

Ciliary Reversal without Rotation of Axonemal Structures in Ctenophore Comb Plates

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ABSTRACT We have used a newly discovered reversal response of ctenophore comb plates to investigate the structural mechanisms controlling the direction of ciliary bending. High K^+ concentrations cause cydippid larvae of the ctenophore *Pleurobrachia* to swim backward. High-speed cine films of backward-swimming animals show a 180° reversal in beat direction of the comb plates. Ion substitution and blocking experiments with artificial seawaters demonstrate that ciliary reversal is a Ca^{++} -dependent response. Comb plate cilia possess unique morphological markers for numbering specific outer-doublet microtubules and identifying the sidedness of the central pair. Comb plates of forward- and backward-swimming ctenophores were frozen in different stages of the beat cycle by an "instantaneous fixation" method. Analysis of transverse and longitudinal sections of instantaneously fixed cilia showed that the assembly of outer doublets does not twist during ciliary reversal. This directly confirms the existence of a radial switching mechanism regulating the sequence of active sliding on opposite sides of the axoneme.

We also found that the axis of the central pair always remains perpendicular to the plane of bending; more importantly, the ultrastructural marker showed that the central pair does not rotate during a 180° reversal in beat direction. Thus, the orientation of the central pair does not control the direction of ciliary bending (i.e., the pattern of active sliding around the axoneme). We discuss the validity of this finding for three-dimensional as well as two-dimensional ciliary beat cycles and conclude that models of central-pair function based on correlative data alone must now be re-examined in light of these new findings on causal relations.

Various modifications in the pattern of ciliary and flagellar beating are known to be caused by transient increases in free Ca^{++} acting directly on the $9 + 2$ axoneme itself (3–6, 15, 17, 20, 24, 34, 54, 56). These Ca^{++} -dependent motor responses include changes in the direction of ciliary beating in protozoa (8, 10, 26, 29, 34), reversal of the direction of flagellar bend propagation in trypanosomes (20), alterations in the symmetry of flagellar wave forms in sperm and algae (3–6, 24, 45), regulation of ciliary beat frequency (8, 9, 30, 31), and arrest of ciliary beating in various animals (12, 17, 32, 54, 56).

Despite their common ionic basis, little is known about the molecular mechanisms and axonemal components responsible for these motor responses. We investigate here the ultrastructural basis of one aspect of the Ca^{++} -regulatory system in cilia—the control of bend direction. This problem is closely related to the basic mechanism of how cilia and flagella beat. Bending is the result of active sliding between adjacent doublet microtubules, driven by dynein ATPase arms (16, 43, 46, 57), coupled with resistance to sliding, which converts translational

movement into local bending (58, 59). All nine doublets appear to be functionally equivalent with respect to sliding, except, in certain cases, for doublets 5 and 6 (25, 35). Because the direction of dynein force generation is unipolar (40), arms on opposite sides of the axoneme would, if active simultaneously, act antagonistically with respect to creating bends. Consequently, only the dynein arms (or resistive elements) on one side of the axoneme are believed to be activated at any one time to produce bends. The direction of bending is therefore assumed to be regulated, directly or indirectly, by some type of switching mechanism which activates or changes the effectiveness of doublet sliding at different sites around the axoneme (5, 7, 29, 36, 37, 39, 40, 44, 53, 55).

However, there is no evidence for such a radial regulatory mechanism operating within a fixed array of outer doublets. The alternative possibility, that changes in bend direction are accompanied by a corresponding rotation of the assembly of doublets, has never been ruled out. Indeed, several reports suggest that axonemal twisting may occur (14, 19, 37).

In previous work on protozoan cilia with a variable direction of beat, rapid chemical fixation was used to "freeze" the cilia for electron microscopy (23, 36, 37, 53). These studies showed that the plane of the central-pair microtubules changes orientation depending on the direction of the effective stroke and stage in the three-dimensional beat cycle.

However, because markers for numbering the doublets are not available in protozoan cilia, the relationship between the orientation of specific doublets and the variable direction of bending could not be determined in these studies. Hence, it could not be decided whether the observed shifts in central-pair orientation were due to rotation of this part alone, or of the entire $9 + 2$ as a unit.

The former possibility, if found to be true, would provide circumstantial evidence for a switching mechanism in cilia. Assuming this to be the case, it was suggested that active rotation of the central pair serves to regulate the pattern of doublet sliding (36, 37, 53). However, the correlation observed between central-pair orientation and bend direction (53) is equally consistent with a passive role for the central pair (53).

Thus, due largely to the inherent limitations and technical difficulties associated with protozoan cilia, it has not been possible to demonstrate the existence of a radial switching mechanism coordinating doublet sliding, nor to determine whether the central pair plays an active role in regulating beat direction.

We therefore developed a new and more advantageous system for analyzing the structural control of bend direction. This system is based on our discovery that comb plate cilia of ctenophores undergo a Ca^{++} -dependent reversal in beat direction. Comb plate cilia possess unique morphological markers, not present in protozoan cilia, for numbering the outer doublets and identifying the sidedness of the central pair. By applying the instantaneous fixation method to the ctenophore ciliary reversal system, we show here that the array of outer doublets does not twist when beat direction reverses. This result provides strong support for the existence of a switching mechanism in cilia.

In addition, because the beat cycle of comb plates is planar, and the directional shift is 180° , this system allows us to distinguish an active (i.e., rotational) response of the central pair from a passive (i.e., no rotation) one. By finding that the central pair does not change orientation during ciliary reversal, we conclude that the switching mechanism that signals the pattern of doublet sliding does not involve rotation of the central pair. A preliminary account of some of these results has been presented previously (49, 50, 52).

MATERIALS AND METHODS

Ctenophores

This report deals with cydippid larvae of *Pleurobrachia pileus*. Similar but less extensive observations were made on cydippid larvae of *Mnemiopsis leidyi*. In addition, behavioral observations were also made on adult ctenophores of both species, as well as on *Lampea pancerina*, a ctenophore from the open ocean.

Sexually mature *Pleurobrachia* were dipped from the sea at Woods Hole, Mass., during early summer, 1978 and 1979. *Mnemiopsis* were collected at Woods Hole during late summer, 1979 and 1980. *Lampea pancerina* were collected in the Sargasso Sea in June 1980 during scuba dives from the R. V. *Oceanus*, operated by the Woods Hole Oceanographic Institution.

To obtain cydippid larvae, freshly collected *Pleurobrachia* or *Mnemiopsis* were placed in large glass bowls of seawater at ambient ocean temperatures. By the following morning, large numbers of eggs in early cleavage stages were found at the bottom of the bowls. The eggs were transferred to fresh seawater and allowed to develop into free-swimming cydippid larvae. 2- to 3-d-old larvae were typically used for most experiments.

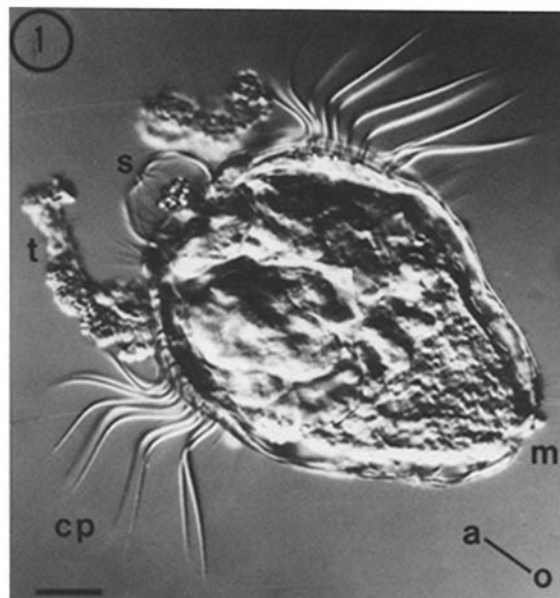


FIGURE 1 Cydippid larva of *Pleurobrachia* swimming forward in seawater. The statocyst (*s*) and mouth (*m*) define the aboral-oral axis (*a-o*) of the larva. At this stage of development, the eight rows of comb plates (*cp*) are grouped into four pairs. On either side of the larva, a pair of closely spaced rows is caught in profile by electronic flash. Because the metachronal waves in each pair are almost in phase, they appear as a "double image." The plates nearest the statocyst have already performed an effective stroke, and are beginning to unroll toward the mouth in the recovery stroke. Plates closer to the oral end are in the late-to-middle phases of the effective stroke. The tentacles (*t*) are partially retracted. Zeiss Nomarski, 1/3000 s. Bar, 30 μm .

Artificial Sea Waters

The ionic basis of the ciliary responses was tested with artificial seawaters (ASW) of different compositions (Table I).¹

Ciliary Responses

Cydippid larvae were collected from the bowls with a braking pipet. Several hundred larvae in excellent condition could be concentrated in 5–10 min by this method.

Ciliary responses of the larvae to different ionic conditions were determined by pipetting 20–30 larvae (in about 1 drop of seawater) into a depression, and then adding an excess volume (~0.5 ml) of the desired ASW. The direction and speed of swimming, as well as the frequency of beating of the comb plates, were followed under a dissecting microscope and quantified by cinemicrography.

Cinemicrography

The patterns of ciliary beating in forward- and backward-swimming larvae were recorded on Plus X Negative film at 200 or 400 frames/s with a Locam 16-mm movie camera (Redlake Laboratories, Santa Clara, Calif.) and a Zeiss Universal microscope ($\times 16$ objective, Nomarski optics). Frame-by-frame analysis of the films was performed with a L-W Photo Optical projector (L-W International, Woodland Hills, Calif.).

Electron Microscopy

A rapid osmium tetroxide fixation method (38), modified for our study, was used to freeze the movements of comb plates in forward- and backward swimming *Pleurobrachia* larvae. This "instantaneous" fixation method has been shown to preserve the pattern of ciliary coordination and the form of ciliary beat in both protozoa and metazoa (28, 41, 47, 53). Relative sliding displacement between outer doublets (42), changes in radial spoke configuration (58), and shifts in

¹ See also G. M. Cavanaugh, editor. 1965. *Formulae and Methods VI*, Marine Biological Laboratory, Chemical Room.

orientation of the central pair (36, 37, 53) are also dynamically preserved by this technique. However, instantaneous fixation does not reveal dynein arm cross-bridging between doublet microtubules, as has been visualized by other methods (14, 61). Rapid fixation appears therefore to preserve the effect of the ciliary

machine but not the working of its parts. Osmium probably acts primarily on the ciliary membrane, leading to a secondary immobilization of axonemal components via the various connections between these structures and the membrane.

About six drops of either Marine Biological Laboratory (MBL) ASW (for

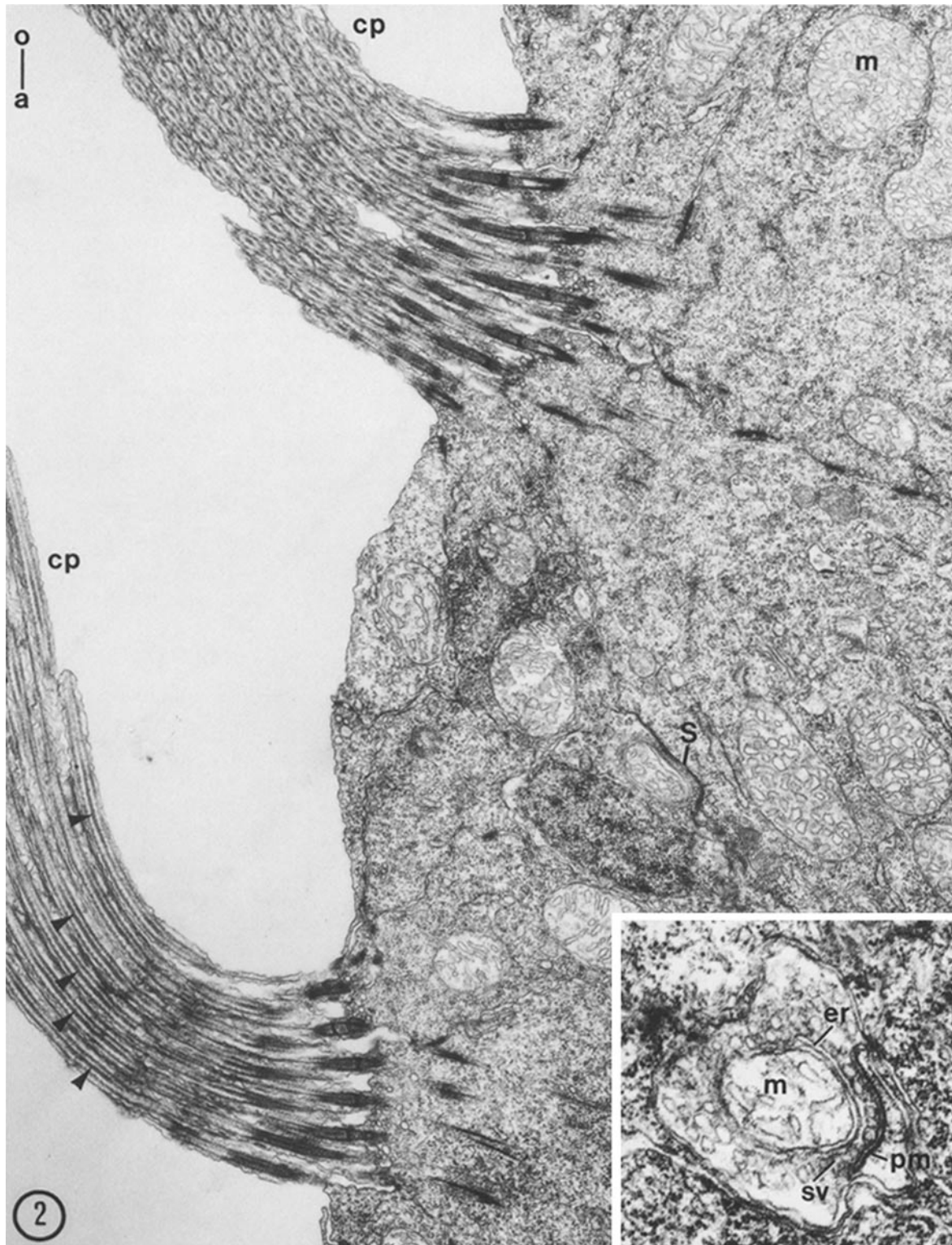


FIGURE 2 Synapse (*s*) of a neurite onto a cell bearing comb plate cilia (*cp*) in a *Pleurobrachia* larva. Note the giant mitochondria (*m*) in the cytoplasm of the comb plate cells. The base of the lower comb plate is bent in the oral direction (*a-o*, aboral-oral axis). Note that the central-pair microtubules are stacked so that only one tubule is seen in longitudinal sections through the center of the cilia (arrowheads). The plane of the central pair is therefore oriented perpendicular to the direction of bending (see also, Fig. 12). $\times 16,600$. *Inset*: a neurociliary synapse at higher magnification. Ctenophore synapses characteristically consist of a synaptic cleft with a thickened postsynaptic membrane (*pm*), a single layer of synaptic vesicles (*sv*), a sac of smooth endoplasmic reticulum (*er*) immediately behind the vesicles, and one or two closely apposed mitochondria (*m*). $\times 41,600$.

forward locomotion) or high-K⁺ ASW (to induce backward swimming) were added to several dozen larvae in 1 drop of seawater in a glass depression. Swimming behavior was observed immediately under a dissecting microscope, and 15–30 s later a tenfold excess of instantaneous fixative was rapidly pipetted onto the swimming larvae. Instantaneous fixative consisted of 2.5% glutaraldehyde, 2% osmium tetroxide, 0.16 M sodium cacodylate buffer, pH 7.4, and 0.16 M NaCl (room temperature). Microscopic observations showed that almost all the larvae in high-K⁺ ASW were swimming backward at the time of fixation, whereas those in MBL ASW were swimming forward.

Larvae were fixed for 5–10 min, then washed in buffer (0.2 M sodium cacodylate [pH 7.4], 0.3 M NaCl) for 30–60 min, and postfixed in 2.5% glutaraldehyde, 0.2 M sodium cacodylate buffer, pH 7.4, 0.14 M NaCl for 60–90 min. Following a second buffer rinse, larvae were dehydrated rapidly in acetone, and flat-embedded in a thin layer of Araldite. At each step of the procedure, solutions were changed in the depression slide without centrifugation.

Individual flat-embedded larvae were examined with a light microscope. Larvae were selected with comb plates fixed in metachronal patterns resembling those seen in single frames from high-speed cine films of forward- and backward-swimming animals (Figs. 3–5). It was necessary to confirm the quality of fixation in this way because not all cilia are instantaneously stopped by osmium, even in the best preparations (27). This variability is well known to workers in the field, and may be due to slight spatial and temporal variations in the concentration of fixative reaching different organisms, or different cilia on the surface of the same individual (28).

Once selected for analysis, the beat pattern of the larvae was recorded, and the animals were cut out of the Araldite and mounted on stubs in known orientation for transverse or longitudinal sections through comb plates in specific stages of the beat cycle. The blocks were trimmed so that the asymmetry of the trapezoid bore a constant relationship to the aboral-oral axis of the larva. This allowed the body axis of the animal, and thus the direction of the effective stroke, to be determined in cross sections through comb plates. Sections were picked up on formvar-coated grids, stained with uranyl and lead salts, and viewed in a Philips 300 electron microscope at 80 kV.

RESULTS

General Morphology

Free-swimming cydippid larvae closely resemble adult ctenophores of the order Cydippida (i.e., *Pleurobrachia*). The mouth defines the oral end of the body, and a prominent statocyst is located at the opposite, aboral end (Fig. 1). 2- to 3-d-old cydippid larvae of *Pleurobrachia* are 200–300 μm long; those of *Mnemiopsis* are about twice as large.

The ciliary system of cydippid larvae is disproportionately large in relation to the body size. Like adults, larvae possess eight rows of ciliary comb plates which run in an aboral-oral direction (Fig. 1). Each comb row contains about half a dozen comb plates at this stage. A single plate consists of hundreds of long cilia, 50–80 μm in length, which beat together as a unit.

A nervous system is already present in larval stages. Synapses with the characteristic ultrastructure of synaptic contacts in adult ctenophores (18, 52) are found onto the comb plate cells of cydippid larvae (Fig. 2).

Pattern of Ciliary Activity

The beat cycle of comb plates takes place entirely in one plane, parallel to the aboral-oral axis. The effective stroke is a rapid swing of the plates toward the aboral end, propelling the animal mouth foremost. In the recovery stroke, the plates unroll in the opposite direction by propagating a bend distally up the cilia (Figs. 1, 3, 5 *a-f*, and 6).

Beat frequency of the comb rows is commonly controlled by the aboral statocyst (Fig. 1), which mediates geotactic responses of the animals (50, 52). Four groups of motile mechanoreceptor cilia in the statocyst act as pacemakers for the four pairs of comb rows (50, 52). Beating starts at the pacemaker cilia and travels as a wave of activity down the comb rows in an oral direction (Figs. 3 and 5 *a-f*). Consequently, the direction of the metachronal waves is opposite to that of the effective stroke (antiplectic metachronal coordination). The comb plates of cydippid larvae are thought to be coordinated by mechanical (hydrodynamic) interaction, as shown to be the case for adult *Pleurobrachia* (48, 50, 52).

Beat frequency of the comb plates may also be regulated by pathways independent of the statocyst (52). For example, temporary inhibition of beating or increases in beat frequency can be elicited by appropriate mechanical stimulation of the larvae. In adult ctenophores, these motor responses are thought to be controlled by nerves (52). The neurociliary synapses in larvae may serve similar functions, but may also be involved in triggering ciliary reversal (next section).

In undisturbed larvae swimming forward in a horizontal plane, the beat frequency of the comb plates is typically 3–5 Hz (cf. Fig. 3).

Ciliary Reversal in *Pleurobrachia* Larvae

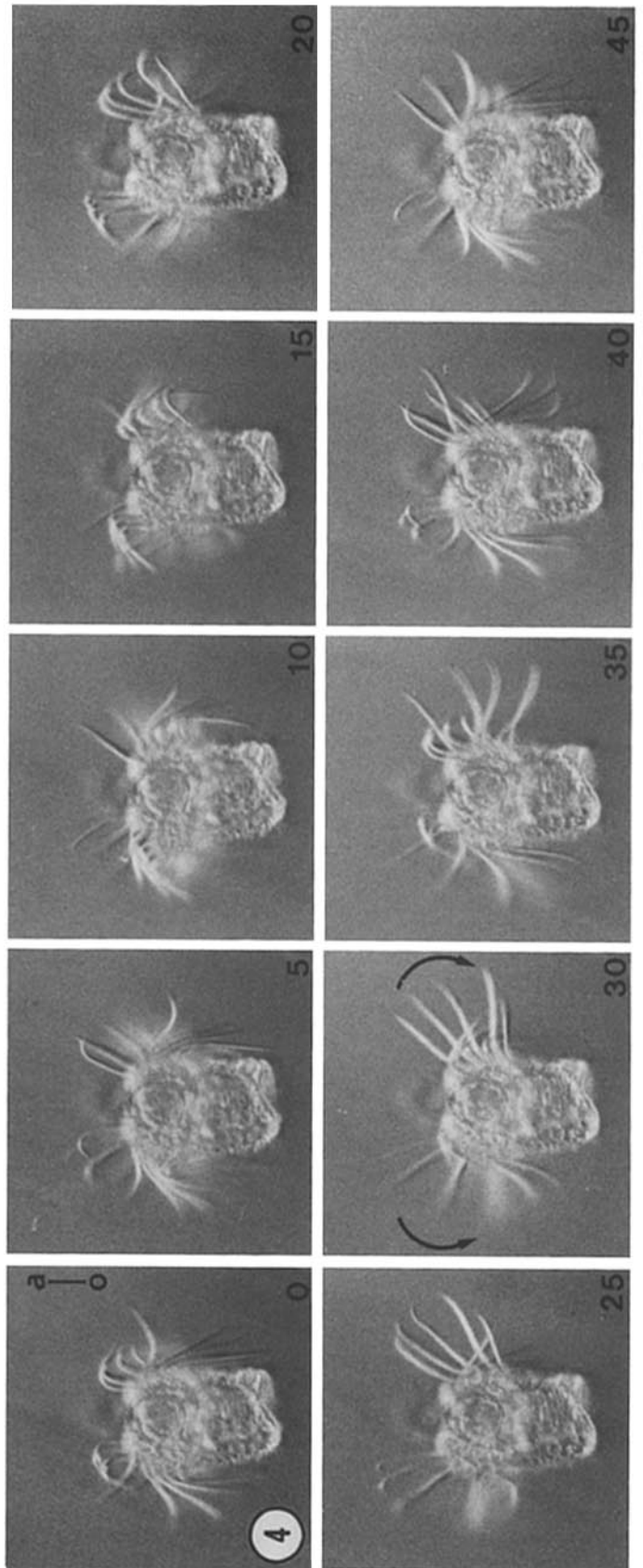
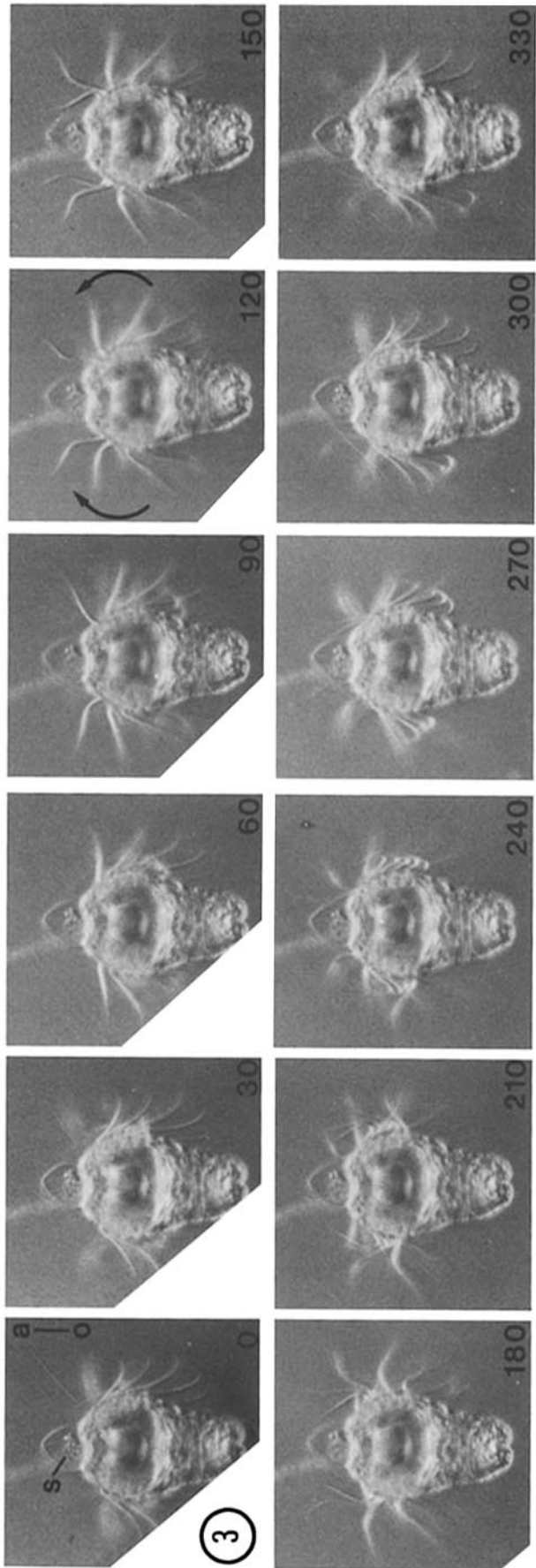
We discovered initially that 50–100 mM KCl in sea water causes cydippid larvae of *Pleurobrachia* to swim backwards for a brief time. Subsequently, isotonic artificial sea water containing 100 mM KCl (high-K⁺ ASW) was routinely used to evoke this response.

Backward locomotion in high-K⁺ ASW is accompanied by an increased beat frequency of 20–25 Hz (Fig. 4), but not by an increase in swimming speed. After 3–5 min in high-K⁺ ASW, the larvae gradually resume forward swimming; however, high beat frequency continues for >30 min.

High-speed cinematography shows that backward swimming is due to a 180° reversal in the beat direction of all comb plates (Figs. 4, 5 *g-l*, and 6). The effective stroke is directed toward the mouth, and the plates unroll in the aboral direction

FIGURE 3 *Pleurobrachia* larva swimming forward in seawater, from a cine film taken at 200 frames/s (fps) and printed at 30-ms intervals (ms at lower right of each print). Note statocyst (s) at aboral end (a-o, aboral-oral axis). The two comb rows of a pair are seen in profile on each side, but they are not beating in phase. However, the in-focus rows on either side are in phase, and these rows are followed here. The figure shows one complete beat cycle, beginning with plates at the end of the recovery stroke, pointing toward the mouth (time 0). The effective stroke takes place in the aboral direction, with the plates nearest the statocyst performing a beat first (60 ms print), followed by plates more orally (arrows). The plates unroll toward the mouth in the recovery stroke (lower row), also in an aboral-to-oral sequence. $\times 135$.

FIGURE 4 *Pleurobrachia* larva swimming backwards in high-K⁺ seawater, from a cine film taken at 400 fps and printed at 5-ms intervals to show one complete beat cycle. The two comb rows on each side are out of phase. At time 0, the plates of the in-focus rows on either side are at the end of the effective stroke, pointing toward the mouth. The plates unroll toward the statocyst during the recovery stroke, with those closest to the mouth initiating the recovery stroke first (5 and 10 ms). The effective stroke (lower row) is reversed 180° toward the mouth (arrows, 30 ms). As in the recovery stroke, plates are triggered to beat in an oral-to-aboral (o-a) sequence (30–40 ms), opposite to the sequence during forward swimming (cf. Fig. 3). $\times 135$.



during the recovery stroke. The form of the beat cycle during backward swimming is similar to that in forward-swimming larvae (Fig. 6). Intermediate concentrations of K and Ca (see below) do not elicit graded shifts in beat direction; instead, ciliary reversal in ctenophores is always a 180° all-or-nothing response.

During backward swimming, the plates are triggered to beat in an oral-aboral sequence, opposite to the usual direction of coordination (Figs. 4 and 5 g-l). Therefore, the type of metachrony does not change, but remains antiplectic during ciliary reversal.

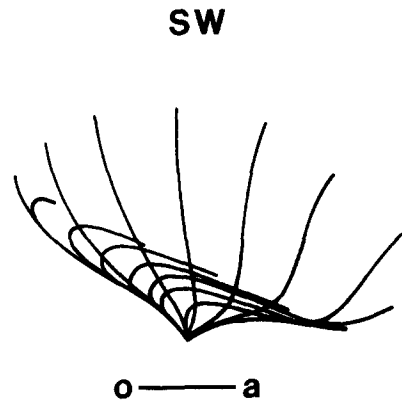
Cydidippid larvae of *Mnemiopsis* also show a 180° ciliary reversal, accompanied by backward locomotion. However, the reversal response in *Mnemiopsis* larvae is not triggered by an increase in KCl concentration, as in *Pleurobrachia*, but by an increase in the external concentration of Ca^{++} (50 mM), even at normal KCl levels.

Ion Dependence of Ciliary Responses

The ionic basis of ciliary reversal in *Pleurobrachia* larvae was investigated by ion substitution and competition experiments using artificial seawaters.

Table I shows that KCl-induced reversal does not occur in Ca^{++} -free high- K^+ ASW. Ciliary reversal is also prevented by the addition of Ca^{++} competitors, such as Mg^{++} , Mn^{++} , and Co^{++} , to high- K^+ seawater. Replacing external Na^+ with choline chloride or Tris-HCl, however, does not prevent backward swimming. Thus, ciliary reversal in *Pleurobrachia* larvae is Ca^{++} dependent, but not Na^{++} dependent.

The close association between ciliary reversal and increased beat frequency (Table I) indicates that beat frequency is also a Ca^{++} -dependent response. Indeed, increasing the external Ca^{++} concentration of high- K^+ ASW appears to cause a greater frequency of reversed beating than does high- K^+ ASW alone. However, increasing the Mg^{++} concentration of high- K^+ ASW results in high beat frequency without ciliary reversal. In addition, the increased frequency of beating in high- K^+ ASW persists longer than does the reversed beating. These examples



KCl-SW

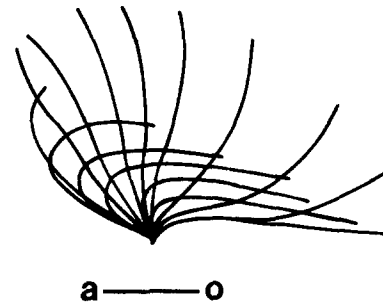


FIGURE 6 Sequence of profiles showing one complete beat cycle of a comb plate from a forward-swimming (top, drawn at 20-ms intervals) and a backward-swimming (bottom, drawn at 2.5-ms intervals) *Pleurobrachia* larva. Both plates are oriented with the effective stroke directed to the reader's right, and the recovery stroke to the left. Note that the form of reversed beating is similar to that of normal beating. SW, seawater; KCl-SW, high- K^+ seawater.

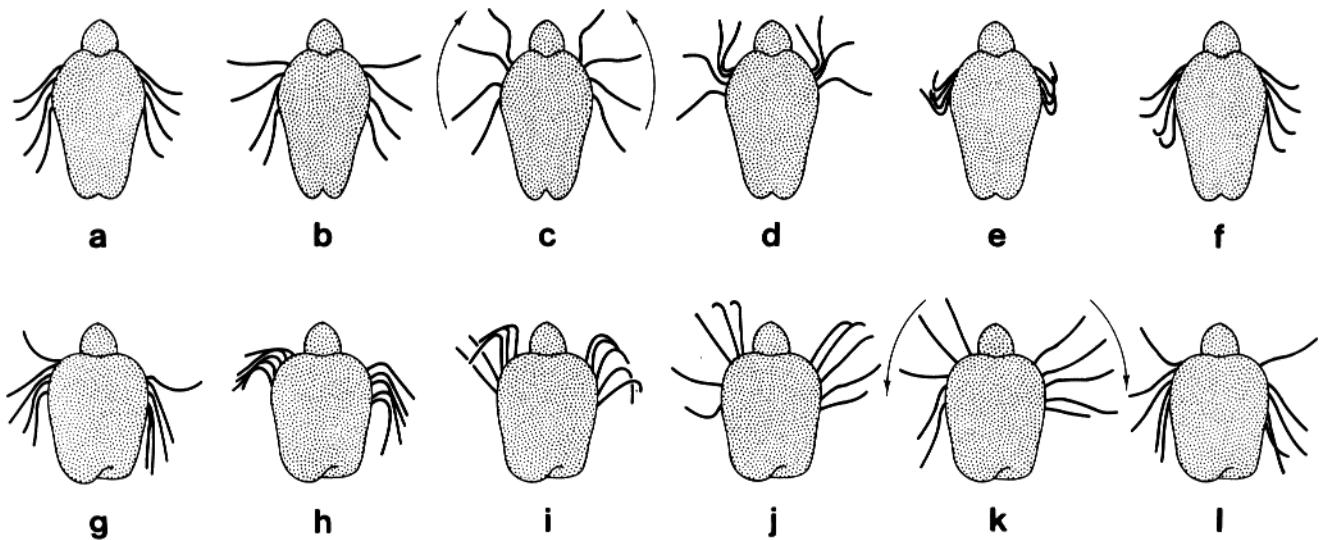


FIGURE 5 Tracings of in-focus rows on either side of the forward (upper row) and backward (lower row) swimming larvae shown in Figs. 3 and 4, respectively. a-f are drawn at 60-ms intervals; g-l are drawn at 8-ms intervals. Orientation of the a-o axis is the same as in Figs. 3 and 4. The direction of effective stroke (arrows c and k) is reversed 180° towards the mouth during backward swimming. Adjacent plates are triggered to beat in an a-o order during forward swimming (a-f), but in an o-a sequence during backward swimming (g-l). Therefore, the pattern of metachronal coordination remains antiplectic during ciliary reversal.

of uncoupling of the directional and frequency responses indicate that the two parameters may be regulated by Ca^{++} in different ways (see Discussion).

Ultrastructural Markers

The comb plate cilia of larvae, like those of adults, possess unique morphological markers for identifying specific outer doublet microtubules and defining sidedness of the central pair (Figs. 7–11). Flangelike longitudinal connections extend from doublets 3 and 8 to the ciliary membrane, linking adjacent axonemes into rows running normal to the plane of bending (Fig. 7). These connections are called compartmenting lamellae (1) because they divide the cilium into two unequal compartments, one containing three doublets (9, 1, 2), and the other containing four doublets (4, 5, 6, 7). The compartmenting

lamellae run the entire length of the cilia, and thus provide unambiguous markers for numbering the nine outer doublets at any level within the comb plate.

The compartmenting lamellae probably help to synchronize the beating of all the cilia within a comb plate, inasmuch as microsurgery on single plates shows that adjacent cilia are synchronized by hydrodynamic coupling between them (51).

An electron-dense body, called a midfilament (1), is found on only one side of the central pair (Figs. 7–11). This structure is generally round or oval in outline, and is present along the whole length of the cilia (Figs. 8–11). A similar but smaller dense dot, termed a midfiber, has been observed in association with the central pair in mussel gill cilia (13). In both cases, it is unclear whether these structures represent continuous longitudinal elements or periodic projections from the central pair. On the opposite side of the central pair, each tubule bears a

TABLE I
Effects of Different Ions on the Beat Pattern of Comb Plates of Pleurobrachia Cydippid Larvae

	Artificial Sea Waters*										
	MBL	High-K ⁺	High-K ⁺ Ca ⁺⁺ -free	High-K ⁺ Mg ⁺⁺ -free	High-K ⁺ High-Ca ⁺⁺	High-K ⁺ High-Mg ⁺⁺	High-K ⁺ High-Mn ⁺⁺	High-K ⁺ High-Co ⁺⁺	High-K ⁺ Na ⁺ -free Choline	High-K ⁺ Na ⁺ -free Tris	High-Mg ⁺⁺
	1	2	3	4	5	6	7	8	9	10	11
Reversed beating	-	+	-	+	+	-	-	-	+	+	-
High frequency	-	+	-	+	++	+	-	-	+	+	-

+ and - indicate the presence or absence, respectively, of ciliary reversal (i.e., backward swimming) or of a frequency of beating higher than in MBL ASW.

* Composition (mM).

- (1) 423 NaCl, 9 KCl, 9 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (2) 333 NaCl, 100 KCl, 9 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (3) 347 NaCl, 100 KCl, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (4) 391 NaCl, 100 KCl, 9 CaCl₂, 51 Na₂SO₄, 2 NaHCO₃.
- (5) 273 NaCl, 100 KCl, 50 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (6) 170 NaCl, 100 KCl, 9 CaCl₂, 100 MgCl₂, 100 MgSO₄, 2 NaHCO₃.
- (7) 333 NaCl, 100 KCl, 9 CaCl₂, 20 MnCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (8) 333 NaCl, 100 KCl, 9 CaCl₂, 20 CoCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (9) 333 Choline Cl, 100 KCl, 9 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (10) 380 Tris-HCl (pH 7.0), 100 KCl, 9 CaCl₂, 23 MgCl₂, 26 MgSO₄, 2 NaHCO₃.
- (11) 270 NaCl, 9 KCl, 9 CaCl₂, 100 MgCl₂, 100 MgSO₄, 2 NaHCO₃.

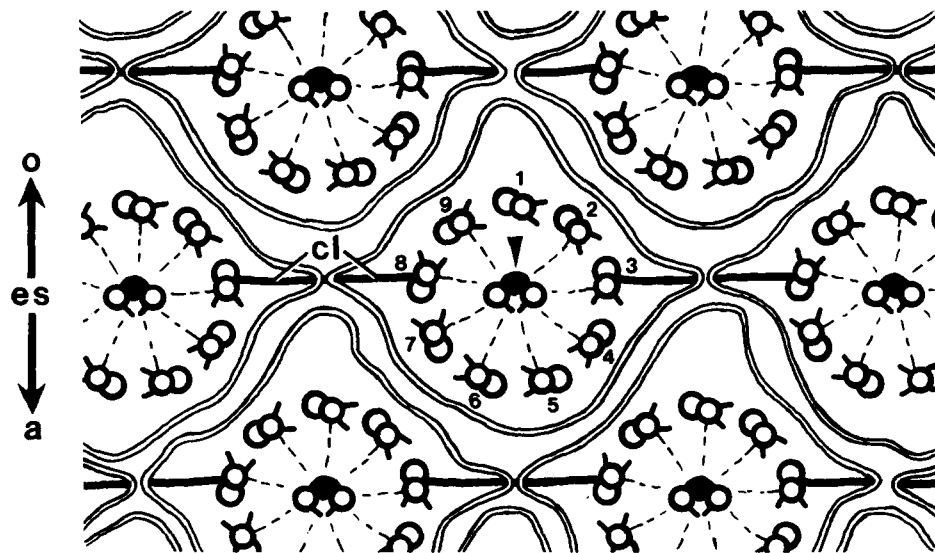
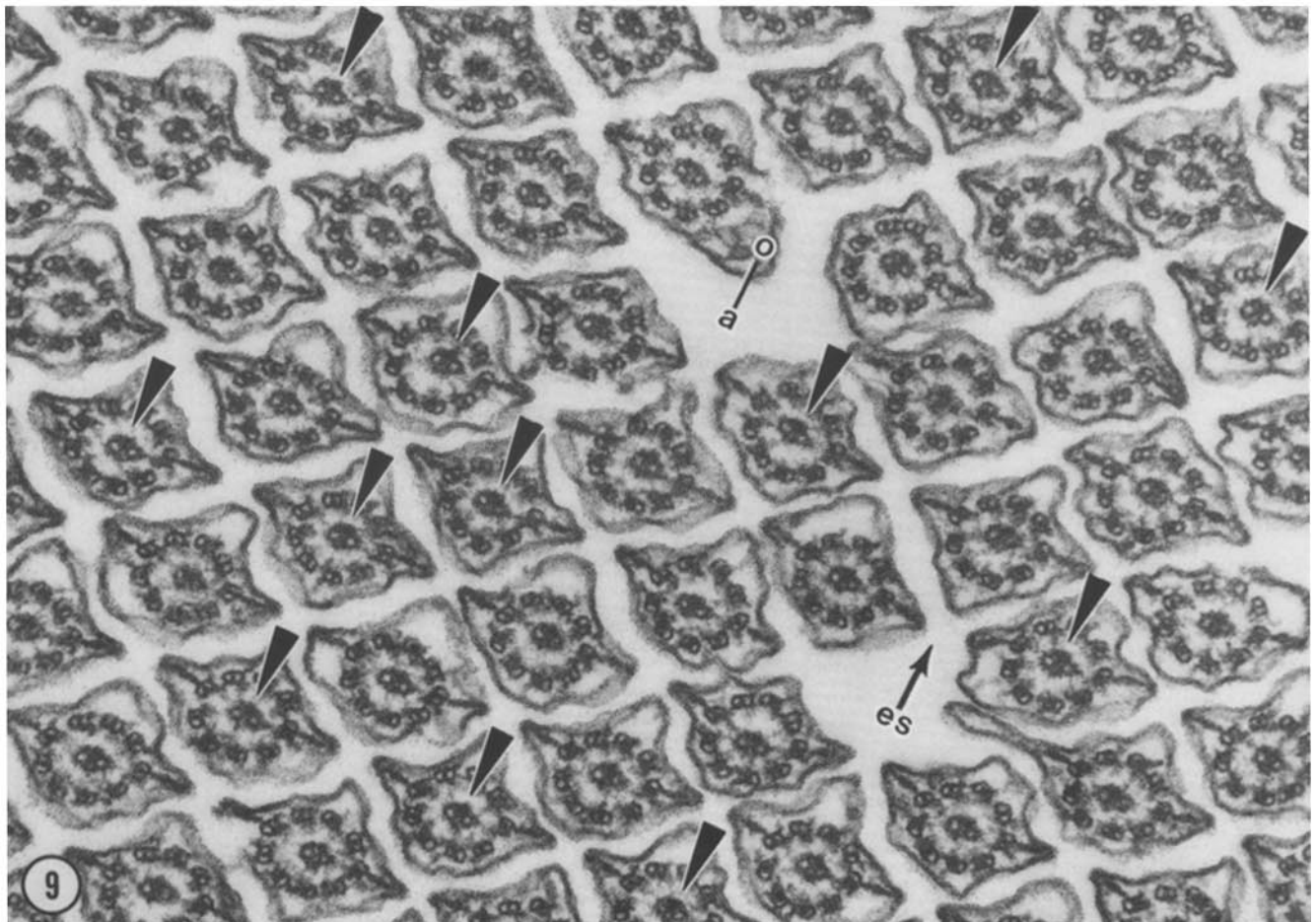
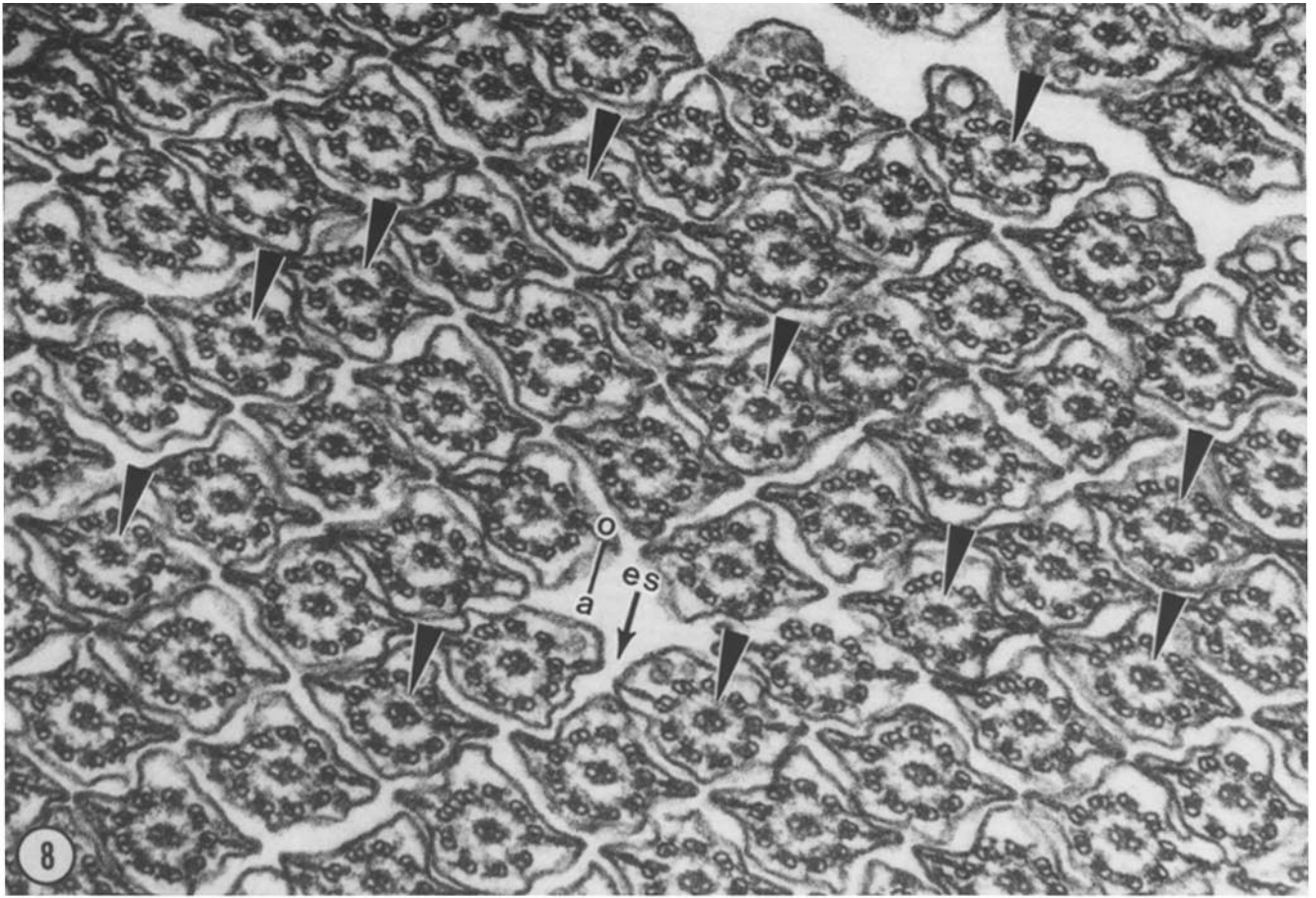


FIGURE 7 Diagrammatic cross-section through comb plate cilia of a cydippid larva. During forward swimming the effective stroke (es) is directed aborally (a); during backward swimming it is reversed 180° toward the oral end (o). Compartmenting lamellae (cl) join doublets 3 and 8 of adjacent cilia into rows running normal to the plane of bending. The cilia are thus divided into a three-doublet side (9, 1, 2) and a four-doublet side (4, 5, 6, 7). A dense midfilament (arrowhead in central cilium) lies on only one side of the central pair. These axonemal markers do not change their orientation when beat direction reverses (cf. Figs. 8–11).



single row of projections which point toward one another, forming an arch (Figs. 7–11). The midfilament in comb plate cilia thus provides a convenient marker for distinguishing one side of the central pair from the other, and allows us to determine whether or not the central pair rotates during ciliary reversal.

Relation between Axonemal Structures and Beat Direction

To determine whether the orientation of the central pair or the outer doublets is correlated with beat direction, the orientation of the axonemal markers was compared in cross sections through plates “frozen” at known stages of the beat cycle in forward- vs. backward-swimming *Pleurobrachia* larvae (Figs. 8 vs. 9, 10 vs. 11). Sections through plates fixed at different stages of the beat cycle (i.e., effective vs. recovery strokes) in forward- and backward-swimming larvae were also examined. In addition, cross sections at different levels from base to tip of the plates were compared (Figs. 8 vs. 10, 9 vs. 11). In contrast to protozoan cilia, the relatively large size and wide spacing of the comb plates allowed direct visualization of the direction and location of bend regions in flat-embedded cilia that were subsequently cross-sectioned.

We found that the axis of the central pair is always perpendicular to the plane of bending regardless of (a) the direction of effective stroke, (b) the stage of the beat cycle, (c) the level from base to tip along the cilia, and (d) whether actively bending vs. straight regions of the plate are examined.

In addition, longitudinal sections were cut through distally propagated recovery stroke bends (Fig. 12), and though the sharply bent region at the base of resting and recovery stroke plates (Fig. 2). Longitudinal sections through the center of the axoneme and parallel to the bend direction show only one tubule of the central pair (Figs. 2 and 12). This confirms that the two central tubules are aligned on a plane perpendicular, not parallel, to the direction of bending. These findings contradict those of Omoto and Kung (37) on *Paramecium* cilia, but agree with most other studies of central-pair orientation in protozoan (53) and metazoan cilia (1, 11, 13, 14, 21).

More importantly, we found no change in orientation of the compartmenting lamellae or the midfilament with respect to the aboral-oral axis in forward- vs. backward-swimming larvae (Figs. 8 and 10 vs. 9 and 11). During both normal and reversed beating, doublet 1 (i.e., the three-doublet compartment) and the midfilament lie on the oral side of the cilia. This finding holds true from base to tip of the comb plates, and in bent as well as in straight regions of the cilia. Therefore, the effective stroke is directed toward doublets 5 and 6 and away from the midfilament during normal beating, but toward doublet 1 and the midfilament during ciliary reversal (Fig. 7).

These results show that the Ca^{++} dependent 180° reversal in beat direction is not accompanied by a corresponding rotation of the central pair or of the assembly of outer doublets. Thus, the pattern of doublet sliding must be regulated around the

axoneme, but the control mechanism does not involve rotation of the central-pair microtubules (see Discussion).

Our findings also correct a minor error made in previous studies on ctenophore comb plates. We consistently found that doublet 1 faces the oral side of the cilia in both larval and adult comb plates of *Pleurobrachia* and *Mnemiopsis*, regardless of the fixation method used. Afzelius (1) reported that doublet 1 lies on the aboral side of comb plate cilia in adult *Mnemiopsis*, so that the normal effective stroke is directed toward doublet 1. This result, later adopted by Horridge (22), was probably due to an error in specimen or section orientation (B. A. Afzelius, personal communication).

Ciliary Reversal in Adult Ctenophores

We have made preliminary observations which reveal that adult ctenophores are also capable of ciliary reversal responses. For example, mature *Pleurobrachia* are sometimes observed to swim backward for a brief time, indicating a temporary ciliary reversal. However, we were unable to induce backward locomotion by high- K^+ seawater, which triggers reversed beating in *Pleurobrachia* larvae. Increasing the Ca^{++} -concentration of seawater to 50 mM, on the other hand, does stimulate ciliary reversal in adult *Pleurobrachia*.

Similarly, we found that high KCl concentrations do not cause backward swimming in adult *Mnemiopsis*, but elevated Ca^{++} levels (i.e., 50 mM) do trigger reversed beating, as in larvae of this species (see above). In addition, high-speed cinematography of *Lampea pancerina*, performed aboard the R. V. *Oceanus*, shows that high- K^+ seawater triggers ciliary reversal in this ctenophore from the open ocean. At present we do not understand the reason for the differences between species, and between larval vs. adult *Pleurobrachia* in the ion-sensitivity of their reversal responses.

Recently, we documented a localized pattern of ciliary reversal that occurs during the feeding response of adult *Pleurobrachia* (50, 52). After catching copepods with one of the long tentacles, the two comb rows adjacent to the food-capturing tentacle temporarily beat in the reversed direction at a high frequency, sweeping the food toward the mouth. This unilateral ciliary reversal is apparently triggered by nervous stimuli from the tentacles (52), and provides a useful system for investigating the neural control of ciliary beat direction.

DISCUSSION

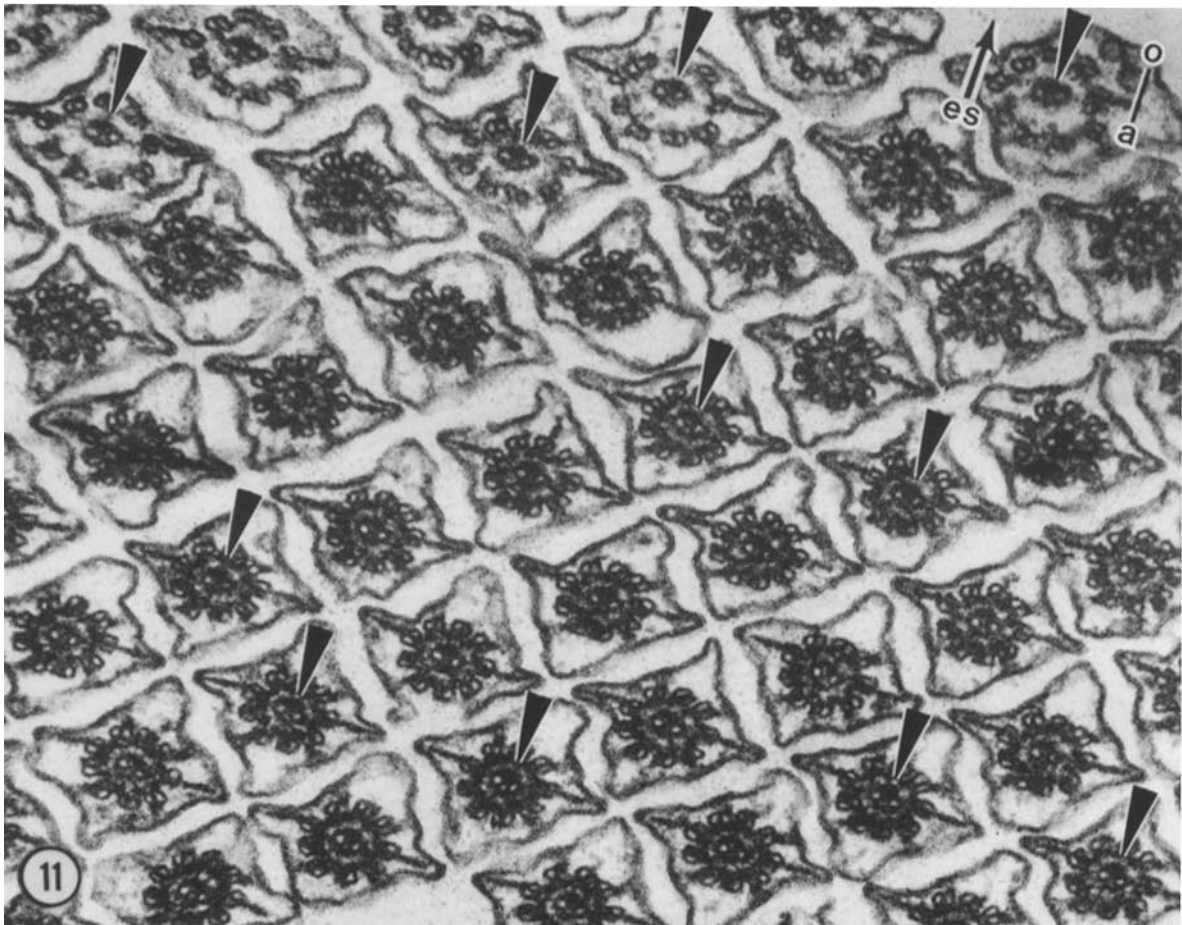
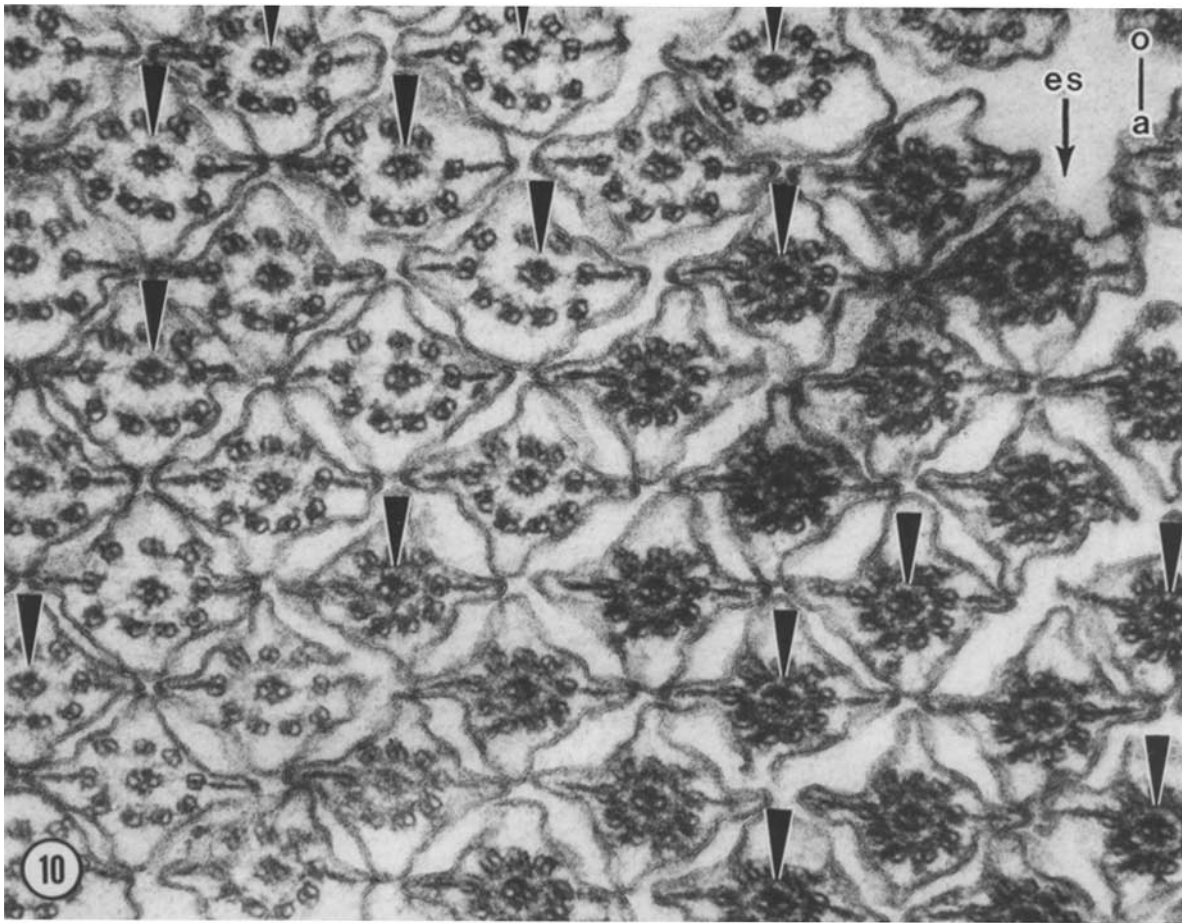
Ciliary Reversal in Metazoa

Changes in the direction of ciliary beating occur commonly in protozoa, as avoidance responses to unfavorable stimuli (33). These directional responses are typically graded with the intensity of the stimulus, and are evident not only by changes in the path of swimming, but by direct visualization of the cilia themselves (28, 53).

Reports of ciliary reversal in multicellular animals are much rarer, and rely on indirect indicators such as backward swim-

FIGURE 8 Cross section midway along the length of a comb plate fixed in late effective stroke from a forward-swimming *Pleurobrachia* larva. Doublet 1 (i.e., the three-doublet side of the cilia) and the midfilament (arrowheads) uniformly face in the oral direction (a-o), opposite to the direction of the effective stroke (es). $\times 82,400$.

FIGURE 9 Cross section midway along the length of a comb plate fixed at the beginning of the effective stroke from a backward-swimming larva. Doublet 1 (three-doublet side) and the midfilament (arrowheads) still face orally, as in Fig. 8, but now point in the same direction as the effective stroke (es). $\times 82,400$.



ming and changes in direction of particle transport or water currents (2). In no case has reversal in the direction of the effective stroke been visualized directly in any metazoan system, and alternative explanations have not been ruled out in certain cases (2). In ctenophores, reversal in the beat direction of comb plates has been claimed by some workers, but denied by others (cf. reference 52 for review). Although indirect and contradictory, these reports strongly suggest that under certain conditions—not clearly defined—ciliary reversal can occur in ctenophores.

By devising ionic conditions under which ciliary reversal is consistently and reproducibly obtained in ctenophore larvae, and by carefully documenting this response with high-speed cinemicrography, we have directly demonstrated the phenomenon of ciliary reversal in ctenophores—and in metazoa—for the first time. By so doing, we have been able to use the unique features of comb plate cilia to analyze the structural control of ciliary bend direction.

Ca⁺⁺-Dependence of Directional and Frequency Responses

Ion substitution and blocking experiments show that external Ca^{++} is required for KCl-induced reversal of beat direction in *Pleurobrachia* larvae. Presumably, Ca^{++} activates the ciliary reversal mechanism itself, as in *Paramecium* (8, 10, 26, 29, 34). The direct involvement of Ca^{++} is now being tested by determining the effects of Ca^{++} on the beat direction of ATP-reactivated, demembrated comb plates (S. L. Tamm. In preparation).

In addition, Ca^{++} may also be necessary for synaptic triggering of reversal, because the comb plate cells are innervated (Fig. 2). In adult ctenophores, the nervous system is thought to mediate both inhibitory and excitatory responses of comb plates, as well as unilateral ciliary reversal which occurs during feeding of *Pleurobrachia* (52; see above). The possible role of neurociliary synapses in triggering the global ciliary reversal of larvae is currently under investigation.

The close association between ciliary reversal and increased beat frequency, together with the even greater beat frequency caused by high Ca^{++} concentrations (Table I), indicate that Ca^{++} regulates both parameters of ciliary activity in *Pleurobrachia* larvae. Under certain conditions, however, the directional and frequency responses of comb plates become uncoupled. In high- K^+ ASW, for example, ciliary reversal lasts only 3–5 min, but the increase in beat frequency persists for a longer time. Raising the Mg^{++} concentration of high- K^+ ASW causes an increased beat frequency without an accompanying ciliary reversal. Similarly, an increase in beat frequency and speed of forward swimming can be elicited by mechanical stimulation of the tentacles (52).

Therefore, the directional and frequency responses may have different thresholds and/or sensitivities to Ca^{++} concentration. High Mg^{++} concentrations presumably compete with external Ca^{++} , thereby decreasing the Ca^{++} influx into comb plate cells

and reducing the internal free Ca^{++} concentration. This possibility suggests that beat frequency, but not ciliary reversal, may have a bimodal dependence on intracellular Ca^{++} concentration, with frequency maxima occurring at both low and high Ca^{++} concentrations. A similar bimodal relation between beat frequency and internal Ca^{++} concentration has been proposed to explain the frequency responses of living and demembrated *Paramecium* (10, 29, 30, 34).

The ionic basis of the reversal response in *Mnemiopsis* larvae has not yet been investigated. Nor is it known why increased concentrations of KCl do not stimulate ciliary reversal in this ctenophore, as in *Pleurobrachia* larvae. However, the ability to induce reversed beating in *Mnemiopsis* by an increase in external Ca^{++} concentration indicates that ciliary reversal in this species is also controlled by Ca^{++} .

Evidence for a Radial Switching Mechanism in Cilia

Because of the single polarity of active sliding between microtubule doublets (40), most investigators assume that beat direction is regulated by some type of switching mechanism that activates or changes the effectiveness of doublet sliding around the axoneme (5, 7, 29, 36, 37, 39, 40, 44, 53, 55).

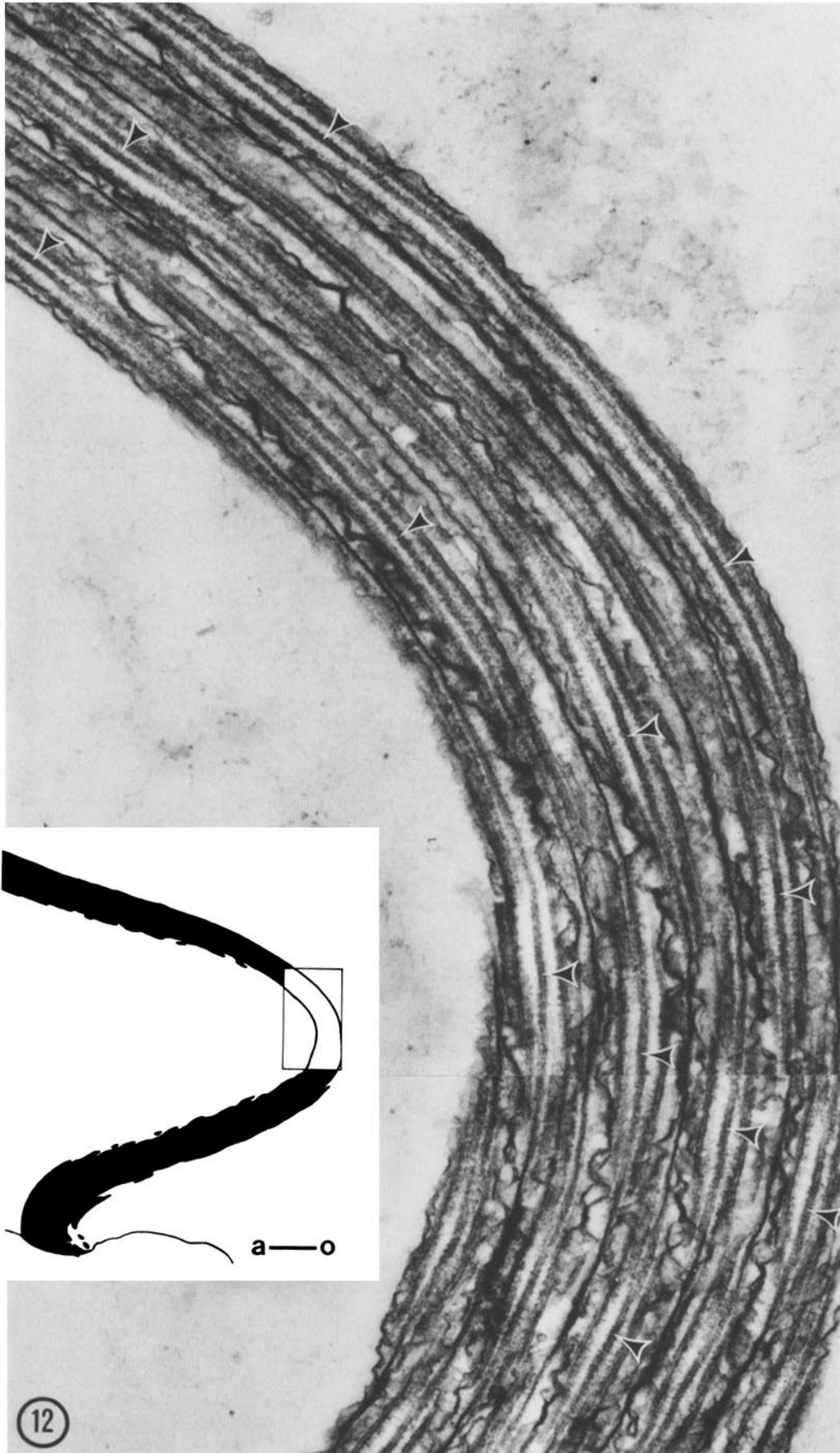
Such a regulatory mechanism has been invoked to explain differentiation of the beat cycle into effective and recovery strokes, as well as angular shifts in the orientation of the entire cycle during ciliary reversal. The role of Ca^{++} in the former case is problematical: intracellular free Ca^{++} concentration does not appear to change during the normal beat cycle. On the other hand, Wais-Steider and Satir (55) have reported that there are two different switching mechanisms in gill cilia, with the switch at the end of the recovery stroke being Ca^{++} -sensitive. Because Ca^{++} has clearly been shown to determine the direction of the beat cycle (8, 10, 26, 29, 34), a switching mechanism controlling ciliary reversal must be Ca^{++} dependent, and is probably distinct from that governing the transition between effective and recovery phases.

Regardless of the number and kinds of switching mechanisms postulated, an obvious prediction of this type of regulation is that the assembly of outer doublets should not rotate with bend direction. This simple prediction has never been demonstrated heretofore. To the contrary, Gibbons (14) reported a systematic twisting of the outer doublets and central pair in sea urchin sperm, suggesting that the doublets twist with respect to the plane of bending in different regions of the flagellum. Woolley (60) found that the plane of each successive bending cycle of golden hamster sperm tails twists as it progresses along the flagellum, and suggested that a twisted-plane wave form containing no twist in the axoneme itself would, if artifactually flattened, give Gibbons's results. Holwill et al. (19) recently reinterpreted Satir's (42) results on *Elliptio* cilia to support a model incorporating axonemal twist during a three-dimensional recovery stroke.

Our earlier work on *Opalina* showed that the shifts in central-

FIGURE 10 Cross section through the base of a comb plate in mid-to-late effective stroke from a forward-swimming *Pleurobrachia* larva. The orientation of the outer doublets and midfilament (arrowheads) with respect to the direction of effective stroke (*es*) and the aboral-oral axis (*a-o*) is the same as in Fig. 8. $\times 82,400$.

FIGURE 11 Cross section through the base of a comb plate at the beginning of the effective stroke from a backward-swimming larva. The orientation of the outer doublets and midfilament (arrowheads) with respect to beat direction (*es*) and the aboral-oral axis (*a-o*) is the same as in Fig. 9. $\times 82,400$.



pair orientation that accompany changes in beat direction also occur near the base of the cilia (53). Because neither rotation of the basal bodies nor a great degree of doublet twisting within a very short basal segment of the cilia seemed likely to us, we inferred that only the central pair, not the entire 9 + 2, rotates with beat direction. Omoto and Kung (37) did not find a large amount of axonemal twisting just above the basal body in *Paramecium* cilia; however, they reported that limited twisting of the outer doublets and central pair does occur, but in opposite senses, leading them also to rule out rotation of the whole axoneme during beating. Nevertheless, the lack of structural markers for numbering the doublet microtubules in protozoan cilia prevented a conclusive answer to this question.

We have overcome this difficulty by using the compartmenting lamellae of comb plate cilia as markers for specific doublets. We found that the array of outer doublets does not twist during the effective and recovery phases of the planar beat cycle, nor does it rotate during Ca^{++} -dependent 180° reversals in beat direction.

Therefore, reversal of the direction of beating, as well as transitions from effective to recovery strokes, must involve changes in activation or effectiveness of doublet sliding on opposite sides of the axoneme. This finding is direct evidence for a radial switching mechanism regulating doublet sliding in cilia.

Active or Passive Role of the Central Pair?

A major result of this paper concerns the nature of the switching mechanism that controls the direction of the ciliary effective stroke. A regulatory role for the central pair microtubules was first suggested by earlier studies on metazoan cilia with a fixed direction of beat. In general, these studies showed that the direction of the effective stroke was perpendicular to the axis of the central pair (1, 11, 13, 21).

Tamm and Horridge (53) tested the validity of this relationship by applying instantaneous fixation to *Opalina* cilia that can change their direction of beating. They found that the central pair was always perpendicular to the plane of bending at any level along the cilium, regardless of 90° changes in the direction of beat, and also during the three-dimensional recovery stroke. This correlation clearly showed that the orientation of the central pair is related to the plane of bending but left unanswered the problem of causal relations. That is, the "angle of the central fibers could be the cause [of the direction of beat] ... which by its rotation releases the bending forces sequentially ... or rotation of the central pair could equally well be a consequence of the bending" (53). Because the directional responses of protozoan cilia are usually <180°, the results were consistent with either an active or a passive role of the central pair (Fig. 13).

Recently, Omoto and Kung (36, 37) have extended these observations to show that the central pair near the base of *Paramecium* cilia also undergoes beat-dependent changes in orientation, and may in fact rotate 360° during each beat cycle. Although these authors did not relate central pair orientation to bend direction during specific parts of the beat cycle, they

reported that the central pair as seen in longitudinal sections was parallel, not perpendicular, to the plane of bending, and thus could not be passively twisted by the motion of the cilium. However, because the cilia in these sections were not identified with respect to stage in the beat cycle or part of the metachronal wave, it could not be directly ascertained that they truly represented instantaneous images of active bends (as in Fig. 12).

Nevertheless, it was argued that rotation of the central pair, rather than being a passive process, may be an active one, at least in certain parts of the beat cycle, and that the orientation of the central pair determines the pattern of active sliding around the axoneme (36, 37).

The ctenophore ciliary reversal system has allowed a direct test of the active role of the central pair in regulating doublet sliding, without the ambiguities associated with correlative studies on protozoan cilia (Fig. 13). The planar beat cycle, ultrastructural markers, and 180° reversal response of comb plates permits the relation between central pair orientation and bend direction to be analyzed on a causal basis for the first time (Fig. 13*a* and *b*). In comb plate cilia, as in most other cilia, the axis of the central pair is always perpendicular to the plane of bending. We reasoned that if the orientation of the central pair actively controls the direction of beating, then it should rotate 180° when the direction of the effective stroke is reversed 180° (Fig. 13*a*). If, however, the orientation of the central pair determines the plane of bending or only represents a passive mechanical response to bending, then it should not rotate during a 180° reversal in beat direction (Fig. 13*b*). In contrast, the possible central-pair orientations during the graded shifts in beat direction of protozoan cilia do not offer as conclusive a test for deciding cause-effect relations (Fig. 13*c* and *d*).

We found that the central pair does not rotate during Ca^{++} -dependent ciliary reversal of comb plates. Therefore, the orientation of the central pair does not regulate the direction of ciliary beating in ctenophores. That is, rotation of the central pair is not the switching mechanism that signals the sequence of doublet sliding around the axoneme. We cannot rule out the possibility that the central pair determines the plane of bending in comb plate cilia, with some other process specifying which direction within this plane. However, such a mechanism seems unnecessarily complex. It seems more likely that Ca^{++} acts directly on the dynein arms themselves, or on the resistive elements which convert sliding into bending, rather than on an intermediary step such as rotation of the central pair. The functional target of Ca^{++} in the axoneme is now being investigated by examining the effects of Ca^{++} on the pattern of ATP-induced tubule extrusion in trypsin-treated comb plate cilia (S. L. Tamm. In preparation). We also found that the central pair does not rotate during the transition between effective and recovery strokes. The orientation of the central pair therefore does not appear to be involved in regulating alternate-side force generation during a single beat cycle.

These findings are relevant to the problem of asymmetry in the bend patterns of cilia and flagella. Because neither the

FIGURE 12 Longitudinal section through a distally propagated recovery-stroke bend in a comb plate from a forward-swimming larva. The profile of only one tubule of the central pair is visible in sections through the center of the axoneme (arrowheads). The plane of the central pair is therefore perpendicular to the direction of active bending (see Fig. 2). $\times 39,100$. *Inset*: tracing of a low magnification micrograph of the proximal part of the plate, showing the location of the bend region (rectangle) illustrated at high magnification.

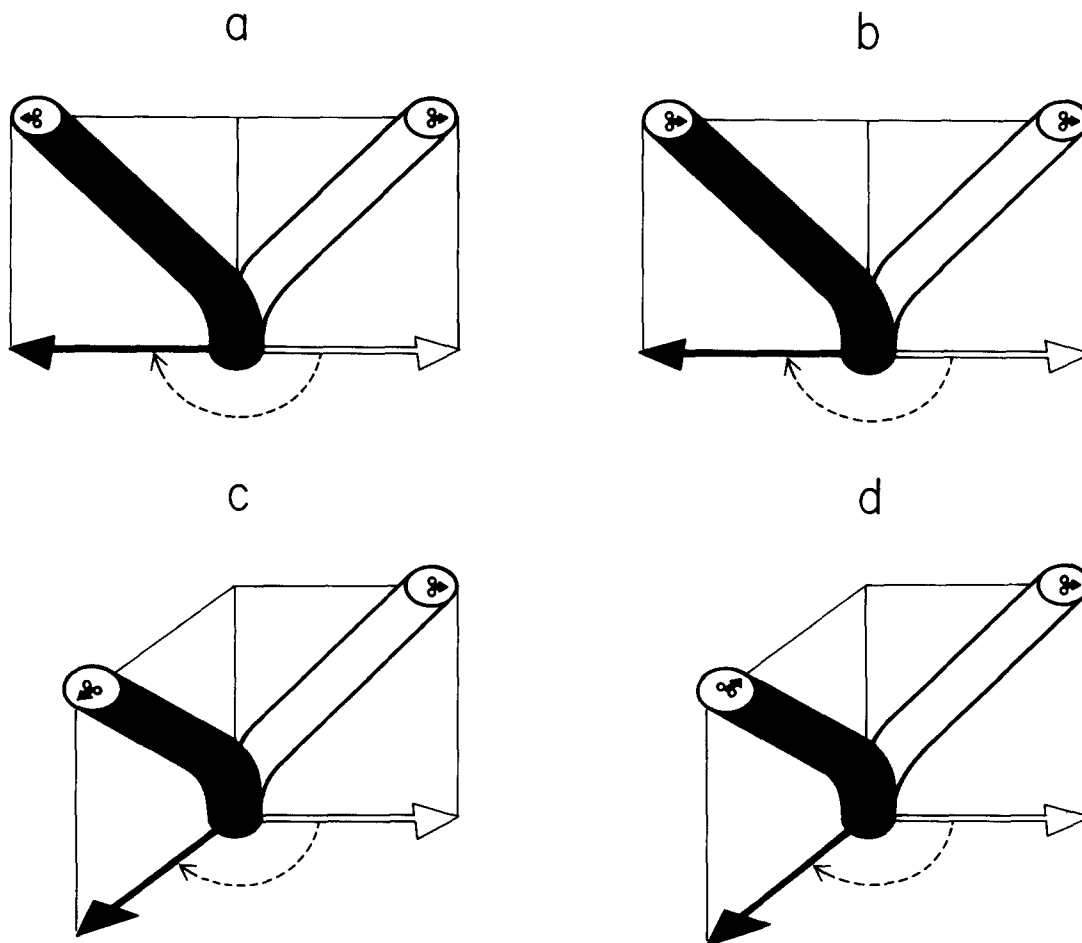


FIGURE 13 Functional significance of possible orientations of the central-pair microtubules during 180° (*a* and *b*) vs. graded (*c* and *d*) directional responses of cilia. The normal plane of the effective stroke is shown by the white cilium and arrow in each diagram; the angular shift in this plane during reversal (dashed arrow) is indicated by the black cilium and arrow. The only constraint on the central pair is that its axis always remains perpendicular to the plane of effective stroke (see also reference 53). The recovery stroke is not considered here. An ultrastructural marker is used to define one side of the central pair (arrow in ciliary cross sections; see also references 36 and 37). For 180° ciliary reversals typical of ctenophore comb plates (*a* and *b*), a corresponding 180° rotation of the central pair (*a*) would indicate an active response controlling bend direction, whereas no rotation of the central pair (*b*) would rule out this possibility. We found the latter to be true. For graded reversals found in protozoan cilia (*c* and *d*), rotation of the central pair so as to coincide with beat direction (*c*) would be consistent with an active or a passive response, and therefore not be conclusive for deciding cause-effect relations; however, rotation of the central pair in the opposite direction (*d*) would rule out an active process. It is not known whether *c* or *d* is true for protozoan cilia.

outer doublet assembly nor the central pair rotates when the beat cycle is reversed, the asymmetric form of ciliary beating—i.e., the effective and recovery strokes—cannot be due to the bilateral asymmetry of the outer doublet array or to structural asymmetries built into the central pair-central sheath complex. This result argues against the view that central sheath asymmetry is responsible for asymmetric bending in *Chlamydomonas* flagella (3). Instead, the form of beat must also be governed by a switching mechanism (55), but with an ionic sensitivity different from that controlling the direction of beat.

What, then, is the functional significance of the beat-dependent changes in central pair orientation observed in protozoan cilia (36, 37, 53)? Until it is shown conclusively that these changes represent rotation of the central pair alone, and ambiguities concerning the relation between the axis of the central pair and bend direction are resolved, an active role of the central pair in regulating beat direction in protozoan cilia must remain doubtful. We believe that our conclusions on ctenophore cilia with a two-dimensional beat cycle also hold for protozoan cilia with three-dimensional beat cycles. In both

cases, the direction of active bending at any level within the axoneme must be regulated, and it seems unlikely that the basic mechanism controlling dynein arm activity or resistive shear interaction would be different, depending on whether the overall motion of the organelle takes place in two or three dimensions. The advantage of analyzing a two-dimensional case such as ctenophore comb plates is that it allows an investigation of causal relations.

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