



Motility of zooxanthellae isolated from the Red Sea soft coral *Heteroxenia fuscescens* (Cnidaria)

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Abstract

Unicellular dinoflagellate algae are among the best examples of organisms that exhibit biological clocks. This study examined the effect of light regime on rhythmicity of motility in the symbiotic dinoflagellate *Symbiodinium* sp., freshly isolated from the soft coral *Heteroxenia fuscescens* (Ehrenberg). Freshly isolated algal cells, placed under a 12-h L:12-h D cycle, exhibited motility with a diel rhythm. This motility occurred only during the period of illumination and lasted 8–9 h, with a peak at 2.5–4 h after lights on. Algal cells placed in an inverted light regime inverted their motility pattern. The response to the L/D regime was very precise, and even a 1-h shift backward or forward affected initiation of motility and time of its maximal peak. When placed in either constant light or dark, algal motility ceased until the L/D cycle was restored. These findings suggest that the rhythm is entrained by light cues and is not due to an endogenous circadian rhythm. Further, we provide evidence that the presence of juvenile hosts does not affect the algal motility pattern. These results offer the first evidence for the lack of impact by the host on rhythmicity of motility of free-living algal cells. The motility pattern found in freshly isolated algae may indicate the presence of light-induced diel rhythmicity in yet-to-be described free-living *Symbiodinium*.

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1. Introduction

The ability of organisms to track time has been of interest to scientists for centuries. Numerous rhythms that control all manner of biological processes have been described

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from organisms in all kingdoms. Recently, a variety of clock genes that underlie some of these rhythms have been described in organisms ranging from yeast to humans (Ederly, 2000). Circadian rhythms are defined as endogenous biological rhythms that (1) cycle over approximately 24 h, (2) continue in the absence of environmental cues, (3) are reset or entrained by environmental cues, and (4) have a constant cycle regardless of temperature change (a.k.a. temperature compensation) (reviewed in Ederly, 2000; Sweeney, 1984).

Free-living dinoflagellates exhibit daily rhythmicity of numerous cellular processes including motility, photosynthesis, bioluminescence, and organelle migration (Roenneberg and Merrow, 2002; Suzuki and Johnson, 2001). Indeed, dinoflagellates served as one of the early models for studies of circadian rhythms. It is now known that some dinoflagellates possess at least two biological clocks that can be entrained by external cues, including blue and far red light and nitrate concentration (reviewed in Roenneberg and Merrow, 2002).

Despite this wealth of information on free-living dinoflagellates, relatively little is known about the rhythmicity of mutualistic dinoflagellates (often referred to as zooxanthellae) engaged in symbioses with various invertebrates, including cnidarians. This is due in part to our poor understanding of the complete life history of these algae. The majority of symbiotic dinoflagellates are members of the diverse genus *Symbiodinium* (Rowan, 1998). In the host, zooxanthellae occur in a nonmotile coccoid form (Trench, 1993), although there are some EM studies that show evidence of flagellae and basal bodies in algae in hospite (Schoenberg and Trench, 1980b; Trench, 1993). Much is known about *Symbiodinium* as a symbiont in cnidarians and other invertebrates. However, virtually nothing is known about *Symbiodinium* in its free-living state—if such a state indeed exists in nature. To date, there is only one report of *Symbiodinium* being isolated from the water column (Carlos et al., 1999), whereas numerous studies have examined cultured *Symbiodinium* spp. isolated from invertebrate hosts (Maclaughlin and Zahl, 1957, 1966; Freudenthal, 1962; Taylor, 1973; Kinzie, 1974; Loeblich and Sherley, 1979; Schoenberg and Trench, 1980a,b, 1987; Fitt et al., 1981; Fitt and Trench, 1983; Lerch and Cook, 1984; Crafts and Tuliszewski, 1995).

Cultured zooxanthellae can occur as coccoid nonmotile cells and as motile swimmers with a characteristic gymnodinoid morphology. Some studies of the cultured phase include descriptions of a diel rhythmicity of algal motility (Maclaughlin and Zahl, 1957; Freudenthal, 1962; Fitt et al., 1981; Lerch and Cook, 1984; Banaszak and Trench, 1995; Crafts and Tuliszewski, 1995). The significance of this motile phase and its rhythmicity in nature remains obscure. It has been suggested that the motile phase is a natural stage in algal symbiont life cycles, although it is not known whether swimmers are a direct result of cell division of the coccoid form (Freudenthal, 1962; Taylor, 1973). To date, the only study to correlate algal cell division and algal motility is that of Fitt and Trench (1983), who reported that the highest frequency of cytokinesis precedes highest motility by 4–7 h. In addition, they found that the ratio of number of dividing cells to number of motile cells recorded was 1:2, suggesting that two motile cells arise from one newly divided zooxanthella. Trench (1980) has speculated that the motile phase could be a dispersal or infectious form that may function in symbiosis onset in those associations where the symbiosis is open or established anew with each host generation.

Our present study provides an in-depth description of motility patterns of zooxanthellae, freshly isolated from the Red Sea soft coral *Heteroxenia fuscescens*. This is the first study to describe thoroughly the diel rhythmicity of motility in these zooxanthellae, including

effects of light, shifts in light duration, inversion of light cycle, elimination of diel light pattern, and host presence. Our results suggest that the rhythm is entrained by light cues and is not due to an endogenous circadian mechanism. Further, we show evidence that the presence of hosts does not affect algal motility pattern. A more complete understanding of zooxanthellae motility will provide insight into its role in the establishment of symbioses between zooxanthellae and sexually produced cnidarian offspring.

2. Materials and methods

2.1. Isolation of algal cells

The algal cells used in this study were isolated from adult parental colonies of *H. fuscescens*, sampled haphazardly from the coral reef (3–8 m deep) across from the Interuniversity Institute of Eilat (IUI). The colonies were transported to Tel Aviv for isolation of algal cells. Ten haphazardly selected polyps from an individual colony were removed for isolation of symbiotic algae. The polyps were homogenized in 1.5-ml microfuge tubes containing 0.5 ml filter (0.2 μm)-sterilized seawater (FSW). The homogenate was then transferred to a 15-ml sterile tube containing 12 ml FSW, and centrifuged for 30 min at 1500 rpm. The supernatant was discarded; the algal pellet was resuspended in 12 ml FSW and centrifuged for a further 15 min (see also Davy et al., 1997). This last procedure was repeated four times in order to obtain clean algal preparations.

2.2. Maintenance of algal cells

Motility of the isolated algal cells was monitored while maintained in 24-well tissue culture plates, each well filled with 3 ml 0.45 μm FSW. In addition, when applicable, motility was monitored in the presence of primary polyps of *H. fuscescens*. For this purpose, planulae of *H. fuscescens* were placed in the culture plates, three planulae per well (72 planulae per plate) filled with 3 ml 0.45 μm FSW, following the methodology of Yacobovitch et al. (2003). Freshly isolated algal cells were introduced into each well at the initiation of planulae metamorphosis, 10–14 days after their release. In both cases, 100 μl of 0.45 μm FSW was removed from each well, replaced by a similar volume of clean fresh algal preparation, yielding a final concentration of $5\text{--}7 \times 10^4$ algal cells per 1 ml FSW in each well. The plates were maintained in an incubator under cool white fluorescent light (30 μmol quanta $\text{m}^{-2} \text{s}^{-1}$), at a temperature corresponding to the ambient seawater temperature at time of collection of the parental colonies (21–28 °C). The light regime in the incubator was a 12-h-light/12-h-dark (12L:12D) cycle, unless specifically stated otherwise. Every other day, half of the 0.45- μm FSW in each well was changed.

2.3. Quantifying algal motility

Except where specifically stated below, motility was determined in algae incubated in the presence of host primary polyps. In order to quantify changes in algal motility

throughout a 24-h cycle (12L:12D), motility was monitored by observing algae inside the wells, using either a dissecting or compound microscope. In each experiment, algal counts were made at 1–2-h intervals during the light phase and in one experiment (see below) during the dark phase at the same time intervals. Wells were kept under the microscope for no longer than 10 min to minimize the possible influence of the light source on motility. Only motile cells were counted, and the degree of motility was categorized into five ranks according to the number of motile algal cells counted in a well at a given time; rank 1: 1 cell, 2: 2–10, 3: 10–100, 4: 100–1000, 5:>1000. Before each count, the wells were screened under a dissecting microscope, and the number of motile cells was estimated. When fewer than 50 motile cells per well were viewed, their precise number was determined. At higher motile cell density, subsamples of 10 μ l each ($n=6$) were viewed by hemacytometer under light microscopy (200 \times), and the number was extrapolated to calculate the average number of motile cells in one 3-ml well. For each motility determination, a random subset of wells, out of the entire population of wells, was sampled (see below). The number of wells monitored in all the experiments depended on primary polyp survival, which decreased with time. Consequently, in some experiments, fewer wells were monitored at the conclusion of the experiment than at the beginning.

In all the assays described below, the pattern of motility was determined according to five parameters (Fig. 1) following Fitt et al. (1981), which were used to analyze the results: (1) time to initiation of motility=time between beginning of the light phase and appearance of the first swimming cells; (2) lag time=time from onset of the light period to the time taken to achieve half-maximum motility; (3) peak motility time=duration of maximum motility; (4) peak duration=duration of half maximum motility or greater; (5) duration of the motile period=time from initiation to termination of all swimming activity. For each experiment, the obtained parameters of motility were presented as ranges of time (hours).

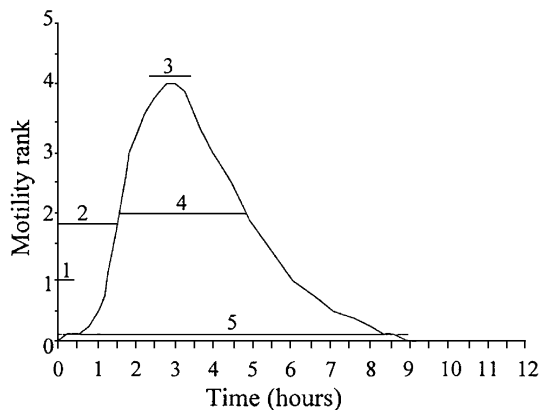


Fig. 1. Schematic representation of algal motility pattern following Fitt et al. (1981). Light was turned on at hour 0 and off at hour 12.

2.4. Examination of rhythmicity of algal motility

To examine diel rhythmicity of algal motility and possible changes in motility with time, algae were monitored for a 5-week period starting on day 1 after inoculation (December 1999–January 2000), under 12L:12D cycle (lights on at 0900 h). In this experiment, motility was examined every 1–6 days for a total of 12 observations (for each date, 12–30 wells were randomly selected from the 144 initial ones).

Experiments manipulating three aspects of the diel light regime were performed in order to determine whether motility patterns were entirely endogenous, or whether they were influenced by a light cycle. First, an inverted light/dark cycle experiment was performed in February 2000, with the plates kept for 3 weeks under a regular cycle before being transferred to an inverted cycle, with lights on at 2000 h for 12 h for 1 week, after which motility was recorded. The control plates were kept for the same period of time under the regular 12L:12D cycle (lights on at 0800 h). In this experiment, 12 out of an initial 48 wells were examined for the treatment and for the control.

Algal motility under continuous dark was examined in an experiment conducted in April 2000. Three plates were transferred to constant dark, after an initial 3-day period under the regular 12L:12D regime. Then, after being under constant darkness for 24 h, 30 out of 72 wells were examined during the next 24 h at 1–3-h intervals. The control plate was maintained under the regular cycle (12 out of 48 wells were examined).

Algal motility under continuous illumination was examined in two experiments conducted in August–September 2000. In the first, three plates were transferred to constant light, immediately after algal cell isolation. In the second, three plates were transferred to constant light after an initial 3-day period under the regular 12L:12D regime. In each of the above two assays, 30 out of 72 wells were examined. In both, the control plates were maintained under the regular cycle (12 out of 48 wells were examined). In addition, a reverse test was made, in which three plates were first kept under continuous illumination for a 3-day period immediately after algal introduction and then transferred to a 12L:12D cycle (30 of 72 wells were examined). The controls (two plates) were maintained under the regular cycle (12 out of 48 wells were examined). All the above experiments lasted for 1 week.

Finally, the lag time between the beginning of the light cycle and maximal motility was examined in May 2000, using three different times of light onset: 0700, 0800 and 0900 h. Each assay lasted 1 week during which motile cells were counted daily in 12–36 out of 96–144 initial wells for each treatment.

2.5. Effect of host presence on algal motility

In order to examine the possible effect of the presence of host primary polyps on motility, two experiments were conducted in August 1999 and February 2000 using wells with algae in the presence or absence of hosts. Each of the two experiments lasted for a period of 1 week under a 12L:12D cycle (light on at 0800 and 0700 h, respectively), starting after the freshly isolated algal cells were introduced into the wells. One group of wells contained algal cells with primary polyps maintained in 0.45 μm FSW (in August 1999, 12–30 out of 96 wells and February 2000, 12–30 out of an initial 144 wells were

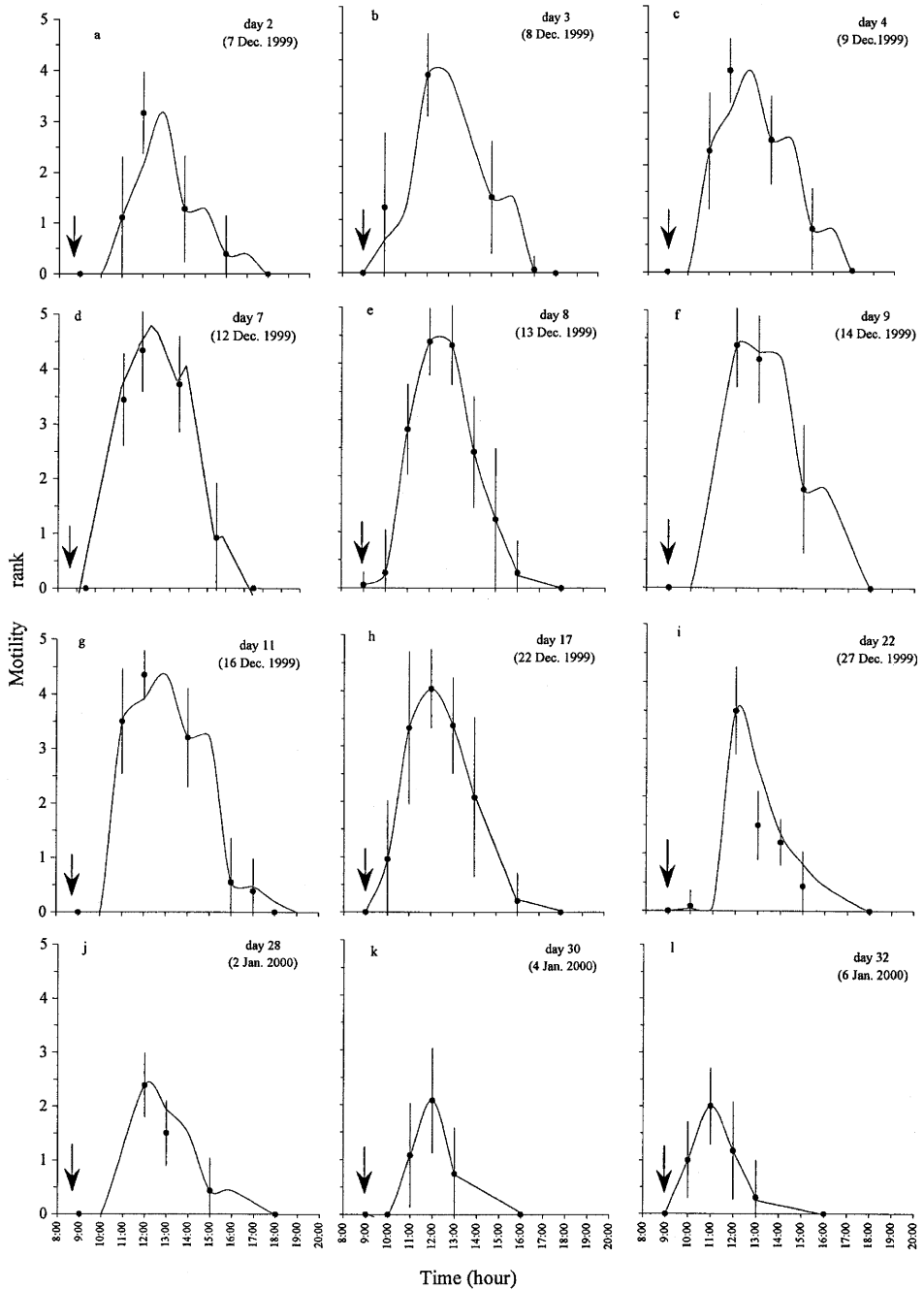


Fig. 2. *H. fuscescens*. Algal motility pattern during a 5-week period starting in December 1999. Light was turned on at 0900 h (arrows). (a–c) first week; (d–g) second week; (h) third week; (i–j) fourth week; (k–l) fifth week. Each point represents the average motility rank (\pm S.D.) of 12–30 out of 144 wells.

examined), while the other group contained only algal cells in 0.45 μm FSW (each time 12 out of 48 wells).

3. Results

3.1. Rhythmicity of algal motility

Motility of freshly isolated algae from mature colonies of *H. fuscescens* exhibited diel rhythmicity under a 12L:12D cycle over a 5-week period (Fig. 2). Algal motility began 0–1.2 h after the light was turned on, and became most intense 1.5–2.25 h after that time (lag time) with a peak duration of 2.5–4 h (Table 1). Peak motility period lasted 1.5–2 h. Motility then gradually decreased and ended 3–5 h before dark. Motility duration was 7–9 h and was never observed during darkness.

Variation in motility with time was noticed over the examined 5-week period (Fig. 2). The highest average motility rank (\pm S.D.) was observed during weeks 2 (Fig. 2d–g) and 3 of the experiment (Fig. 2h) after adding algal cells to the wells, with a maximum rank of 4.39 ± 0.59 ($n=24$ wells) and 4.04 ± 0.71 ($n=18$), respectively. Later, motility gradually decreased, until it achieved a maximum of only 2.33 ± 0.85 ($n=12$) in the fifth week (Fig. 2k–l). There was no significant difference in maximum motility rank between the second

Table 1
H. fuscescens

Type of assay examining motility	Time to initiation of motility	Lag time	Peak motility time	Peak duration	Duration of motile period
Diel rhythmicity (5 weeks)	0–1.2	1.5–2.25	1.5–2	2.5–4	7–9
Inverted light/dark cycle	0–1	1.5–1.6	2	3.5–4	9
Constant dark	0	0	0	0	0
Constant light	0	0	0	0	0
Constant light \rightarrow 12:12 h L/D	0–1	1.5–2	2–2.5	3.5–4	8–9
<i>Shifting time of light onset</i>					
0700 h	0–1	1.5–1.8	2–2.5	4–4.5	8–9
0800 h	0–1	1.5–1.8	1.5–2.2	3.5–4	8–9
0900 h	0.5–1	1.5–1.8	1.5–2	3.5–4	8–9
<i>Presence of host primary polyps</i>					
August 1999					
Primary polyps	0–1	1.25–1.5	1.5–2.5	3–3.5	8–9
Algae only	0–1.2	1.5–1.6	1.5–2	3–3.5	8–9
February 2000					
Primary polyps	0–1	1.5–1.8	2–2.5	4–4.5	9
Algae only	0–1	1.5–1.8	2–2.5	4–4.5	9
Summary	0–1.2	1.5–2.25	1.5–2.5	2.5–4.5	7–9

Parameters of motility (following Fitt et al., 1981) found in the different assays. Numbers denote ranges (in hours) of all available data from each assay. Summary = the minimum and maximum values of all ranges in each parameter.

and third week and between the fourth (Fig. 2i–j) and fifth week of the experiment (t -test, $p > 0.05$). However, there was a significantly higher average motility rank in the combined weeks 1–3 compared to weeks 4 and 5 (t -test, $p < 0.001$). Motility pattern remained the same throughout the entire period (Fig. 2), though the duration of motile period was shorter in the fifth week and lasted for only 7 h (Fig. 2, Table 1).

Inverting the light phase to the night hours (2000–0800 h) and the dark phase to the day hours (0800–2000 h) also affected the timing of motility (Fig. 3). In this experiment, motility occurred only during the light phase, and its temporal pattern and parameters of motility remained as above (Figs. 2 and 3; Table 1). Interestingly, there was no significant difference between motility ranks of the experimental and the control wells (t -test, $p > 0.05$).

No motility occurred when algal cells were kept under continuous dark. Furthermore, no motility occurred when algal cells were kept under continuous illumination immediately after isolation or after initial 12L:12D cycle for 3 days and then transferred to constant light (Table 1). However, after being under constant light for 3 days and then transferred to a

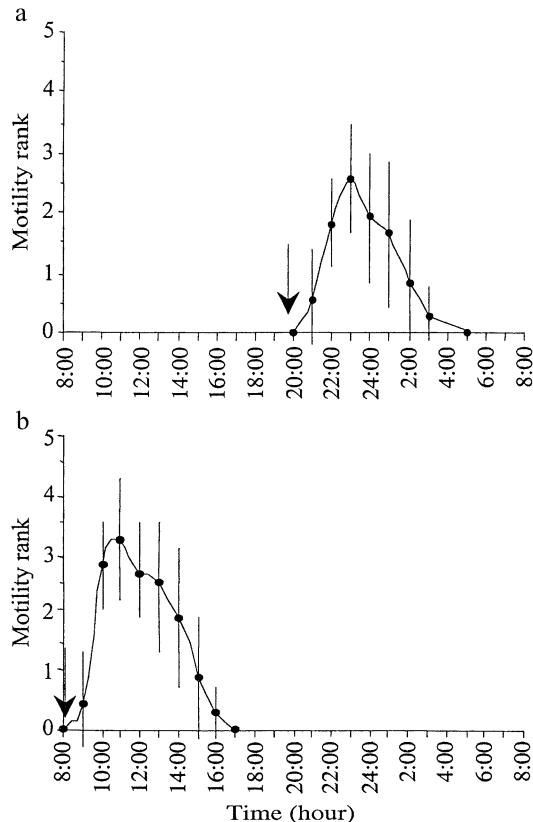


Fig. 3. *H. fuscescens*. Algal motility pattern under (a) inverted light regime: lights on from 2000 to 0800 h and (b) regular light regime: lights on from 0800 to 2000 h. Each point represents the average motility rank (\pm S.D.) of 12 out of 48 experimental wells and 12 out of 48 control wells. Arrows indicate onset of light.

regular 12L:12D cycle, motile cells reappeared the next day, with parameters of motility as above.

Shifting the time of light onset 1 h backward, from 0800 to 0700 h, or 1 h forward, from 0800 to 0900 h, shifted the peak time of motility accordingly, while the motility pattern remained similar (Fig. 4). In all three experiments, motility appeared 0–1 h after the light was turned on and lasted for 8–9 h (Table 1), with the same temporal pattern described above

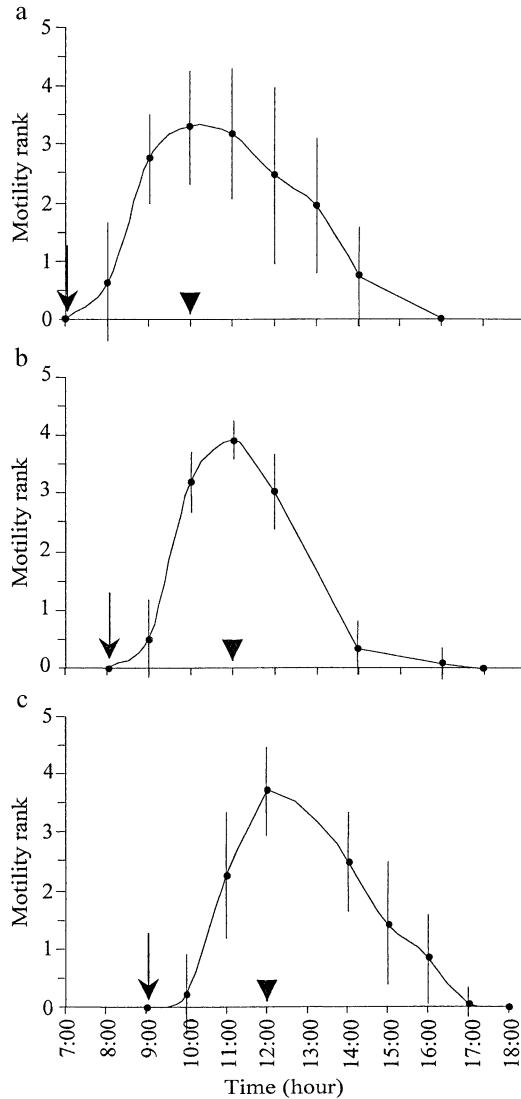


Fig. 4. *H. fuscescens*. Motility pattern at three different times of light onset (thick arrows). (a) Lights on at 0700 h; (b) at 0800 h; (c) at 0900 h. Each point represents average motility rank (\pm S.D.) of 12–36 out of 96–144 wells. Thin arrows indicate light onset, arrowheads indicate peak motility time.

(Figs. 2 and 3; Table 1). When the light was turned on at 0700, 0800 or 0900 h, maximum motility rank was 3.29 ± 0.98 ($n = 18$ wells), 3.88 ± 0.33 ($n = 24$), and 3.73 ± 0.77 ($n = 30$), respectively, with no significant differences among the values (one-way ANOVA, $p > 0.05$).

3.2. Algal motility in the presence of host primary polyps

There were no significant differences (two-way nested ANOVA, $p > 0.05$) in average motility rank between the experimental wells, which contained primary polyps, and the control ones in the two experiments, whether light was turned on at 0800 or at 0700 h (Fig. 5a and b). Motility pattern and the parameters of motility resembled those described above (Figs. 2–4; Table 1).

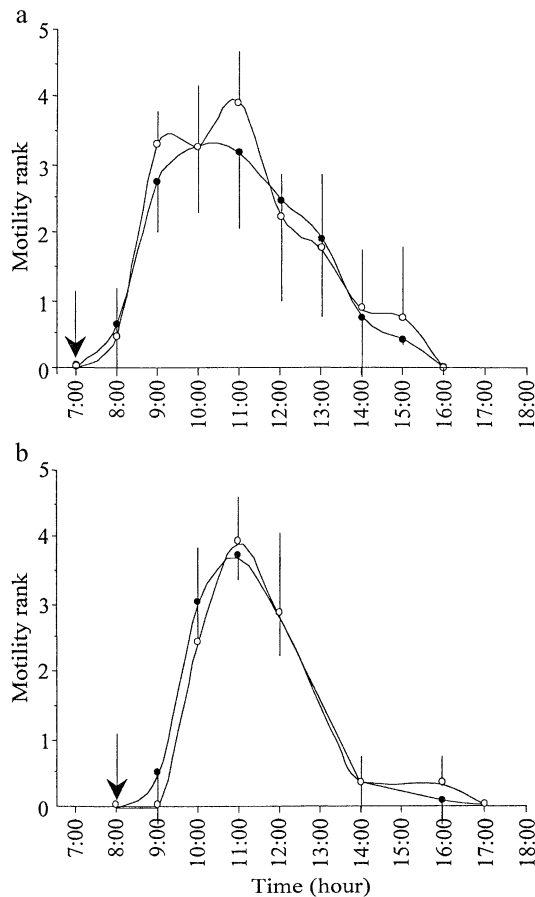


Fig. 5. *H. fuscescens*. Algal motility pattern in the presence (closed circles) and absence (open circles) of host primary polyps. (a) Lights on at 0700 h (arrow, February 2000); (b) light on at 0800 h (arrow, August 1999). Points represent the average motility rank (\pm S.D.) in (a) of 12–30 out of 144 experimental wells and 12 of 48 control wells and (b) of 12–30 out of 96 experimental wells and 12 of 48 control ones.

4. Discussion

Freshly isolated symbiotic algal cells from the soft coral *H. fuscescens* exhibit motility with a diel rhythm. Examining motility pattern in algal cells isolated from *H. fuscescens* parental colonies in our experiments (Figs. 2–5), revealed only slight differences among the ranges of each parameter of motility. Thus, these ranges are summarized for all experiments, giving the minimum and maximum periods found for each parameter (Table 1). This is the first study to describe rhythmicity of motility in heterogeneous population of algae freshly isolated from an invertebrate host. However, similar rhythmicity has been reported previously for cultured algae isolated from other invertebrates, including the anemones *Aiptasia pallida* and *Aiptasia tagetes* (Fitt et al., 1981; Lerch and Cook, 1984), the scyphozoans *Cassiopeia frondosa*, *Cassiopeia xamachana*, and *Linuche unguiculata* (Fitt et al., 1981; Crafts and Tuliszewski, 1995), and the giant clam *Tridacna gigas* (Fitt et al., 1981).

4.1. Effect of light on rhythm of algal motility

In this study, when cells were placed under an alternating light cycle, motility occurred only during the period of illumination. This is similar to previous findings on algal motility in other symbioses (Fitt et al., 1981; Lerch and Cook, 1984), with the exception of cultured algae from *L. unguiculata* which initiated motility prior to light onset (Crafts and Tuliszewski, 1995). In our study, response to an L/D regime was very precise, motility pattern in algae placed in an inverted light regime became correspondingly inverted (Fig. 3) and, further, even a 1-h shift backwards or forwards affected the initiation of motility and time of peak motility (Fig. 4).

Algae lost motility when placed in either constant light or constant dark (Table 1). This is in marked contrast to other *Symbiodinium* motility studies, which have shown a cessation or severe attenuation of motility pattern in constant light but a continuation of motility rhythms in constant dark (Fitt et al., 1981; Lerch and Cook, 1984; Banaszak and Trench, 1995; Crafts and Tuliszewski, 1995). The cessation of motility in the absence of exogenous cues violates the second criterion (listed in the Introduction) of a circadian rhythm. Further, in *H. fuscescens* algae, rhythmicity of motility reoccurred only when algae were placed back into an L/D cycle. These findings suggest that a change in light intensity is required for rhythmicity of motility in algae freshly isolated from *H. fuscescens*. Therefore, at present, although we have strong evidence that light is involved in setting a diel rhythm, we do not show evidence that rhythmicity is controlled by an endogenous clock, as rhythmicity disappears in the absence of changes in light.

Many organisms possess light receptors that entrain endogenous rhythms including diel rhythms. The two best studied are the phytochromes and cryptochromes. Phytochromes are proteins that detect red light and are found in chlorophytes including green algae (Cashmore et al., 1999, reviewed in Briggs and Olney, 2001). Cryptochromes are blue light-sensing flavoproteins that are homologous to photolyases and

are important in entraining diel rhythms in a broad range of taxa including higher plants and animals (reviewed in Cashmore et al., 1999). Algae from a variety of taxa also use blue and red light in controlling cellular processes such as motility, photosynthesis, chloroplast movement, and gamete release. In a few green algal species, phytochrome (Kidd and Lagarias, 1990; Winands and Wagner, 1996) and cryptochrome (Small et al., 1995) homologs have been identified and characterized. Free-living dinoflagellates are among the algae for which entrainment of a clock by blue and red light has been demonstrated (see Introduction); however, to date no phytochrome or cryptochrome homologs have been identified (Roenneberg and Merrow, 2002). Our studies on *Symbiodinium* freshly isolated from *H. fuscescens* suggest that changes in light control patterns of rhythmicity of motility. Future studies investigating the specific effects of blue and red light and the presence of phytochrome and/or cryptochrome homologs would contribute greatly to our understanding of the control of rhythmicity in dinoflagellates.

4.2. Effect of host presence on rhythm of algal motility

Kinzie (1974) was the first to raise the question of whether the infection of polyps by algal cells is controlled by the algae, by the host, or by both; a question that has remained open. *H. fuscescens* broods and releases azooxanthellate offspring that acquire symbionts both in the laboratory and in the field soon after metamorphosis from planulae into primary polyps (Benayahu et al., 1989a,b; Yacobovitch, 2001; Yacobovitch et al., 2003). This study shows that the pattern of algal cell motility is not affected by the presence of these juvenile hosts (Fig. 5). Freshly isolated algae show rhythmicity of motility in both the presence and absence of juvenile hosts. This is the first direct evidence for the lack of impact by the host on symbiont motility appearance and pattern. Hosts do affect the directionality of swarmer swimming. Indeed, aggregation of zooxanthellae swarmer in the vicinity of the mouth of azooxanthellate primary polyps of *H. fuscescens* was followed by onset of symbiosis between the algae and the juvenile hosts (Yacobovitch, 2001). Similarly, zooxanthellae isolated from several hosts (e.g., stony corals, gorgonians, and a giant clam) were observed swimming towards the mouth of primary polyps of the gorgonian *Pseudopterogorgia bipinnata*, followed by infection of these juvenile hosts (Kinzie, 1974). In another study, zooxanthellae from a variety of hosts were likewise attracted to several types of aposymbiotic adult hosts (Fitt, 1984). Therefore, although rhythmicity of motility may remain independent of host presence, algal motility itself appears to play an important role in symbiosis onset in *H. fuscescens*.

What is the significance, therefore, of the diel rhythmicity of cultured and freshly isolated *Symbiodinium* motility? The free-living phototrophic dinoflagellate *Gonyaulax polyedra* undergoes diel vertical migration from high light, nitrogen-poor shallow water during the day to deeper nitrogen-rich water at night (reviewed in Roenneberg and Merrow, 2002). This migration is controlled by one or more light- and nitrogen-sensitive clocks that affect motility. Rhythmicity of motility in freshly isolated and cultured *Symbiodinium* could be evidence that a similar diel motility pattern occurs in the yet-to-be described free-living *Symbiodinium* in nature.

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