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The kinematics of phototaxis in larvae of the ascidian *Aplidium constellatum*

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Abstract Although phototaxis has an important influence on the vertical distribution and settlement of marine invertebrate larvae, few studies have explored the mechanisms of taxis in larvae at the organismal level. We examined how phototaxis changes over ontogeny in larvae of the ascidian *Aplidium constellatum* and experimentally tested hypotheses about the kinematics of oriented swimming. By video recording their swimming movements at regular intervals over their ontogeny, we found that larvae switched from positive to negative phototaxis. We tested hypotheses about the kinematics of phototaxis by recording the three-dimensional movement of larvae in response to a change in the direction of illumination and by tracking the tail motion of tethered larvae in response to sinusoidal changes in light intensity. Larvae swimming with negative phototaxis changed their rate of rotation about their antero-posterior and dorso-ventral axes in response to a change in the direction of illumination. These changes in the rates of rotation caused the axis of the helical trajectory to orient away from the light source. Tethered larvae oscillated their tails at a constant tail beat frequency and with a slow periodicity that was correlated with the stimulus frequency. These findings suggest that ascidian larvae orient by changing tail motion in proportion to perceived changes in light intensity. This method of orientation predicts that larvae achieve the switch from positive to negative phototaxis by changing the delay of their kinematic response to changes in perceived light intensity.

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Introduction

A broad diversity of marine invertebrate larvae are capable of oriented swimming toward or away from vector cues such as gravity and light (for reviews see Thorson 1964; Young and Chia 1987; Forward 1988; Young 1995). Such tactic swimming influences the vertical distribution of larvae (e.g. Crisp and Ghobashy 1971; Forward 1984; Forward 1985; Young 1986; Olson and McPherson 1987; Stoner 1992; Shanks 1995b) and their selection of a microhabitat for settlement (e.g. Thorson 1964; van Duyl et al. 1981; Durante 1991; Svane and Dolmer 1995). Despite widespread interest in understanding how changes in larval behavior affect dispersal (e.g. Scheltema 1986; Davis and Butler 1989; Stoner 1990; Shanks 1995a), few studies have considered the kinematic mechanisms of tactic behavior at the organismal level. An exception is Mast (1921), which investigated phototaxis in the ascidian *Aplidium constellatum* and proposed a model for the kinematic response to a light stimulus that facilitates orientation. In the present study, we quantified the kinematics of phototaxis over ontogeny in *A. constellatum* and tested Mast's (1921) model by experimentation on individual larvae.

Like other species of compound ascidian, larvae of *A. constellatum* switch from positive to negative phototaxis during a brief dispersal phase. Upon hatching, these 2 mm long larvae initially swim away from their parent colony by moving toward light (Grave and Woodbridge 1924). Shortly after hatching, larvae switch to negative phototaxis, which is how they swim for the rest of the 10 to 100 min larval stage. Qualitative observations suggest that ascidian larval swimming becomes slower, more intermittent, and less directed with age (e.g. *Trididemnum solidum*, Bak et al. 1981; *Podoclavella moluccensis*, Davis and Butler 1989; *Chelyosoma productum,* Young and Braithwaite 1980). We tested whether larvae of *A. constellatum* exhibit these changes as they age by measuring swimming trajectories of individual larvae at hatching and just prior to settlement.

According to Mast (1921), a larva is capable of phototaxis because it moves its tail laterally with a large-amplitude tail beat in response to a particular rate of change in perceived light intensity (Fig. 1). Between these tail flicks, body rotation occurs about the antero-posterior axis (rotation known as roll) and the larva follows a straight trajectory. Because the larva possesses a single ocellus that is directed toward one side of the body, roll exposes the ocellus to temporal oscillations in light intensity. These oscillations are low in amplitude when the body is aligned with a light source, but large when the body moves at an angle with respect to the source. In response to a large-amplitude change in intensity, the larva flicks its tail and changes the direction of swimming. During tail flicks, the body rotates strictly around its dorso-ventral axis (rotation known as yaw). Mast (1921) suggested that positive phototaxis is achieved by this mechanism when the tail flicks toward the abocular side of the body in response to a decrease in light intensity and toward the ocular side in response to an increase. Later in ontogeny, this response is reversed to achieve negative phototaxis.

We evaluated Mast's (1921) model in *A. constellatum* by testing three hypotheses. (1) By video recording the movement of larvae in three dimensions, we tested whether larvae rotate strictly by roll and thereby swim straight when not turning. (2) By rapidly changing the direction of illumination while larvae are swimming, we tested whether their bodies rotate primarily by yaw to cause a sharp change in direction during turning maneuvers. (3) By tethering larvae and stimulating their ocellus with sinusoidal changes in light intensity, we tested whether tail flicking occurs in response to changes in light intensity.

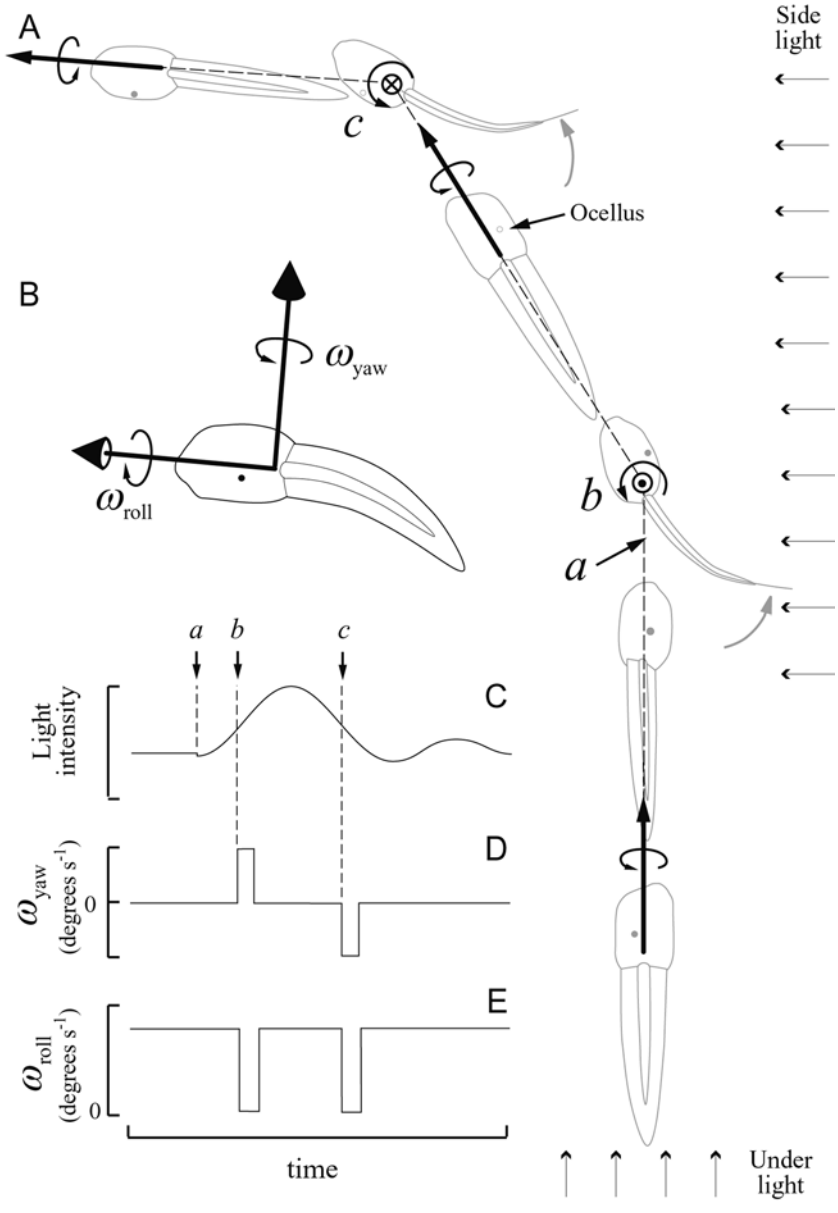

Fig. 1A–E The mechanism of phototaxis proposed by Mast (1921). **A** In an experimental situation, this negatively phototactic larva begins by swimming up and away from a source of illumination that is beneath it (the "under light"). At *a*, the under light turns off and the side light turns on. In response, the larva flicks its tail toward the right and ocular side at *b*, which directs swimming toward an oblique angle to the illumination. After one half of a body rotation, the tail flicks at *c*, toward the right, which is then the abocular side of the body. This directs swimming nearly in the opposite direction of light. **B** During periods of straight swimming, the larva rotates around the antero-posterior axis of the body at the roll rate, ω_{roll}, and during turns, the body rotates around the dorso-ventral axis at the yaw rate, ω_{yaw}. **C** With the change in light direction at *a*, the perceived light intensity oscillates because of the rotation of the body. The amplitude of these oscillations decreases, after *c*, as the body is directed away from the side light. **D** The yaw rate remains at a value of zero during periods of straight swimming but changes during the turns stimulated at *b* and *c*. At *b*, the body turns to its left and therefore has a positive value for yaw rate. At *c*, the body turns to its right, which requires a negative yaw rate. **E** The roll rate remains at a steady value during straight swimming but decreases to zero during the turns stimulated at *b* and *c*

Materials and methods

In the month of August 2000, colonies of *Aplidium constellatum* (Verrill) were shipped from the Woods Hole Marine Biological Laboratory (Woods Hole, Mass.) and emptied into a recirculating seawater tank with water temperatures between 16 and 18°C. To stimulate release of larvae, colonies were exposed to sunlight through a window after being kept in darkness overnight (Cloney 1987). We checked for released larvae at intervals of ≤ 15 min during all experiments and the age of each larva was measured relative to the time of collection. Individuals were used in one of the four experiments described below. All experiments used digital video cameras capable of recording at frame rates between 30 and 500 frames s^{-1} (Redlake PCI Mono/1000S Motionscope, 420×480 pixels per camera).

Two-dimensional kinematics

Once every 15 min, individual larvae were moved by pipette from a petri dish (that was concealed from light) into a 66×26-mm tank with a water depth of 20 mm and a single illuminated wall. This wall, made of translucent white Plexiglas, made up one of the short sides of the tank. Water temperature was kept between 16 and 20°C by frequently replenishing the tank with cool water. A cool-burning compact fluorescent light bulb (Superlite PL-25EJ, 110–130 V, 60 Hz) was used as a source of illumination, which made temperature gradients in the tank undetectable with a digital thermocouple (Wescor, Inc., TH-65) at a resolution of 0.1°C. We prevented illumination of the tank from above by using opaque plastic baffles.

We recorded the swimming of larvae from above (at 60 frames s^{-1}) and analyzed their movement with an automated computer program. We began tracking the position of larvae about 1 s after they were placed in the tank. At this time, larvae were no longer visibly influenced by the flow generated by placing them in the tank. Swimming was measured for a duration of 10 s or until larvae reached the illuminated wall or the far wall of the tank, whichever occurred first. The position of the center of the trunk of a larva was found in each frame of video using a particle-tracking algorithm (using Matlab, v.5.3 with the image-processing toolbox on a Gateway E-4200, Pentium II 350 MHz). This algorithm found the centroid of the group of high luminance pixels that described the trunk within a region of interest (see Russ 1999). We verified the algorithm by visually inspecting the measured position of the trunk overlaid on the video frames.

Because the axis of a helical trajectory points in the net direction of movement of the swimmer, we used the orientation of the axis of the helix as a measure of heading direction. Using position data with the frequency components due to tail beating and body rotation filtered out (see "filtering kinematic data" below), we calculated the instantaneous velocity vector (**U**) as the product of the displacement between video frames and the frame rate. The swimming direction of the larva relative to the source of illumination was measured by the heading angle (ϕ) between **U** and a vector **L**. **L** points toward the source of illumination and has a magnitude of 1. Heading angle was calculated by taking the arccosine of the dot product of **U** and **L**, divided by the product of the magnitudes of **U** (U) and **L** (L). This calculation is shown in the following equation (Hill 1996):

$$\phi = \cos^{-1}\left(\frac{\mathbf{U}\cdot\mathbf{L}}{UL}\right), \quad (1)$$

We defined positive phototaxis as movement with a mean heading angle of less than 90° and negative phototaxis as having a mean heading angle of greater than 90°.

We measured the swimming behavior of larvae at 15 min intervals after their release from the colony and compared the behavior of larvae at hatching to the last age measured before settlement. Three parameters were used to describe the movement of larvae. First, the percentage of time spent swimming ($T_{\%}$) was calculated as

$$T_{\%} = \frac{f}{T_s r} \times 100\%, \quad (2)$$

where f is the total number of video frames in which larvae traversed a distance greater than 0.5 pixels, T_s is the total duration (in s) of the video recording, and r is the frame rate (frames s^{-1}). Displacements greater than 0.5 pixels (approximately 0.10 mm) could be distinguished from variation due to digitizing error because our particle-tracking algorithm had sub-pixel resolution. Second, when larvae were found to move more than 0.5 pixels, their swimming speed was recorded. The swimming speed for a video sequence was calculated as the mean value of these measurements of speed. Third, the standard deviation of the instantaneous measures of heading angle provided a measure of the variability of swimming direction. Because the literature on ascidian swimming behavior (e.g. *Tridemnum solidum*, Bak et al. 1981; *Podoclavella moluccensis*, Davis and Butler 1989; *Chelyosoma productum,* Young and Braithwaite 1980) provided us with predictions for how behavior changes with ontogeny, we tested for significant changes in behavior using a paired, one-tailed Student's t-test (Sokal and Rohlf 1995) in Microsoft Excel 2000.

Three-dimensional kinematics of the center of the body

To test how larvae respond to a rapid change in the direction of illumination, we recorded the movement by the center of the body (at 125 frames s^{-1}) in three spatial dimensions using two video cameras in the arrangement described by Crenshaw (1991). These cameras were positioned with orthogonal views of a 1.7×1.7×2.0 mm tall volume of water in the center of an aquarium with the inner dimensions of 3×3×6 cm tall. This aquarium was built with a separate outer chamber into which chilled water flowed from a water bath with a thermostat (VWR Scientific, 1166) to maintain a water temperature of 17°C.

The source of illumination during these experiments was switched in approximately 0.06 s using two fiber-optic illuminators (Cole Parmer, 9741-50) modified with a mechanical switch (Fig. 2A, B). Larvae were initially illuminated from below (the "under light" in Fig. 2), but as they moved into the field of view, the first illuminator was turned off and a second illuminator, positioned to the side of the tank, was turned on (the "side light" in Fig. 2). Illuminance from the top, bottom, and side of the tank was measured with a photodiode (Micro, ML308) calibrated with a digital light meter (INS DX-100), under both conditions of illumination (Fig. 2C, D).

The position of larvae was first approximated using the same Matlab program used in the two-dimensional kinematic analysis (described above). After finding this point in both camera views, we used a boundary pattern algorithm to find a more accurate center for the trunk. This algorithm (also programmed in Matlab) found the edge coordinates of high luminance pixels describing the periphery of the trunk. We found the ellipse that best represented the shape of these coordinates using a least-squares algorithm (Fitzgibbon et al. 1996). The center of the trunk was measured as the center of the fit ellipse. The X coordinate for the trunk's center was measured from the horizontal axis of one camera ("camera 1" in Fig. 2E), the Z coordinate was measured from the vertical axis of the same camera, and the Y coordinate was measured from the horizontal axis of the second camera ("camera 2" in Fig. 2E).

Using these position data, we measured how larvae changed the rotation of their bodies and how this rotation affected heading angle in response to changing light direction. Using a Matlab program (written by H. Crenshaw and C. Ciampaglio) based on the method of finite helix fit described by Crenshaw et al. (2000), we calculated yaw rate and roll rate. The finite helix fit method estimates the curvature (κ, which measures deviation from a line in degrees mm^{-1}) and torsion (τ, which measure deviation from a plane in degrees mm^{-1}) of a three-dimensional curve from discrete data. Under the assumption that the antero-posterior axis points in the same direction as velocity,

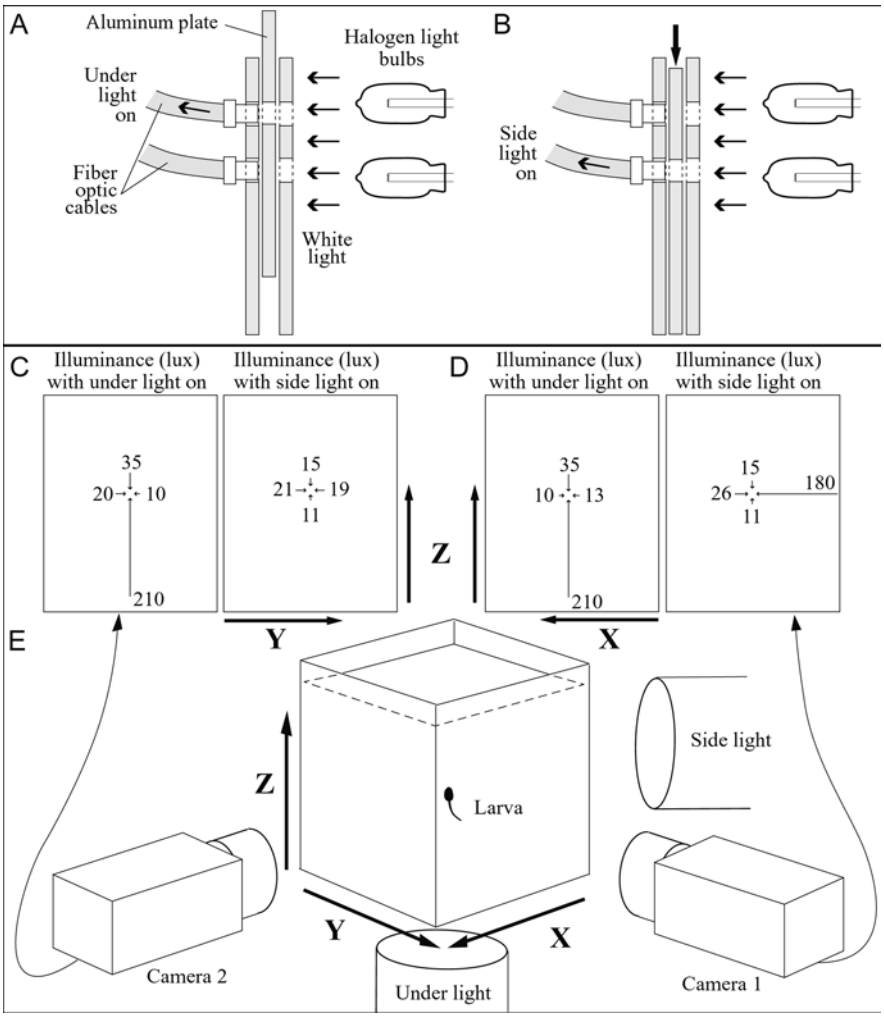

Fig. 2A–E The experimental setup for recording the response to changing the direction of illumination in the three dimensions. **A**, **B** A mechanical switch directed illumination away from the fiber-optic cable connected to the under light to the cable connected to the side light by quickly lowering an aluminum plate with a hole in its center. Two halogen light bulbs (110/120 VDC, 60 Hz) continuously illuminated the ends of two fiber-optic cables. **A** During experiments, the aluminum plate initially was raised such that a hole was aligned with the cable to the under light. **B** When larvae were within view of both video cameras, the plate was quickly lowered, which caused illumination to be directed to the side light. **C** The illuminance measured in two spatial dimensions (Y and Z) when the under light is on or when the side light is on. **D** The illuminance measured in the X and Z dimensions with the under light or side light on. **E** The relative positioning of the cameras, observation tank, and lights

the rate of body rotation about the antero-posterior axis is equal to the product of torsion and speed (s, mm s^{-1}). This is known as the roll rate (degrees s^{-1}), ω_{roll}, and it is calculated as

$$\omega_{roll} \approx s\tau. \quad (3)$$

If we assume that yaw rate (degrees s^{-1}), ω_{yaw}, the rate of rotation about the dorso-ventral axis, is much larger than pitch rate, the rate of rotation about the medio-lateral axis, then the following approximation holds true:

$$\omega_{yaw} \approx s\kappa. \quad (4)$$

From the instantaneous measures of speed, curvature, and torsion provided by the finite helix fit method, roll rate and yaw rate were calculated for each frame interval of a video sequence.

We compared temporal changes in roll rate and yaw rate with changes in heading angle to determine how larvae changed the rotation of their bodies to change their heading direction. We calculated heading angle as described above (Eq. 1), but in this case the vector **L** was directed toward the light at the side of the tank (the "side light" in Fig. 2) throughout the experiment. With this arrangement, heading angle was expected to decrease in larvae with positive phototaxis and increase in those with negative phototaxis after changing the direction of illumination from beneath the tank to the side of the tank.

After dividing larvae into three age classes of equal duration (68 min), we tested whether larvae within each class showed a significant change in heading angle in response to the change in light direction. The rate of change in heading angle was calculated by subtracting the mean heading angle after the change in light direction from the mean heading angle prior to the change and dividing that difference by the elapsed time. Durations of 0.5 s before and after the change in light direction were considered transitional periods and we therefore excluded measurements of heading angle during these periods. Using a two-tailed Student's t-test, we tested whether larvae in each age class showed a rate of change in heading angle that was significantly different from zero (Sokal and Rohlf 1995).

Tethering experiments

Individual larvae were tethered to a glass micro-pipette (using light suction from a modified mouth pipette) and positioned with micromanipulators to allow the tail's position to be recorded while the ocellus was stimulated with a focused source of white light (Fig. 3A, light generated by a light-emitting diode or LED, Jameco, 142893). As in most ascidian species, *A. constellatum* larvae exhibit a "shadow response" behavior, where they initiate swimming in response to a decrease in ambient light (Grave 1941; Crenshaw et al. 2000). Using the shadow response as an assay of light perception, larvae were found to be insensitive to a decrease in the infrared (IR) light generated by the IR LED used to illuminate the tail, but they responded to changes in white light (Fig. 3B). A video camera was mounted on a dissecting microscope (Wild, M5A) beneath the tank containing the larva. This camera was sensitive to IR light, which allowed the tail to be recorded at a frame rate of 250 Hz. We stimulated larvae with sinusoidally oscillating light at frequencies of 1, 5, and 10 Hz using a function generator (LG Precision Co., FG-8002).

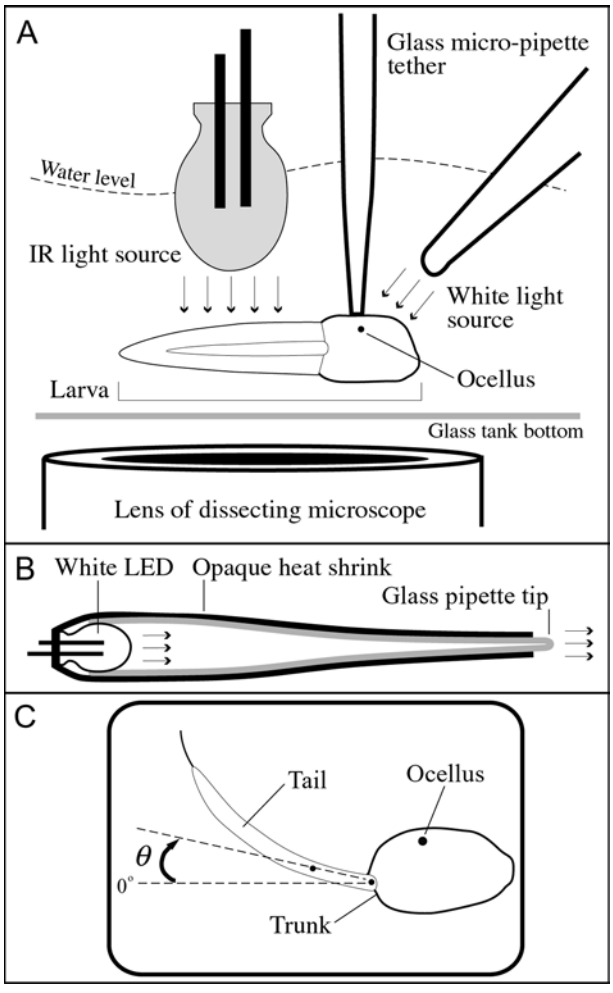

Fig. 3A–C The setup for experiments on tethered larvae. **A** Larvae were tethered to a glass micro-pipette with light suction, and movement by the tails of larvae was recorded by a high-speed video camera mounted on an upside-down dissecting microscope using the illumination provided by an infrared (*IR*) LED. During experiments, the ocellus of larvae was stimulated with a custom-made illuminator. **B** This illuminator was constructed by enclosing a white LED (4.0 V) in a glass pipette with a fused end. The illumination was concentrated at the glass tip by shielding the entire construction with opaque heat shrink. **C** Measurements of trunk angle were made from video recordings of the ventral or dorsal side of the tail, as seen through the dissecting microscope

While the larvae were stimulated with light, the position of the tail was measured using a program written in Matlab. This program followed the tail by tracking the pixel of greatest luminance at a distance 0.12 mm from the base of the tail. The position of this point was described by the vector **T**, which had its origin at the intersection of the trunk and tail. Because the trunks of larvae were positioned parallel to the length of the video frame, the axis of the trunk (**R**) was approximated by the horizontal axis of the video frame, directed posteriorly (Fig. 3C). We characterized tail position by the angle between **R** (having a magnitude of R) and **T** (with a magnitude of T), known as the trunk angle (θ, McHenry 2001). The magnitude of trunk angle was calculated with the following equation (Hill 1996):

$$\theta = \cos^{-1}\left(\frac{\mathbf{R}\cdot\mathbf{T}}{RT}\right). \quad (5)$$

Values for trunk angle were negative when the tail was on the abocular side of the body and positive when on the ocular side.

To test whether larvae vary tail motion with the same frequency as the stimulus, trunk angle data were analyzed with a fast Fourier transform (FFT) using Matlab. An FFT determines the relative contribution (or power) of individual frequencies to time-varying data (Duhamel and Vetterli 1990). We predicted the tail-beat frequency to be a dominant frequency in the trunk angle data and therefore expected a large value for power at that frequency. According to Mast's (1921) model, larvae flick their tails at the stimulus frequency. Therefore, a second peak in power was predicted at the stimulus frequency. We verified whether high frequency peaks were equal to the tail-beat frequency by comparing it to the average tail beat frequency, which was measured as the total number of tail beats, divided by the duration of swimming (in seconds). Low-frequency peaks were tested for significant difference from the stimulus frequency using a paired, two-tailed, Student's *t*-test (Sokal and Rohlf 1995).

Filtering kinematic data

Studies of swimming in ascidian larvae (Crenshaw et al. 2000; McHenry 2001) have shown that the position of the center of the body varies at three frequencies. Tail beating causes the center of the body to move laterally at a tail-beat frequency between 15 and 30 Hz (McHenry 2001). The center of mass also varies position at the frequency of body rotation (between 1 and 3 Hz). Finally, as a larva changes the direction of swimming, the axis of the helical trajectory changes direction and thereby creates the slowest harmonic in the kinematics. Larvae of *Botrylloides* sp. change the direction of swimming by 45° in about 2.5 s, which corresponds to a rate of turning of 0.05 Hz (Crenshaw et al. 2000).

To focus on specific aspects of the motion of larvae, we applied a low-pass digital filter (second-order Butterworth with zero phase offset using Matlab with the signal-processing toolbox, version 4, Mathworks) to our kinematic data. To filter out variation in the body position data due to tail beating, and thereby track only the shape of the helical trajectory, we used a cut-off frequency equal to one-fourth the average tail-beat frequency. Cut-off frequencies greater than this introduced variation from tail beating and frequencies lower than this showed deviation from the position of the body averaged over a tail beat. To approximate the shape of the axis of the helix, we filtered out variation in the data due to body rotation by using a cut-off frequency equal to one-fourth the average frequency of body rotation. The average frequency of body rotation was calculated as the number of times that the body was observed to rotate divided by the duration of the swimming sequence. Cut-off frequencies greater than this introduced variation from body rotation and frequencies immediately lower than this did not substantially influence the resulting trajectory.

Results

Ontogenetic changes in phototaxis

Larvae exposed to light (Fig. 4A), swam with positive phototaxis upon hatching, but they switched to negative phototaxis later in ontogeny (Fig. 4B). Therefore, the mean heading angle for swimming sequences early in ontogeny was less than 90° (Fig. 4C), but was between 90° and 180° late in ontogeny (Fig. 4D). With the exception of 1 larva in a sample of 13, larvae did not revert back to positive phototaxis after the switch to negative phototaxis. Although the greatest number of larvae made the switch to negative phototaxis between the ages of 15 and 30 min, the timing of this change was highly variable and it occurred in some larvae beyond the age of 90 min (Fig. 5). All larvae either settled or otherwise

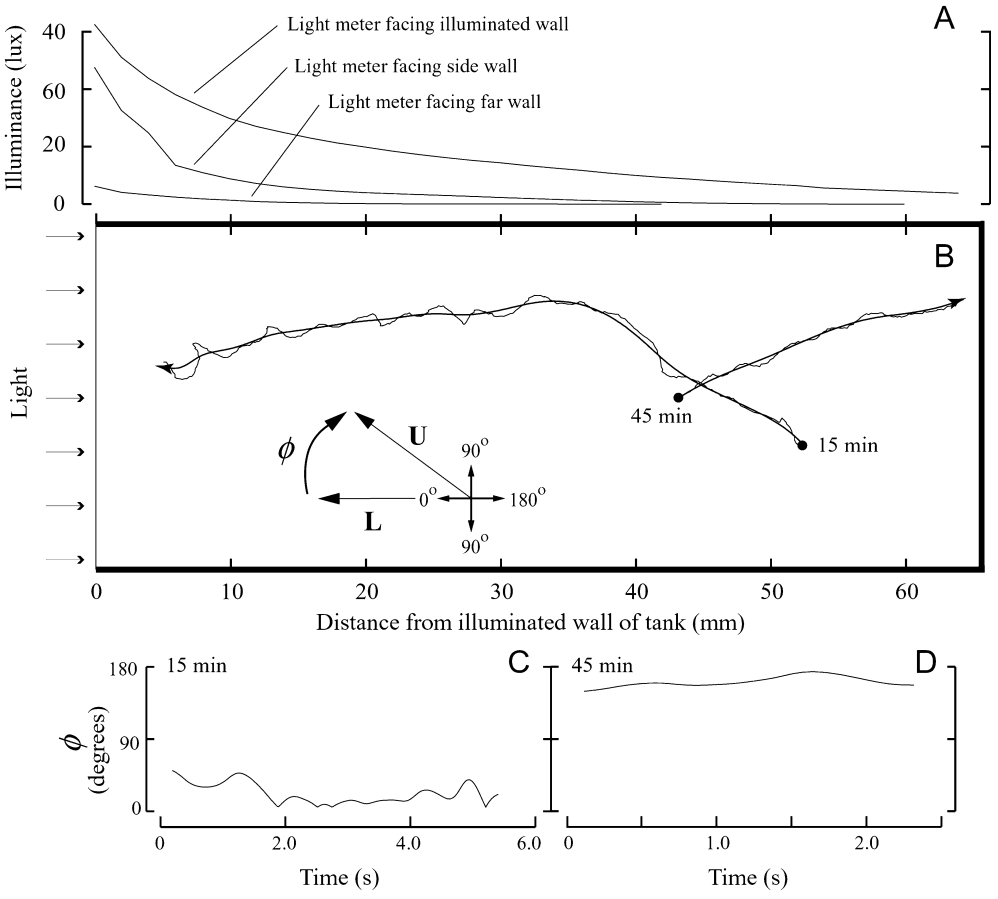

Fig. 4A–D Typical movement by a larva when positively phototactic early in ontogeny and negatively phototactic late in ontogeny. **A** Light intensity as a function of position along the length of the tank was measured by positioning a light meter toward the illuminated wall, then toward the side wall, and then toward the far wall. Larvae were released in a tank under this illumination condition at regular intervals throughout the larval stage. **A–D** Swimming trajectories for the same larva at the age of 15 min and at 45 min. **B** The *thin lines* show the video-recorded trajectories of swimming for a larva at 15 min and 45 min after release from the parent colony, starting at the *filled circles*. The *thick lines* are the same data after filtering out oscillations due to tail beating and body rotation. These lines approximate the axis of the helical trajectories. The *inset* shows the measurement of heading angle, ϕ, from the velocity vector, **U**, and the vector **L** directed toward the light. The orientation of larval movement with respect to illumination is expressed by the heading angle. **C**, **D** The variation in heading angle with time illustrated for the trajectories shown in **B**. **C** Positive phototaxis is shown by values of heading angle that are below 90°. **D** During the period of negative phototaxis, values for heading angle are above 90°

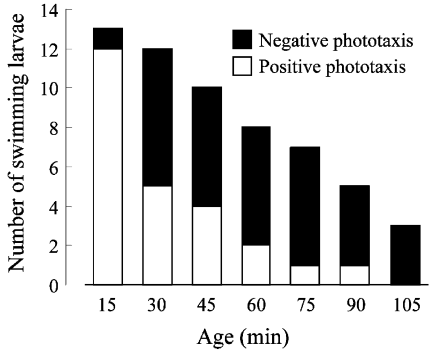

Fig. 5 The number of larvae exhibiting positive and negative phototaxis over ontogeny. Larvae settled at a regular rate throughout the larval stage, which can be seen by the gradual decrease in the total number of swimming larvae

ceased swimming by the age of 120 min. There was an ontogenetic increase in the proportion of larvae swimming with negative phototaxis because the number of larvae switching to negative phototaxis exceeded the number that ceased to swim at each interval (Fig. 5).

In addition to changing the direction of taxis, larvae altered their swimming behavior between hatching and just prior to settlement. We found that larvae within 15 min of settlement spent a smaller percentage of their time moving ($P = 0.018$, $n = 15$, one-tailed t-test; Fig. 6A), moved at a lower mean swimming speed ($P = 0.032$, $n = 15$, one-tailed t-test; Fig. 6B), and had a greater standard deviation in heading angle ($P = 0.007$ $n = 15$, one-tailed t-test; Fig. 6C) compared to larvae of the age of 15 min.

Three-dimensional kinematics of the center of the body

The center of the body of *Aplidium constellatum* larvae moved with three periodicities as they beat their tails,

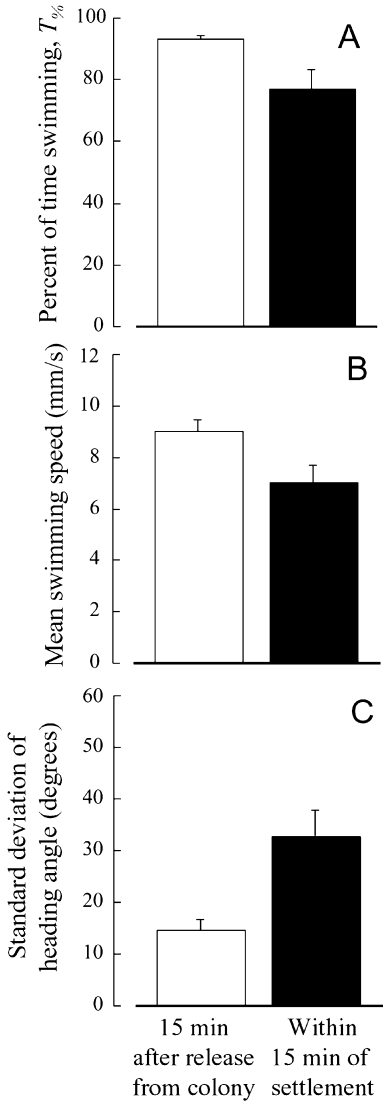

Fig. 6A–C Changes in swimming behavior. Compared to when they are released from their parent colony, larvae just prior to settlement **A** spent a lower percentage of their time swimming, **B** swam at a slower mean speed, and **C** had a greater standard deviation in heading angle. All of these differences are statistically significant (see Results for details)

rotated their bodies, and changed their direction of swimming (Table 1). Larvae had a mean tail beat frequency of 25.2 Hz and a mean body rotation frequency of 1.7 Hz. This suggests that there were approximately 15 tail beats for each rotation of the body. Larvae stimulated to change their heading angle showed a mean rate of turning of 0.076 Hz, which means that a 45° change in heading angle was achieved over the course of about 2.7 body rotations and 41 tail beats. Therefore, changes in swimming direction during tactic orientation occur over multiple body rotations and tens of tail beats. This means that larvae swimming away from a light source would momentarily move toward the source while swimming through loops of the helical trajectory (as in Fig. 7).

Table 1 Average kinematics. The mean and standard error of parameters measured from video recordings of freely swimming larvae. The rate of turning was calculated as the rate of change in heading angle in response to a change in illumination (data for $68 < \text{age} < 136$ min in Fig. 7E), divided by 360°. The unfiltered swimming speed was measured from three-dimensional position data of the center of the trunk. Filtered swimming speed was measured from the same data, after being filtered with a low-pass filter at one-fourth the frequency of body rotation (see Methods)

Variable	n	Mean ± 1 SE
Tail beat frequency (Hz)	16	25.2 ± 0.9
Body rotation frequency (Hz)	10	1.7 ± 0.2
Rate of turning (Hz)	10	0.076 ± 0.002
Unfiltered swimming speed (mm s^{-1})	18	14.3 ± 1.52
Filtered swimming speed (mm s^{-1})	18	4.47 ± 0.33

In the middle age class of the larval stage, individuals were responsive to rapid changes in the direction of illumination. These negatively phototactic larvae would typically swim up and away from the light source beneath the tank (the "under light" in Fig. 7A) but would redirect their swimming away from a new light directed to the side of the tank (the "side light" in Fig. 7A) in less than 1 s. This change in swimming direction could be seen by an increase in heading angle after the switch in light direction (Fig. 7B). Changes in the shape of the helix were achieved by a large, rapid increase in yaw rate (Fig. 7C) and a larger negative change in roll rate (Fig. 7D). Negative values of roll rate suggest a momentary switch from a right-handed to a left-handed helix. This kinematic pattern was typical of larvae between the ages of 68 min and 136 min, and it was only in this age class that we found a significant response in larvae to a change in the direction of light. Only in this class was the mean rate of change in heading angle significantly different from zero ($P = 0.002$, $n = 3$, two-tailed t-test; Fig. 7E). Neither the larvae younger than 68 min ($P = 0.301$, $n = 4$, two-tailed t-test; Fig. 7E), nor the larvae older than 136 min ($P = 0.315$, $n = 3$, two-tailed t-test; Fig. 7E) moved with a rate of change in heading angle significantly different from zero.

Swimming kinematics in tethered larvae

The tail of tethered larvae oscillated both with the high frequency of tail beating and a relatively slow periodicity (Fig. 8A, B) that approximated the frequency of the light stimulus. We found this correlation between the slow-bending frequency and the stimulus frequencies at 1, 5, and 10 Hz (Fig. 8D, Table 2). The standard error of slow-bending frequency increased with greater stimulus frequencies, but the slow-bending frequency was not found to be significantly different from the stimulus frequency ($P < 0.05$, two-tailed Student's t-test).

This correlation suggests that slow bending was stimulated by variation in perceived light intensity. In free swimming, such oscillations in light intensity likely result from the rotation of the body. We found the

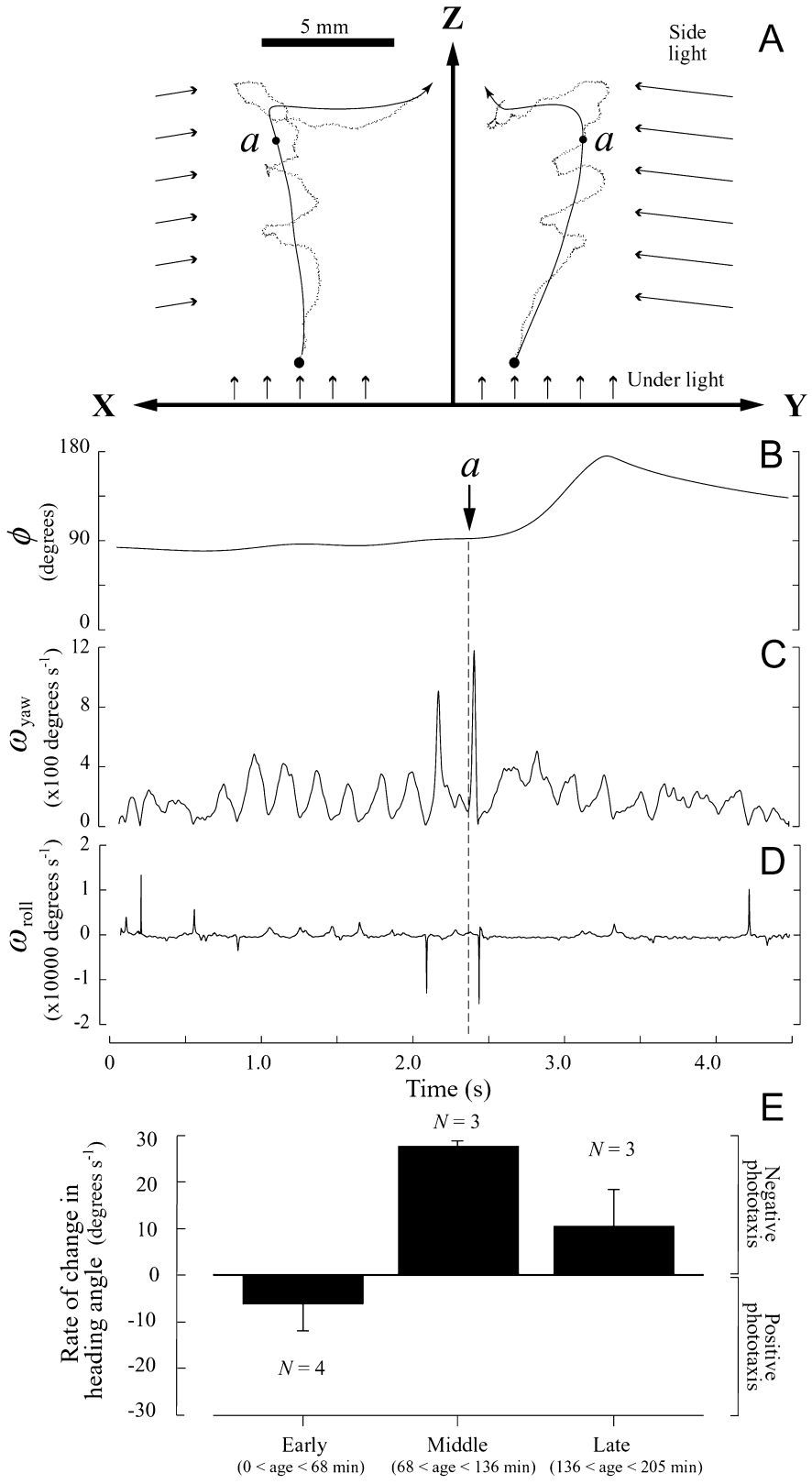

Fig. 7A–E Swimming kinematics typical of a larva responding to a change in light direction. **A** The swimming trajectory for one larva, as viewed from two orthogonal perspectives. *Points* show the movement by the center of the trunk of a larva, beginning at the *filled circle*. The axis of this helical trajectory is approximated by the *line* running through the points, which was found by filtering the position data (see Methods for details). At the position *a*, the under light was turned off and the side light was turned on. Shortly after this switch, the axis of the helix changed direction. **B** This change is expressed by heading angle, ϕ, which increases shortly after the switch in illumination. **C**, **D** Changes in the direction of the axis of the helix were caused by changes in the rates of rotation by the body. **C** Yaw rate, ω_{yaw}, shows one rapid increase prior to the change in heading angle and at the same time, **D** roll rate, ω_{roll}, momentarily switches direction and achieves large negative values. Not all larvae oriented their swimming to the side light in these experiments. **E** The mean and standard error of the rate of change in heading angle within three larval age classes

closest match between the slow-bending frequency and the stimulus frequency at 1 Hz (Table 2). Since the average frequency of body rotation was 1.7 Hz (Table 1), it appears that larvae are best able to match slow bending to stimulus frequency within close range to the frequency of body rotation.

We did not observe the rapid, large-amplitude tail flicks reported by Mast (1921). Although it remains

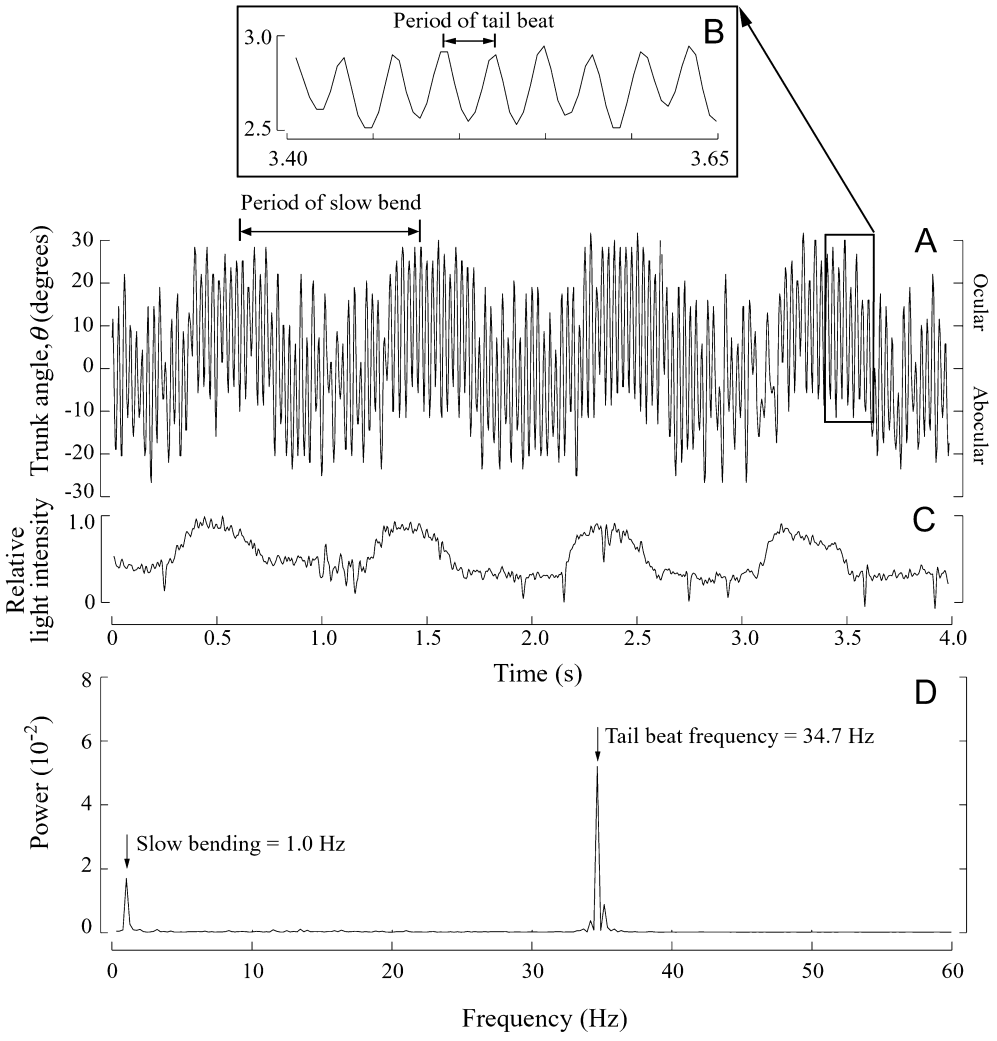

Fig. 8A–D Typical tail motion in a tethered larva stimulated with 1 Hz oscillating illumination. **A** Changes in trunk angle (θ) with time show rapid oscillations due to tail beating and a slow-bending oscillation. **B** Detail of the rapid changes in tail position due to tail beating. **C** Sinusoidal light stimulus directed at the ocellus of the larva. **D** A fast Fourier transform of the tail position data in **A** shows peaks at both the tail beat and slow-bending frequencies

Table 2 Frequency of slow bending in tethered larvae. The *P*-values given are the results of a *t*-test to determine whether the frequency of slow bending is significantly different from the stimulus frequency (see Methods). The frequency of slow bending was not found to be significantly different from any of the stimulus frequencies

Stimulus frequency (Hz)	*n*	Mean ± 1 SE (Hz)	*P*
1	8	1.35 ± 0.25	0.89
5	4	6.59 ± 0.71	0.94
10	8	7.35 ± 1.60	0.07

possible that we did not reach a great enough rate of change in light intensity in our experiments to elicit tail flicking, two facts suggest otherwise. First, this same source of illumination was capable of stimulating a shadow response from larvae when dimmed (see Methods), which suggests that larvae could perceive changes in light intensity. Second, we did observe oscillatory changes in the tail position that were correlated with the stimulus frequency (Fig. 8), which suggests that larvae were responding to oscillatory changes in the stimulus.

Discussion and conclusions

Ontogenetic change in phototaxis

We found support for existing hypotheses about ontogenetic change in the swimming behavior of ascidian larvae. Our results verified earlier reports (e.g. *Tridemnum solidum*, Bak et al. 1981; *Podoclavella moluccensis*, Davis and Butler 1989; *Chelyosoma productum,* Young and Braithwaite 1980; *Polyandrocarpa zorritensis*, Vazquez and Young 1998) that ascidian larval swimming becomes slower, more intermittent, and less directed with age. We found that prior to settlement, larvae spent a smaller percentage of their time swimming (Fig. 6A), swam at a lower mean speed (Fig. 6B), and varied their swimming direction more (Fig. 6C), compared to when they were first released from the parent colony. As reported by Grave (1920), larvae switched from positive to negative phototaxis early in their ontogeny (Fig. 5). To understand how larvae achieved this switch, it is first necessary to establish how larvae are capable of orienting to light.

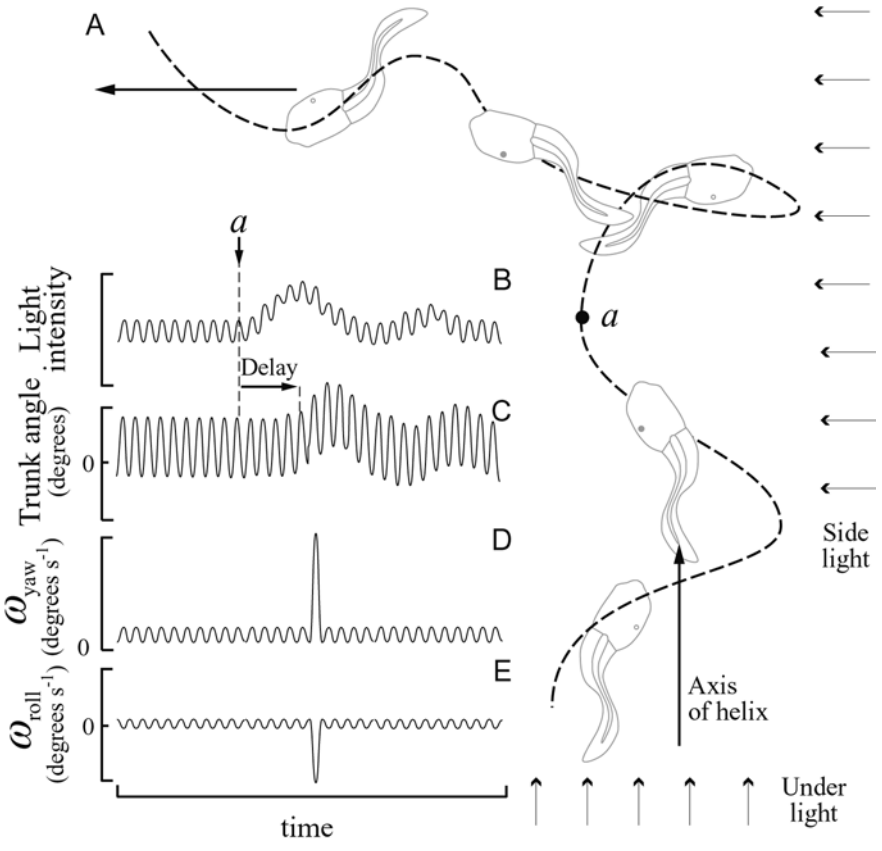

Fig. 9A–E Phototaxis by helical klinotaxis in ascidian larvae. **A** In this experimental situation, the larva begins by swimming away from the under light but swims away from the side light when it is turned on and the under light is turned off (at *a*). Prior to the light switch, this larva swims in a helical trajectory (the *dotted line*) with its axis oriented vertically and parallel to the under light. When the light direction switches at *a*, the shape of the helical trajectory changes such that the axis points to the left, away from the side light. **B–E** All variables oscillate rapidly at the tail-beat frequency. **B** With the under light initially below the larva, light intensity oscillates around a constant value, but when the lights switch, the light intensity oscillates due to the rotation of the body. **C** These oscillations generate a linear response in the slow bending of trunk angle after a delay. **D**, **E** The slow bending of trunk angle influences the rotation of the body such that rapid changes in yaw rate, ω_{yaw} (**D**), and roll rate, ω_{roll} (**E**), change the direction of the helical trajectory

The Mast model of phototaxis

Our results did not support any of the three hypotheses consistent with Mast's model. (1) By recording the trajectories of larvae, we found that they did not rotate strictly by roll or swim along straight lines. (2) Larvae did not execute turning maneuvers strictly by yaw. (3) Although we did find a correlation between the frequency of slow bending and the stimulus frequency in tethered larvae (Fig. 8, Table 2), this motion did not occur in rapid tail flicks. These results suggest that Mast's (1921) model does not accurately describe the kinematics of phototaxis in ascidian larvae.

Prior to the present study, published observations on the kinematics of swimming in ascidian larvae challenged Mast's (1921) model. Grave (1926) reported on the helical swimming of larvae of *Mogula citrina*. Such a trajectory requires that the swimmer not swim in straight lines and that body rotation occur about at least two of the orthogonal body axes (Crenshaw 1993a). McHenry (2001) reported that larvae of *Botrylloides* sp. undulate their tails asymmetrically during steady swimming, which is unlike the symmetrical oscillations observed by Mast (1921). Furthermore, McHenry (2001) suggested that swimming becomes redirected in the direction of large-amplitude motion. For example, a tail beat toward the right would generate a rotation about the center of mass that causes swimming to be directed to the right. This is the opposite direction proposed by Mast (1921), who suggested that larvae turn away from the direction of tail flicks.

Phototaxis by helical klinotaxis

Although our results refute many components of Mast's model, it remains likely that larvae of *A. constellatum* orient to light by coordinating a motor response with the temporal changes in perceived light intensity, which is a form of orientation known as klinotaxis (Frankel and Gunn 1940). Rather than follow straight lines punctuated by rapid changes in body orientation, ascidian larvae orient by changing the geometry of a helical trajectory over the course of many tail beats (Table 1, Fig. 7). This suggests that larvae may be able to orient

by the mechanism of helical klinotaxis. Crenshaw (1993b) demonstrated that it is theoretically possible for helical swimmers to orient to vector cues if their rates of body rotation (e.g. roll rate or yaw rate) are simple functions of perceived stimulus intensity. We found that by rapidly changing yaw rate and roll rate, negatively phototactic larvae redirected their swimming away from illumination (Fig. 7) in a pattern similar to that measured in *Chlaymdomonas reinhardtii*, a flagellate thought to orient by helical klinotaxis (Crenshaw 1996).

We propose that ascidian larvae orient through helical klinotaxis by varying the motion of the tail in proportion to the changes in perceived light intensity resulting from body rotation (Fig. 9A). According to this model, the ocellus is exposed to slow oscillations in light intensity as the body rotates. These changes in perceived intensity are small when the axis of the helical trajectory is parallel to the light source and are large when the axis points at an oblique angle to the light (Fig. 9B). Oscillations in perceived intensity stimulate proportional change in slow tail bending (Fig. 9C). These oscillations by the tail cause changes in yaw rate and roll rate that serve to align the axis of the helical trajectory with the direction of the light source (Fig. 9D, E).

Whether the axis of a helix becomes directed toward or away from the source of illumination during a turning maneuver is determined by the delay between the perceived stimulus and the resulting changes in yaw rate and roll rate (Crenshaw 1993b). Therefore, our model predicts that the switch from positive to negative phototaxis in ascidian larvae may be achieved by altering this delay. The necessary change in the duration of this delay depends on the orientation of the ocellus with respect to the body (see Crenshaw 1993b).

Phototaxis on the organismal level

To distinguish between different mechanisms of taxis or between taxis and kinesis, it is necessary to perform experiments on individual larvae. The direction of taxis is commonly measured by the distribution of larvae in an aquarium with either a single illuminated end or with illuminated and shaded sides (e.g. Crisp and Ghobashy 1971; Forward 1984; Orlov 1997). By these approaches, it is often assumed that the distribution of larvae is due to phototaxis because light, like gravity, possesses directional information. However, unlike gravity, light also varies spatially in magnitude (e.g. Fig. 4A), which allows some organisms to orient by kinesis (Frankel and Gunn 1940). In a classic example, Ullyott (1936) demonstrated that the planarian *Dendrocoelum lacteum* varies its frequency of turning in response to changes in undirected light intensity such that it congregates in the dark regions of a light gradient. Such a kinematic pattern could operate in marine invertebrate larvae to affect their distribution in a light gradient. Therefore, distributions of larvae that are attributed to phototaxis (e.g. Crisp and Ghobashy 1971; Forward 1984; Orlov 1997) may actually be the result of photokinesis. In the present study, the maneuvers that resulted by changing the direction of light (Fig. 7) suggest that larvae of *Aplidium constellatum* orient by taxis. However, it remains possible that these larvae also employ photokinesis in situations where illumination varies spatially in magnitude but does not have strong directionality. Ideal experiments on the relative contribution of kinesis and taxis would decouple directional and intensity cues and test their individual effects (as attempted by Ryland 1960).

In summary, we found that larvae of *A. constellatum* orient to light by taxis and that larvae switch from positive to negative phototaxis over ontogeny. Although our results support the idea that larvae orient by coordinating their swimming with perceived changes in light intensity, the measured kinematics of swimming do not agree with the model proposed by Mast (1921). We propose that larvae orient through helical klinotaxis by varying tail motion in proportion to perceived changes in light intensity. According to this model, the switch from positive to negative phototaxis is achieved by increasing the delay between the stimulus and motor response. Testing such tactic mechanisms and distinguishing between kinesis and taxis requires further organismal-level investigation.

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