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The responses of a lotic mayfly *Nousia* sp. (Ephemeroptera: Leptophlebiidae) to moving water and light of different wavelengths

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SUMMARY

1. Although laboratory studies of the behaviour of aquatic macroinvertebrates are common, there has been little critical evaluation of the importance of test conditions to them. We used a common Australian leptophlebiid mayfly, *Nousia* sp., to investigate responses to light, wavelength of light, presence or absence of cover and still or flowing water.
2. *Nousia* sp. showed substantial qualitative differences in behaviour, as measured by movement, when there was no refuge (in the form of a crevice beneath a tile) present in the experimental arena.
3. We found no evidence of diel periodicity in activity in *Nousia* sp.
4. *Nousia* sp. did not respond to infra-red, red or green light at a flux density of 18–19 μmol m^{-2} s^{-1}.
5. Nymphs were three times more likely to remain stationary in flowing water (mean velocity 0.10 m s^{-1}) than in still water.
6. We conclude that generalized assumptions about test conditions for experiments designed to quantify laboratory behaviour in benthic macroinvertebrates are unjustified and that evaluation of the individual requirements of test species should be conducted routinely.

Keywords: behaviour, Leptophlebiidae, light, lotic macroinvertebrate, phototactic response

Introduction

Making detailed observations of behaviour has become an increasingly important component of many studies of freshwater organisms (e.g. Elliott, 1968; Corkum, 1978; Allan, Flecker & McClintock, 1986; Casey, 1987; Wilzbach, 1990; Sih, 1993; McIntosh, Crowl & Townsend, 1994). However, observing the behaviour of benthic stream macroinvertebrates presents difficulties that frequently appear to be ignored. For example, numerous researchers have assumed or claimed, that dim red light does not affect the behaviour of their test species (e.g. Chaston, 1968; Elliott, 1968; Bailey, 1981; Casey, 1987; Huhta, Muotka & Tikkanen, 1995). However, few workers have provided rigorous statistical evidence to substantiate this claim and Heise (1992) demonstrated that dim visible red light, as used by many researchers, caused two species of heptageniid mayflies, *Stenacron interpunctatum* (Say) and *Stenonema vicarium* (Walker) to move away from the light rapidly; such evidence suggests that even dim red light may affect detailed behavioural observations.

Likewise, maintaining water flow is likely to be important for species that use this cue for orientation and Elliott (1968) showed that withholding flow can

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change the taxes of some mayfly species. Shelter may also be important and Sjöström (1985) criticized the conclusion reached by Molles & Pietruszka (1983), that foraging success in two species of predatory stoneflies was not influenced by presence or absence of light because of the absence of shelter. He reported abnormal searching behaviour by *Dinocras cephalotes* (Curtis) in full light when shelter was absent.

Clearly then, light, flow and the provision of shelter may influence the results of behavioural observations of lotic macroinvertebrates. The dearth of well-controlled studies that examined these factors prompted this investigation. Our particular aim was to determine how flow, light and light wavelength interacted in the presence of a refuge to influence the movements of nymphs of a leptophlebiid mayfly, *Nousia* sp., a member of a commonly encountered genus in temperate Australia.

Methods

Ten small, recirculating laboratory flumes (Vogel & LaBarbera, 1978) incorporating the diffusers and collimators recommended by Lacoursière & Craig (1990) to minimize turbulence, were set up in a constant temperature room with lighting control. The dimensions of the test section with a fully developed, turbulent boundary layer were 700 mm long × 130 mm wide × 120 mm deep. Water temperature was 12 ± 1 °C, which was similar to daily maxima (range 10–14 °C during this investigation) within Hobart Rivulet (147°16′45″ E 42°54′30″ S) from which filtered water and animals were collected. Water depth throughout the trials was maintained at 80 mm and mean velocity at 0.10 m s^{-1} (the approximate mean free stream velocity in riffles and runs in Hobart Rivulet), measured using a miniature propellor flow meter (MiniWater2 Micro 661/22, Schiltknecht Messtechnik, Gossau, Switzerland).

Nymphs of the most abundant mayfly *Nousia* sp. (Leptophlebiidae) were collected 1–2 days before use from Hobart Rivulet from October–December 1996 and maintained in aquaria with aerated, circulating water containing algae covered rocks and detritus for food. Only nymphs with wing-pads were used as they were easily distinguishable from nymphs of other sympatric leptophlebiids. However, mature nymphs with darkened wing-pads were excluded as they were close to emergence.

We conducted two quantitative experiments: first, to determine whether nymphs showed diel periodicity in activity; and, second, to investigate positional responses to wavelength of light in the presence or absence of flow. These experiments were preceded by qualitative observations that demonstrated the need for animals to be provided with a refuge. A refuge consisted of a 60 mm × 60 mm clear perspex tile attached to the bottom of the flume with four 10 mm × 10 mm velcro pads affixed to each corner of the square. This allowed a 4-mm deep gap which easily accommodated the largest animals used in these experiments. The perspex was roughened on both sides with sandpaper to help the animals cling to it.

To investigate periodicity, the activity of individual nymphs was recorded as the number of activity units in 5 min at 15 min intervals for 1 h, starting at 10:00 (daylight), 20:00 (1 h after dusk) and 24:00 h (midnight). The photoperiod was 13 h L:11 h D. One activity unit was defined as any movement (lasting 10 s or less) of a nymph that resulted in a change of position (Elliott, 1968; Bailey, 1981); there were no movements that lasted >10 s that involved changes in position. To ensure independence of observations, 10 different nymphs, in individual flumes, were used for each time-period; flumes were prepared 24 h before observations began and nymphs were provided with food in the form of some algal and detrital material lodged against the left-hand side of the perspex tile. Observations were made with an infra-red light source and video camera. Data were analysed as a one-way, fixed factor analysis of variance; inspection of residuals showed that all assumptions were met and no transformation of the data was necessary.

To investigate whether nymphs responded to light of different wavelengths and whether flow influenced their response, we transferred a nymph to the underside of a refuge tile using a pipette and then gently seated the tile in the flume using the velcro pads. If the treatment was allocated to flowing water, the flow was increased gently over 15 s from still water to 0.10 m s^{-1}. Placement was carried out in dim red light, after which the light was switched off. After 2 min the designated light source was turned on for 2 min or if the treatment was 'no light' the switch was flipped on but no light source was connected. At the end of the 2-min exposure period, the response of the nymph to the treatment was recorded as a binary variable:

'moved' – all movements that could be interpreted as the nymph reorienting its body so that its eyes were away from the light; and 'did not move' – the nymph remaining stationary. If the nymph deserted the tile (< 1% trials), the trial was discarded. For the 'no light' treatment, responses were recorded by turning on the dim red light at the end of the exposure period and recording whether the nymph had repositioned itself; any repositioning that occurred as a result of turning on this light was clearly distinguishable from repositioning during the preceding dark period because of the time lag between a stimulus being applied and the response of the nymph. For the infra-red treatment, observations were made using a video camera and a monitor.

The light sources were light-emitting diodes and three wavelengths were used: 950 nm (infra-red), 635 nm (red) and 568 nm (green). Light sources were located underneath the flume so that light fell on the eyes of the *Nousia* nymphs attached to the undersurface of the tile. Visible light sources incident on the bottom of the flume were measured using a Li-Cor LI 190SZ quantum sensor (Li-Cor, Lincoln, NE, U.S.A.) with a sensitivity range 400–700 nm. The photon flux density was 18–19 μmol m^{-2} s^{-1}.

Our experimental design consisted of four light treatments crossed with two flow treatments (0.00 and 0.10 m s^{-1}), with a dichotomous response variable as the dependent variable. Our desired outcome was to identify a combination of lighting and flow conditions that resulted in least disturbance to the animal, i.e. 'success' was defined as 'did not respond'. We used hierarchical logit analysis (Tabachnick & Fidell, 1996), using SYSTAT version 8.0 (SPSS, 1998), to identify the simplest model that fitted our data adequately. Confidence intervals for the proportions meeting our success criterion were calculated according to Zar (1984).

The use of a binary response variable requires large numbers of independent observations to achieve reasonable power. We decided to collect sufficient observations so that we could distinguish between 50 and 20% success rate under any two treatment combinations. From a preliminary analysis of the first 20 trials of dark versus light and setting the type I error (α) = 0.05 and type II error (β) = 0.1, the sample size necessary for each cell in our design was 58 (Zar, 1984). Accordingly we used 60 different mayflies in each combination of light and flow, and randomized the order of allocation of these treatment combinations. No nymphs were re-used in any trials.

Results

Extensive preliminary observations were conducted in the laboratory during daytime in full light (30 trials) and during night time using infra-red video (28 trials), in which nymphs were placed in separate flumes together with the tile refuge but outside it. Half the trials were conducted with still water and the other half with running water (0.10 m s^{-1}). Initially, nymphs moved rapidly in short bursts, either running or swimming with these bursts often punctuated by reorientation of the body. These larger scale movements were still apparent, but ceased once the nymph found the crevice beneath the tile. Twenty-nine of the 30 nymphs in the daytime trials and all of the night time nymphs found and remained under the crevice within 2 min of introduction to the flume. Once under the tile, movements in the initial 5–10 min were generally limited to minor changes in orientation of the body. The few occasions when the nymph deserted the tile were nearly always associated with an accidental knock to the flume (four out of five occasions); the nymph resumed its position in the crevice within 3 min on all occasions. This lack of large scale movement once the tile was found contrasted with earlier attempts (>12 trials) to place nymphs on the bottom of the flume in the absence of a refuge. In both still and moving water, the nymphs moved, ran and swam for much of the 20 min observation period, with movements becoming slower and more atypical as time progressed. In some moving water trials, nymphs became trapped on the exit screen of the flume. These observations, combined with the fact that this species is found in rocky bottomed streams where crevices and refuges are always present, justify the use of the refuge in the remaining experiments.

There were no significant differences in the number of activity units per individual between any of the time periods ($F_{(2,27)} = 0.154$; $P = 0.90$); means for 10:00, 20:00 and 24:00 h were 2.65, 2.48 and 2.58 activity units 5 min^{-1} interval. Clearly, there was no pattern of diel activity and accordingly, it was not necessary to incorporate daytime versus night time in the formal design of the second experiment.

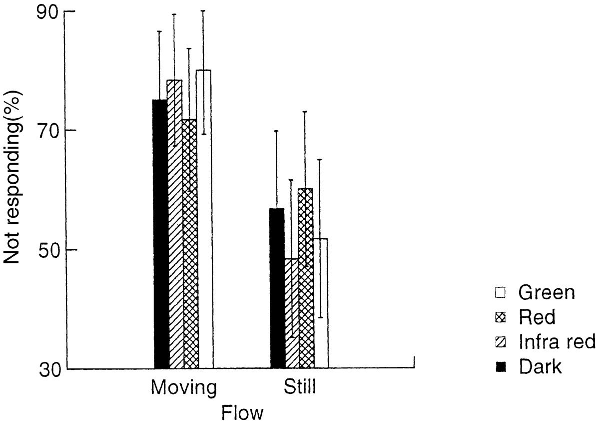

Fig. 1 The percentage of nymphs that were undisturbed by a light source in moving (0.10 m s^{-1}) and still water. Trials were conducted in darkness (black shading), infra-red (diagonal hatching), red (double cross-hatching) and green (white shading) light sources. Values are mean ± 95% confidence intervals calculated according to Zar (1984).

Hierarchical logit analysis led to a final model that included no terms associated with the type of lighting; however, omitting the term incorporating the effect of flow on the response led to a significant deterioration in the fit between the model and the data (likelihood ratio $\chi^2_{(10)} = 28.83$, $P = 0.001$), whereas including flow yielded a well-fitted model (likelihood ratio $\chi^2_{(9)} = 3.2308$, $P = 0.95$). Under all lighting conditions, animals showed an increased tendency to remain immobile (i.e. 'not respond') in flowing water (Fig. 1). Expressed in terms of the odds ratio, in flowing water the odds of success, i.e. of a nymph not responding, were 3.4 to 1, whereas the odds were 1.2 to 1 in still water; thus nymphs were about three times more likely to remain under the tile in flowing water.

Discussion

Nousia sp. showed no diel pattern of activity, whereas Bailey (1981) found that the closely related *Atalophlebioides* sp. was most active at night. His observations were comprehensive, being based on measurements of activity units in a laboratory stream, counts of the numbers of nymphs on the upper surfaces of rocks in both laboratory and field streams, and on drift rates in a natural stream. In all cases nymphs were exposed to flowing water and had access to food and refuge. He concluded that this periodicity was probably because of an endogenous rhythm rather than a response to changed light intensities. In examinations of the propensity of mayfly nymphs to be on the upper surfaces of rocks, other species of leptophlebiid have shown no diel periodicity (Kohler, 1983) or have increased nocturnal activity only in the presence of predatory fish (Culp, Glozier & Scrimgeour, 1991). Other factors that have been shown to affect measures of activity include lighting conditions (Glozier & Culp, 1989; Huhta *et al.*, 1995) and the stage of development (Culp & Scrimgeour, 1993; Huhta *et al.*, 1995).

We found no evidence to suggest that nymphs of *Nousia* sp. reacted adversely to lighting conditions. Indeed this species and other congeners have been observed regularly on the upper surfaces of rocks during daylight in a wide variety of Tasmanian streams (W. Elvey, University of Tasmania, unpublished data). This apparent insensitivity to light was surprising, given that most species of Ephemeroptera examined, in a wide range of families, have been found to be negatively phototactic (reviews: Bishop, 1969; Bishop & Hynes, 1969). In contrast, Hughes (1966) found that *Baetis harrisoni* Barnard was positively phototactic under flowing laboratory conditions and found this behaviour consistent with their elevated abundances in unshaded streams in South Africa. We saw no evidence that *Nousia* sp. was attracted or repelled by light. Even shining a bright white flashlight on nymphs ensconced in the refuge did not elicit a response. By contrast, nymphs of some species have been reported to retreat from bright light in the field although the presence of the observers confounded observations (Allan, Flecker & Kohler, 1991). The only other laboratory examination of a leptophlebiid, *Atalophlebioides* sp., by Bailey (1981), found that some nymphs did not respond to substantial increases in light (from <1 to >250 lx) in flowing water.

Our light intensities may have been substantially lower than those used in other laboratory studies. However, it is difficult to make quantitative comparisons because there is little consistency in the measurement of light 'intensity'. Some authors merely state the wattage of the light source whereas others have measured light photometrically. Photometric measurements are weighted for the spectral sensitivity of the human eye and are likely to underestimate the photon flux at the red end of the spectrum; this makes it difficult to convert measurements based on candelas, lumens or lux to photon flux density (Jones, 1992). We agree with Heise (1992) who argued that it is preferable to measure photons because it is the number of photons rather than the power of each photon that is important for insect vision. Heise (1992) reported his light 'intensity' in mW and so the maximum photon flux density that his mayflies could have experienced at the front of his experimental chamber was ≈11 μmol m^{-2} s^{-1}, which was slightly less than we used. As Heise observed significant responses to this intensity, we feel that there was sufficient light to elicit a response in our nymphs.

The presence of moving water, however, was clearly much more important to the behaviour of *Nousia* sp. nymphs than the type of lighting. Nymphs showed more small-scale movements in still water than in moving water. This suggests that they require moving water to orient and stay attached to the substratum, although the flow through the refuge must have been much less than the mean free-stream velocity of 0.10 m s^{-1}.

Heise's (1992) experiments remain the clearest demonstration of the generally unquestioned claim that mayflies can respond to dim, visible light. There are two aspects of his experiment that may have influenced his results. First, the experimental conditions included no refuge for either of the species he tested, although both normally reside on the undersides of rocks. Our results suggest that the lack of a suitable attachment site may have increased the propensity of his species to move. Second, the same mayfly individuals were exposed to each of the lighting conditions on successive nights. Although appropriately analysed using repeated measures techniques, the light treatments were not presented in random order. The sequence was always green, red, infra-red and then darkness; thus the two treatments that showed the lowest response were always the last to be used. However, the response to red light (the second treatment) seemed to be on average greater than that to green (the first treatment), but not significantly so. We cannot therefore discount the possibility that the order of the lighting treatments was confounded with some endogenous change in the nymphs because of their repeated use in the same experiment.

Our observations indicate that the procedures used to study behavioural responses need to be tailored to meet the range of behaviours and habitat requirements of the organism under study. This may seem intuitively obvious but it is clear that many studies ignore these requirements. It was apparent from our initial observations that *Nousia* sp. behaved abnormally in the absence of any refuge and spent much more time swimming, walking and running around the flume bed than when any shelter was provided. We also attempted quantitative observations with some other species but found this type of flume unsuitable. An undescribed species of *Baetis*, for example, moved by detaching from the substratum and actively swimming upwards so that it was usually washed into the exit screen of the flume. The large predatory stonefly, *Eusthenia costalis* Banks (Eustheniidae), by contrast would settle in some part of the flume and remain stationary for long periods of time (>3 h); even shining bright white light and tapping the flume sides or proffering wriggling prey would not stir them. Allan *et al.* (1991) similarly appealed for more attention to be paid to identifying and quantifying behavioural features relevant to the question being asked. They demonstrated that counts of animals on the upper surfaces of rocks in a stream were strongly affected by human observers making simple visual inspections and higher abundances were recorded when they used time-lapse cinematography.

As far as we know, the spectral sensitivities of immature benthic insects have not been examined. This has been carried out for adult dragonflies (review: Yang & Osorio, 1991) and some non-insect arthropods (unionicolid water mites; Dimock & Davids, 1985). Such research could settle the issue of whether benthic insects can perceive red light mechanistically, but the diversity of the fauna and the resources necessary to measure these phenomena make this impractical for field ecologists who merely wish to be assured that their observational procedures are not causing unwanted artefacts. Although further investigations into responses to different light sources may be warranted for more eco-physiologically oriented research, getting the conditions right for an appropriate test can be time-consuming and perhaps impossible for some species. Alternatively, as ecologists, we could start to get reliable answers to our ecological questions more quickly if we can afford the resources to take advantage of advances such as time-lapse photography (Kohler, 1985), the use of infra-red light sources and videos (Culp *et al.*, 1991) and endoscopes for interstitial observations (Wilzbach, 1990).

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