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The use of computer-assisted motion analysis for quantitative studies of the behaviour of barnacle (*Balanus amphitrite*) larvae

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Abstract
The effects of larval density and age on pre-settlement swimming behaviour of *Balanus amphitrite* cyprid larvae were studied with the aid of computer-assisted motion analysis. Swimming behaviour was monitored in individual, in groups of 10–15 and in groups of 50–100 cyprids. There was a small, but significant effect of larval density on swimming speed and no effect on two other quantitative measures: rate of change of direction and net-to-gross displacement ratio. There was also small but significant variation in swimming speed between different batches of cyprids over the course of 2 years. Swimming behaviour of individual cyprid larvae was also monitored daily for 7 days, with the larvae maintained in the cold and dark between measurements to prevent settlement and metamorphosis. There were no significant behavioural differences observed over time indicating that larvae may be held in this manner experimentally without affecting these parameters.

Keywords: *Crustacean, barnacle, larvae, cyprid, swimming behaviour, video tracking, marine biofouling*

Introduction

Computer-assisted motion analysis has proven to be an effective tool for study of swimming behaviour, chemo-attraction and phototaxis in bacteria, microalgae and macroalgal spores (e.g. Sager et al. 1988; Zacks and Spudich 1994; Amsler 1996; Lee et al. 1999; Iken et al. 2001, 2003; Greer and Amsler 2002). Dodson et al. (1995) were perhaps the first to apply this technology to motile invertebrates, characterizing the swimming behaviour of *Daphnia pulex* individuals in response to pesticides and kairomones/natural chemical signals.

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O'Keefe et al. (1998) also used the same motion analysis system to investigate swimming behaviour of *Daphnia* clones with respect to predation risk. Faimali et al. (2006) documented with motion analysis swimming speed changes in the nauplii of *Balanus amphitrite* with respect to sublethal concentrations of potential biocides. Thus, motion analysis has been shown to be a useful tool enabling quantitative, ecologically relevant interpretations of the behaviour of swimming micro- and meso-organisms. Applying this technology to motile larval stages of biofouling oysters, annelids, bryozoans, and as in this study, barnacle cyprids, may provide insight to controlling their settlement and hence biofouling.

Marine larval settlement, metamorphosis and biofouling have been the focus of many studies (Hadfield and Paul 2001). It is well-established that in barnacles, the free-swimming larval barnacle stage, cyprid, searches out a settlement location using a combination of substrate chemistry, texture and the presence of conspecifics. Light and water flow also influence settlement. There is evidence that increasing age reduces a cyprid's discrimination of settlement cues (Knight-Jones and Crisp 1953; Crisp 1974; Rittschof et al. 1984; Rittschof et al. 1998). Thomason et al. (2002) have suggested that cyprid search behaviour can serve as an indicator of the efficacy of anti-fouling paints and coatings based on field bioassays and our group has shown that computer-assisted motion analysis techniques can be used in identification of and bioassay-guided fractionation of compounds bioactive as anti-foulants for algal spores (Iken et al. 2003; Greer et al. 2006). Quantitatively studying cyprid swimming behaviour using similar techniques (in the absence of deliberately applied cues) may yield useful baseline information and lead to a better understanding of the subsequent search and settlement behaviour exhibited by cyprid larvae.

Crisp was among the first investigators to describe and study cyprid movement in a quantitative fashion. His early tracking methods were based on dedicated observations and documented by camera lucida drawings of the paths cyprids travelled (reviewed by Crisp 1976). Recently, Marechal et al. (2004) studied settlement behaviour of cyprids of *B. amphitrite* with a computer-assisted motion analysis system marketed as EthoVision. This system, designed for movement analysis of single, larger animals like mice (Noldus et al. 2001), was only able to track a single cyprid isolated in separate wells. Head et al. (2004) investigated gregarious settlement in cyprids and found a threshold of as few as 5 larvae/mL induces gregarious settlement and suggested that for bioassay studies concerned with settlement inhibition or induction that a comparison between both single and multi-cyprid assays is worthwhile. Monitoring the movements of a single cyprid could factor out behaviour based on a prospective gregarious effect if no difference in baseline behaviours was detected. This may be possible with the ExpertVision motion analysis system which has been used previously in studies of bacteria, algae and *Daphnia*, and is capable of tracking multiple moving objects within the same field. The aim of our study was to document that ExpertVision can be used to track multiple cyprid larvae simultaneously and to compare the swimming behaviour of *B. amphitrite* cyprids *in vitro* as a function of (1) larval density and (2) changes with cyprid age. Specifically we quantify the effect of larval density and cyprid age on swimming speed, rate of change of direction (absolute value of angular velocity) and net-to-gross displacement ratio.

Methods

Balanus amphitrite. Darwin cyprids were supplied from the culture facility at Duke University Marine Laboratory (Beaufort, NC, USA). Larvae generated from a mixed

broodstock of several hundred adults within 2 days of moulting from the final nauplial stage, cyprids were shipped at a temperature of $6 \pm 2^{\circ}$C. Cyprids were maintained in artificial seawater and held at 6°C in complete darkness before and after video monitoring. These conditions slowed further development and settlement (Mary et al. 1987) and ensured actively swimming cyprids for several days of experiments from a single shipment. This commonly accepted practice has been used in many studies (e.g. Ritschoff et al. 1984; Pechenik et al. 1993; Holm et al. 2000).

For use in experiments, cyprids were allowed to slowly warm to and acclimate to room temperature in a light-protected dish. Active cyprids attracted to a pinpoint light source were selected and pipetted into Falcon 1143 plates (24 mm diametre/well). Three different density treatments were employed: 1, 10–15 and 50–100 cyprids/well for individual ($n = 30$ wells), low- and high-density treatments, respectively ($n = 3$ wells/experiment treatment). Well volumes were adjusted to 1 mL of artificial seawater and the cyprids acclimated to 26°C in a darkened incubator for 2 h prior to each experiment.

All experiments were conducted under red light to mimic darkness for cyprids and to minimize cyprid swimming behaviour as a function of phototaxis. (Crisp 1955; Eckman et al. 1990). The multi-welled plate was placed in a custom water bath that maintained the well temperature at 26°C. Cyprids were videotaped with an Olympus dissecting microscope coupled to a Cohu High Performance CCD video camera. The camera was positioned at a standardized height above the well to maximize recording area coverage within the well. After a 2-min equilibration period, each well was videotaped for 5 min at 30 frames/s.

Cyprid behaviour was monitored on 3 days (29 August 2000, 6 June 2001 and 20 July 2001), each within several days of their receipt. On all three experimental dates we compared cyprid behaviour at both low (10–15 cyprids) and high density (50–100 cyprids). The last experimental date incorporated a three-way comparison between individual cyprids maintained in single wells in addition to both low- and high-density wells.

A time series was conducted to test for changes in cyprid swimming behaviour as a function of age. Four cyprids were recorded for the first three consecutive days (17–19 July 2001). Another four cyprids were recorded for the last three consecutive days (21–23 July 2001).

Finally, we investigated the variability of cyprid behaviour with time on an individual basis. Actively swimming cyprids were selected as aforedescribed from the same stock that served as the source for the final density experiment. The single cyprids were maintained and filmed as described earlier but each in a separate well. Swimming behaviour was recorded once a day on three different days throughout a week.

Recorded swimming behaviour was analyzed with a Motion Analysis Corp (Santa Rosa, CA, USA) VP110 video processor and ExpertVision Software (EV, Motion Analysis Corp.) The theory governing video image capture and more detailed methods are published elsewhere (Sager et al. 1988). Briefly in our study, five 10 s portions of videotape (frame rate of 30 frames/s) from each well were haphazardly selected for digitizing. Within a 10 s video, an individual cyprid was identified such that the software recognized it as a unique image and tracked movement through a maximum of 300 frames to yield a path.

Data acquisition from the digitized images required defining the following constants: centroids, the geometric center of the cyprid image, were based on a neighborhood width and height of each 2 pixels; minimum no. of pixels = 4, maximum no. of pixels = 50. Cyprid paths were derived using a search mask size 25 pixels, minimum path duration = 5 frames, average minimum movement = 0 pixels/frame, maximum interpolation = 0 frames and a prediction percentage = 50%. Paths were generated for each cyprid analyzed. From these paths, cyprid swimming behaviour was quantified in terms of: cyprid swimming speed (SPD), net-to-gross displacement ratio (NGD) and rate of change of direction (RCD).

Swimming speeds are expressed in micrometres per second. NGD is an index of path twistedness and is expressed as a ratio ranging between 0 (circular motion) and 1 (linear motion). RCD is a measure of the degrees of swimming direction change per unit time. Values vary (at 30 frames s^{-1}) from 0 (linear motion) to (hypothetically) 5400° s^{-1} (which would represent 180° of turn each and every frame). ExpertVision detection of angular travel is limited to 180°/frame. All variables are based on the video magnification of 1.92 μm/pixel. (The VP110 and EV software are no longer commercially available but Motion Analysis Corporation has a new Windows-based software package called CellTrak to be released in 2006 that replaces both. We have tested the Beta version of CellTrak with cyprids and it has all the features necessary to perform these bioassays.)

Statistical analyses were performed with SPSS software (SPSS 10.0, SPSS Inc., Chicago, IL, USA). Data for all experiments were not normally distributed and thus normalized by Tukey ranking prior to applying specific statistical tests. Overall effect of low-density *versus* high-density treatment was analyzed by two-way ANOVA. The comparison of cyprids swimming as individuals, in low-density and in high-density treatments was analyzed by ANOVA. Behaviour of individuals in the time series was analyzed with a GLM repeated measures ANOVA and a two-tailed pairwise comparison.

Results

From three dates, 30 individual cyprid paths, 499 low-density cyprid paths and 469 high-density cyprid paths were digitized and analyzed. These paths do not all represent unique cyprids but most do and each are unique motion events within a treatment. Combining the data from the two density treatments for each of the experiments, we found that cyprid density does not affect the swimming behaviours NGD or RCD (Table I). This trend is

Table I. Two-way ANOVA comparisons of normal-scored swimming behaviour of cyprids of *B. amphitrite* with brood batch treated as a random effect.

Source of variation	Type III sum of squares	df	Mean square	*F*	Sig.	Noncent parameter	Observed power[a]
Dependent variable: Tukey normal-scored SPD							
Density	4.211	1	4.211	8.196	0.051	8.196	0.558
Batch	105.12	2	52.60	116.68	0.008	233.378	0.997
Density *Batch	0.901	2	0.450	0.529	0.589	1.058	0.138
Error	891.065	962	0.851				
Dependent variable: Tukey normal-scored NGD							
Density	1.987	1	1.987	0.873	0.438	0.873	0.094
Batch	11.875	2	5.938	2.355	0.298	4.71	0.156
Density *Batch	5.042	2	2.521	2.623	0.73	5.247	0.523
Error	924.552	962	0.961				
Dependent variable: Tukey normal-scored RCD							
Density	2.05×10^{-3}	1	2.05×10^{-3}	0.001	0.975	0.001	0.050
Batch	54.741	2	27.27	14.198	0.066	28.397	0.533
Density *Batch	3.855	2	1.928	2.098	0.123	4.197	0.432
Error	883.717	962	0.919				

[a]Computed using $\alpha = 0.05$.

also observed graphically (Figure 1) but the statistical power in our analysis is very low. Density does have a marginal effect on cyprid swimming speed ($P = 0.051$) but there is no consistent trend. In two of the three experiments, the larvae in the low-density treatment swam on average slower than those in the high-density treatments, however, overall, the cyprids in the low-density treatment swam on average slightly faster than those in the higher density treatment.

Our three experiments include cyprids from two consecutive years, therefore two different reproductive seasons. Batch does have a significant effect on speed (two factor ANOVA: $P = 0.008$; Table I). Batch does not have a significant effect on RCD. NGD is not influenced by batch and remained within a consistent range as a function of both density and batch (Figure 1).

Thirty individual cyprids were observed over 7 days, but none were considered active enough for videotaping on all days. One common cyprid behaviour observed in this study was for the larva to remain in one spot and simply twitch occasionally without changing location. Any motion analysis protocol requires motion in order to conduct an analysis, so

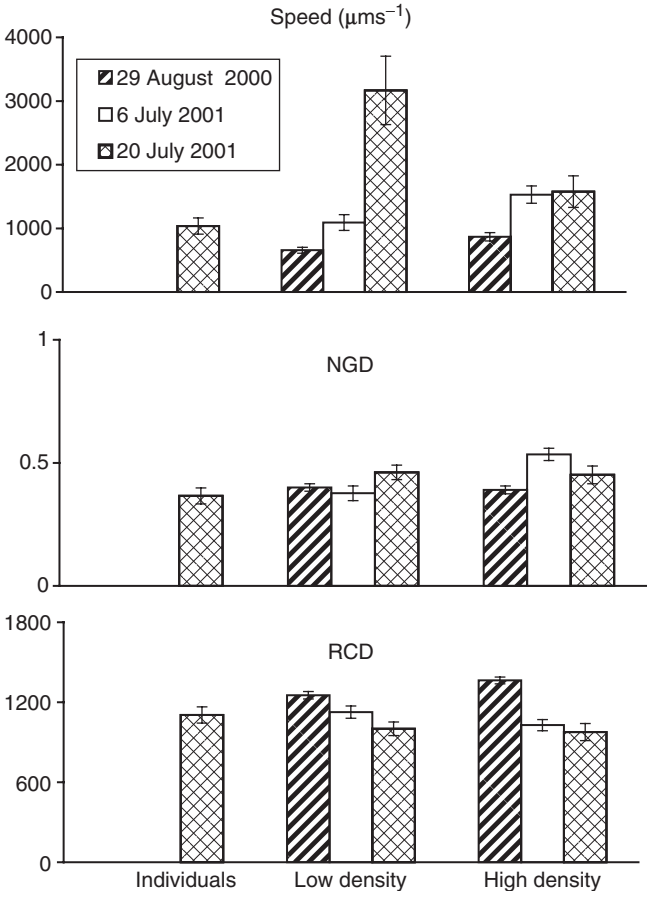

Figure 1. Effect of larval density on swimming behaviour of cyprids of *B. amphitrite* analyzed with computer-assisted motion analysis (means ± SE). Bar shading as per legend. NGD = net-to-gross displacement; RCD = rate of change of direction.

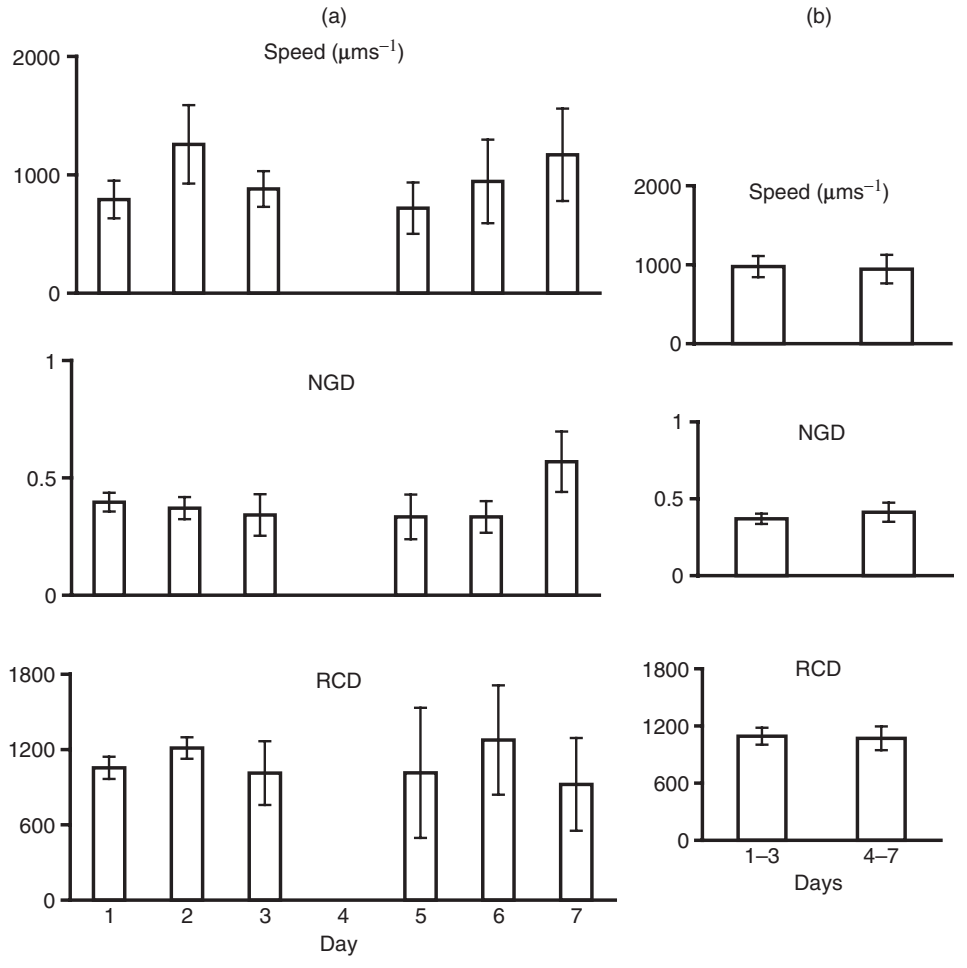

Figure 2. (a) Effect of larval age on swimming behaviour of cyprids of *B. amphitrite* analyzed with computer-assisted motion analysis. Mean ± SE of four individuals recorded 17–19 July 2001 and four different cyprids of the same broodstock recorded 21–23 July 2001. (b) Insert graph is mean ± SE of combined days as indicated.

this behaviour is quantitatively no different from that of an inanimate object. Thus, only portions of the data set in which larvae were moving have been statistically analyzed. During the first three days of the experiment, four individual cyprids swam consistently enough to monitor their behaviour. Similarly, the last three days of the experiment, a different group of four individuals swam consistently (Figure 2a). There was no significant difference in cyprid swimming behaviour whether monitored on the first or the last days of the experiment (days 1–3: speed: $F_{2,6} = 0.929$, $P = 0.445$; NGD: $F_{2,6} = 0.164$, $P = 0.852$; RCD: $F_{2,6} = 0.478$, $P = 0.642$; Days 5–7: speed: $F_{2,6} = 0.569$, $P = 0.594$; NGD: $F_{2,6} = 1.808$, $P = 0.243$; RCD: $F_{2,6} = 0.352$, $P = 0.790$). To evaluate the effect of cyprid age on behaviour we then pooled each set of four larvae to compare behaviour at the beginning *versus* the end of the experiment (Figure 2b). We found no significant differences in cyprid behaviours during these 2 time periods (speed: $t_{11} = 0.170$, $P = 0.868$; NGD: $t_{11} = -0.559$, $P = 0.093$; RCD: $t_{11} = 0.125$, $P = 0.903$).

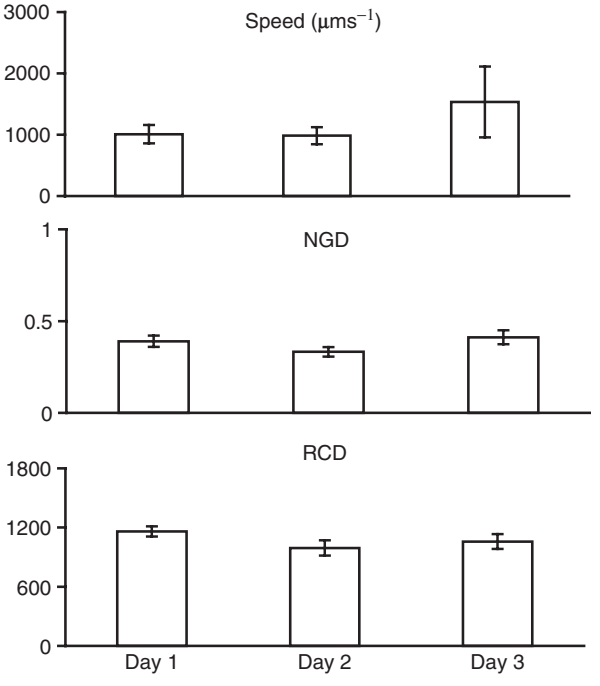

Figure 3. Swimming behaviour of individual cyprids of *B. amphitrite* analyzed with computer-assisted motion analysis on three haphazard days of July 2001 experiment (mean ± SE).

Lastly, the variability of cyprid behaviour with time on an individual basis was evaluated from a total of 25 cyprids recorded on three days (Figure 3). These days were not necessarily consecutive within a single cyprid or overlapping amongst the total group. Nonetheless, in each case, the swimming behaviour of each particular individual remained unchanged throughout the three random days of monitoring (Wilks' Lambda values range from 0.233 to 0.693). We then compared the average value of each swimming behaviour for all 25 cyprids as a function of day and again found no significant differences (speed: $t_{24} = 0.388–0.914$, $P = -0.879–0.109$; NGD: $t_{24} = 0.177–0.634$, $P = -1.391–1.361$; RCD: $t_{24} = 0.122–0.572$, $P = -0.573–1.603$).

Discussion

Crisp (1976, 1984) described the pre-settlement behaviour of cyprids, identifying two distinct phases as the cyprids explored potential settlement surfaces. In the first, ''wide searching'' phase, cyprids move in a more-or-less straight path with relatively few alterations in speed or direction. In motion analysis terms, this would translate into a relatively higher speed, greater NGD and lower RCD. When the larva transitions to the second, ''close searching'' phase, the cyprid pauses more often and frequently pivots (Crisp 1976, 1984). Again, in motion analysis terms, this would result in a relatively lower speed and NGD and higher RCD. Thus, motion analysis of cyprid swimming can be studied in a quantifiable manner providing interpretable data of pre-settlement cyprid behaviours. Although our data were obtained under artificial conditions, the technique of video motion analysis could be applied in a natural setting and provide equally interpretable data.

Faimali et al. (2006) suggest that monitoring swimming performance of aquatic larvae can yield valid ecological insight. These authors used an unidentified motion analysis system to monitor swimming behaviour of multiple nauplii of *B. amphitrite*. Swimming speeds were measured after a 24 h exposure to different sub-lethal concentrations of various toxins. The resulting swimming speed inhibition rates were much more informative and sensitive than a standard mortality test. The authors state that swimming speed may be the most pertinent behavioural measurement reflecting the physiological state of a larva.

Marechal et al. (2004) demonstrated that motion analysis technology can be applied to studying the settlement behaviour of *B. amphitrite* cyprids. The technology utilized in that study only allowed the movement of individual cyprids to be tracked. Our results complement this investigation by also video tracking the behaviour of single cyprids, but have the marked advantage of allowing us to quantitatively track multiple cyprids within a single treatment.

It has been reported that the number of cyprid larvae attaching and settling increases with increasing cyprid density (Clare et al.1994). Holm et al. (2000) determined that as few as 4 cyprids/mL induce a significant increase in settlement rate of *B. amphitrite* larvae. Head et al. (2003) determined a threshold of five or more cyprids per milllilitre elicits a gregariousness settlement response. Based on these previous studies one might predict an effect of gregariousness/density on the pre-attachment swimming behaviour. Our results, with a range of 10–100 cyprids/mL, reveal only a very slight effect of density, on one measured parameter—swimming speed. Head et al. (2003) report a 10-fold effect of settlement between single cyprids and five cyprids in the presence of the settlement promoter IBMX. Although our study did not employ settlement promoters or inhibitors, the results suggest that differences in settlement behaviour in larvae maintained at high density are not reflected by differences in swimming behaviour.

In a study investigating variation in *B. amphitrite* attachment, Holm et al. (2000) reported significant differences in cyprid attachment rates conducted in different years. The authors suggested that the observed differences between their larval batches were possibly the result of either genetic or maternal effects influencing larval response to settlement cues or surfaces. This could similarly explain our swimming speed results which varied as a function of experimental date/brood year. Caution should be used when comparing quantifiable swimming responses of cyprids produced in different years.

Crisp (1988) reported that cyprids can be maintained indefinitely in their pre-metamorphic form if stored in the dark at 6°C. In the same study, it was reported that increasing cyprid age lead to reduced discrimination in settlement. Rittschof et al. (1984) reported that *B. amphitrite* ability to metamorphose decreases dramatically by day 11 and Crisp (1988) reported that cyprids that were maintained for extended periods often did not complete metamorphosis. Head et al. (2004) investigated gregarious settlement as a function of age and assay duration in cyprids of *B. amphitrite*. Larvae were maintained individually and in groups of up to 10 cyprids per treatment exposed to a 15:9 h light : dark regime during the 48 h settlement experiments. This study found that increasing both larval age and experiment duration increased settlement rates. In our experiment, during the 7 days of recording single cyprids, we found no significant differences in swimming behaviour. This suggests that swimming behaviour does not correlate with this aspect of settlement behaviour, but it does indicate that swimming behaviour measurements can be made across multiple days if larvae are stored at 6°C and kept in the dark. In a novel field study, Hills et al. (2000) recorded *Semibalanus balanoides* cyprids swimming behaviour *in situ* during the day and at night with the aid of red light. Their results indicated that cyprids were more active at night, swimming at higher speeds, than during the day.

Our experiments were performed in red light in order to mimic darkness because we could not provide a completely uniform spherical light field and, therefore, we could not have controlled for phototactic swimming behaviour had the experiments been done using white light. However, the results of Hills et al. (2000) demonstrate that darkness is indeed an ecologically relevant period in which to study cyprid swimming behaviour.

We have demonstrated with computer-assisted motion analysis that swimming behaviours of cyprid larvae can be quantified. Under proper conditions, individual cyprids can be monitored for a period of several days without a significant change in their swimming behaviour. Holding the cyprids in the cold and in dark between experiments apparently delayed development sufficiently that experiments could be performed over multiple days on larvae at the same developmental state, at least with respect to these behavioural parameters. Similarly, Satuito et al. (2005) found that veliger larvae of the biofouling mussel *Mytilus galloprovincialis* can be maintained at low temperatures and in darkness for up to 4 months without any noted changes in bioassay experimental responses. Importantly, we have further shown that based on measurable parameters, individual *B. amphitrite* cyprids may swim in a manner similar to those in a group. This observation could be of great value to future studies evaluating the effects of bio-active compounds on cyprid swimming. Importantly, experimental setups can be scaled down conserving both bio-active compounds and reducing numbers of required larvae for tracking purposes.

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