpoint that is often
affected by chemicals at concentrations where few other responses are observed.
Watts et al. (2001) and Dussault et al. (2008) both found that development rate in
Chironomus species was the most sensitive endpoint to EE2. This contrasts with the
result described in Chapter 2, where EE2 did not affect the development rate of any
common chironomid taxa. Food limitation, on the other hand, caused significant
developmental delays in Chironomus tepperi (Chapter 4) and was the most sensitive
endpoint measured in that experiment. Interestingly the development rate of the sexes
differed when food became limited. In the second generation, delayed development
was even more pronounced in females than males when food was limited. In several
chironomid species female larvae and pupae are known to be larger and have greater
energy reserves (Atchley, 1971; Servia et al., 2006; Benbow, 2008), probably because females require more energy to produce eggs. This suggests that females may be more susceptible to stressors that impede development, particularly those that could exert indirect effects by altering the abundance of chironomid prey species. Although development rate cannot be measured in the field, it can be readily incorporated into laboratory studies and field-based experiments if these are frequently sampled.

One of the key findings presented in this thesis concerns the presentation of data that are novel for Australian species. Many of the guidelines we use to assess toxicity are derived from single species tests using international data, which may not be relevant for Australian conditions. These data need to be tested using Australian species and under local conditions before they can be deemed relevant for local situations. In the case of toxicity tests the use of local fauna is vital, but the procedures for conducting toxicity tests are optimised for international species (e.g. US EPA, 2002; OECD, 2004a, 2004b), which may not be suitable. For example, the reconstituted water recommended by Martin et al. (1980) for many Australian Chironomus species has higher concentrations of sodium chloride than those recommended for European species (Martin, 2009). The OECD guidelines (2004a, 2004b) recommend the optimal amount of food for Chironomus riparius is 0.5 – 1.0 mg larva⁻¹ day⁻¹, and note that this may need altering for other test species (C. tentans and C. yoshimatsui) mentioned in the guidelines. In testing the effects of food limitation in Chapter 4 it was shown that the optimum level of nutrition for C. tepperi was 0.5 mg larva⁻¹ day⁻¹ for late instar larvae; even animals fed the standard amount, which was the upper value recommended for C. riparius by the OECD guidelines, did not fare as well for some measures. This demonstrates the importance of optimising our culture methods and toxicity tests for Australian species.

The situation is different for biomonitoring in that approaches are designed and tolerance values are defined specifically for Australian taxa. However, tolerance values are usually assigned for families or subfamilies (e.g. Chessman 1995; Barmuta et al. 1998). Although family and subfamily assessments require less time, taxonomic expertise and can still demonstrate sensitivity to toxicants, the assumption that all species within a family exhibit similar tolerances can be wrong and lead to inaccurate assessments of ecosystem condition (Lenat and Resh, 2001). Biomonitoring and field-
based microcosm experiments have demonstrated that chironomid species differ in their responses to various types of pollution (e.g. Pettigrove and Hoffmann 2005; Carew *et al.* 2007). The composition of chironomid assemblages can therefore be used to indicate different types of pollution, although more data regarding how different species respond to other pollutants are required first. Studies elucidating the effects of different pollutants on chironomid assemblages are valuable. The results of the experiments from Chapters 2 and 3 provide some of this data; chironomid assemblages are unlikely to be affected by EE2, tenufenozide or diuron at ecologically relevant concentrations of each chemical, although diuron contamination may result in an increase in the number of chironomid species present. Experiments such as these contribute substantially to our knowledge of the factors affecting chironomid assemblages, and as more data become available it may one day be possible to ascribe tolerance values for specific pollutants to individual species.

Another important biomonitoring area where information is lacking is the use of adult endpoints. Endpoints that show an organism has successfully survived its environment through its entire life cycle are important in assessing ecotoxicity (Bouchard and Ferrington, 2011) and are subsequently included in laboratory tests (e.g. Watts *et al.*, 2001; Rakotondravelo *et al.*, 2006) and field-based experiments (e.g. Pettigrove and Hoffmann, 2005; Sharley *et al.*, 2008). To a large extent these endpoints are ignored in biomonitoring, where larvae are predominantly used to indicate ecosystem health. In Chapters 5 and 6 the development of a quantitative approach for capturing adult chironomids *in situ* is described. The method chosen was yellow sticky traps, which have been successfully used in terrestrial biomonitoring (e.g. Thomson *et al.*, 2004) and ecological surveys of chironomids (King and Wrubleski, 1998). These were effectively used to intercept adult chironomids at field sites and were able to measure differences in fauna between sites. Exploratory analyses showed some of the data correlated with zinc pollution in sediments, highlighting the biomonitoring potential of this method. Further work to distinguish what environmental factors are affecting the chironomid assemblages will be valuable for improving the ability of the method to identify aquatic ecosystem degradation and its causes. There were some problems with the method that are common for many adult capture techniques, especially uncertainty as to whether the chironomids emerged from the site of interest (Verneaux and Aleya, 1999), but with continued
testing, comparisons to larval surveys and tolerance values reported in the literature, and an increased understanding of the behaviours of different species will address this limitation.
Conclusions

By assessing responses of particular species to different stressors, testing a variety of endpoints, and developing new approaches for measuring these endpoints, bioassessments based on chironomids can be expanded to indicate specific types of impairment. Two areas of research presented in this thesis that show promise are sex ratio skewing and field surveys of adult chironomids. Comprehensive data using these approaches are still lacking. The routine inclusion of sex ratio measurements in studies should help to build up information on this endpoint. Biomonitoring with adults now requires rigorous testing before it can be routinely used. With improvements in our understanding of chironomid biology and responses to stressors, biological assessments with chironomids will become increasingly powerful and useful for identifying, evaluating and predicting the impairment of aquatic ecosystems.

References


