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Ciliary Reversal and Locomotory Control in the Pluteus Larva of Lytechinus pictus

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Ciliary reversal and locomotory control in the pluteus larva of *Lytechinus pictus*

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SUMMARY

Normal swimming behaviour of *Lytechinus pictus* larvae and the effects of selected drugs are described, based on direct observation and videotapes of free-swimming and tethered larvae. The principal effector response is a coordinated ciliary reversal that enables larvae to back away from obstacles and avoid entanglement. The effect is best seen in the epaulettes, whose large size makes the pluteus an especially favourable subject for behavioural observation and tests.

Reversals and sustained arrests can be induced by various cholinergic agonists, notably nicotine, which is active to concentrations of 0.2 μM. Dopamine and adrenaline cause reversals and arrests as well, but they act initially on the response as a whole, increasing its frequency, rather than directly on ciliary beat. The data suggest a two-step control sequence, with an initial catecholamine-dependent step that triggers a cholinergic effector response.

1. INTRODUCTION

Relating form and function in the sea-urchin pluteus larva is a challenging task. The larva is elaborately shaped, with an ingeniously arranged system of ciliary bands that enable it to feed while swimming, despite the fact that the band cilia beat away from the mouth. Feeding has been the subject of considerable study, and there is disagreement about the precise mechanism, especially with regard to the role of local ciliary reversals that may occur in response to particle contact (Strathmann 1975; Gilmour 1985, 1986). Our concern here is with locomotion, specifically the avoidance response, which involves coordinated, large-scale ciliary reversals, and the means by which this is controlled.

The pluteus represents one variant of a basic plan shared with other echinoderm larvae, but its ciliary band is not as suitable for detailed ultrastructural analysis as, for example, the bipinnaria band (Lacalli *et al.* 1990). This is because many of the cells involved in band innervation lie elsewhere, outside the band, at some distance from their site of action (Bisgrove & Burke 1987). The pluteus is nevertheless the most suitable of the four main types of echinoderm larvae for behavioural studies, and the work we have done on it complements our more limited observations on the behaviour of the other larval types. The main advantage of the pluteus is the ease with which the beat pattern of the epaulette cilia can be monitored, even at comparatively low magnification, to assess the effect of experimental treatments, including drug effects. The results reported here are on normal swimming, the avoidance response, and ciliary reversal, as observed in free-swimming larvae and in video images of free-swimming and tethered specimens.

2. METHODS

Adult *Lytechinus pictus* (Verrill) were obtained from commercial suppliers, spawned, and the larvae reared in the laboratory following methods described previously (Gilmour 1985). Larvae were fed suspensions of *Dunaliella tertiolecta* at concentrations of *ca.* 5000 cells/ml, and transferred to fresh cultures at 2–3-day intervals to ensure optimal growth. With this culture régime, at 16 °C, the larvae began metamorphosing at 28 days. Most of our observations are on 12–18-day larvae. Cultures were established weekly over a period of several months, often from repeated spawnings of the same individuals by injection of 1 ml of acetylcholine chloride (0.1 M) in saline. This provided a reliable means of obtaining 12-day pluteus larvae at the beginning of each week for experiments, so that a prolonged series of observations could be carried out.

Free-swimming larvae were observed by dissecting microscope at low magnification in small dishes, and video recordings were made of both free-swimming and tethered larvae, the latter attached to suction pipettes using the apparatus described by Gilmour (1989). All drug studies were done on groups of five or six larvae maintained in small dishes, with two or three replicates at each concentration. Solutions were freshly made, with ascorbic acid added in approximately equimolar concentration as an antioxidant in solutions of aminergic transmitters and tubocurarine. Sodium metabisulphite proved unsuitable as an antioxidant for reasons discussed in §3*a*.

3. RESULTS

Figure 1 shows a 16-day *Lytechinus* larva. It has projecting arms supported by internal skeletal rods,

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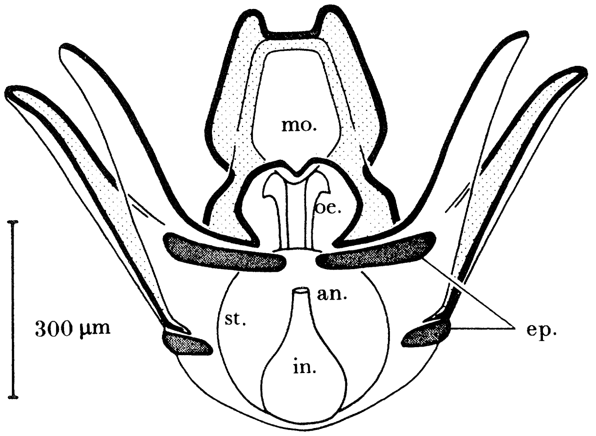


Figure 1. Arrangement of the ciliary band in the pluteus larva of *Lytechinus pictus*, a posterior view showing the ciliary band (heavy line), which defines the oral field (light shading), the epaulettes (ep.), and the digestive tract: mouth (mo.), oesophagus (oe.), stomach (st.), intestine (in.) and anus (an.).

and a well-developed oral hood. As in other echinoderm larvae, there is a single circumoral ciliary band that defines the margin of the oral field. Cilia on the band beat away from the mouth while those in the oral field beat towards the mouth. The circumoral band in pluteus larvae runs along the sides of the arms, so the oral field lies essentially within them. In advanced larvae, the main locomotory force is provided by the epaulettes, which develop as loops from the band that pinch off and develop further independently. In both the circumoral band and the epaulettes, the ciliated cells are tapered and their tips compressed so that a dense ciliary array is produced. This, together with the length of the epaulette cilia, is why the epaulettes are such good subjects for observation.

(*a*) *Normal swimming and the role of ciliary reversal*

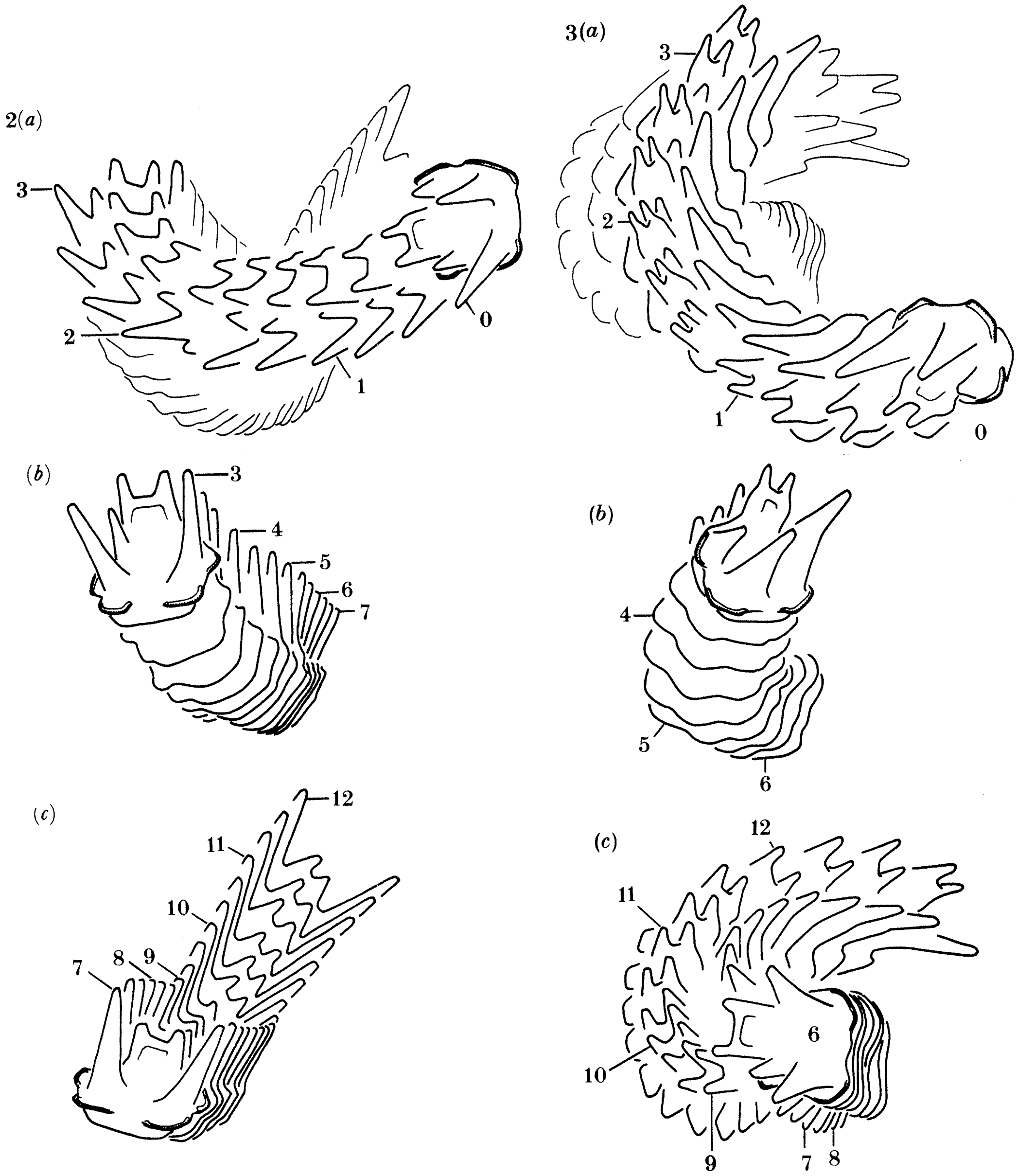
Our observations reported here are on 12–18-day larvae and agree in most respects and with previous reports (e.g. Strathmann 1971). The larvae swim steadily when undisturbed, with continuous metachronal waves visible along the band and epaulettes, and they rotate slowly as they swim. The main behavioural response is a coordinated reversal of the cilia evoked on contact with obstacles, e.g. suspended particles, sides of the dish, the water surface, or other larvae. We have not been able to determine which parts of the body are most sensitive to contact. The tips of the arms are clearly involved in many instances, and so must be sensitive, but it seems that particle contact elsewhere on the body surface often induces reversals as well.

Typical reversal sequences are shown in figures 2 and 3. Initial contact is followed immediately by a period of backward swimming, usually several seconds in duration. The backing slows, and is followed by a period of relative immobility during which the larvae may begin to sink. They then start suddenly forward. Even momentary sinking will reorient a larva, so it starts off in a new direction. The overall result is thus a backing and avoidance response much like the better known avoidance response of *Paramecium* (Wichterman 1986). Reversals during a period of rapid swimming can give the impression that the larvae are bouncing off obstacles, but analysis of the video images shows that this is a visual illusion, due to the fact that the larva's speed during backward swimming is, at onset, nearly as rapid as its original forward speed. Viscous effects dominate over inertial ones in this situation (Sleigh 1984), so each backward movement requires a period of strong reversed ciliary beat.

How cilium beat changes during reversal can be seen in tethered larvae (figures 4 and 5). Onset is rapid, within the interval of a single video frame ($\frac{1}{30}$ s). There is then a period of strong reversed beat followed by a transitional phase and return to normal. The reversals observed in tethered larvae are typically very brief, lasting *ca.* 1 s. They occur singly or in series. We see only the beat envelope in our recordings, but the pattern of cilium beat inferred from this is essentially the same as that recorded in high-speed cine films of cilium beat in *Hemicentrotus* larvae by Baba (1975). During both normal and drug-induced ciliary arrests, the aboral cilia are mostly strongly affected. Cilia along the oral margin of the band continue to beat feebly in some instances (figure 5*d*) and recover first, before the aboral cilia. There appears to be no fundamental difference between reversals and arrests in the pluteus and calcium-dependent reversals and arrests in bivalves and tunicates (Sleigh & Barlow 1982; Takahashi 1982).

The significance of avoidance behaviour to the larvae can be appreciated by observing what happens when reversals are blocked. The most effective blocking agent we have so far discovered is verapamil, which interferes with calcium transport. Similar effects are obtained with isosmotic $MgCl_2$ (Mackie *et al.* 1969), which probably acts at the level of neural transmission, and in calcium-free seawater (Baba 1975). At 5 μM, verapamil reversibly blocks all reversals, but does not otherwise impair swimming. In fact, swimming speed usually increases. On approach to the water surface, such larvae veer away from the surface and cruise along just beneath it without making contact. When the larval arms do make contact, however, whether with the water surface or the sides of the dish, the larvae get trapped. Typically, within a short time of treatment, most or all of the larvae will be found hanging motionless from the surface or trapped in the meniscus as shown in figure 6. The band cilia continue to beat, and when freed, the larvae swim off immediately at normal speed. Treated larvae also frequently become entangled with each other and swim locked together, in circles. Clearly, larvae without a functional avoidance response face hazards which, from the point of view of survival, are extremely serious.

Sodium metabisulphite provides an alternative, but apparently more damaging, way to block reversals. We discovered this while testing antioxidants for use with some drugs. The eventual effect is the same as in verapamil, but develops progressively over *ca.* 30–40



Figures 2 and 3. Normal swimming and reversal in *Lytechinus*, traced at $\frac{1}{3}$ s intervals from video recordings of free-swimming larvae. In both figures, (*a*) shows the sequence in detail up to the onset of reversal, at the arrow head, with the remainder of the sequence sketched in outline, and continued in detail in (*b*) and (*c*). Numbers give elapsed seconds starting at zero for each sequence.

min in a 0.001 % solution. In the early stages, sensitivity to contact is reduced so that reversals occur, but they are comparatively feeble and begin a second or more after initial contact. It seems the initial phase of the response, which occurs immediately on contact and produces the strongest reversals, is the part most sensitive to metabisulphite.

The effects of various drugs on ciliary beat are described below. Significant alterations in swimming behaviour were induced by both cholinergic and aminergic agents. GABA, glutamate and glycine were also tried, at concentrations up to *ca.* 25 μM, but had no noticeable effects.

(*b*) *Cholinergic agents*

Carbachol is a convenient and stable agonist for demonstrating ciliary reversals and arrests. The other cholinergic agents produced similar effects, varying mainly in degree as discussed below, and all were reversible on removal of the drug. There is first a low concentration threshold (*ca.* 2 μM for carbachol) at which older larvae stop swimming and, over a period of seconds, sink to the bottom of the test dish. The epaulette cilia continue to beat, but show varying degrees of impairment, e.g. metachronal waves are disturbed or absent, and parts of the band exhibit periods of reversed or transitional beat. Young larvae (less than 11 days, or younger without epaulettes) continue to swim, and it was not clear whether their cilia were much affected. The greater apparent sensitivity of older larvae could simply to be due to their greater reliance on the unimpaired normal function of the epaulettes in order to swim at all. A

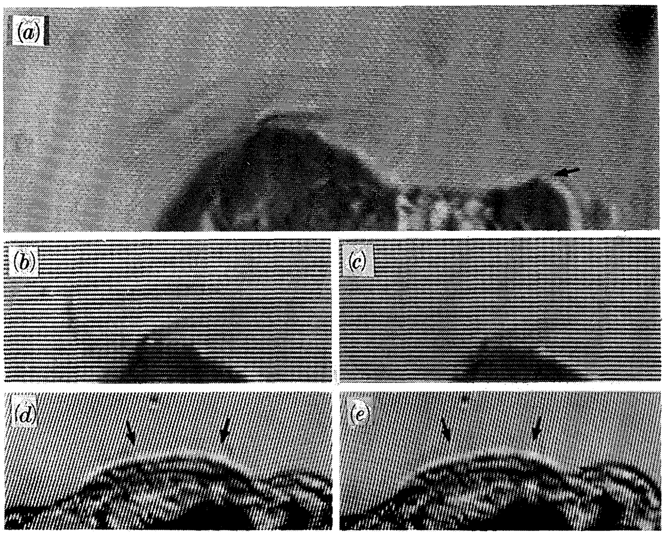


Figure 4. Video images of epaulette beat in tethered larvae. Figure 5 shows corresponding traced images. (*a–c*) A reversal sequence, with the direction of normal beat from right to left. (Magn. × 670.) (*a*) Normal beat, $\frac{1}{4}$ s exposure to show the beat envelope, equivalent to frame 5 in figure 5*a*. An adjacent part of the circumoral band is included (arrow), but its cilia are not visible in still images. (*b*) Single frame $\frac{1}{6}$ s into the reversal. This would be frame 5 in figure 5*b*; compare with the aggregate tracing for frames 2–10. (*c*) Single frame during the transition phase before resumption of normal beat. This falls between frames 20 and 23 in figure 5*c* when the cilium has a helical motion that is difficult to image.

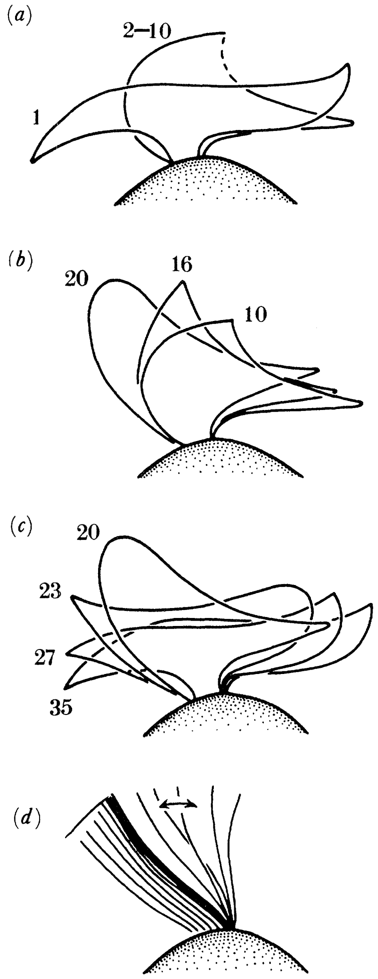


Figure 5. Tracings of a reversal sequence from video images like those in figure 4. Direction of normal beat is from right to left. Frame number is indicated, and the interframe interval is $\frac{1}{30}$ s. (*a*) First $\frac{1}{3}$ s showing the onset of reversal followed by a period of continuous reversed beat. (*b*) Second $\frac{1}{3}$ s showing entry into the transitional phase. (*c*) Transitional phase and return to normal beat. (*d*) Cilium posture during an arrest. Cilia along the oral margin of the band may continue to beat feebly (arrow), but the majority of the cilia are essentially immobile.

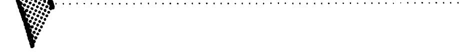


Figure 6. Trapped larvae; a sketch to show what happens when ciliary reversals are blocked: the larvae typically become trapped either at the edges of the dish, in the meniscus, or at the water surface.

muscular response was also observed at low doses, involving repeated muscular twitching of the oral hood and peristaltic swallowing movements of the oesophagus. At higher does, typically *ca.* 5 μM, ciliary bands in both old and young larvae go into an arrest, with occasional bouts of normal beating in some instances. The threshold for arrest differs between larvae by a factor of 2–3 times, and also differs along the band in a given larva. For example, as shown in figure 4*d* and *e*, the centre of the epaulette has a lower arrest threshold than the ends.

Other agonists tested were: nicotine (0.2 μM threshold for the first signs of the low-concentration effect, 1 μM for arrest); PTMA (phenyl trimethyl ammonium chloride, 2 μM threshold for impairment, complete arrests at 10–20 μM); DMPP (dimethyl phenyl piperazinium chloride, similar to PTMA, but arrests need *ca.* 50 μM). The sensitivity to nicotine suggests that a nicotinic receptor is involved. PTMA and DMPP are nicotinic agonists with mainly neuromuscular and ganglionic actions, respectively, and the former seems to be the more effective agonist. Various antagonists were tested. Atropine, a muscarinic blocker, tested at up to 10 μM, had no effect by itself on ciliary beat and gave no protection against carbachol. Of the nicotinic antagonists tested, tubocurarine chloride, gallamine, decamethonium bromide and pentolinium, tested at up to *ca.* 50 μM, only the last significantly raised the carbachol threshold, by a factor of 10 times in some instances, but the effect was quite variable between larvae. Pentolinium is selective for ganglionic nicotinic receptors, while the other three have mainly neuromuscular actions, and of the latter only gallamine had even moderate protective ability. Eserine sulphate, a cholinesterase inhibitor, had similar effects to the agonists, producing some beat impairment at 2 μM, and sustained arrests at 8 μM. As expected, larvae introduced into a solution containing eserine show no swimming impairment until the first normal contact-induced event. They then either remain impaired, at low dose, or arrested, at higher dose.

In summary, cholinergic agents induce, with increasing concentration, first ciliary reversals, then arrests. This occurs at micromolar concentrations. The effect has nicotinic properties, but is sufficiently different from better-known nicotinic responses that conventional nicotinic blocking agents were not very effective.

(*c*) *Aminergic agents*

Three catecholamines were tested: dopamine, adrenaline and tryptamine. The first two had nearly identical effects and thresholds. At very low concentrations, *ca.* 0.2 μM, both dopamine and adrenaline increased the frequency at which bouts of ciliary reversal occur, as if larval sensitivity to contact were being affected. Repeated sequences of reversals and recovery occurred, giving the impression that larvae were bouncing back and forth off unseen obstacles. The bouncing increased in frequency with increasing concentrations until, at *ca.* 2 μM, there was a sustained arrest. Larvae added directly to test solutions with concentrations of 2 μM or greater approached arrest via a similar series of increasingly frequent reversals, each of which was otherwise normal. This presumably reflects the rate of penetration of the drug. Oral hood twitching and swallowing motions also occurred. Tryptamine, tested at up to *ca.* 60 μM, had no effects on larval swimming.

Three aminergic antagonists were tested. Sulpiride, a dopamine antagonist, induced the same back-and-forth bouncing behaviour as dopamine, but only at relatively high concentrations (*ca.* 100 μM), and it did not reliably protect larvae against dopamine-induced arrests. Propranolol, a β-adrenergic agent, produced very similar effects to sulpiride at similar (i.e. relatively high) concentrations, but provided no protection against adrenaline-induced arrests. Prazosin, an α-adrenergic blocker, produced some impairment of ciliary beat and was generally rather toxic, but at low concentrations (*ca.* 5 μM) there was a degree of protection against adrenaline. The threshold for adrenaline-induced arrest was raised by a factor of 2–3 times. Because we were unable to discover an effective, specific block to carbachol, it was not possible to test whether the aminergic effects operate independently of the cholinergic ones.

In summary, aminergic agents induce reversals and arrests that mimic normal behaviour, but at greater than normal frequencies. This suggests that they are involved in triggering the response as a coordinated whole, rather than acting at the level of individual cells to initiate reversals directly. Both dopamine and adrenaline were effective at similar concentrations, but the best block was obtained with an α-adrenergic antagonist against adrenaline.

4. DISCUSSION

Different types of echinoderm larvae use the ciliary band in different ways (Strathmann (1971) and our own observations, reported here and unpublished). Coordinated reversals involving the whole band or large parts of it are important in both echinoid and ophiuroid pluteus larvae and the auricularia. We have observed all three engaged in periods of backward swimming lasting from one to several seconds, and both the echinoid pluteus and auricularia show contact responses involving momentary reversals. The echinoid pluteus responds by backing up. The auricularia uses coordinated reversals in feeding when young (Gilmour 1990), whereas advanced larvae have prominent lateral ridges that respond to contact with a reversal that bounces the body sideways off obstacles. All three types of larvae are sufficiently complex morphologically that the value of this type of response, to avoid entanglement, is obvious. The bipinnaria shows neither reversals nor a pluteus-type avoidance response (Lacalli *et al.* 1990), but its body is more smoothly contoured than the other three larval types during much of its development, so it may be less subject to entanglement. All the reversals we observed in this study, in the Pluteus, were coordinated once involving large segments of the band.

Various neurotransmitters are known from adult echinoderms, including acetylcholine, which is involved in neuromuscular control (Cobb 1987), and both acetylcholine and serotonin are implicated in control of muscle contraction in the pluteus (Gustafson *et al.* 1972*a*, *b*). Ciliary reversal appears also to be neurally controlled, at least in the epaulettes, from which electrical potentials can be recorded during reversal (Mackie *et al.* 1969). Previous work does not identify the transmitters involved, but the band does stain intensely for cholinesterase (Gustafson *et al.* 1972*b*; Ryberg 1973), which suggests the involvement of acetylcholine. Examples of cholinergic control over ciliary arrest occur in related organisms, e.g. in tunicates (Arkett *et al.* 1989; Mackie *et al.* 1974).

Our behavioural tests show two separable effects evoked by different categories of drugs. The aminergic agents, notably dopamine and adrenaline, evoke sequences of apparently normal reversals whose frequency increases with concentration until an arrest results. Cholinergic agonists simply impair ciliary beat until, with increasing concentration, the cilia arrest. This suggests a two-step control process in which acetylcholine is the transmitter directly responsible for ciliary reversal and arrest, with pattern and quantity of release controlled, under normal circumstances, through a catecholamine-dependent step. The difference between ciliary reversal and arrest can be interpreted as a concentration effect. Increasing concentrations of cholinergic agents, in experimental treatments, cause first reversal and then arrest. The same sequence is seen over time, in single experiments, as the drugs penetrate. Failure to obtain a specific block of the cholinergic response means we cannot rule out a direct effect by catecholamines on ciliary cells, but morphological results on bipinnaria bands also support the idea of a two-step control sequence, involving aminergic sensory input and cholinergic effector neurons (Lacalli *et al.* 1990).

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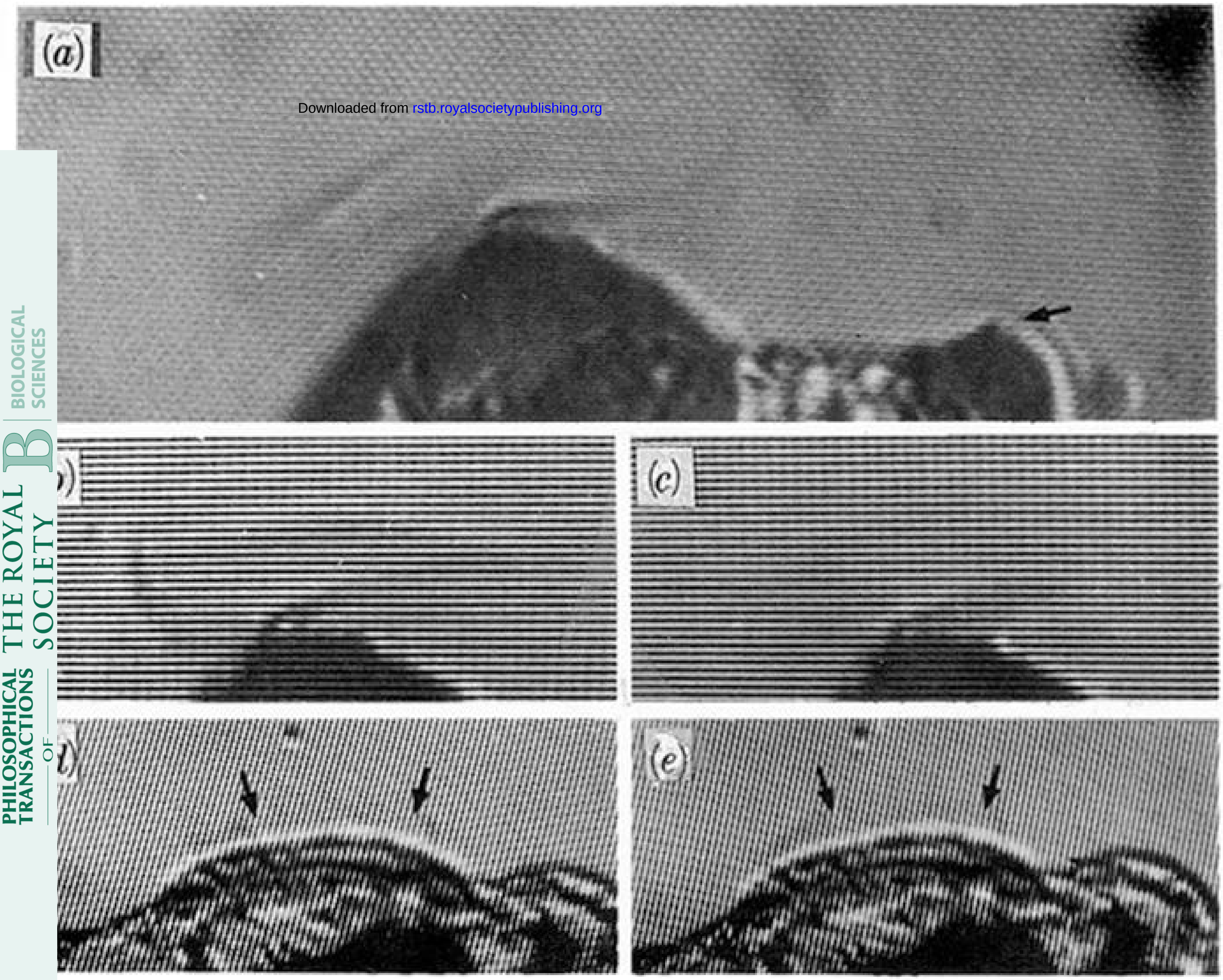

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