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Complex sexual courtship displays by luminescent male marine ostracods

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SUMMARY

In the western Caribbean Sea, about an hour after the sun sets, a complex and ritualized light show of precise, vertically placed luminescent pulses erupts over shallow grassbeds. These are among the most complex displays known in marine systems. Displays consist of repeated trains of secreted bioluminescent pulses in a specific pattern ejected into the water column as courtship signals by male *Vargula annecohenae*, which are small (<2 mm) myodocopid ostracod crustaceans. Although these animals display in near darkness, we have used image intensification and infrared videography and three-dimensional analysis in the lab to demonstrate that each luminescent display train, which can be up to 60 cm long, consists of two distinct luminescent and swimming phases. The first, or 'stationary,' phase consists of three (usually) bright, longer pulses placed close together, with the male swimming in a looping pattern. We hypothesize that this pattern acts as an attention-grabbing signal for receptive females. The stationary phase is followed by the 'helical phase,' which consists of about a dozen evenly placed dimmer, shorter pulses secreted by an individual male rapidly spiraling upward in a helical pattern. We hypothesize that this phase, which has very uniform interpulse intervals and distances, helps an approaching female target and intercept the rapidly moving male. Here we provide details of these two phases, and produce a three-dimensional model of a multiply-displaying male.

Key words: bioluminescence, courtship, ostracod, mating display.

INTRODUCTION

Where mate choice is exhibited by one or both sexes, a signal must be detected by the receivers. Signals can be vocal, olfactory, tactile or visual, and can range from simple, such as a pheromone plume, to highly complex, as found in the visual and auditory displays of some birds (Andersson, 1994). In order to infer which aspects of a signal are important, signal characteristics must be quantified.

Visually based mating systems are more common in diurnal than nocturnal animals. However, some nocturnal organisms, such as fireflies, utilize visual displays involving luminescent signals. Firefly studies include descriptions of the luminescent display patterns (Lloyd, 1966), reveal which aspects of the displays are attractive to females (e.g. Branham and Greenfield, 1996; Lewis and Wang, 1991; Michaelidis et al., 2006; Vencl and Carlson, 1998) and examine male–male interactions (Buck, 1988; Copeland and Moiseff, 1997a; Copeland and Moiseff, 1997b). Other firefly studies describe mimicry and tracking by extra-specific females for predation purposes (Lloyd, 1975; Lloyd, 1980; Lloyd and Wing, 1983).

Although the number of luminescent marine species exceeds luminescent terrestrial species (Hastings and Morin, 1991), luminescent sexual displays in marine environments are not well known, largely because of the difficulty of *in situ* observations. Research on syllid polychaetes ('fire-worms') reveal that a female, either swimming at the surface or rising from the benthos will glow for many seconds or minutes to attract conspecific males, which intermittently and rapidly flash when approaching (Markert et al., 1961; Tsuji and Hill, 1983) (T.J.R. and J.G.M., personal observation).

The luminescent courtship displays of male cypridinid ostracods (Myodocopida, Ostracoda, Crustacea) in the Caribbean have proved to be much more complex, more akin to firefly displays than 'fire-worm' displays (Morin, 1986; Morin and Cohen, 1991; Herring, 2000). Over a dozen species of ostracods can be found in specific habitat types (e.g. gorgonian patches, coral types, sand patches, grassbeds, etc.) within a single Caribbean reef system. Each species has a dramatically different light display. Each display train is secreted as a series of multi-component packets into the water column (Morin, 1986; Morin and Cohen, 1991). The luminescent compounds are synthesized in a luminescent organ made up of long secretory or exocrine cells, with each one extending the entire length of the light organ and terminating at nozzles on the upper lip (Huvard, 1993; Abe et al., 2000). Muscle bands around and through the light organ apparently contract, squeezing the compounds into the water (Huvard, 1993) to produce controlled species-specific patterns. Depending on the species, males can display while swimming upwards, downwards, diagonally, or horizontally, with bright blue pulses that vary from about 100 ms to >10 s depending on the species. Of the more than 60 known species of displaying ostracods in the Caribbean, there is no known case of a luminescent 'duet' or 'dialogue' between males and females. Females do not produce light during courtship bouts, although both sexes and all instars luminesce when attacked by a predator (Morin, 1986; Morin and Cohen, 1991). The lack of dialogue between the sexes, the complexity and diversity of the signals among species, and the fact that the luminescence is an extracellular secretion set these cypridinid systems apart from other luminescent courtship systems currently known. Morin (Morin, 1986) tentatively classified the mating system as a spree, or temporal lek (*sensu* Walker, 1983), with individuals only entering the water column (lek area) for courtship during a specific twilight time window. Fertilization is internal, females brood young within a brood pouch, and males provide no parental care (Cohen and Morin, 1990; Gerrish and Morin, in press), and there is evidence of female choice (Rivers and Morin, 2006).

Previous research on luminescent ostracods primarily addressed questions regarding their systematics, phylogeny, display patterns and distributional differences among species (Morin and Cohen, 1991; Cohen and Morin, 1990; Cohen and Morin, 2003; Torres and Cohen, 2005; Torres and Morin, 2007). Because of the difficult nature of this system [i.e. working with small (~2 mm), fast-swimming (up to 15 cm s^{-1}) marine crustacea that intermittently luminesce in the dark in the open sea], little is known of their detailed courtship mating system beyond basic descriptions of the luminescent patterns in the field. While it is clear that the signals are important in species and probably mate-quality recognition by females, which components of the display trains are involved in the recognition are unknown. Based on a series of laboratory experiments, this paper provides the first detailed quantitative documentation of the characteristics of these trains of pulses and what individual males are doing between pulses. These data are essential in order for us to be able to address questions concerning pattern recognition and its mechanisms by females and other males.

We discovered that infrared (IR) light reflects sufficiently off the carapaces of individual ostracods freely swimming in clear acrylic sea-water tanks to enable the use of low-light CCD cameras to examine individual ostracod behavior during courtship displays. Using this experimental approach we address the following questions. (1) What are the actual swimming patterns of the males as they produce their luminescent pulses? (2) What are the quantitative characteristics of the pulses themselves, the relationships among pulses, and among different parts of each display train? (3) How much variation do we find in all these characteristics both within and between displays? (4) What are the probable functions of each phase of the display? This paper is the first of four papers that focuses on the luminescent behavior of one signaling species, *Vargula annecohenae* (Torres and Morin, 2007), in which we tease apart the details of this fascinating mating system through field documentation and laboratory experiments.

Background of the life history patterns and luminescent displays of *Vargula annecohenae*

V. annecohenae (Torres and Morin, 2007) is one of the most abundant western Caribbean luminescent ostracod species. This species is the only luminescent ostracod found in abundance in grassbeds in Belize and can be collected in great numbers (both juveniles and adults) using special traps baited with fish muscle. As with all other cypridinid ostracods, *V. annecohenae* has a life cycle that includes reproduction by copulation with internal fertilization, brooding by females, crawl-away juveniles (i.e. there is no planktonic larval stage), and five discrete juvenile instars that lead to a single terminal adult stage (Cohen, 1983; Cohen and Morin, 1990; Gerrish and Morin, in press). There is clear sexual dimorphism, with females being much larger than males: males are 1.62±0.05 mm (± s.d.) in length whereas females are 1.99±0.05 mm (± s.d.). The entire life span in the lab can be up to nine and a half months, within which the time from brooded embryo to adulthood is about 3 months (Gerrish and Morin, 2008).

The courtship displays of *V. annecohenae* are trains of vertically placed short pulses of light that are easily quantifiable in space and time. The display periods are synchronized with the darkness, with the activity occurring either when the moon is not present or is low in the sky; no courtship activity occurs only during the two nights around full moon (Gerrish et al., 2008). At a precise 'dark threshold', approximately 1 h after sunset or moonset, whichever occurs later (Gerrish et al., 2008), males participate in mating displays above the grassbeds of Belize for approximately an hour.

Males can exhibit one of several alternative mating tactics: (1) initiate a display on their own, (2) entrain (synchronize) their flashing pattern on that of an already displaying male, or (3) sneak silently above a luminescing male (Rivers and Morin, 2004).

In this paper we show that each display train appears to have two distinct phases: a stationary and a helical phase. The initial *stationary phase* consists of three to four (variable) bright pulses with some interpulse interval variation, and occurs at or just above the top of the grass (~15–20 cm above the substratum). These pulses show no distinct upward movement, although some lateral movement may occur. The second, more uniform (in space and time) portion, which we call the *helical phase* (see below for explanation), occurs as a series (10–15) of somewhat dimmer, upwardly placed shorter pulses with more consistent interpulse intervals and interpulse distances. The total vertical length of a display train is about a maximum of 60 cm upward in the water column. These two phases are variants of the *shortening* and *trill phases*, respectively, observed in other ostracod courtship displays that have been previously documented in the Caribbean, based on field observations and recordings (Morin, 1986; Morin and Cohen, 1991; Cohen and Morin, 1993; Torres and Cohen, 2005; Torres and Morin, 2007).

MATERIALS AND METHODS

Field experiments and observations

Collection of ostracods

Ostracods for lab trials were collected off the southwest shore of Southwater Caye, Belize (16.801°N lat., 88.083°W long.) between 15 January and 10 February 2006. We used small (4 cm diameter × 8 cm long) PVC-pipe traps with a 500 μm mesh funnel at each end, and fish muscle as bait, similar to methods described by Cohen and Morin (Cohen and Morin, 1986). Males were maintained in seawater in 750 ml Gladware (Oakland, CA, USA) containers until used in a trial. The ostracods were returned to the grassbeds following the experiments.

Collection of ostracods during luminescent displays

A 500 μm mesh cloth sweep net (25 cm diameter, 50 cm length) was used to collect ostracods during the displays. To avoid the effects of the moon, we did all our sampling and experiments during the waxing phase of the moon when there was not visible moon in the sky at sunset. We would wait underwater to sweep until a male started the helical phase of the display (usually the third pulse), thus minimizing collecting unwanted particulates such as grass blades and other organisms in the net, and then raised the net around the display from below and twisted close the net after each sweep. We repeated this procedure throughout the display period. The netted males and females were placed in fresh seawater in a bucket and taken to the lab where they were sorted, separated by sex, counted, and their average numbers per display calculated. They were then stored in the Gladware 'aquaria'. The ratio of males to females provided us with the operational sex ratio (OSR) for the proximity around the displays.

Field male display density

A 0.25 m^2 square quadrat (50 cm side) made of 1.25 cm diameter PVC pipe was haphazardly placed on the grassbed in ~2 m of water off the south beach of Southwater Caye, Belize. A diver, either on snorkel or using scuba, rotated on the sea surface with eyes closed, tossed the quadrat and let it settle to the bottom, and then recorded how many displays (including displays that were entrained with

earlier displays) were observed in the water column directly above the quadrat in a 3 min period. For non-random, high-density sampling, the quadrat was placed on the grassbed adjacent to a small (1 m×0.5 m) dead section of coral rubble where we had observed consistently high numbers of displays over multiple nights. We again recorded the number of displays in the quadrat observed in a 3 min period. We counted three (two random, one nonrandom) quadrats within the first 30 min after the first displays started, and again after 60 min from the start of the first display. We used a log-transformed random effects mixed model (SAS 9.1) to compare the densities between random and nonrandom samples.

Number of pulses per display

In situ videos of the courtship displays were recorded using a Dark Invader Generation II night-vision device (NVD; B. E. Meyers & Co., Inc., Redmond, WA, USA) attached to a Sony DCR VX-2000 camcorder (New York, NY, USA), in a custom Aquavideo (Weston, FL, USA) underwater video housing and positioned perpendicular to the displays and parallel to the sea floor. The numbers of pulses per display from individual displays were recorded from the video files. We also performed field censuses by counting the pulses from individual displays while we were either on snorkel or on scuba, and writing the results on an underwater slate.

Lab experiments and observations

Two-dimensional recordings

To control the start of displays in the lab, males were maintained in the Gladware 'aquaria' under ambient light conditions from the night of their collection through the next day, and then under a 15 W fluorescent light until use during the second night after collection. All trials were performed at night. For each trial, at least four males were placed in a clear acrylic tank with dimensions of either 60 cm×70 cm×15 cm (height×width×depth; hereafter called the large tank) or 60 cm×15 cm×16 cm (hereafter called the small tank) filled with clean seawater collected off the dock on the lagoon-side of Southwater Cay near the display grounds. We used a minimum of four males because it was difficult to elicit displays consistently with fewer than four. For each experimental trial, a 15 W fluorescent light was kept on above the tank for 20 min and then extinguished. We began recording when the displays commenced, usually within 10 to 45 min. If 45 min passed without displays, new males were substituted. Infrared illumination for filming was supplied by a rheostat-controlled 15 W red frosted incandescent bulb further restricted by an IR barrier filter situated 1 cm above the waterline. The output from a high-sensitivity (0.00015 lux) low-light 1.25 cm CCD camera (Watec LCL-902K, Orangeburg, NY, USA) with a 12 mm aspherical low-light TV lens [Computar HG1208FCS-HSP, CBC (America) Corp., Torrance, CA, USA] situated about 2 m away and on the side of the tank was fed into a Sony DCR VX-2000 miniDV camcorder, which we used as a VCR. This system allowed us to follow most of the behavioral activity of each of the males in the tank during and between displays. Trials were recorded for either 30 or 60 min.

Three-dimensional recordings

To observe the display in three dimensions, two low-light (0.00015 lux) CCD cameras (Watec LCL-902K) with low-light aspherical lenses were used to film the top and bottom of the front of the tank, while a third, more distant CCD camera, similarly equipped, filmed the side of the tank (Fig. 1). In addition, a Dark Invader Generation II NVD equipped with a 3 mm BG-39 barrier filter (to block out IR light) fed into a Sony DCR VX-2000 camcorder and also recorded the same field as the side camera. All four images were connected to a 30 frames s^{-1} black-and-white digital quad-processor. This arrangement made it possible to display all four cameras on one screen; a Canon ZR 85 Mini-DV camcorder was used as a VCR. Two of the CCD cameras were closer in front (1 m), and one CCD camera was aligned in tandem with the NVD system farther away (2 m) on the side (Fig. 1). The IR light was placed as in the two-dimensional arrangement. With this method we could follow the individual activities of each male with the IR light (the three CCD cameras) and the luminescent displays [NVD-equipped camera] simultaneously in three dimensions.

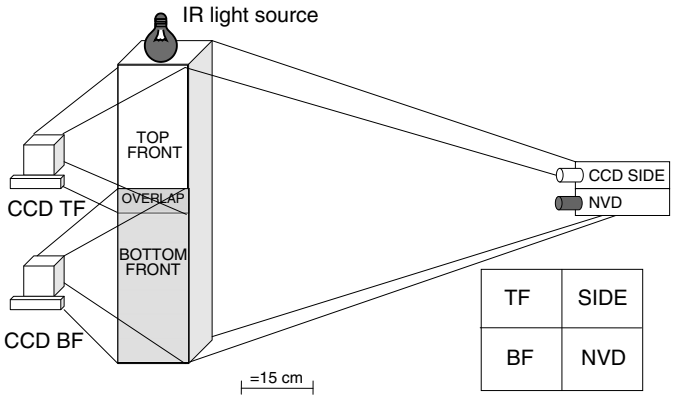

Fig. 1. Setup for three-dimensional video analysis. For close viewing, two low-light CCD cameras were positioned to cover (with overlap) the front of the tank (TF, top front; BF, bottom front), and one CCD camera (CCD=SIDE) and a video camera with night-vision device (NVD) and infrared barrier filter in tandem recorded movement and luminescence, respectively, from the side. The image panel box at the lower right indicates the image partitioning of the recordings.

Photomultiplier tube (PMT) setup and analysis

For all experiments involving the use of light-intensity recording, we used a horizontally placed RCA 931-A (Burle Industries, Lancaster, PA, USA) photomultiplier tube (PMT), covered by an Andover (Salem, NH, USA) 039FG11-50 3 mm IR barrier filter, at a distance of 76 cm from the experimental tank. The PMT was powered by an Emco (Sutter Creek, CA, USA) Ca12N high voltage converter (set to 1000 V). The PMT output was connected to a Dataq DI-158U analog data acquisition device and set to a gain of 8. Data were recorded at a rate of 240 data points per second on a Dell laptop computer (Austin, TX, USA), using the waveform analysis program WinDAQ. Using this program we were able to determine relative intensity, pulse duration and interpulse intervals of the displays.

Data analysis of the luminescent displays

Maximum luminescent intensities

The maximum pulse intensities of 78 displays over five trials were used to determine variations within and among displays. We calculated the maximum variation in light intensity that could be attributed to location in the tank and distance from the PMT by using the inverse square law (intensity at a given distance = source intensity/4π distance^2) for distances between 78–82.4 cm (tank minimum to maximum) from a display in the small tank to the PMT, and the attenuation of light passing through seawater (the maximum distance of 21.5 cm × the coefficient of 0.00015 yields an attenuation value of 0.003). Subtracting this value from the variation found in

intensities of the displays gave us the variation in displays due to luminescence output among displaying males.

Interpulse distances and intervals

For interpulse intervals, our aim was (1) to determine the differences between the stationary and helical phases of individual display trains and (2) to characterize the differences in intervals within the helical phase.

We calculated interpulse intervals using two separate methods. First, to obtain the most accurate intervals, we used the waveform data of 20 representative display trains (longer, uninterrupted and with clean peaks for analysis) from the PMT data (at a resolution of 240 data points per second) to find the interval from the beginning of each pulse to the beginning of the next. Because variations were relatively low, these results were used to produce a model for a typical display. Second, to determine the amount of variation among individual males, we analyzed two-dimensional videos of male behavior during 85 displays in five trials in the small tank. Since we had only video images and not waveform data that came from known different individuals, the resolution was restricted to the speed of our DV camera (single frame = 1/30th second = 33 ms). The projected image of the male's location was marked on a projection board, digitized, and analyzed with ImageJ software. Because the stationary phase is more variable than the helical phase and the helical phase is evident by a distinct change in pattern, starting the analysis with the helical phase rather than the first pulse of the stationary phase provided a more accurate estimate of the variations. We used a random effects mixed model (SAS 9.1) to correct for multiple observations of the same male within treatments.

For interpulse distances, we analyzed two-dimensional vertical and horizontal distances between pulses on the projection board. The scatterplot of our data showed a parabolic trend, so we used a quadratic, rather than linear, equation in a random effects mixed model analysis (SAS 9.1).

After obtaining the mean interpulse intervals and distances from laboratory trials, we used these to extrapolate the display duration and length of laboratory and field displays with the mean and maximum number of pulses per display. We had to extrapolate our data beyond the 10–11 data points for individual display interpulse intervals and interpulse distances in the lab because it is necessary to have a sufficient intensity of IR light in order to observe the swimming patterns of ostracods at the bottom of a tank. Once they reach about two-thirds of the way up the tank, the IR is brighter than the luminescence and the camera is unable to pick up the luminescent signal. The reason extrapolation was necessary for field observations was that with a moving camera in the field, multiple nearby displays and the large depth-of-field, accurate determinations of interpulse intervals and distances were difficult. We counted the number of pulses per display of 23 displays in the field, by eye, while snorkeling, then used the mean intervals and durations between each pulse to calculate the mean and maximum display lengths and durations.

Three-dimensional swimming patterns and speeds

In the small tank, the helical portions of display trains of eight males and the entire display trains of four additional males were analyzed in three dimensions in order to determine the pattern of swimming of both the stationary and helical phases, and to compare to the two-dimensional helical calculations of swimming speed during the helical phase. The two front cameras and one side camera were size-standardized in ImageJ, and the male's position (in three-dimensional space) was marked every two frames (1/15th second=67 ms). Cartesian coordinates in three planes were plotted and point-to-point distances and speeds were subsequently calculated. Owing to the nature of the recordings and tanks, in order to observe the stationary phase we had to choose only those males (N=4) that started their displays high enough off the bottom and far enough from the sides of the tank to prevent potential edge effects. Since these males were higher in the tank, their displays only consisted of 9–10 pulses before reaching the surface, rather than the 15–19 possible from males starting their displays at the bottom of the tank. A paired t-test was used to compare mean actual three-dimensional swimming speeds with the mean two-dimensional transformed data during the helical phase (N=8).

Swimming speeds with respect to pulse production

In order to accurately describe the swimming speeds and patterns of males before, at, and after the release of luminescence, we placed our three-dimensional camera setup close to the tank (10–15 cm distance) on the front and sides, until the cameras had a 15 cm field-of-view. The visible portions of 10 displays were analyzed as outlined above, with a total of 50 pulses analyzed. The swimming speeds at 0.1 s prior to luminescence, at the first sign of luminescence, and 0.1 s after luminescence, were analyzed by matched-pair comparisons (we treated each individual train as one replicate, N=10).

RESULTS

Based on field observations and recordings and laboratory experiments, our analyses of the luminescent courtship displays of the cypridinid ostracod *Vargula annecohenae* show consistent patterns with respect to habitat and display period in the field, and display train and pulse characteristics (Tables 1, 2, Fig. 2). A single display, which consists of a series of discrete extracellular pulses of light-producing products, shows two major phases: (1) an initial stationary phase composed of three to four brighter, closely placed and slightly longer pulses, followed by (2) a more conserved and less variable helical phase composed of many dimmer and shorter regularly spaced pulses of similar intensity and duration to one another in both space and time, secreted by a spirally swimming male (Figs 2, 3). Our definition of 'stationary' does not mean the male itself is stationary, but that the males are looping about 2–3 cm upward and then back to nearly the same location and secreting the subsequent pulse near the previous one, so that the luminescent pulses do not seem to be moving in any particular direction.

Display period and density

In the field, the display arena occurs in the water column immediately above the seagrass bed. Displays begin from 0 to 10 cm above the tops of the *Thallasia testudinum* seagrasses and, extrapolating from laboratory interpulse distance data, proceed upward for a mean distance of approximately 35 cm, with a maximum of approximately 61 cm (Table 1). Displays commence toward the end of twilight (~45 min after sunset) or near the end of moonset, whichever occurs later, and last for about 60 min. There is an abrupt initial increase and later a gradual decrease in display densities over this period. These luminescent courtship displays occur abundantly over the shallow grassbeds at Southwater Cay, Belize, and, based on random field samples, average about seven displays per square meter per minute during peak activity in this area. Specifically, based on 33 random counts, we found 1.78±0.23 (± s.e.m.) displays per 0.25 m^2 quadrat in the more homogeneous grassbed area. Where unattached, but at least temporarily stable, coral heads were situated within this homogenous environment, we documented 14.37±2.35 (± s.e.m.; N=13) per 0.25 m^2, or nearly

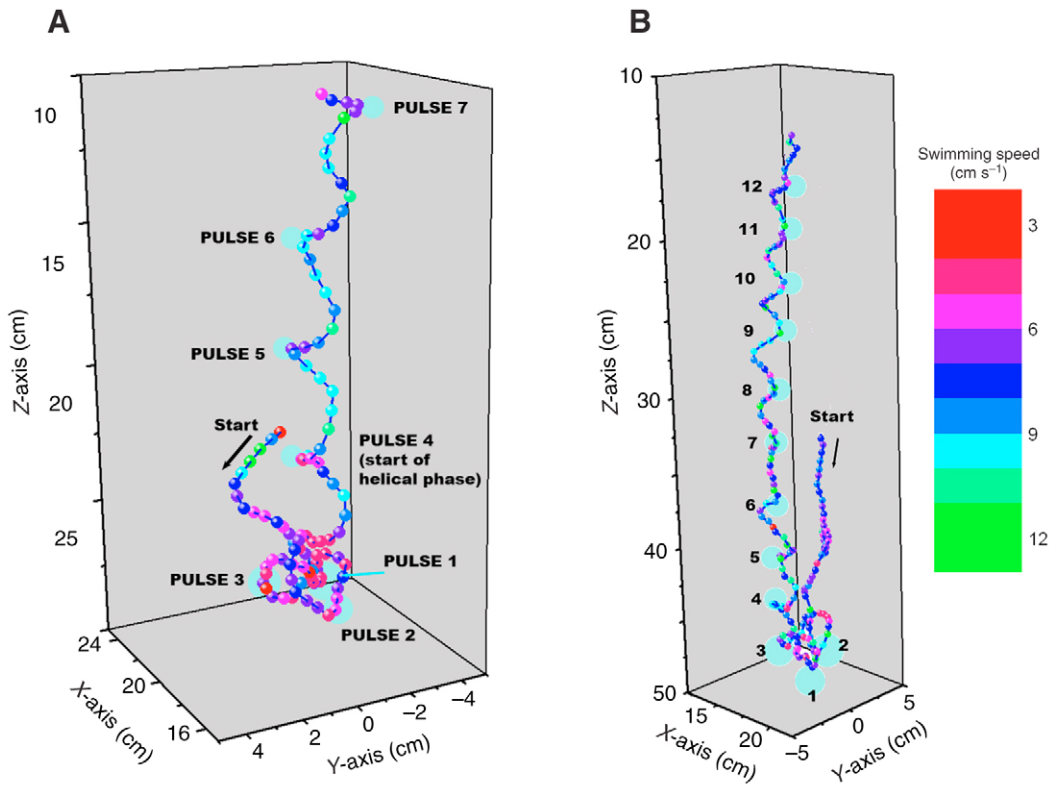

Fig. 2. Two three-dimensional examples of male ostracod displays, showing luminescence, swimming patterns and speeds. (A) Close-up of the first seven pulses of a display. (B) Example of an entire display. In the stationary phase males swim slower than when in the helical phase (see also Table 2, Fig. 4D and Fig. 5). Data points are every 67 ms. Large blue circles indicate the location of each luminescent pulse. The color of the small spheres indicates the swimming speed of the ostracod at that given point in time. The z-axis is in centimeters from the top of the tank, the x-axis is the distance from the left side of the tank, and the y-axis is the distance in depth from the first data point.

60 displays m^{-2} min^{-1} in these 'hotspot' areas, which is significantly higher than the homogeneous grassbed numbers ($F_{1,32}$=29.02, P<0.0001 between the log-transformed data from randomly thrown quadrats and the known high-display-density area near the coral head). However, this species does not display in areas far from seagrasses, such as sand areas, cobble or coral, all of which have their own habitat-specific displaying species.

Although the overall population has an even male:female sex ratio (Gerrish and Morin, in press), luminescent courtship display arenas are highly male biased. We collected an average of about two and a half males from each display, but only slightly more than one female in every 100 displays (sweeps from 868 displays yielded a mean number of 2.47±0.18 males and 0.014±0.004 females per display). This difference yields a

Table 1. Display train characteristics

	Lab*				Field			
	Mean	s.e.m.	Max	*N*	Mean	s.e.m.	Max	*N*
Total train (includes both stationary and helical phases)								
Pulses per train	12.79	0.30	19	161	12.26	0.91	19	23
Mean and max train length (vertical) (cm)†	37.29	–	60.7	–	35.29	–	60.7	–
Mean and max train duration (s)	10.86	0.29	16.3	85	10.10†	–	14.12†	–
Swimming speed (cm s^{-1})	7.76	0.55		4				
Stationary phase								
Swimming speed (cm s^{-1})	7.16	0.137	9.2	4				
Distance: horizontal (cm)	–0.08	0.240	5.26	85				
Distance: vertical (cm)	–0.31	0.21	3.59	85				
Helical phase (see also Fig. 6)								
Pulse number after which helical phase starts	2.85	0.02	6	85				
Swimming speed (actual) (cm s^{-1})	8.39	0.192	13.3	8				
Vertical swimming speed (apparent) (cm s^{-1})‡	5.41							
Mean width of helix (cm)	0.73	0.02	0.83	8				
Length of one spiral (cm)	1.96							
Duration of one spiral (ms)	390							
Spirals per interpulse	1.92							
Mean pulse duration (ms) (from Table 2)	210	03		129				
Mean interpulse interval (ms) (from Table 2)	750	07		494				
Mean interpulse distance (cm) (from Table 2)	3.77	0.05		494				

*Lab observations with five males (four of them participating in courtship behavior) in the aquarium per trial.
†Distances and durations extrapolated after pulse 11 with mean lab interpulse interval and distance of the helical phase (because these values were extrapolated, s.e.m. and *N* values are not applicable).
‡The slope of the mean interpulse intervals plotted against mean interpulse distances r^2=0.999 (see also Fig. 5).

Table 2. Characteristics of pulses and interpulses in the luminescent courtship displays of the cypridinid ostracod *Vargula annecohenae* based on laboratory recordings

Pulse number	Intensity (as % of pulse 1)	Pulse duration (ms) (*N*)	Interpulse interval (s) (*N*)	Interpulse distance (vertical; cm) (*N*)	Interpulse distance (horizontal; cm) (*N*)
1	100	430±20 (16)			
2	57.3±5.5 (19)	330±18 (16)	1.30±0.05 (85)	–0.07±0.24 (85)	–0.28±0.21 (85)
3	35.34±3.4 (19)	260±10 (16)	0.98±0.04 (85)	1.55±0.17 (85)	0.32±0.15 (85)
4	30.31±4.4 (19)	260±10 (16)	0.83±0.01 (85)	2.53±0.17 (85)	–0.10±0.16 (85)
5	23.77±4.7(19)	240±9 (16)	0.76±0.01 (83)	3.41±0.15 (83)	0.21±0.19 (83)
6	20.35±4.3(18)	220±10 (16)	0.73±0.01 (81)	3.81±0.15 (81)	0.24±0.24 (81)
7	15.68±2.7(17)	210±6 (16)	0.73±0.01 (76)	4.05±0.15 (76)	0.38±0.33 (76)
8	15.32±3.2 (17)	210±10 (15)	0.69±0.01 (69)	3.92±0.18 (69)	–0.13±0.07 (69)
9	14.53±1.3(17)	190±7 (15)	0.71±0.01 (51)	4.04±0.19 (51)	0.45±0.46 (51)
10	14.13±3.4 (14)	180±8 (15)	0.69±0.03 (43)	3.73±0.19 (43)	0.00±0.07 (43)
11	12.30±2.5(12)	190±9 (13)	0.67±0.01 (21)	3.57±0.23 (21)	–0.26±0.08 (21)
12	13.75±3.2(9)	180±11 (8)	*	*	*
Stationary phase (mean)		340±13 (48)	1.23±0.04 (170)	0.74±0.16 (170)	0.02±0.13 (170)
Helical phase mean	18.41±1.5(122)	210±3 (129)	0.75±0.007(494)	3.77±0.05 (494)	0.23±0.10 (494)

Pulse numbers 1–3 are in the stationary phase and 4–12 are in the helical phase.
Values are means ± s.e.m. *No data available.

female:male operational sex ratio (OSR) of 0.00567, or 176 males per female.

Display characteristics

Each train is quite predictable, uniform and repetitive. It is composed of an initial stationary phase followed by a rapid upward production of slightly dimmer, more regular pulses in a helical phase. In the field, there was a mean of 12.26±0.91 (± s.e.m.; N=23) total pulses per display, with a maximum of 19 pulses (Table 1). Using the mean interpulse interval durations collected in the lab (since these data are more accurate than field data as discussed above), the mean duration of each display train (from the beginning of the first pulse to the end of the last pulse) was calculated to be 10.1 s in the field (Table 1). The mean *interdisplay* interval (the time between the end of one train to the beginning of the next) with five males in a tank per trial (four of them actively participating in courtship behavior), was 19.15±2.09 s (N=85), with a maximum of 67.5 s once displays had started. Because intensities, pulse duration, interpulse intervals and interpulse distances plateau by about pulse 12 and because the field and lab recordings are virtually identical, with low standard error, we used the helical pulse lab data to project the characteristics of the remaining seven helical pulses to obtain the maximum length and duration of trains in the field (Table 1). Based on the results of our random effects mixed-model analysis of 85 male displays (SAS 9.1), overall there was a consistent, distinct vertical component to male display trains, with each display terminating about 60 cm above the top of the grassbed, but there was no detectable horizontal component to the displays (Fig. 4D). Furthermore, the within-train interpulse vertical distance follows a weak quadratic pattern where distances within the train first increased slightly to a maximum at interpulse number x and subsequently decreased slightly. Both the interpulse interval ($F_{1,362}$=165.23, P<0.0001) and the square of the interpulse interval ($F_{1,364}$=102.00, P<0.0001) are required to accurately describe the vertical distance pattern, using the following equation:

$$\text{Distance} = 1.9385 + 0.8379(\text{interpulse interval}) - 0.07067(\text{interpulse interval})^2. \quad (1)$$

Stationary phase

The initial stationary phase of the display does not demonstrate any distinct spatial pattern other than issuing a luminescent ‘call’ followed by the male looping up and back down a short distance and then luminescing again, often in nearly the same location or slightly lateral to the preceding pulse (Fig. 2). During the three to four (usually) pulses of this phase, intensities, durations and interpulse intervals all decline from one pulse to the next (Table 2). The mean intensity of luminescent pulses decreased (Fig. 3, Fig. 4A) and dropped to below 40% of the first pulse intensity by the third pulse (Table 2, Fig. 4A). The mean pulse duration also decreased during the stationary phase from about 0.4 s to 0.25 s (Table 2, Fig. 4B), and interpulse intervals decreased by about half from more than 1 s to about 650 ms (Table 2, Fig. 4C). There was no trend for any vertical or horizontal movement during the stationary phase (Table 2, Fig. 4D). The mean three-dimensional swimming speed of males in the stationary phase was 7.16 cm s^{-1} (Table 1).

Helical phase

During the helical phase, especially compared to the stationary phase, the display is quite regular. Field data indicate there can be up to 16 pulses in the helical phase. Using the mean interpulse

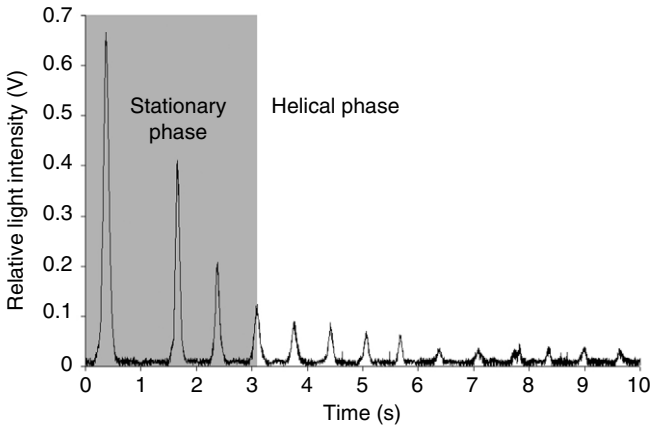

Fig. 3. Typical photometric waveforms of a single display train. The first three to four bright pulses are typical of the stationary phase and the remaining pulses are typical of the helical phase. The mean duration of each pulse decreases during the stationary phase, but becomes more consistent during the helical phase (see also Fig. 4).

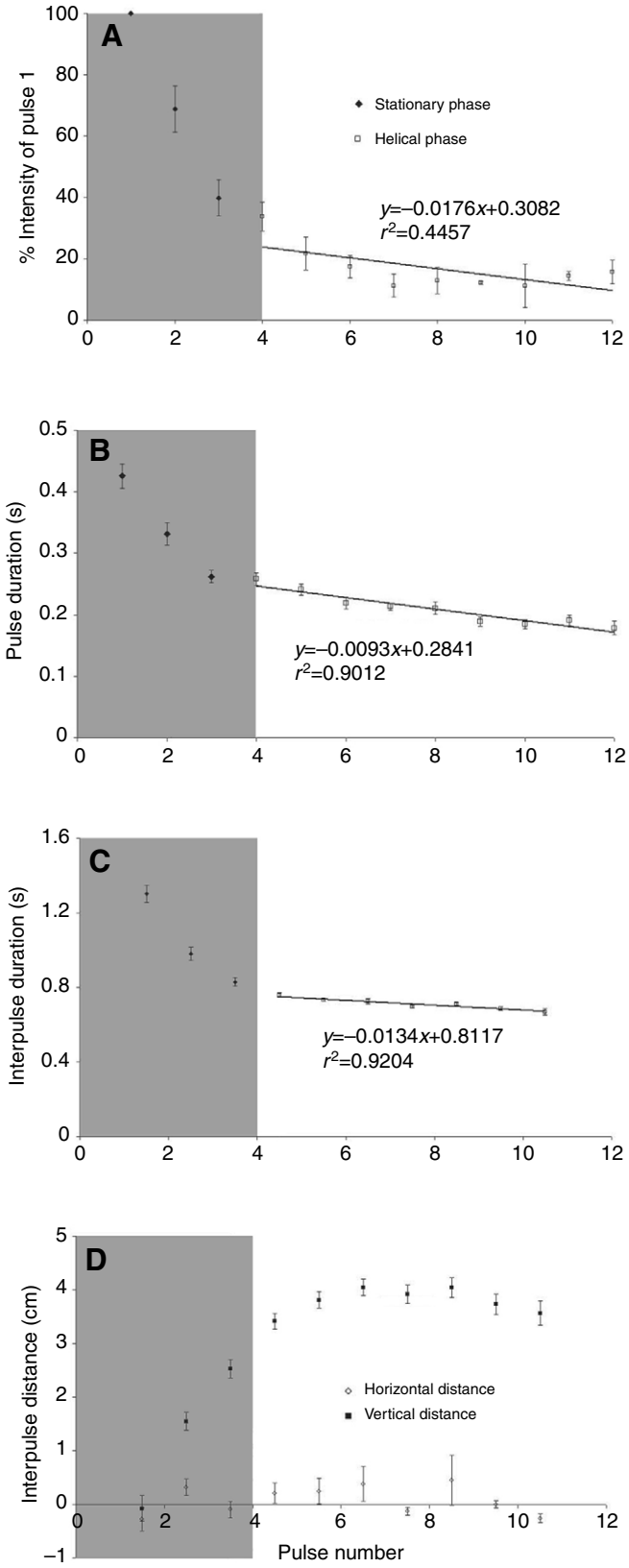

Fig. 4. Characteristics of a luminescent display train during stationary (shaded) and helical (nonshaded) phases. (A) Mean pulse intensity (as a percentage of first pulse). Helical phase pulse intensities are approximately 10% of the initial stationary pulse (N=16). (B) Mean pulse duration: pulse duration decreases rapidly during the stationary phase, then only slowly during the helical phase (N=16). (C) Mean interpulse interval. Interpulse intervals decrease rapidly during the stationary phase, but are highly conserved during the helical phase (N=85 pulse 1, see Table 2 for remainder). (D) Mean interpulse distances. Vertical distance increases during the stationary phase, then levels off during the helical phase, but there is no trend for horizontal movement during the course of a display (N=85 pulse 1, see Table 2 for remainder). Bars indicate standard errors. For C and D the points are *interpulse* values so points occur *between* pulse numbers.

distance from laboratory trials, (3.77±0.05 cm), the helical phase extends a maximum vertical distance of 60.7 cm. Based on laboratory data on the first 12 pulses, after the fourth pulse (the end of stationary phase): (1) intensities decrease only slightly and are constant during the last seven to eight pulses; (2) pulse duration declines only slightly and at a constant rate; (3) interpulse intervals are very constant, and (4) interpulse distances decrease only slightly if at all (Table 2, Fig. 4). The pulse intensity during the helical phase decreased with pulse number, as in the stationary phase, but only from about 24% of the first pulse to about 14% at the twelth pulse (with some of this variation possibly due to distance of the pulse to the PMT; Fig. 4A, Table 2). The mean pulse durations decreased from 260 to 180 ms, with a mean decrease of 9.5 ms per pulse (r^2=0.90). Interpulse intervals remained fairly consistent with a mean of 0.75±0.007 s (Table 2, Fig. 4C) and showed only a slight decrease with increasing interval number (r^2=0.92). During the helical phase the vertical interpulse distance was fairly constant at 3.77±0.05 cm, but with a slight parabolic trend (Table 2, Fig. 4D).

The vertical (apparent) speed of a display production during the helical phase remained fairly constant, at 5.41 cm s^{-1} (Table 1, Fig. 5), but the mean three-dimensional helical phase (actual) speed, i.e. the male swimming in a tight upward spiral, was 8.39±0.19 cm s^{-1} (Table 1) with the width of the helical cylinder being about 7.3 mm. Since there was no significant difference between three-dimensional actual display speeds and a helical swimming speed calculated from two-dimensional analyses using a paired t-test (t_7=–0.986, P=0.357), the two-dimensional calculations are representative of true mean swimming speed during the helical phase of the display and were used for most analyses from a single-angle camera. Observations of the three-dimensional swimming pattern of males during the helical portion of the display suggest that there is no chirality; males are swimming in both right-handed and left-handed helices, but whether individuals always exhibit the same handedness in their spirals is unknown.

Individual variations among displays

Although the spatial and temporal structure of displays is quite uniform overall, individual displays can vary significantly in brightness, interpulse intervals, and possibly interpulse distances. Based on controlled laboratory observations, across 78 displays, the brightest first pulse was 84±4% brighter than the dimmest first pulse. Using the dimensions and distances of the photomultiplier from our observation tank and the inverse square law, we calculated the maximum difference due to variation in distances from the display to the PMT to be 15%. Therefore, for each case, at least 69±4% of the variation of signal intensities between display trains during a trial can be directly attributed to the variation in actual display luminescence intensities. Because intensities could only be measured photometrically and not by video, we were unable to match displays to individual males during these tests, so we do not know whether the intensity variation occurs only between males, or may even occur between sequential display trains in a single male.

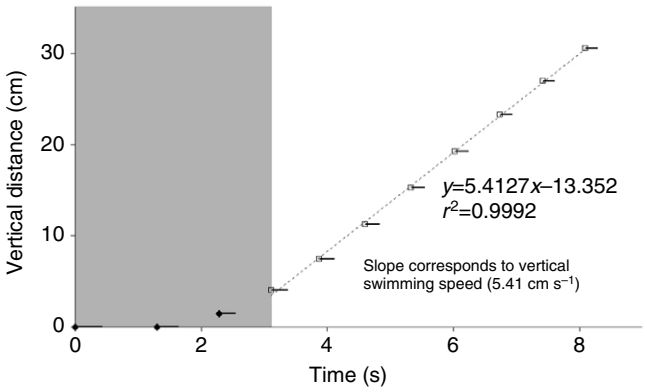

Fig. 5. Time–distance relationships of luminescent courtship display trains in *Vargula annecohenae* (from Table 2). Data points represent vertical distance traveled after each flash: filled diamonds indicate the stationary phase (shaded area) and open squares indicate the helical phase of the display. Horizontal solid lines correspond to pulse duration. The vertical swimming speed (5.41 cm s^{-1}) was calculated from the regression line (dotted line) during the helical phase, which is consistent over the course of a display. Since we used the mean time and distances (Table 2), there are no error bars.

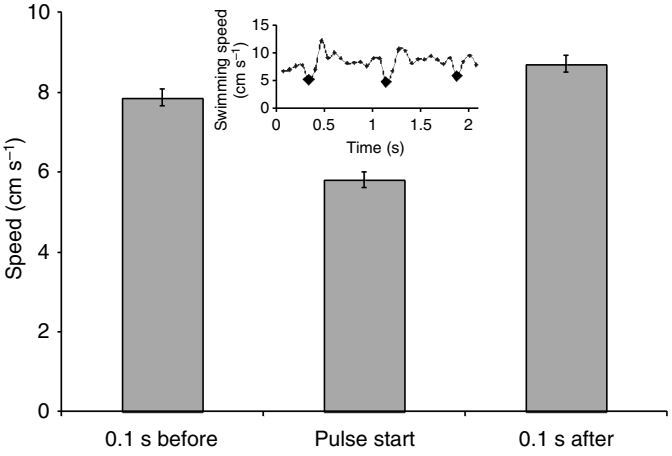

Fig. 6. Mean swimming speeds of luminescing males before, at and after a luminescent pulse. The inset graph shows a representative male releasing three pulses during a 2 s period. Males swim slowest when starting a luminescent pulse (black dots), and fastest immediately after releasing a pulse (N=50).

By observing the interpulse intervals of individual males (through the use of the low-light CCD camera coupled with low-intensity infrared light), we found that the duration of interpulse intervals *within* individual trains differed enough among individual males to be significant ($F_{18,101}$=2.13, P=0.0095), even though there is relatively little overall variation between displays (Table 2). Similarly there may be differences in the interpulse distances *within* individual trains among males but there were not enough degrees of freedom to run this test.

Timing of the light emitting product secretion

During both the stationary and helical phase, the males are slowing significantly around the time of pulse production (Fig. 6). At 0.1 s before the first sign of luminescence, males were swimming at a mean rate of 7.97±0.17 cm s^{-1}; they were swimming at a mean rate of only 5.83±0.15 cm s^{-1} at the first sign of luminescence, and then 8.67±0.17 cm s^{-1} 0.1 s after luminescence (N=50). Matched-pair comparisons showed that swimming speeds are significantly different between the point of luminescence and 0.1 s before (t_9=–7.78, P<0.0001) and 0.1 s after the luminescence (t_9=10.09, P<0.0001). The swimming speeds of a male 0.1 s before, and 0.1 s after, luminescence are also significantly different from each other (t_9=2.25, P=0.0504), with a male swimming faster immediately after luminescing than before.

DISCUSSION

Ostracod luminescent display patterns are unique among known courtship displays in a number of ways. First, the luminescent courtship displays are extracellular and secreted into the water column by rapidly swimming males, who (although averaging over 40 body lengths per second while swimming) are significantly slowing to eject their luminescent pulses (Fig. 6); males may actually even be stopping completely at the point of luminescence secretion (from personal observations), but our camera recording speed of only 30 frames per second limits our accuracy. The purpose of the decrease in speed may be to help keep the luminescence in a discrete packet, which would both optimize the intensity of the luminescent pulse and, given their intermediate Reynold's numbers when swimming, prevent the luminescence from trailing along behind them in the viscous boundary layer medium in which they swim. Because of this speed, by the time the luminescence has reached its peak intensity (100–150 ms), the male is already about 5–10 mm away from the pulse. Further, since there is no luminescent dialogue between males and females (unlike fireflies) where males and females might orient to each other using reciprocated signals, there is the question of how a female can get close enough to a chosen male to mate. Our observations of females, and also competing males, indicate that they approach a displaying male silently (i.e. without luminescing) and in a stereotypical pattern for interception (Rivers and Morin, 2006). We hypothesize that the two dramatically different but predictable phases of the display, the stationary and helical phases, impart different information to both responsive females and 'eavesdropping' males.

Our laboratory experiments indicate that the swimming pattern is not predictable during the stationary phase; thus, it would appear to function as an attention-grabbing signal and imparts little information for orientation to observers. Thus, with this hypothesis the stationary phase is functionally an alerting and species assessment (or call) phase because it appears to alter the behavior of both receptive females and competing males but not their orientation (Rivers and Morin, 2004; Rivers and Morin, 2006). It notifies conspecifics that a new display by a male *V. annecohenae* is about to commence. This phase takes place at or just above the tips of the seagrass blades, and the pulses are longer lasting and up to 85% brighter than those in the later helical phase (Fig. 4A, Table 2). Furthermore, there is some variation in pulse number and interpulse intervals, so that during this phase it is difficult to predict precisely where along the top of the grassbed the displaying male is located immediately after a pulse. This lack of horizontal reference does not allow for precise localization of the signaler, but it does both alert attracted parties to the presence of a pending display and the general vicinity of the event. The observations that females require at least two luminescent pulses before responding to a display (Rivers and Morin, 2006) and that other males need at least two to three pulses before starting an alternative mating tactic (Rivers and Morin, 2004) (T.J.R. and J.G.M., manuscript submitted) lend further

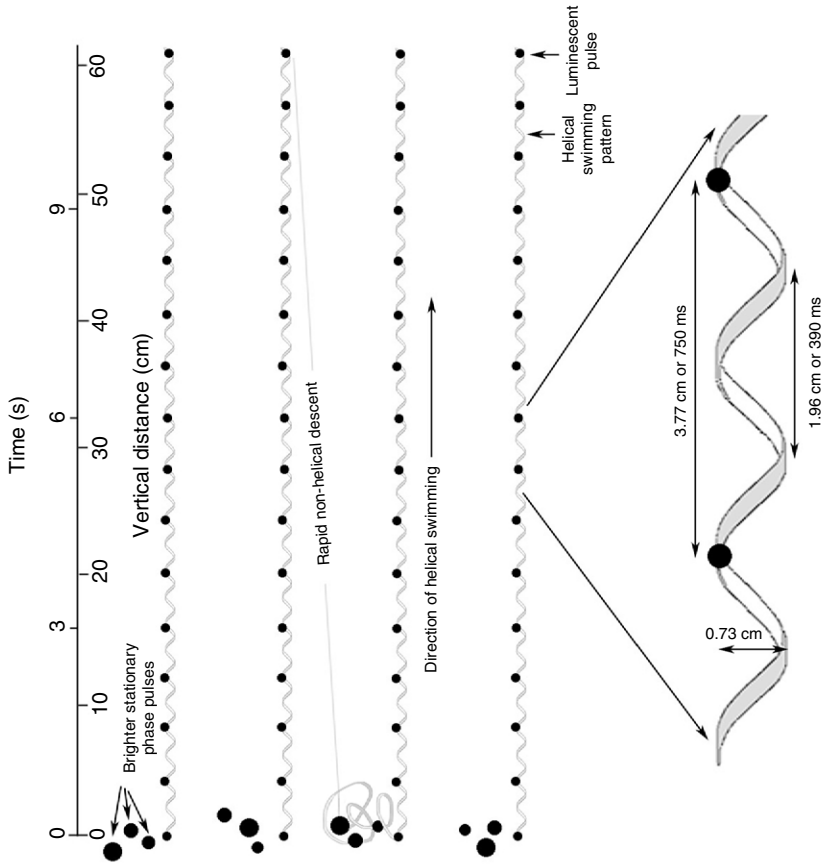

Fig. 7. Model of four luminescent display trains produced by a single *Vargula annecohenae*, based on the interpulse intervals, interpulse distances, pulse intensities, and swimming patterns of males displaying in the lab (Tables 1 and 2). The first three pulses in each train are the stationary phase; the remaining pulses are the helical phase. Black dots indicate the location of the light pulses (their size corresponds to relative intensity) and the faint lines indicate swimming trajectory (only the third display train shows the stationary phase swimming pattern). Once a male reaches the top of the train, it swims rapidly downward without spiraling, and then commences displaying again.

support that this phase is an alerting signal. In addition, general bioluminescence in grassbed areas is also not restricted solely to ostracod mating displays; there may be potentially other luminescent signals from dinoflagellates, syllid polychaetes, occasionally displays from other ostracod species, or from predation attempts on ostracods (personal observation). Responding erroneously to these spurious luminescent signals could be prevented by requiring at least two to three pulses before males and females commit to a response. Thus, the stationary phase probably also serves as a species recognition signal.

The helical phase of the display, however, provides a highly conserved stereotypical pattern (in both space and time) that conspecifics can use to extrapolate the displaying male's future position (Figs 5, 7). Thus, this communication hypothesis posits that this helical phase functions as an orientation phase wherein approaching conspecifics can use the predictive aspects of the signals to accurately approach and intercept a displaying male. The display arena is the water column above the grass beds, which means that responding individuals (both male and female) could be anywhere in three-dimensional space, although females are likely to be above or lateral to the displays (Rivers and Morin, 2006). Therefore, it is more crucial during this phase for the male to behave in a predictable way to be able to maximize its likelihood for successful interception by, and copulation with, a receptive female. Females approach luminescent signals by swimming in a trajectory to intercept the male above the most recent pulse, which is where he will be within a fairly narrow spatio-temporal range, thus supporting this hypothesis (Rivers and Morin, 2006). In addition, our experimental lab data show that the majority of responding males that perform alternative mating tactics (entraining or sneaking) begin *after* the helical phase starts, indicating the shift to the helical phase is important for competing males as well (Rivers and Morin, 2004; Rivers and Morin, 2008).

From our laboratory studies of interpulse interval durations, distances, two and three-dimensional swimming patterns, and intensities, and because of the consistency among all trains, we have been able to construct a model of the average luminescent courtship display behavior of a male *V. annecohenae* (Fig. 7) that closely resembles actual three-dimensional display and swimming patterns (e.g. Fig. 2). When a male begins displaying, he either drops to or loops at the level of the seagrass, releasing a bright pulse of luminescence from a downward trajectory. At the point of luminescence, the male changes its direction and swims upward, making (sometimes not immediately) a vertical loop, which orients him facing downward once again, where he releases his next pulse near the bottom of the loop. The three to four pulses secreted in this manner yields the attention-grabbing stationary phase. Next the male swims vertically in a tight helical pattern (the helical phase) with predictable interpulse distances and interpulse intervals (Figs 5, 7). The display helix is about 7.5 mm wide, each cycle is about 2 cm long (or 0.4 s) and the pulses are produced approximately once every two cycles (Fig. 7). Thus, the *apparent* swimming speed, which is based on field or lab recordings of the rate of pulse production, represents only about two thirds of the *actual* swimming speed of the individual male (Table 1).

If a display is successful in attracting a female, it ends. Termination of an unsuccessful display appears to occur when a male either reaches the maximum number of pulses per train (19) or the sea surface; interference from other males also may cause display termination. Next, the male swims directly down, without a spiral, to the top of the seagrass and often starts again. We have

documented repeated displays multiple times in the lab, which suggests that males behave similarly in the field.

Before a male's display can be used for courtship, it first must be recognized as a signal from a conspecific and not a spurious signal from another ostracod species or other luminescent organism. Pattern recognition of visual and auditory signals by members of the same species has been extensively studied amongst many organisms (crickets, frogs, fish, birds, etc.), with call frequency, intervals, intensity and pattern providing important cues (Becker, 1982; Doherty and Hoy, 1985; Michaud, 1962; Morris and Fullard, 1983). The courtship displays most akin to ostracod displays are produced by fireflies, and we hypothesize that similar means of coding species identity may be used in both cases. The interpulse intervals of multiple species of *Photinus* fireflies have been found to be integral to female response to luminescent cues; if the intervals are outside a critical range (either too long or too short), there is no female response (Lloyd, 1966; Michaelidis et al., 2006), which may imply females are not recognizing such a signal as a courtship signal. *Photinus* fireflies respond, in laboratory settings, to a stationary flash, without needing other spatial cues such as distances traveled between flashes, etc. for pattern recognition. We hypothesize, however, that the spatial patterns in ostracods will prove to be as important as the timing for species and mate recognition. Ultimately, by modifying displays in laboratory settings using LED lights (e.g. Rivers and Morin, 2006), we should be able to determine the thresholds and other pattern characteristics that *V. annecohenae* recognize as a display emitted by a conspecific.

Once a signal has been recognized as a conspecific mating display, various aspects of the signal should impart information regarding the quality of the displayer, which could then be used for female choice (for a review, see Andersson, 1994). The probability of female choice in the *V. annecohenae* mating system is quite high as suggested by the skewed operational sex ratio, the ability of females to avoid unwanted copulation, and the precise female behavior of tracking and intercepting light displays, although the skewed OSR may also serve to make female choice more difficult because of the sheer numbers of males in the water column (Rivers and Morin, 2006). For female choice to occur there must be some variation among displaying males (Shuster and Wade, 2003) and they could be the same parameters involved in species recognition: frequency, intervals, intensity and patterns.

The intensity of a display (visual, auditory or chemical) has been hypothesized to be a character on which females exhibit choice, and has been found to be important in a wide variety of organisms (Arak, 1983; Bailey et al., 1990; Moore, 1988) (for a review, see Andersson, 1994), including fireflies (Cratsley and Lewis, 2003; Vencl and Carlson, 1998). We have observed a wide variety of luminescent intensities in *V. annecohenae* (with some displays over 70% brighter than others), and although we were not able to simultaneously track and record individual luminescence intensities, based on our observations we expect that individuals will show significant intensity differences.

In addition, there is evidence from fireflies that female *Photinus consimilis* prefer faster flash rates (which corresponds to shorter interpulse intervals) (Branham and Greenfield, 1996). In ostracods, although interpulse interval and interpulse distance variations may seem to be relatively small (Fig. 4C, Table 2) in comparison to the variation in display intensities, there are still significant differences among individual males with respect to at least interpulse intervals and perhaps interpulse distances as well. The variability of these parameters between displays would then be features that may be used for female choice in addition to species recognition and orientation as discussed previously. We have evidence that female *V. annecohenae*, at least, use these characteristics to approach and intercept a chosen male (Rivers and Morin, 2006), but further research is necessary to determine the presence or absence of choice on them between competing signals. Complicating all of these interactions is the confounding possibility that the high male to female OSR may also make implementation of female choice more difficult by being duped by large numbers of sneaking males in the water column.

Field display distribution

On first observation of the high-density displays in the field, it is difficult to detect how displays are dispersed throughout the grassbed habitat. Although grassbeds are for the most part homogenous in their composition, we found that there are three separate display density phenomena: (1) lower-density display areas that cover huge swaths of seagrass beds, and are the most common type of display, (2) predictable hotspots and (3) ephemeral hotspots. The predictable and ephemeral hotspots may be formed for entirely different reasons. Predictable hotspots are occasionally found adjacent to semi-stable intrusive reef materials (e.g. a dead coral head), which tend to collect high levels of biological activity. The ostracods (both male and female) could be drawn to these sites as food-rich areas, or they could provide access to their (as of yet unknown) diurnal resting places, which may increase the probability of encountering females. This high display activity could form as predicted by the 'hotspot' model which states that display arenas are chosen for reasons such as being on or near female feeding grounds (Bradbury and Gibson, 1983; Bradbury et al., 1986). However, the formation of ephemeral hotspots may be due to the attractiveness of certain signalers to not only females, but to competing males. Since multiple males respond to a single display in the surrounding area (Rivers and Morin, 2004; T.J.R. and J.G.M., manuscript submitted), this clumping could then further induce a cascade of clustering of male displays in the homogenous grassbed areas. Therefore, the formation of ephemeral hotspots may be more in line with the 'hotshot' hypothesis, where males cluster around displaying 'hotshot' males (Beehler and Foster, 1988). Regardless of what causes the clustering of male displays in both predictable and ephemeral hotspot areas, the high display numbers may attract females at a higher rate, thus allowing them more opportunities for choice in a small area. Although a hotspot area may increase the number of females that may potentially respond to a signal, there is also a concomitant increase in competing males. If there is a large variation in male fitness in the population (which is likely given the skewed OSR) with displaying males tending to have higher reproductive fitness than sneakers, there may be a potential downside in hotspot activity in that a female may be more likely to be intercepted by sneaking males than in more homogeneous, lower-density situations.

Conclusion

The luminescent displays of Caribbean ostracods are the most complex found in the marine environment to date, and, based on hundreds of *in situ* observations of over 65 species (Morin and Cohen, 1991), suggests that they rival or even exceed those of terrestrial fireflies. The grassbed species *Vargula annecohenae* is found in prodigious quantities and produces huge numbers of displays nearly every night of the year throughout the grassbed habitats of Belize and probably beyond (Gerrish et al., in press). The extremely skewed male:female sex ratio (~176:1) in the water column indicates high levels of male competition and probably significant female choice.

The two phases of the display trains appear to serve to first attract the attention of receptive females and competing males and then provide a predictable target for approaching females. Although the displays overall are quite conserved with respect to the general parameters of a display train, there is display intensity variation. The complexity of the display trains we have described in this paper allows for the possibility of complex behaviors and decision-making by responding males and females extending beyond simple female choice. The lack of a visual dialogue between males and females necessitates finely-tuned tracking and interception of intermittent visual signals in three-dimensional space by females (Rivers and Morin, 2006). The complexity and uniqueness of many aspects of the courtship behavior of *V. annecohenae*, coupled with our ability to observe and manipulate it in controlled laboratory settings, has given us the opportunity to expand our understanding of the mating behavior in marine organisms that utilize luminescence for courtship and in crustaceans in general.

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