

CONSTRUCTION AND EVALUATION OF A NEW LABORATORY SYSTEM FOR REARING MAYFLIES

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ABSTRACT

A new laboratory system was developed for rearing adults of Australian Leptophlebiidae (Ephemeroptera). The space-efficient chambers, made from readily available materials, are an inexpensive alternative to conventional rearing systems and worked just as well, and in many cases better, than a comparable larger system. Genera differed significantly in rearing success. No genera were significantly affected by the rearing conditions except *Austrophlebioides* Campbell and Suter (1988), which responded strongly to all external factors. This suggests that a more restricted environmental regime may be required to maximise emergence success for this genus. Sex ratios were biased towards females for all genera, indicating the possible occurrence of parthenogenesis in Australian Leptophlebiidae.

INTRODUCTION

Positive identification of species requires examination of all life stages for most aquatic insects. However, in most cases, identification of species has been based on the nymphs or adults only with no association being made between the two (Hynes, 1970; Smock, 1996; Merritt *et al.*, 1996). Field collecting of nymphs and adults in one location is an accepted method of identifying all insect life stages but has the inherent problem with discriminating between different species, especially if one has to rely on immature nymphs for initial identification. An insect reared from an immature stage to an adult, with the subsequent larval skin moult kept for comparison, provides the definitive association.

Many authors have made suggestions for rearing aquatic insects to adults (see review by Merritt *et al.*, 1996). The two main approaches are field and laboratory rearing. Most field rearing techniques involve a mechanism for containing the nymphs within the existing water body and providing room for the animal to emerge while safe from drowning (Speith, 1938; Fremling, 1967; Day, 1968; Schnieder, 1967; Edmunds *et al.*, 1976). An alternative is the use of emergence traps (Hynes, 1941; Southwood, 1978; Merritt *et al.*, 1996). Despite the relative

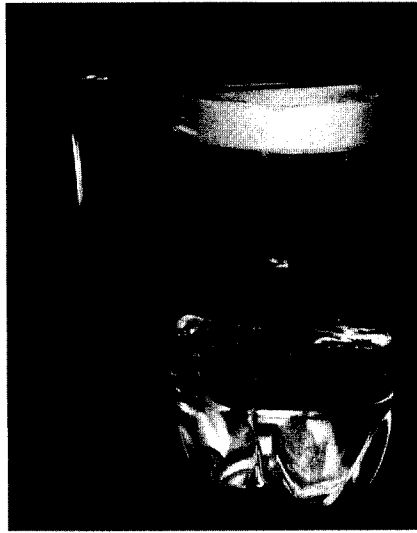


Fig. 1. The new rearing chamber. The aquarium tubing is connected to an air supply.

simplicity of design, the disadvantage of field rearing is that it may require the researcher to be away from the workplace for extended periods. Laboratory methods range from simple to extremely complex as authors have tried to address the problem of recreating stream flow conditions. Covered aquariums are used in conjunction with various methods of inducing a current such as a magnetic stirrer (Mason and Lewis, 1970) or directed air (Craig, 1966). Gravity flow systems were first used by Hynes (1941) and improved upon by Mason and Lewis (1970). Later came the development of large flow tanks powered by propellers (Vogel and La-Barbera, 1978) and complicated systems designed more specifically for the purposes of toxicity testing (Buikema and Voshell, 1993). All have the disadvantage of being suitable only for mass rearing and often requiring large inputs of electrical power. None address the issues of cost-effectiveness and simplicity in a field where rearing is likely to be of secondary concern.

The rearing of mayflies can be especially difficult because of the presence of a fragile subimago stage which has characteristics different to those of the adult. With all these factors in mind I have designed and tested a new laboratory rearing system for mayflies. Each chamber houses one individual and allows the animal to pass through the stages of nymph and subimago without disturbance. The chambers are made from readily obtainable material and are easy to construct. They are space-efficient and inexpensive, costing less than US\$2.00 each when an air supply is available. Such systems have been described before in the literature (Merritt *et al.*, 1996) but I have yet to find a published account of their use.

An analysis of the success of the new rearing system by imago emergence success rates in relation to genus, sex, photoperiod, year of collection, temperature, and for an alternative chamber type has been conducted. The influences of altitude of collection and refrigerated storage of nymphs, which was often necessary after a prolonged collecting trip, were also investigated. These data were collected as a consequence of rearing mayfly adults for taxonomic review rather than experimental purposes. The data collected provides indications of how these genera may respond to factors influencing emergence in the field, however, the laboratory outcomes are, at present, analysed at genus level only. Within each genus there may well be species with very different responses to these environmental factors. Further, only one controlled temperature room was used for this work, therefore all individuals from each

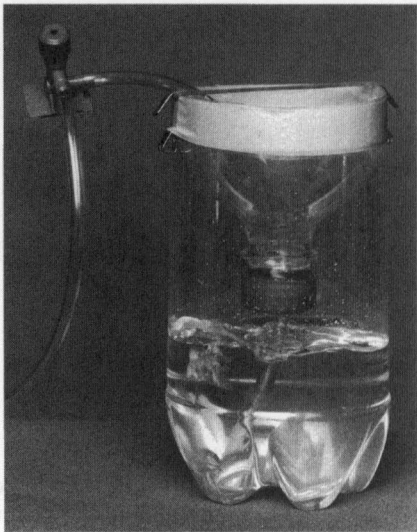


Fig. 1. The new rearing chamber. The aquarium tubing is connected to an air supply.

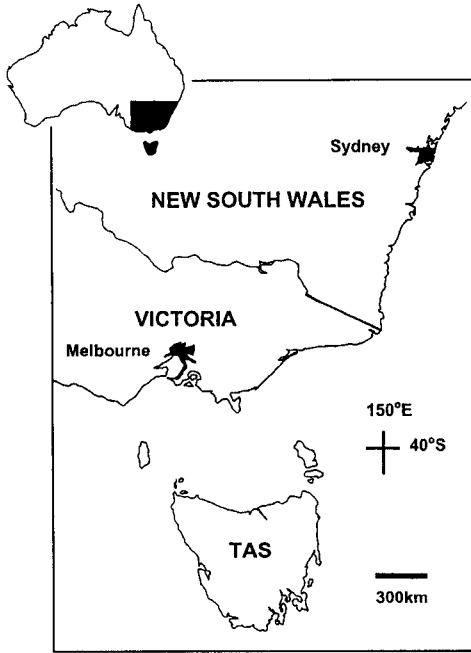


Fig. 2. South-east Australia: area from which mayflies were collected.

separate field collection were placed in the same temperature and photoperiod regimes. As a result the species compositions of the groups receiving each set of conditions may well have been different. In these circumstances it is difficult to be confident that the results obtained mirror responses in the field. Nevertheless, these observations may be of interest to workers concerned with field responses of mayfly nymphs to environmental conditions.

METHODS

Chamber Design

The new rearing chamber (Figure 1) was constructed from a 1.25 litre plastic soft drink bottle. This was cut in two at about two-thirds its length, at the point where the sides start to converge towards the lid. The open container was lined with nylon mesh, which can either be glued in place or simply wetted. Two hooks were attached facing outwards and opposite each other on the outside of the bottle using electrical tape, so that a rubber band could be stretched between them across the open end. A small hole (diameter of 6-7 mm) was drilled in the plastic bottle lid. The top third of the bottle with the lid was then inverted to sit in the chamber, lid downwards, and secured by the rubber band. The chamber was then half filled with water. Compressed air was supplied to the chamber by means of PVC aquarium tubing (interior diameter 4 mm) attached to a pump or laboratory air supply. Up to ten chambers can be aerated from one small 240V air pump linked through aquarium tubing, although each chamber requires a two-way controller so flow can be balanced. It is also advisable to attach a plastic micropipette tip to the end of the tubing to restrict the flow of bubbles to a small stream. Glass pipettes proved too fragile and, being heavier, were prone to blockage by resting on the chamber bottom.

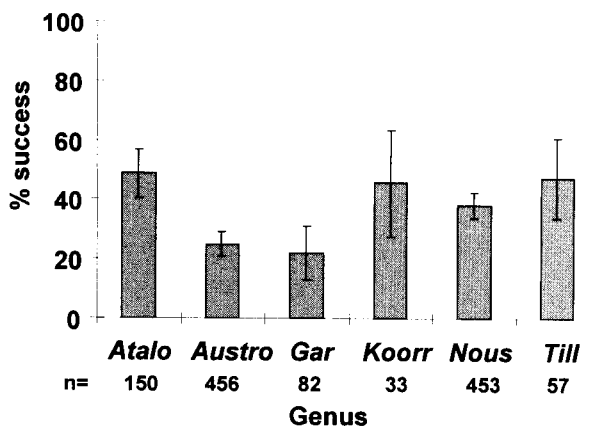


Fig. 3. Imago emergence success by genus.

Abbreviations for genera are as follows: Atalo=*Atalophlebia*, Austro=*Austrophlebioides*, Gar=*Garinjuga*, Koorr=*Koornonga*, Nous=*Nousia*, Till=*Tillyardophlebia*. Bars represent 95% confidence intervals.

Data Collection

Mayfly nymphs from the family Leptophlebiidae were collected over two extended summer periods; October 1996 to April 1997 (year 1) and October 1997 to April 1998 (year 2) from 123 sites throughout Victoria, Tasmania and New South Wales (Figure 2). Animals were collected from altitudes on the shoreline (<10 m) to near the summit of Australia's highest point, Mt Kosciusko (1650m), and therefore represent species from a wide range of climatically diverse regions. Two closely related Leptophlebiids, *Nousia* Navás (1918) and *Koornonga* Campbell and Suter (1988), were targeted for collection as part of a larger taxonomic study being undertaken, although all Leptophlebiidae were collected if found. *Nousia* and *Koornonga* are relatively common in stream riffles in association with logs and organic matter (Peters and Campbell, 1991), so the sampling regime favoured these areas.

Late instar nymphs were carefully removed from the substrate with a paintbrush and placed in a bottle of the stream's water. The bottle was sealed and placed on ice for transportation to the laboratory. During the day the water was adequately aerated through the motion of the vehicle but at night a battery-operated pump was employed to aerate each chamber.

Laboratory Rearing

Each rearing chamber was half filled with water from a particular site; and one late instar individual from that site added. Twigs collected from the site were added to each chamber for the insect to use as a food source and as a platform for emergence. Each chamber was attached to an air supply and placed in controlled temperature room environments at 16°C, 18°C, 20°C or 22°C. Photoperiods of 12 hours daylight and darkness (12:12) or 14 hours daylight, 10 hours darkness (14:10) were used. Some nymphs were placed in much larger chambers designed by Campbell (1983). These were made from a cube-shaped frame of wood to which fly-screen or mesh is stapled on all sides. A container, which can hold volumes up to 500 ml, was placed inside the frame and connected to an air supply by aquarium tubing through a small hole. Surplus nymphs were stored in an aerated container refrigerated to 7°C. In the controlled environment the nymphs were checked every second day and the life cycle stage of the individual noted. Once emergence (or death)

occurred the animals were removed and genus and sex determined by observation using a stereomicroscope. Empty chambers were thoroughly washed and nymphs replaced from refrigerated stock. These new nymphs were acclimatised to the controlled temperatures for a period of 20 to 30 minutes.

RESULTS

The full data set included 1251 individuals of which *Austrophlebioides* and *Nousia* predominated, comprising 36.5% and 36.2% respectively. Next came *Atalohplebia* Eaton (1881) at 12%, then *Garinjuga* (Campbell and Suter (1988)) at 6.6%, *Tillyardophlebia* Dean (1997) at 4.6%, *Koornonga* (Campbell and Suter (1988)) (2.6%) and *Ulmerophlebia* Demoulin (1955) at 0.9%. A few individuals of other genera, such as *Atalomicra*, *Jappa*, *Kirrara* and an undescribed one, were also collected and represented the remaining 0.6%. Of the ten named genera in south-east Australia, all were sampled except *Neboisophlebia* Dean (1988), despite this genus having been found previously in many of the sites where I collected (Dean, 1988). Data analysis will focus on the six most prevalent genera.

Emergence Success

Imago emergence success rate for the full data set was 34.1% with 10.0% reaching the subimago stage before dying and 55.9% dying as nymphs. Individual genera, however, differed significantly in emergence success ($\chi^2=46.071$, $df=5$, $p<<0.001$, Figure 3). *Atalophlebia* was reared most successfully with 48.7% becoming imagos. Other highly successful genera were *Tillyardophlebia* (47.4%) and *Koornonga* (45.5%). The genus with the lowest success rate was *Garinjuga* (22.0%) followed by *Austrophlebioides* (25.0%).

The new rearing chamber was compared with that designed by Campbell (1983). The new chamber produced higher imago emergence rates compared with the 'old' one for all the genera examined (except *Nousia*) (Table 1). A significant difference was found only for *Austrophlebioides* ($\chi^2= 5.993$, $df=1$, $p=0.014$), where the emergence success rate more than doubled in the new chambers (27.1% versus 12.7%).

Emergence success did not differ between the sexes for any genus except *Tillyardophlebia* (Table 1) where a much greater proportion of females (58.8%) than male (30.4%) emerged successfully ($\chi^2= 4.435$, $df=1$, $p=0.035$). The effect of varying the photoperiod could be analysed only for year 1 as there were no individuals reared under 12:12 conditions during year 2. Within the restricted data set a significant difference due to photoperiod was found only for *Austrophlebioides* ($\chi^2= 17.810$, $df=1$, $p<<0.001$, Table 1) yet this went against the trend for all other genera where success rates, although not significantly different, were higher under a 12:12 cycle. Similarly, the effect of year of collection could only be examined in relation to photoperiod 14:10. Again there was a significant difference between year 1 and 2 only for *Austrophlebioides* ($\chi^2= 56.936$, $df=1$, $p<<0.001$, Table 1). There was no apparent trend for the other genera.

For most genera the proportion successfully emerging was highest at 18°C (for *Nousia* this occurred at 16°C but the difference in success rate from 18°C was very slight; 0.1%). The temperature which produced the lowest proportion of successful emergence was 22°C. The effect of temperature was significant for both *Austrophlebioides* ($\chi^2= 47.838$, $df=3$, $p<<0.001$) and *Nousia* ($\chi^2= 10.517$, $df=3$, $p=0.015$, Table 1). The effect of temperature was also considered in relation to time spent in the rearing system for a restricted number of genera (Figure 4). Time taken to emerge successfully was greatest at 18°C followed by 16°C, 20°C then 22°C. Although patterns of response to temperature were similar for each genus, the time taken to reach outcome varied considerably. For example, at 18°C mean time to emerge varied from 7.7 days for *Nousia* to 12.9 days for *Atalophlebia*. Data were log transformed to meet the assumption of normality and an ANOVA run to test for a significant effect of temperature. All genera had significant temperature effects (*Nousia*, F-ratio = 0.635,

Table 1. Percentages of successful emergences as affected by collection and rearing conditions

	<i>Atalo-phlebia</i>	<i>Austro-phlebioides</i>	<i>Garinjuga</i>	<i>Koornonga</i>	<i>Nousia</i>	<i>Tillyard-phlebia</i>
Chamber Type						
p value	0.628	0.014	**	**	0.926	**
n	150	454	82	33	453	57
% success old cage	44.4	12.6	15.4	37.5	38.7	50.0
% success new cage	49.6	27.1	23.2	48.0	38.1	47.0
Sex						
p value	0.795	0.909	0.542	0.435	0.234	0.035
n	150	456	82	33	453	57
% success female	47.8	24.8	20.0	40.0	40.0	58.8
% success male	50.0	25.3	25.9	53.8	34.2	30.4
Photoperiod (year 1) *						
p value	**	0.000	**	**	0.293	**
n	62	164	82	3	166	14
% success 12:12	62.3	24.0	28.2	100.0	46.8	53.8
% success 14:10	33.3	56.5	18.0	100.0	38.4	0.0
Year of Coll (photoperiod 14:10)						
p value	**	0.000	**	**	0.687	**
n	97	377	50	31	391	44
% success – year 1	33.3	56.5	13.0	100.00	38.5	0.0
% success – year 2	42.0	16.1	22.2	40.00	36.2	46.5
Temperature						
p value	**	0.000	**	**	0.015	**
n	150	456	82	33	452	57
% success 16°C	43.4	23.6	20.0	42.1	46.4	51.4
% success 18°C	59.0	45.4	25.6	100.0	46.3	53.8
% success 20°C	50.0	24.3	42.8	0	38.9	25.0
% success 22°C	38.2	10.8	9.5	40.0	29.3	0
Altitude						
p value	0.126	0.000	**	**	0.118	**
n	150	456	82	33	453	57
% success <400m	46.9	33.1	24.5	12.5	39.4	50.0
% success 400-800m	44.4	22.5	-	54.5	34.5	16.7
% success 801-1200m	73.3	9.6	0.0	-	15.4	100.0
% success >1200m	-	6.1	19.0	66.7	56.3	-
Storage						
p-value	**	0.000	**	**	0.856	**
n	150	456	82	33	453	57
% success 0-2 days	43.8	32.3	20.9	50.0	38.5	62.5
% success 3-5 days	75.0	19.8	33.3	33.3	35.0	28.6
% success > 5 days	62.5	12.0	16.7	50.0	39.1	27.8

Effect of each factor analysed by χ^2 . Significant p values (at < 0.05) shown in bold.

* analysed on restricted data set as there were no mayflies reared under the 12:12 regime in year 2.

** one or more categories with small sample size: χ^2 test not reliable.

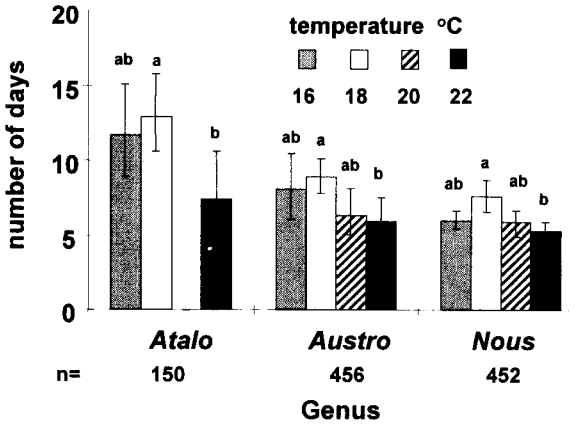


Fig. 4. Effect of temperature on time taken to successful emergence by genus. Abbreviations for genera as in Figure 3. Data back transformed from logged values. Bars represent back transformed standard error. Within each genus temperatures without a letter in common are significantly different (p-value less than 0.05) by Tukeys HSD tests.

df=3, p=0.010; *Austrophlebioides*, F-ratio=4.269, df=3, p=0.007; *Atalophlebia*, 20°C degrees removed, F-ratio = 4.354, df=2, p=0.017) although multiple r^2 showed that very little variability (around 10% or less) in rearing time was explained by temperature differences. Tukey's HSD tests showed significant differences between the temperatures 18°C and 22°C only for all genera (Figure 4).

The effect of altitude of collection on rearing success by genus was examined by chi-squared for 4 altitude categories. (1. <400 m, 2. 400-800 m, 3. 801-1200 m, 4. > 1200 m) and was found to be significant only for *Austrophlebioides* ($\chi^2= 29.826$, df=3, p<<0.001, Table 1) where the highest success rates were found at progressively lower altitudes.

Division of storage time into three categories (1. 0-2 days, 2. 3-5 days, 3. >5 days) and subsequent analysis by chi-squared also showed a significant effect only for the genus *Austrophlebioides* ($\chi^2=18.297$,df=2, p<<0.001, Table 1) with a higher success rate for progressively less days in storage. However, altitude of collection and storage time were positively correlated as it took longer to return to the laboratory from high altitude collection sites. A logistic regression of both factors against emergence success showed altitude probably was more influential than storage.

Sex-Ratios

Ratios of males to females showed a female bias for all individual genera in the range of 1: 1.5 for *Tillyardophlebia* to 1: 2.1 for *Nousia* (Figure 5). Chi-squared analysis was used to determine departure from the expected 1:1 sex ratio and was found to be significant for all genera except *Koornonga* and *Tillyardophlebia*.

DISCUSSION

The overall imago emergence success rate was 34%. Clearly, however, success rates depend on the genus in question. Taxa preferring slow waters would be expected to emerge more successfully in aerated tanks with low flow regimes (Edmunds *et al.*,1976). Therefore,

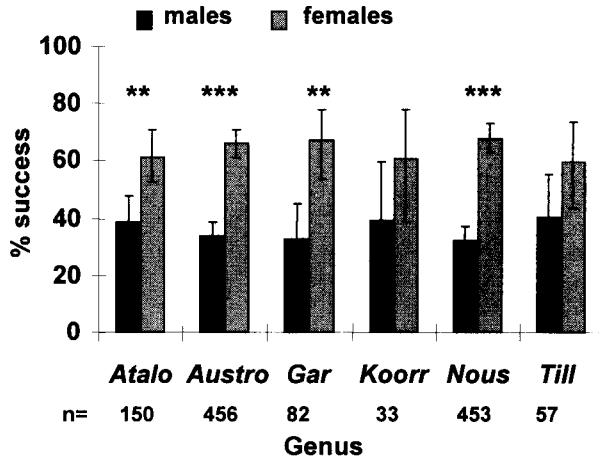


Fig. 5. Mayfly sex-ratios of six genera captured over 2 extended summer periods. Abbreviations for genera as in Figure 3. Bars represent 95% confidence intervals. Asterisks indicate significant departures (at less than 5% significance level) from 1:1 ratio by chi-squared.

it is not surprising that *Atalophlebia*, predominantly found in standing or slowly flowing waters (Peters and Campbell, 1991), had the greatest proportion of imagos that emerged. The relatively low success rates of *Garinjuga* may be due to the presence of a possibly new high altitude species for which the emergence success was very low, thereby reducing the average for the whole genus.

Overall, the new rearing system was a success in that imago emergence rates were generally higher in the new chamber compared with the older one for all genera. In particular, the success rate for *Austrophlebioides* was much greater in the new chamber. The considerable advantages of the new chambers in terms of time saved in construction and set-up, space efficiency and low cost indicate that the system could be widely used even if just for routine species identification.

In general, few factors had an effect on overall emergence success for any genera other than *Austrophlebioides*. Sex appeared to affect *Tillyardophlebia* but, because this was a relatively small data set, may not prove to be biologically significant. There are also some temperature effects for *Nousia* and *Atalophlebia* which must be considered. (Table 1, Figure 4). Most striking, however, were the results for the genus *Austrophlebioides* which appeared to be affected strongly by all factors except sex. This is almost certainly related to habitat requirements. For example, the greater rearing success in smaller chambers with much less water could be indicative of an inherent need for highly oxygenated water in this genus. Neither chamber adequately simulates current flow, but the new chamber may provide more oxygen per volume than the 'old' chamber. A high rate of water movement may be necessary for the development of this genus. Similarly, the highly positive response to a 14 hour photoperiod, which is typical of an Australian summer light regime, may be required as a cue for *Austrophlebioides* to emerge. If this is so, this is the first record of an enhanced emergence response to photoperiod for Australian Leptophlebiidae as photoperiod has been shown previously to have no effect on mayfly egg hatching or emergence (Brittain, 1982; Suter and Bishop, 1990; Newbold *et al.*, 1994) despite the suggestion that it is important for aquatic insects in general by Hynes (1970).

Austrophlebioides fared better in year 1 possibly because they were stored for much less time (0.1 mean days in year 1 compared with 4.9 mean days in year 2). Progressively shorter storage times produced significantly higher success rates. Another contributing factor

may be that altitude range for each year was considerably skewed, with animals being collected no higher than 500 m in year 1, yet up to 1560 m in year 2; progressively lower altitudes produced greater emergence success rates.

It is acknowledged that animals do not respond to altitude *per se* but rather environmental variables associated with altitude (see reviews by Minshall, 1988; Power *et al.*, 1988) such as temperature, substrate, dissolved oxygen and hydraulic variation. One can speculate that there is greater temperature differential between higher altitude sites and the laboratory compared with lower altitude sites, possibly making the physiological stress on the animals greater.

Temperature not only determines abundance, distribution and diversity of stream insects (Hynes, 1970; Ward and Stanford, 1982; Zamora-Muñoz *et al.*, 1993) but is considered to be one of the most important influencing factors affecting insect development (Corkum, 1978; Elliott, 1978; Brittain, 1982; Wallace and Anderson, 1996). Indeed, in this study, temperature was the only external factor significantly affecting emergence success of a genus other than *Austrophlebioides*. Success rates were much higher at 18°C than at 22°C, yet summer water temperatures would be within the range 16-22°C for all but the highest altitudes; so it is puzzling to find such a restricted temperature preference for emergence. This restricted preference also applied to the length of time taken to emerge. It is possible that the nymphs have a narrow temperature requirement for development which is in line with the theory of Sweeney and Vannote (1978) that an optimal thermal regime exists for a given species. Adult size and fecundity and, presumably emergence rates, may diminish outside the bounds of the optimal regime for the species.

Trends in the data are not significant with the clear exception of *Austrophlebioides*. Species level data within this genus would therefore be especially valuable.

Sex-Ratios

Insect sex-ratios in nature are generally expected to be 1:1 although skewed ratios due to inbreeding occur and will be biased towards females (Thornhill and Alcock, 1983). Female biased sex ratios in Ephemeroptera have been recorded only for parthenogenetic taxa of which 50 species are known worldwide (Brittain, 1982) and only in 3 or 4 families (McCafferty and Huff, 1974). It appears obligatory in only a few species (Peters and Campbell, 1991). In general, parthenogenetic eggs develop more slowly, causing a delayed female bias in the sex ratio of the nymphs which is perpetuated through the life cycle. For example Harker (1997) found sex ratios of *Cloeon similae* (Baetidae) increased from 1:1 in the summer to 2:1 in Spring and early Winter over 13 consecutive years due to a longer development time for the unfertilised eggs and subsequent late appearance of parthenogenetic progeny (females). Data for the present study were collected from a wide range of sites over two years, so it is possible that the observed sex ratio actually reflects what occurs in nature. As far as I am aware there are no previous records of parthenogenesis occurring in Australian Leptophlebiidae.

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