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FIVE DIFFERENT STATES OF CILIARY ACTIVITY IN THE EPAULETTE OF ECHINOPLUTEI

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Summary

Movement and spontaneous responses of the epaulette cilia of free-swimming eight-armed echinoplutei were analyzed by means of high-speed video microscopy. In addition to the two cyclic activities, normal beating for forward swimming and reversed beating for backward swimming, we identified another cyclic and two noncyclic states of ciliary activity. Intermediate beating was found during the transition from reversed beating to normal beating. One of the noncyclic states, the upright-inactive state, was found only after reversed beating and then changed to another noncyclic state, the inclined-inactive state, prior to a return to normal beating. Transitions within the cyclic and noncyclic states occurred in both directions, while transitions between the cyclic and the noncyclic states were in one direction.

Introduction

The locomotor activity of sea urchin larvae changes during ontogeny. Movements increase in complexity during larval morphogenesis, which makes it easier for larvae, after certain developmental stages, to escape from predators (Rumrill *et al.* 1985) and to position themselves in a water column (Mogami *et al.* 1988). Because larvae use cilia for propulsion, changes in locomotor responses are presumably largely due to changes in ciliary activity.

Previous papers have demonstrated that, at the pluteus stage, the cilia of sea urchin larvae acquire the capability for reversed beating, resulting in the typical swimming behaviour of echinoplutei (Baba, 1975; Degawa *et al.* 1986; Baba and Mogami, 1987). In the present study we focus on the responses of the cilia of the epaulettes, prominent ciliary bands that form in the six- to eight-armed stages of echinopluteus development. High-speed video microscopy recordings of the epaulette cilia at this stage showed a variety of newly differentiated responses in addition to the reversed beating developed earlier. They also provided new

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information on a bimodal diaplectic metachrony unique to the epaulettes of echinoplutei (Knight-Jones, 1954).

Materials and methods

Sea urchin larvae of *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus* were grown in the laboratory (Degawa *et al.* 1986). Pluteus larvae (2 days after insemination) were transferred to 1 l jars at 5×10^2 larvae l^{-1} and reared at 17 °C with constant stirring (1 rev. min^{-1}). Diatoms (*Chaetoceros gracilis*) were added to the culture once every 3 days. Fifty percent of the culture volume was replaced by fresh, filtered natural sea water every 2 days. Under these conditions, larvae grew to eight-armed plutei within a month of insemination.

To observe and record the movement of epaulette cilia, larvae were introduced into a small chamber containing artificial sea water (Jamarin-U, Jamarin Lab., Osaka). The chamber was made of a slide and cover glass separated by a silicone rubber spacer 2–3 mm thick. A frame-shaped spacer (internal dimensions about 10 mm×10 mm) was used to allow the larvae to swim freely. For axial views, a larva was confined to a small chamber of twice the larval size (i.e. 1 mm inner diameter) restricting the reorientation of the larval axis.

Ciliary movement was recorded by stroboscopic high-speed video microscopy (200 fields s^{-1}, MHS-200, Nac Inc., Tokyo) with phase contrast optics (NH, Olympus, Tokyo). Recorded images were analyzed field by field with the aid of a video motion analyzer (SUM-1110, Sony, Tokyo) and video copy processor (SCT-70P, Mitsubishi Elec. Co., Tokyo).

Results

Epaulettes of echinopluteus

In the eight-armed echinopluteus two systems of circumferential ciliated bands occur; the anterior epaulette, at the base of the postoral and postdorsal arms, and the posterior epaulette, at the posterior end of the body (Fig. 1A). In the anterior epaulettes of *H. pulcherrimus* and *P. depressus*, four small ciliated regions develop initially at the bases of the four arms. At the six-armed pluteus stage these extend towards the mid-line on both dorsal and ventral sides until they meet (Fig. 1B). In the posterior epaulettes, two ciliated regions develop initially on the lateral surface; these extend dorsally and ventrally to form U-shaped bands connecting with each other at the mid-line of both dorsal and ventral sides (Fig. 1B).

Larval locomotion appears to be dependent on the beating activity of the epaulette cilia. When swimming forwards (arms leading), the effective beating stroke is directed posteriorly (normal beating), whereas the beat reverses (reversed beat) when swimming backwards. Swimming speed is roughly proportional to the beat frequency. In terms of responsiveness, the cilia of the different epaulettes appeared to be identical, and the responses described in this paper were evoked almost simultaneously in all of them.

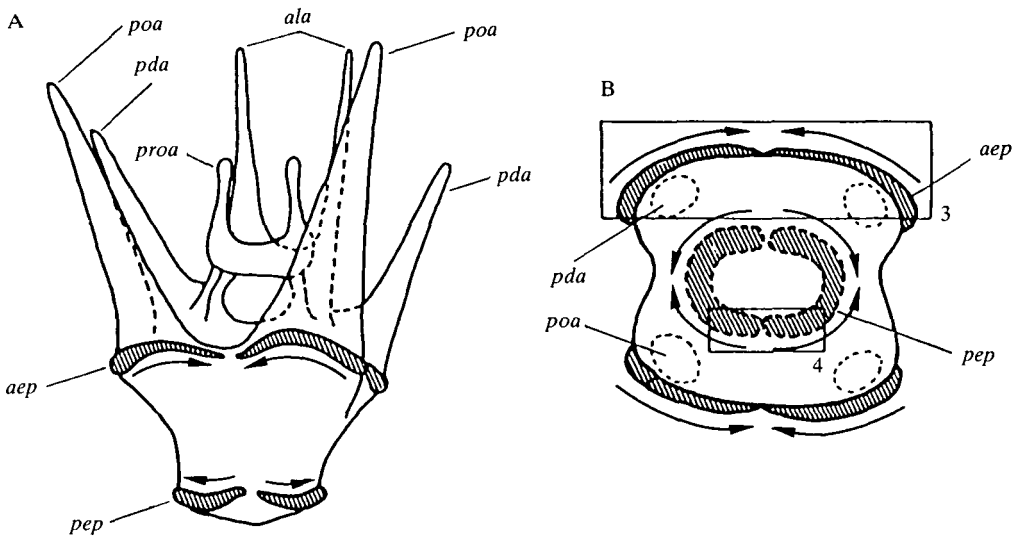

Fig. 1. (A,B) Schematic drawings of an eight-armed pluteus of *Hemicentrotus pulcherrimus* in ventral view (A) and frontal view (B). *pda*, postdorsal arm; *poa*, postoral arm; *ala*, anterolateral arm; *proa*, preoral arm; *aep*, anterior epaulette; *pep*, posterior epaulette. Arrows in A and B indicate the direction of metachronal wave propagation. Metachronal waves in numbered frames in B are shown in figures of the same numbers.

The cilia of the epaulettes of the two species examined in this study were about 50 μm in length, twice the length of other cilia of the same larva.

Ciliary activities

Epaulette cilia showed a ciliary reversal response, as reported previously (Mackie *et al.* 1969; Strathmann, 1971). It was usually evoked when the larva encountered objects such as the chamber walls. The cilia changed their beat pattern, making hook-shaped recovery strokes in the opposite direction to the normal beat after a short transition time of several tens of milliseconds (Fig. 2, upper row). Beat patterns in the normal beat, as well as in the reversed beat, appeared almost identical to those recorded in the cilia on the body surface of four-armed plutei (Baba, 1975; Baba and Mogami, 1987). Following several cycles of reversed beating, the cilia resumed normal beating. The recovery of normal beating was associated with a transition (intermediate beating) in which less-polarized beats continued with rather symmetrical beats propagating at the same speeds in both strokes (Fig. 2, middle row). The intermediate beating lasted for several hundreds of milliseconds and could shift either to the normal beat or, sometimes, to the reversed beat again. No transition from the normal beat to the intermediate beat was observed.

Cyclic beating activities were performed with metachronal coordination. As noted by Knight-Jones (1954) and confirmed by Strathmann (1971), the cilia on one side of the body show laeoplectic waves and those on the other side show

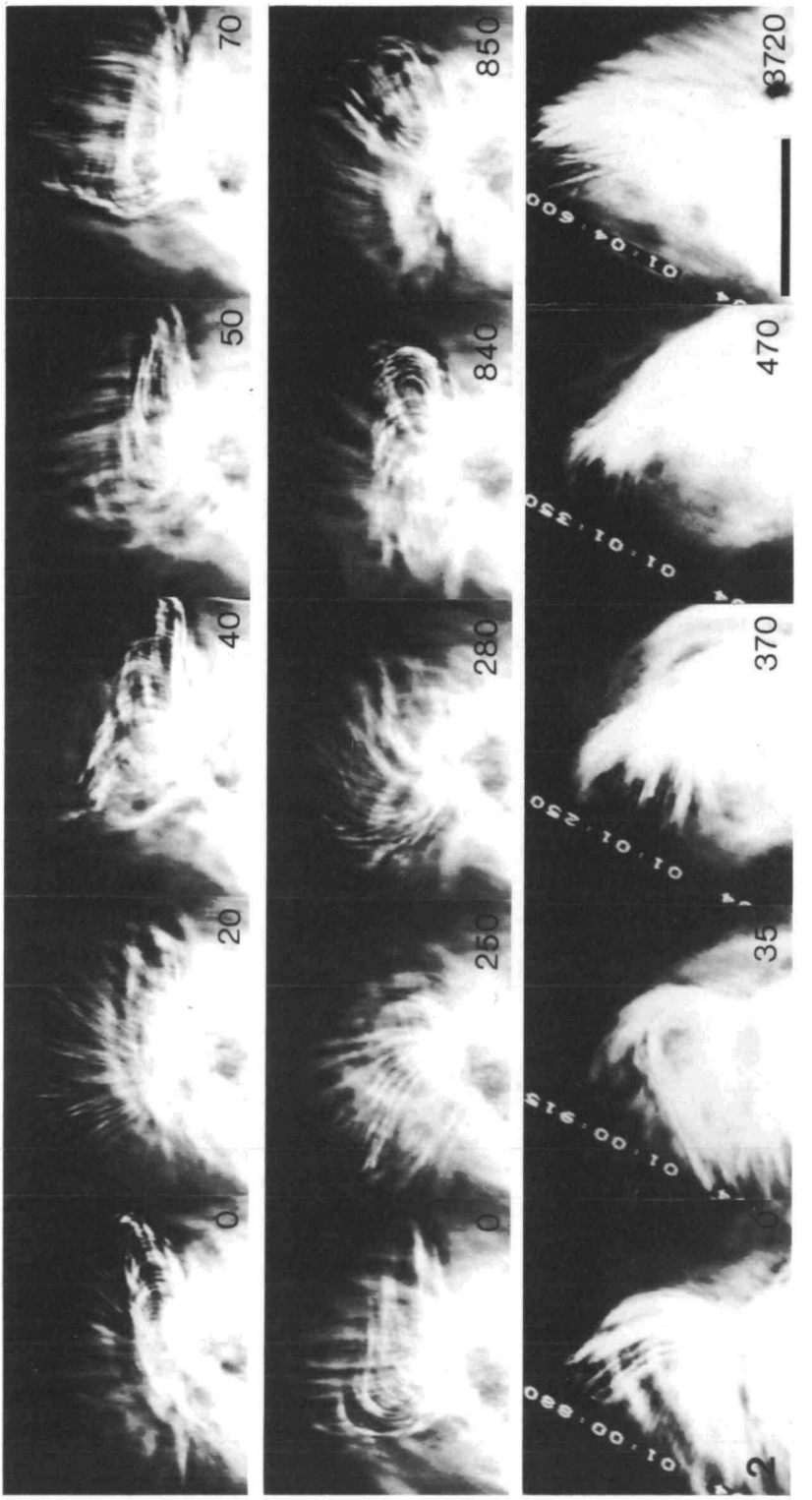

Fig. 2. Sequential images of high-speed video microscopy of stereotyped responses of the anterior epaulette cilia. The upper row shows the fast transition from normal beating (0 and 20) to reversed beating (50 and 70). The middle row shows the reverse transition from reversed beating (0) to normal beating (840 and 850) *via* intermediate beating (250 and 280). The lower row shows the slow transition from reversed beating (0 and 35) to the upright-inactive state (470 and 3720). For ease of discrimination of ciliary shape in normal beating, reversed beating, intermediate beating and the upright-inactive state in this figure (and the inclined-inactive state in Fig. 4) the reader is referred to the drawings in Fig. 7. The numbers at the lower right of each frame represent time in milliseconds, where zero time is arbitrarily chosen at the first frame in each row. The effective stroke of normal beating was towards the left of the figure in the upper and middle rows and towards the right in the lower row. Scale bar, 50 μm.

Fig. 3. Frontal views of metachronal waves of normally beating cilia in the anterior epaulettes. Hard copies from high-speed video microscopy are shown in sequence (from top to bottom) at intervals of 10 ms. Arrowheads indicate the identical wave crests in propagating waves. Scale bar, 50 μm.

dexioplectic waves. Eight-armed plutei of *H. pulcherrimus* and *P. depressus* also exhibit such a combination of metachronal waves. As shown in Fig. 3, metachronal waves of the anterior epaulettes moved towards the mid-line on both the dorsal and ventral sides of the body. In the posterior epaulettes, in contrast, the waves moved from the mid-line towards the centre of the U-shaped ciliated bands (Fig. 4A). In each quarter of the body divided by dorso-ventral and mid-lateral lines, metachronal waves propagated in opposite directions in the anterior and posterior epaulettes (Fig. 1B). The waves propagating in opposite directions showed mirror-image patterns between the two adjacent epaulettes. The directions of wave propagation were preserved during reversed beating (Fig. 4B). Since the direction of the effective stroke was reversed, the dexioplectic waves changed to laeoplectic waves in the same ciliated bands, and *vice versa*. The metachronal waves during reversed beating had bigger crests than during normal beating.

After several successive transitions from normal to reversed beating, the last cycle of reversed beating terminated at the end of the recovery stroke (Fig. 2, lower row). A weak bend in the middle of the ciliary shaft gradually decayed and

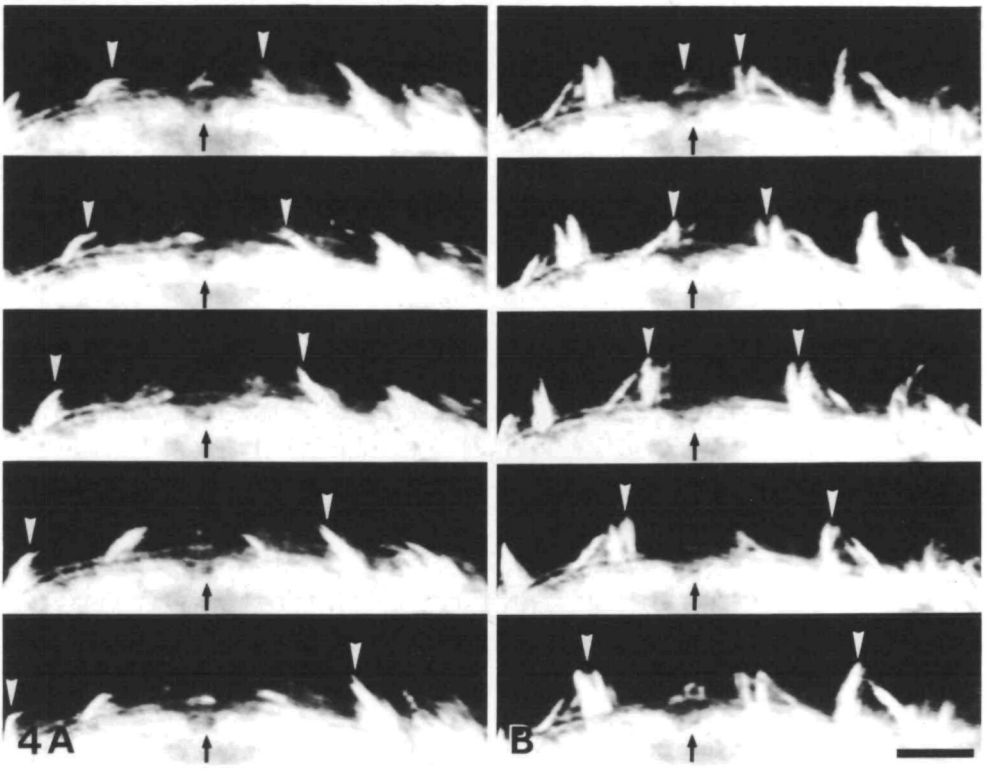

Fig. 4. Frontal views of metachronal waves of cilia of posterior epaulettes beating in normal (A) and reversed (B) directions. Hard copies from high-speed video microscopy are shown at intervals of 10 ms (from top to bottom). Arrowheads indicate the propagating wave crests, as in Fig. 3. Arrows indicate the boundaries of two adjacent epaulettes. Scale bar, 50 μm.

the cilia finally stood in the upright position and became motionless (Fig. 2, latter half of lower row). The quiescent cilia then slowly inclined posteriorly towards the end of the effective stroke of the normal beat (Fig. 5A–C), where they became quiescent again, although they occasionally showed spasmodic vibrations of small amplitude. The cilia maintained the inclined posture for several seconds and then suddenly flipped back to the upright position (Fig. 5). The flip-back movement was faster than the previous inclination, and consistently terminated within several tens of milliseconds, in contrast to the inclination movement, which occurred at various rates (Fig. 6). It should be noted that the time for the flip-back movement was almost equal to that for the transition from normal to reversed beating. We tentatively refer to the cilia in the upright position as being in the 'upright-inactive state' and those in the inclined position as being in the 'inclined-inactive state'. After several repetitions of this flip-flop between the upright-inactive state and the inclined-inactive state, the cilia resumed normal beating following the las

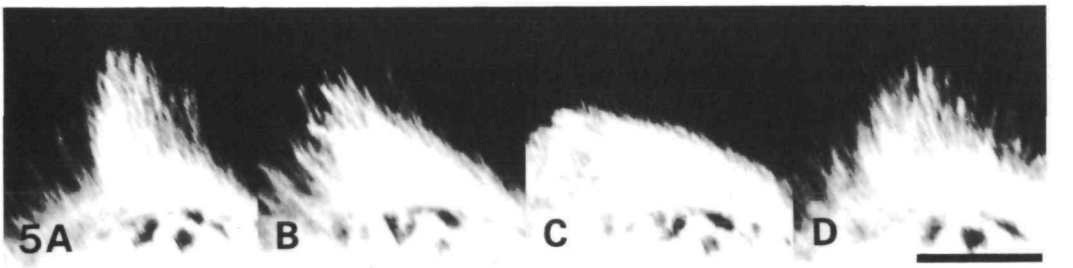

Fig. 5. Sequential images from high-speed video microscopy of transitions between the upright-inactive state (A and D) and the inclined-inactive state (C) in the posterior epaulette cilia. The effective stroke of normal beating was towards the left of the figure. Time and angular position for each frame are indicated by arrows on Fig. 6. Scale bar, 50 μm.

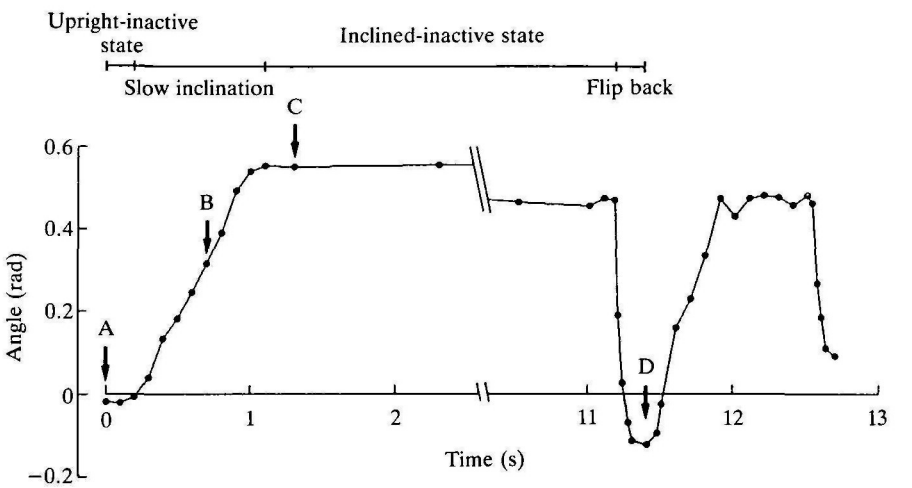

Fig. 6. Time course of angular changes of the ciliary shaft during transitions between the upright-inactive state and the inclined-inactive state. The angles are those between the distal half of the cilia and the perpendicular to the cell surface (positive for angles in the direction of the effective stroke of the normal beat). Marked arrows correspond to frames shown in Fig. 5.

inclined-inactive state. Direct transitions from the upright-inactive state to normal beating or to reversed beating were not observed.

The stereotyped responses of epaulette cilia and the sequence of transitions are summarized in Fig. 7. It should be pointed out that the cilia, once in the upright-inactive state after reversed beating, did not resume normal beating *via* reversed beating, but passed through the inclined-inactive state. In addition, the transitions from left to right of the figure, i.e. from normal beating to reversed beating as well as from the inclined-inactive state to the upright-inactive states, were significantly faster than those in the reversed direction.

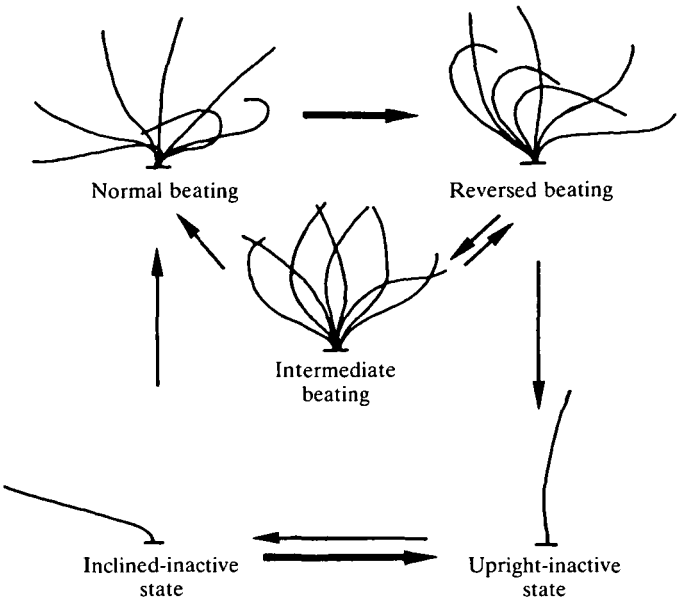

Fig. 7. Schematic drawings of the sequence of stereotyped responses of the epaulette cilia. Arrows indicate the direction of observed transitions. Thick arrows indicate faster steps.

Discussion

Five different states of activity have been distinguished in the epaulette cilia of echinoplutei, of which three showed cyclic motile activity and two did not. The normal and reversed beats of epaulette cilia showed similar patterns and beating forms to those of cilia in earlier developmental stages. Intermediate beating patterns have also been observed in the cilia of larvae at the late eight-armed pluteus stage, though they persisted for a shorter period than in the epaulette cilia. Upright-inactive and inclined-inactive states have not been identified in the earlier stages, even in cilia capable of a reversal response. The upright-inactive state in the epaulette was similar to the primitive response in the blastula and gastrula reported previously (Baba, 1975; Baba and Mogami, 1987), but it differed in temporal sequence. The primitive response occurs by an abrupt interruption of the effective stroke of the normal beat and is followed by direct recovery to the normal beat. The upright-inactive state occurs between a ciliary reversal and an inclined-inactive state. The inclined-inactive state of the epaulette cilia could be clearly distinguished from the primitive response not only because of a more inclined posture but also because of the sequence described above.

In ascidians and *Mytilus edulis*, cilia showed two types of inactivity (Takahashi *et al.* 1973; Mackie *et al.* 1974). The first stimulus produced a rapid arrest of beating in a deeply inclined posture in the direction of the recovery stroke; the arrested cilia slowly moved to the relaxed position with a rather perpendicular

posture, and subsequent stimuli caused a rapid flip back to the arrest position. The inactive state of the cilia in these organisms is quite similar to that of epaulette cilia, with the exception that epaulette cilia stop in a position largely biased in the direction of the effective stroke of normal beating and they perform reversed beating prior to inhibition of the beat. Comb plates of the ctenophore *Pleurobrachia* also exhibit similar types of inactivity as the first response to stimuli. Following the inhibitory phase, they perform reversed beating *via* a preparatory phase (called 'laydown' by Tamm and Moss, 1985), where the relaxation occurs near the base (Sleigh and Barlow, 1982; Tamm and Moss, 1985; Moss and Tamm, 1986). Sleigh and Barlow (1982) suggested that an arrest response normally occurs before the commencement of reversed beating. Thus, epaulette cilia of echinoplutei seem to be exceptional in having a short period of transition between normal and reversed beating, without any detectable cessation, as shown in early pluteus larvae (Baba and Mogami, 1987), and a long period of ciliary inactivity only after the reversed beat.

As shown in Fig. 7, transitions between normal beating and reversed beating occur in both directions, with intermediate beating occurring in the course of the return from reversed beating. Transitions between the upright-inactive state and the inclined-inactive state are also reversible. However, transitions from reversed beating to the upright-inactive state, as well as from the inclined-inactive state to normal beating, occur in one direction only. The two irreversible steps appear to divide the states of ciliary activity into two categories, one with, and the other without, cyclic beating, corresponding to the upper and lower halves of Fig. 7, respectively. In each category the cilia undergo a reversible transition between the states shown in the left and right halves of the figure.

Previous studies have revealed that ciliary reversal in echinoplutei is accompanied by bioelectric activity of ciliated cells (Mackie *et al.* 1969; Baba, 1975). It is inferred from the similarities in time course that the transitions between the upright-inactive and inclined-inactive states are also dependent on bioelectric control mechanisms. Reorientation or inclination responses in the absence of cyclic beating have been induced by electrical stimulation in the stigma cilia of *Ciona intestinalis* (Takahashi *et al.* 1973) and in the lateral gill cilia of *M. edulis* (S. A. Baba, unpublished observation). In both cases the electrical stimulation-induced inclination occurs in the direction of the recovery stroke and is much faster than the recovery from the inclined posture. Similarly, Mackie *et al.* (1974) reported that electrical activity occurs coincidentally with the first flip-back movement from the relaxed to the arrested position. Since ciliary reversal in echinoplutei is induced by intraciliary Ca^{2+} (Degawa *et al.* 1986), the fast transition from the state on the left to that on the right in Fig. 7 might be mediated by Ca^{2+}, which enters following membrane excitation.

The irreversible transitions between the states shown in the upper and lower halves of Fig. 7 could be compared to the deactivation and reactivation of beating in the lateral gill cilia of *M. edulis*. Inactive lateral cilia can be activated by the application of 5-hydroxytryptamine (5-HT) (Aiello, 1960) and/or by stimulating

the branchial nerve (Takahashi and Murakami, 1968). Nervous control of beating activity, including the initiation and cessation of cyclic movement, is a possibility, since several types of neurones have been identified in eight-armed plutei of *Strongylocentrotus droebachiensis* (Bisgrove and Burke, 1987), and at least two nervous components (most probably serotonergic and dopaminergic) occur in the epaulette of *H. pulcherrimus* (Nakajima, 1987). In fact, our preliminary experiments show that the swimming behaviour of plutei of *H. pulcherrimus* changes in the presence of 5-HT or dopamine at physiological concentrations in the external medium. The beating activity of the lateral gill cilia is controlled regardless of regenerative ciliary membrane activity (Saimi *et al.* 1983). The cyclic activity of epaulette cilia could be regulated by a separate system(s) from the system(s) for reversible transitions, which might be accompanied by transient influx of Ca^{2+} into the ciliary space.

The echinopluteus is an extraordinary example of metachrony because of the coexistence of laeoplectic and dexioplectic metachronal waves in the same organism. It has been demonstrated previously that diaplectic metachrony is intimately correlated with three-dimensional hydrodynamic interactions between moving cilia (Machemer, 1974; Sleigh, 1974). The coexistence of dexioplectic and laeoplectic metachrony in the epaulette cilia may, therefore, suggest that the cilia in adjacent epaulettes have a mirror-image organization in their internal motile machinery for different types of three-dimensional beating. Because the direction of metachronal wave propagation is preserved during reversed beating (Fig. 4), additional specialization might be required in the machinery, which makes it possible to switch the mode of three-dimensional movement following the conversion between normal and reversed beating.

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