Initial observations of the effect of electric fields on ciliary motion in *Paramecium* and *Stylonychia*

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Introduction.

The Victorian scientist Karl Ludloff was amongst the first to note the effect of DC electric fields on the action of cilia, particularly its effect on the locomotory cilia of *Paramecium*, and the resulting change in their swimming behaviour. The effect is sometimes named after him (the Ludloff effect). It is an example of a taxic response (whole organism movement), and the response to electric fields is known as galvanotaxis. This response appears to have no significance for ciliates in the wild, but may shed some light on the ion exchange mechanisms controlling ciliary motion. In laboratory situations involving ciliate movement, many swimming paths are recorded and subject to computer analysis [1-4]. This large-scale data collection enables statistical analysis to reveal significant changes in behaviour. The small-scale study presented here is limited to fewer 'manual' observations and measurements. Its aim is to try to reveal interesting aspects of cellular behaviour and to determine what aspects might be taken forwards.

Links to videos recording typical behavioural responses may be found at the end of this paper.

Methodology

1. Cultures

Paramecium cells were grown in a small loosely covered aquarium containing 3000 ml rainwater and 0.5 g of oats. Constant aeration was provided using a small USB powered air pump. The aquarium was left to generate bacterial growth for 1 week at room temperature (16 - 20 °C), and then inoculated by micropipette from a wild population. An additional 0.3 g of oats was added at 2 weekly intervals. Evaporation losses were made good, and 400 ml of water replaced to reduce solute build up at similar intervals. *Stylonychia* were grown in a loosely covered vessel containing 1000 ml of rainwater, and 0.2 g oats. A large surface area (300 cm^2) ensured an adequate rate of gas exchange. The culture fluid was left to generate bacterial growth for 1 week at room temperature (16 – 20 °C) and then inoculated from a wild population. The culture was supplemented with 0.1 g oats at 2 weekly intervals. Evaporation losses were made good, and 100 ml water replaced to reduce solute build up at similar intervals.

2. Microscopy

All observations were made with a Brunel SP300 microscope, with infinity corrected plan-achromatic objectives and oblique or oblique Rheinberg illumination. Results were recorded using a Canon M50 mk 2 camera with direct projection in 24 fps HD. The internal microphone/sound recording facility on the camera was used so that changes in the field direction or application of the field could be noted for later matching to the recorded behaviour. If needed, video editing software was then used to remove the soundtrack.

An experimental cell (Fig. 1) was created on a glass slide using sticky-back copper tape and crocodile clip terminated leads to which a voltage supply could be connected. This tape has a total thickness of about 70 µm; the copper is some 50 µm thick, and the glue layer about 20 µm. It is widely available and sold as a slug repellent. This set up used 6 mm wide tape in 2 strips with an 18 mm gap between them, which allowed a 2 mm overlap with the cover slip at each side. Two

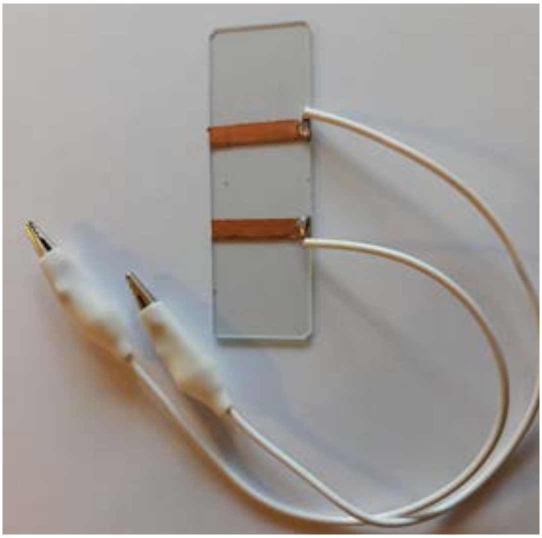

Fig. 1. Experimental cell, showing copper tracks and input leads.

leads with crocodile clips were soldered along the bottom edge of the electrodes. The transient heat of soldering did not damage the glass of the slide.

For the videos acquired to illustrate some of the behaviours discussed later, 3% methyl cellulose was used to retard motion and aid tracking. Similar sized drops of sample and methyl cellulose solution were mixed on the slide with a mounted needle prior to adding the cover slip. The final concentration of the methyl cellulose was about 1.5%. For more detailed observations oat ‘milk’ was added in addition to the methyl cellulose (oat ‘milk’ was found to have less tendency to curdle than cow’s milk). A couple of small drops were mixed into the sample before the cover slip was added. While the cilia were difficult to see directly, the milk enabled the flow they create to be visualised. This helped enable ciliary augmentation rates to be measured. Other results were obtained from videos where the test cell was loaded only with the culture media, and were recorded in real time. Video frame stepping and manual tracing of cell tracks onto tracing paper clipped to the computer screen enabled measurements to be taken with normal drawing instruments. Only those organisms with a relatively clear area of open water into which to move were measured, since avoidance reactions would otherwise confuse the results [2,3]. Care was taken to ensure that the volume of fluid in the cell was such that with the coverslip in place contact was made with the copper electrodes, but not so much that the depth of the fluid made observation difficult.

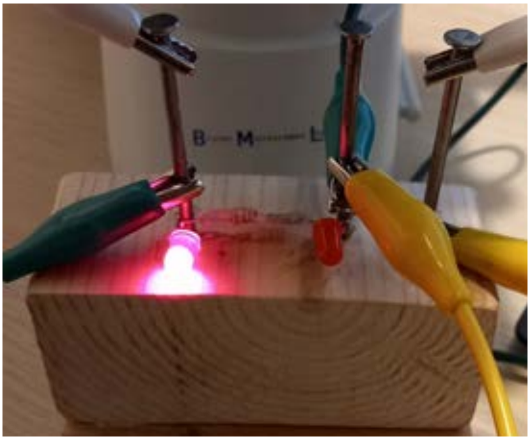

Fig. 2. DC connection block. The right hand LED is on, indicating that the middle pin is positive.

3. Electric field generation

Observations were made using both DC and AC electric fields. A DC field was created by applying a constant voltage across the observation cell. Reversing the polarity reversed the field direction. AC fields were created by the application of an AC voltage across the slide. The initial observations presented here were limited to the effect of DC at one voltage with changing polarity, and AC at one voltage with varying frequency.

DC fields

A connection block was made using nails as connection pins (Fig. 2). The circuit connecting leads were clipped to these nails to take the weight of the leads off the slide and soldered joints. Two of the nails support LED’s acting as polarity indicators and the third was used to connect a DC ammeter. A double pole double throw switch (DPDT) was used to make a polarity reversal circuit. This was mounted on a simple construction of plywood offcuts, hot melt glued together (Fig. 3). The centre position was off, with no connection to the slide, and the top and bottom positions reversed the output polarity. Crocodile clip terminated leads were used to connect the system. A mains powered DC supply was used which maintained a constant and stable voltage at a selectable output level. The applied field was 4 V cm^{-1}. Fig. 4. Shows the circuit arrangement for DC experiments. A multi-meter configured as a DC ammeter was included in series with the output after the polarity indicating LED's. This allowed the current flowing through the cell to be measured to check that the cell was conducting and working. The main problems encountered were insufficient liquid to ensure contact with the electrodes, electrolysis producing gas bubbles, and the copper electrodes becoming dirty or tarnished. These effects reduced conductivity. The tape was easily cleaned with a gentle rub with alcohol. Fig. 5 shows an overall view of a typical apparatus setup for DC experiments.

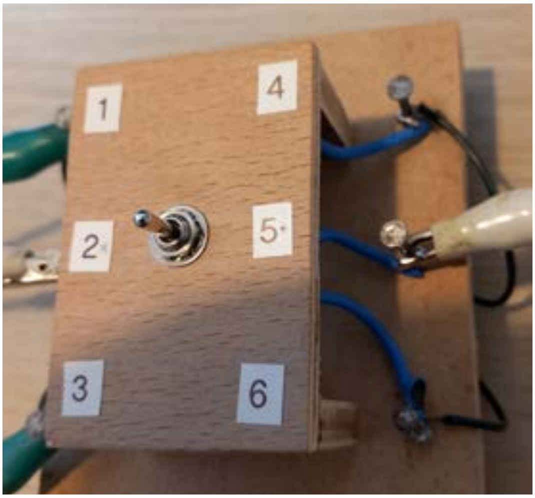

Fig. 3. Polarity reversal switch for DC observations.

AC fields

When selecting a circuit design for these experiments of this type, it is necessary to ensure that the output is actually bipolar, rather than waveform modulated DC. An astable circuit

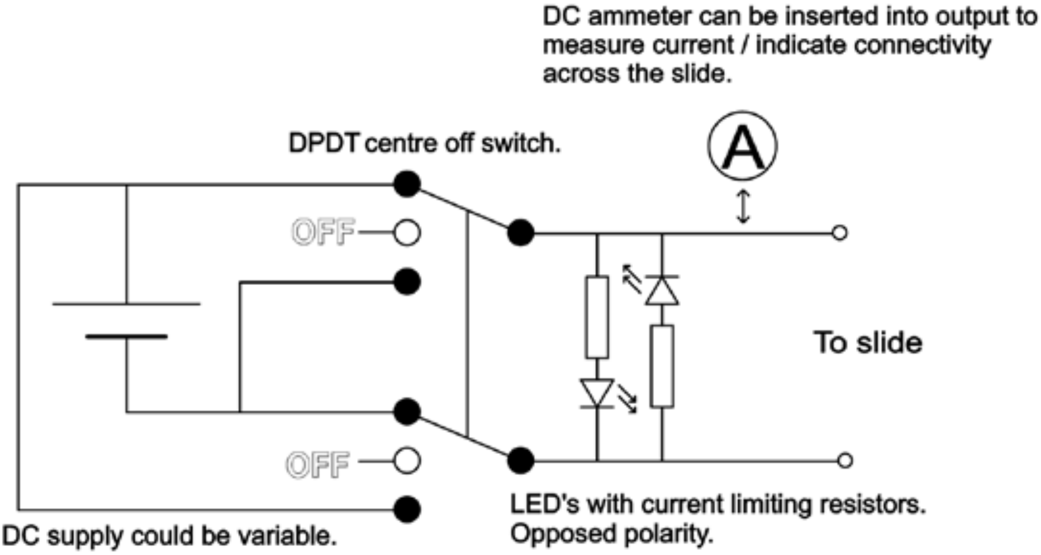

Fig. 4. Schematic diagram for DC field generation.

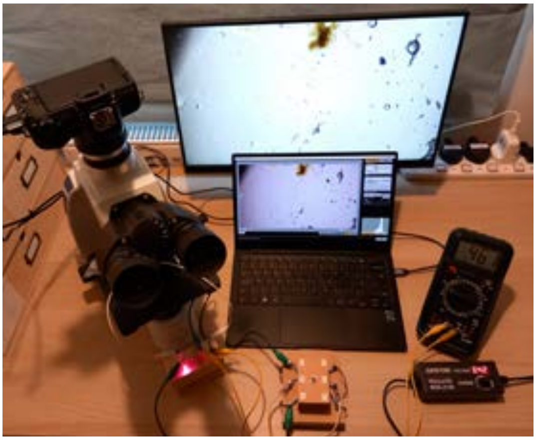

Fig. 5. Bench view of equipment configured for DC experiments.

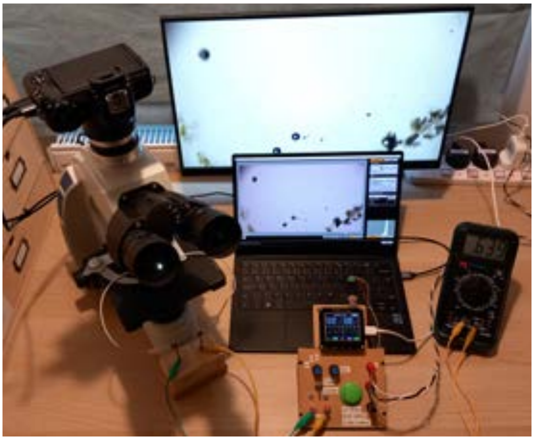

Fig. 7. Bench view of equipment configured for AC experiments.

with a fixed 50% duty cycle (1:1 Mark: Space) was built used 741 op amp chips. A multipole switch selecting a range of timing capacitors to set the output frequency, which could be varied between 1 Hz and 2800 Hz. The output was switchable between continuous and intermittent operation through a push button switch (Fig. 6). The circuit was mounted in a housing constructed from plywood offcuts using hot melt glue. A dual polarity power supply with switchable output voltages enabling control of the peak-to-peak voltage, and the applied field strength was 5 V cm^{-1} peak. The AC connection block had only 2 pins since there was no need for polarity indicator LED's. The AC ammeter was connected directly from the astable output, through the meter and straight to one of pins. The role of the ammeter was the same as that outlined for the DC setup. An oscilloscope module was also incorporated to view the waveform and measure its frequency. A typical bench setup for AC work shown in Fig. 7.

Paramecium: Introduction and DC observations

Ciliary augmentation and stroke direction reversal

The electrolytes present in the observation cell make it electrically conductive and the application of an external electric field causes a potential difference to develop resulting in polarisation. The

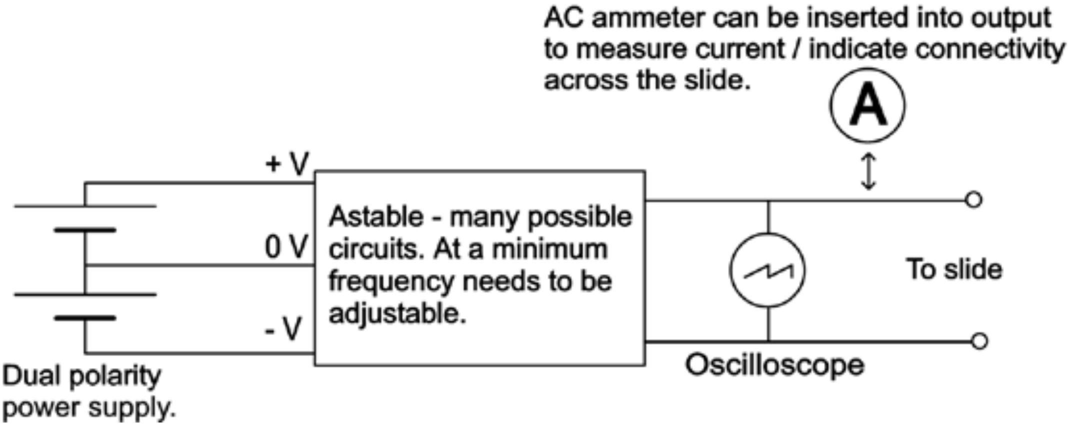

Fig. 6. Schematic diagram for AC field generation.

end facing the anode becomes depolarised while that facing the cathode becomes hyperpolarised. The ciliary membrane houses voltage sensitive ion channels [2,4,5]. At the *depolarised* end Ca^{2+} channels open and calcium ions enter the cell, thus raising their intracellular concentration. Above a certain threshold this produces 2 ciliary effects: augmentation (they beat faster) and reversal of their stroke direction [6,7]. At the *hyperpolarised* end K^+ channels open which allows these ions to leave the cell, decreasing the internal concentration. This produces ciliary augmentation without reversal. The rate of augmentation was measured in 6 cells by frame stepping and measuring on screen the movement of the cell / milk suspension before and after field application. It varied between 110% and 350% with a mean of 214% This is broadly in line with other published data [8,9].

Cell reorientation towards the cathode

There is a very significant enhancement of the thrust the cilia produce under the influence of DC fields, resulting in 2 forces acting on the cell [6]. At the depolarised end an enhanced and redirected thrust acts backwards, while from the hyperpolarised end there is an enhanced forwards thrust. If these forces were acting in direct opposition and of equal intensity the cell would simply compress. The accompanying video illustrates that compression can be observed if cell reorientation is hindered in high viscosity medium, but this is not generally the case. One reason may be that the motile cilia are not evenly distributed across the cell: the cilia of the oral groove, for example, mainly generate feeding vortices. The motile cilia are arranged in longitudinal rows which run at an angle to the anterior/posterior axis. This produces an asymmetric thrust which contributes to the well-known *Paramecium* 'corkscrew' swimming action [5]. There is some evidence that ciliary reversal may occur in a geometrical pattern which produces a thrust which contributes to the turning moment. Cyclical nucleotides may also play a role [10]. The net overall response to a DC field is to generate a torque which orients the cell towards the cathode. The cell then swims with its usual corkscrew motion in that direction until the field is either removed or reversed.

***Paramecium*: AC observations**

At frequencies below 2300 Hz, Paramecium cells turn and reverse swim perpendicular to the field, while above 2300 Hz the cells swim as normal. All frequencies below the 2300 Hz limit seem to cause ciliary augmentation, with enhanced motion in the form of reverse swimming, greater anterior/posterior rolling rate, and greater yawing motion. The amplitude of the yawing motion was the easiest effect to measure, and is discussed below.

Yaw angle measurements

325 yaw angles (Fig. 8) were measured across a range of frequencies, and as with the DC experiments results were taken only from those cells with a clear area of open water before them. The initial 2 or 3 yaws were often not typical and rather depended on the initial orientation of the cells with respect to the field. A stable pattern of yawing was therefore allowed to develop before measurements were made. Below 1800Hz there were about 5 – 10% non-responders, while above this frequency it could rise to over 50%. Non-responding cells are not included in the measurements. At higher frequencies, yaw angles above 180° often turned into full rotations. This 'spinning' could not be measured as a simple yaw angle and was also not included. The low cell response rate at higher frequencies also limited the number of readings which could be gathered. More observations at these frequencies

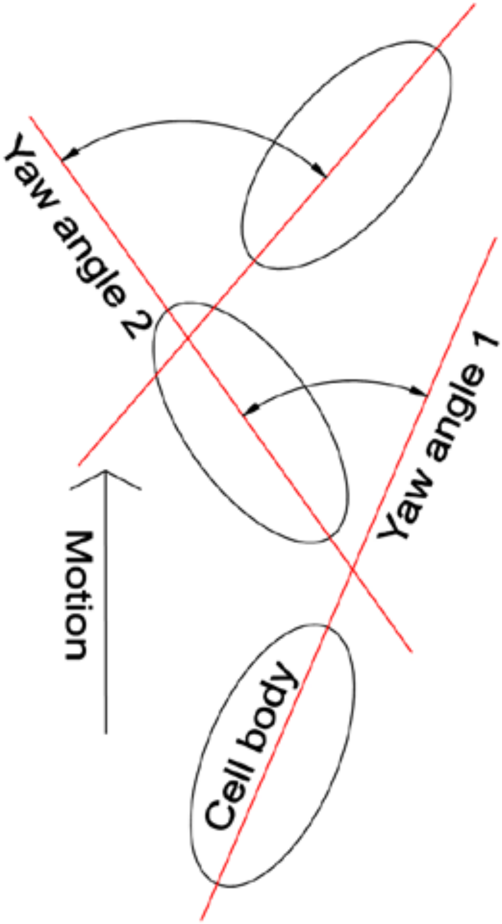

Fig. 8. Geometry for measuring yaw angles

would be the ideal situation, however a general trend appears to have emerged. The results (Fig. 9) suggest that increasing frequency results in greater yaw angles. The wide spread of results is not surprising given that the responses were for of a population of free-swimming living organisms. For example, they have a range of sizes, the initial ion balance across the ciliary membrane would vary between individuals, and they would be at different stages of their cell cycle.

Stylonychia: Introduction and DC observations

Stylonychia is a hypotrich ciliate and its cilia have a complex arrangement. Some are grouped together to form 'leg like' bundles known as cirri. These in turn are organised into discrete clusters

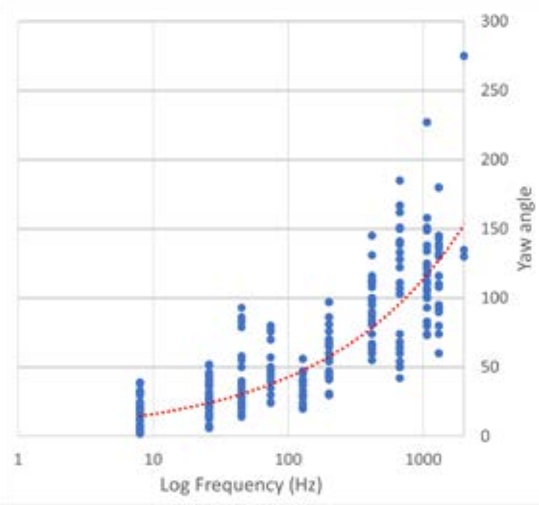

Fig. 9. Effect of frequency on yaw angles for Paramecium (some points represent multiple observations).

on the ventral surface. Other cirri are arranged around the cell margin, while some form a series of continuous membranes. *Stylonychia* cells can use the ventral cirri to 'walk' across a solid surface and they can also free swim. The aim of these observations is to compare their free-swimming galvanic response to that seen with *Paramecium*. *Stylonychia* cells show a helical 'corkscrew' swimming motion similar to that observed in *Paramecium*. This swimming motion is thought to be due to an asymmetrical thrust around the anterior/posterior axis. A large proportion of the locomotory force is generated on the ventral surface. Fig. 10 shows a schematic ventral view of the distribution of groups of cirri in a *Stylonychia* cell. Judging from their positions, the frontal, right marginal, front ventral, postoral, and pretransverse cirri probably produce more thrust on the right hand side of the cell, with the buccal, caudal and transverse cirri providing central thrust along the anterior-posterior axis. In addition, the right marginal group occupies the whole of the right-hand cell periphery, while the left marginal group only occupies the the lower half of the left hand periphery. This difference may also contribute to thrust asymmetry. Cirri associated with the undulating and adoral area membranes are likely to be involved in feeding vortex generation.

Directional response

The most obvious difference between the two organisms is that *Stylonychia* cells swim towards the anode (positive galvanotaxis), whereas *Paramecium* cells swim to wards the cathode (negative galvanotaxis). Since the presence of ion channels in *Stylonychia* has been confirmed in previous investigations [13], this opposite response could be due to the distribution of the voltage sensitive membrane ion channels (K$^+$ and Ca $^{2+}$) being reversed on the anterioir-posterior axis compared to Paramecium.

Ciliary augmentation.

The augmentation of the rate of beating of *Stylonychia* cirri was measured across 6 cells. The range was 187% to 380% with a mean of 240%. No published data was available for comparison, but this is similar to the effect seen in *Paramecium*. This suggests the clustering of cilia into cirri does not significantly alter this aspect of their response. In addition to augmentation the use of milk tracer shows evidence of a differential cirri response. Some areas exhibit the cirri reversing thrust direction, other areas continue beating in their usual direction. It strongly suggests that a similar turning mechanism to that seen in *Paramecium* is occurring i.e. the differential response of the cirri produces a turning torque orienting the cell towards the anode. Owing to the depth of fluid needed to ensure free movement of the cells it was not possible to determine which specific cirri are involved.

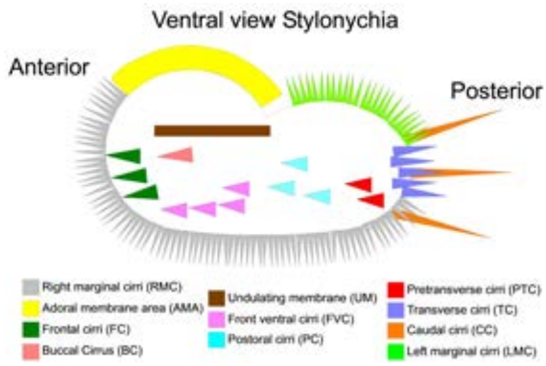

Fig. 10. Ventral arrangement of groups of cirri on *Stylonychia*

***Stylonychia*: AC observations**

The two main responses noted were reverse swimming in near straight lines, and circling. There was some evidence of accommodation, with a prolonged stimulus or repeated short stimuli producing a smaller response in some cells. The accommodation response could be a result of the same mechanism previously discussed for *Paramecium*. Compared to *Paramecium*, *Stylonychia* showed a reduced response at higher frequencies. Some *Paramecium* cells responded up to 2300 Hz, while *Stylonychia* cells rarely responded above 2000 Hz.

Quantification of circling behaviour

On manual tracings of cell tracks, the area enclosed by the circling behaviour was found to vary with frequency. Since the enclosed areas were irregular and did not easily lend themselves to counting squares on graph paper, the traced areas were cut out and weighed on milligram resolution scales. A known area of the same paper was then weighed, and this area was converted into an 'on slide' area. 69 enclosed areas were processed. A cell had to complete at least 2 circles to qualify for tracing and measuring. Only those movements completed in relatively open water were used. At stimulation frequencies above 800 Hz, some cells responded by circling for a few rotations, then a short burst of straight travel, followed by more circling. In this case only the circling sections were processed. At both ends of the frequency range the circling becomes 'tighter' and the enclosed areas smaller, often comprising little more than rotation around the centre of the cell (Fig 11).

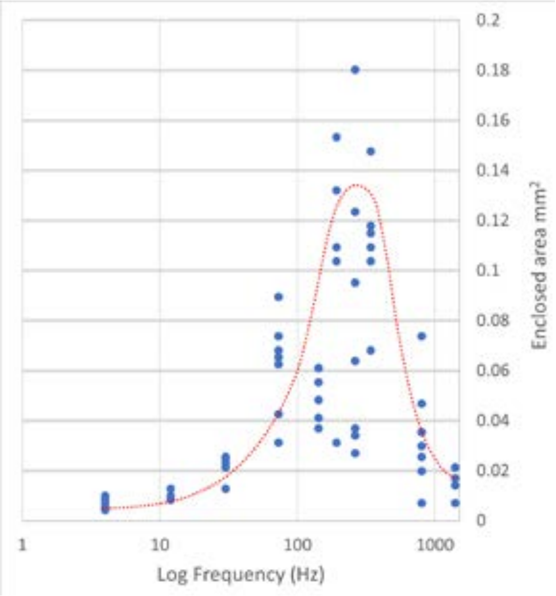

Fig. 11. Frequency dependence of the area enclosed by circling manoeuvres in *Stylonychia.*

Discussion

1. *Paramecium*

For the *Paramecium* response to DC electric fields, the question arises that once the cells are re-orientated towards the cathode, why does continued swimming occur? One possibility is that once cells are oriented parallel to the field, the induced polarisation is less as the surface area of the cell perpendicular to the field is at a minimum. The voltage sensitive ion channels would then reduce their activity, and this together with the cellular mechanisms which exist to restore both ion gradients (either direct pumping or internal sequestration) rebalance the ion gradient to enable a more normal form of swimming with all cilia beating in the normal direction. In this scenario, forward motion in a corkscrew fashion would transiently angle the cell with respect to the field and expose it to enhanced polarisation. Renewed stimulation of the ion gates would result in cell reorientation back to parallel motion with the applied field, resulting in swimming towards the cathode. The effect of AC electric fields on *Paramecium* is less studied. While it has been reported [11] that *Paramecium* will align and swim perpendicular to an intense static magnetic field, this effect is probably due to the diamagnetism of the cells and is a non-physiological response. It is probably not significant to the current arrangements as any induced magnetic fields will be tiny compared to those used experimentally. The ciliary augmentation and reversal of swimming direction may have the same basic mechanism as that seen with DC fields i.e. raised intracellular Ca^{2+} levels and lowered internal K^+ caused by voltage sensitive ion channels. With an AC field the polarisation is reversed every cycle. Initial application of the field causes the usual turning response. The anterior / posterior rolling would in effect 'even out' the fluctuating polarization around the cell's circumference. This could result in the observed perpendicular alignment to the field. Perhaps the availability of Ca^{2+} to flow into the cell is greater than the internal availably of K^+ to leave the cell. The relative fluid volumes are very different. The exit of K^+ through the activated gates might temporarily exhaust the internal supply of these ions resulting in a reduction of the ciliary augmentation producing the forward thrust. The reverse thrust produced by the anterior cilia continues as more calcium is available externally. This would then dominate as the driving force and the cell would swim backwards. It has been noticed that with repeated stimulation some cells show accommodation and their response to the field declines. This might be caused by the mechanisms responsible for restoring the normal ion balance, such as ion pumping and internal

internal sequestration, not being able to keep up with the ion flows produced by the electric field. After a period of rest the response returns, perhaps suggesting that the normal ion balance has been restored. The lack of response above 2,300 Hz may indicate that period of the field reversal exceeds the response time of the augmentation mechanism. The increased yawing effect seen at higher frequencies could, very speculatively, be a sort of 'beat frequency' response i.e. a frequency generated by the interference between 2 or more frequencies combined. Perhaps at higher frequencies the field changes do not produce a response until they coincide with some point in the cycle of other events e.g. the cilia's metachronal wave frequency, and /or the rate and position of the cell when rolling along it anterior – posterior axis.

2. Stylonychia

The lack of published data means that this discussion is very speculative, and other contributions are very welcome. Perhaps the circling behaviour of *Stylonychia* cells is a product of the uneven distribution of cirri. During normal swimming there must be co-ordinated action between the various cirri groupings to partially balance this out and enable relatively straight motion. It probably results in the corkscrewing noted in the DC response. Perhaps AC disrupts this 'normal' operation so that motion of the cirri is augmented but lacks co-ordination. This disruption does not end forward thrust but reduces the corrective action which would normally produce straighter swimming. For example, if the ventral contribution to thrust was reduced the remaining forward thrust could drive the cell around in circles as a continuous turning torque would develop. In this case the more disrupted the ventral cirri action the tighter the turning. This is only one possible example of how cirri disruption might produce the observed response. The circling response also appears to be frequency dependent. As with *Paramecium* this could be due to an interference effect between the applied field and some internal cellular frequency e.g. cell rotation or the normal pattern of cirri beating. Following on from the above perhaps the mid-range frequencies produce less interference with the normal pattern of cirri beating enabling a better balance of thrust and reduced tight circling resulting in a higher enclosed area? Perhaps natural variation in the number and distribution of ion channels may account for the lack of a circling response in some cells. Hopefully, further study may clarify these matters. The reduced high frequency response of *Stylonychia* compared to *Paramecium* might be attributable to the fact that the cirri have a much larger bulk and mass compared to individual cilia (as found on *Paramecium*). Both have a sensory function as well as providing propulsion. In their membranes they host the ion channels which respond to the applied field and produce the ionic gradients which form the basis of the response. Perhaps the inner cilia of the cirri bundle are not as able to respond to the stimulus as their ion channels and access to ions are to some extent masked by the surrounding ones? This would have in effect a reduced surface area to volume ratio.

In conclusion

While the results presented here may have generated more questions than answers, they suggest that complex galvanotaxic responses in protozoa merit further investigation. The next stage planned is to develop a more sophisticated voltage generator to allow detailed control of the waveform shape and timing, both for AC and DC fields. Hopefully, this may reveal more insights into the mechanism producing the responses in *Paramecium* and *Stylonychia*. There are of course numerous other organisms to which these techniques could be applied.

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Video links

Paramecium DC response -
https://youtu.be/b-7mj3PLtAM
Paramecium AC response -
https://youtu.be/UcO0Ldp4aio
Stylonychia DC response -
https://youtu.be/w31UWDDYFmk
Stylonychia AC response -
https://youtu.be/CnNFzGiwR1k

https://www.quekett.org/members/videos/other-videos

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