

## A study of synchronisation between the flagella of bull spermatozoa, with related observations

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### SUMMARY

**Flagellar synchronisation has been observed between bull spermatozoa as they swam in a viscous medium, confined to a glass surface. This process is of interest in understanding the regulation of flagellar oscillation in general. Exact and persisting synchrony between bull spermatozoa occurred only when the spermatozoan heads were tightly coupled mechanically. For these cells, viscous coupling between the flagella was not by itself sufficient to establish synchronisation. Immediately on synchronisation, with the spermatozoan heads superposed, the paired spermatozoa showed rises in conjoint beat frequency, wave velocity and swimming velocity, i.e. in nearly all cases, the new conjoint values were greater than those shown by either of the two singleton spermatozoa. In our interpretation of these results, we put forward hydrodynamic arguments for seeing the primary change as a rise in wave velocity, *via* a decreased viscous resistance to bend propagation. Mechanistically, the rise in beat frequency is mysterious unless, as we suggest, it is consequential to the rise in wave velocity, and mediated by an as-yet-unknown mechanical feedback process. The rise in swimming velocity is not surprising given the rise in wave velocity but there is evidence for an additional influence due to a subtle re-orientation of the conjoint spermatozoan heads, such that they experienced less frictional drag.**

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### INTRODUCTION

The movement of an individual flagellum, or a cilium, is characterised by rhythmic beating (Bray, 2001). The mechanism that controls this oscillatory motion of the axoneme is still debated but it must, in some way, repeatedly switch the pattern of coordinated action of the dynein motors (Brokaw, 2009). Any circumstance that produces a sudden change in the switching rate (which is double the value of the beat frequency) may perhaps, when analysed, shed light on the general control mechanism of the oscillation. One such circumstance is the phenomenon of flagellar synchronisation.

As a general statement, when two flagella that have different beat frequencies come to lie close to each other, there is a tendency for them to adopt a common beating frequency. Also, at the same time, they will come to beat in phase. Thus, they will soon beat exactly in unison, as if they were a single organelle. Synchronisation, then, is the process of adjustment whereby a common frequency and a common phase are adopted.

This phenomenon has long been recognised (Perrin, 1906; Gray, 1928; Taylor, 1951; Rothschild, 1955; Machin, 1963; Jahn and Bovee, 1967; Machemer, 1974). Until now, however, flagellar synchronisation, as distinct from ciliary synchronisation, has not been analysed experimentally, except for a recent study by Riedel et al. (Riedel et al., 2005).

Provisional explanations of synchronisation seem to propose that two hydrodynamical principles apply. One principle is that the mutual influence between the two flagella is transmitted through the fluid ('viscous coupling') rather than by direct mechanical contact. This idea was advanced by Gray (Gray, 1928); it has

received support from experimental studies of the mass synchronisation of cilia [see Tamm (Tamm, 1984) and references cited therein]; it also features in theoretical analyses (Priel and Tsoi, 1990; Gueron et al., 1997; Vilfan and Jülicher, 2006; Niedermayer et al., 2008; Yang, X. et al., 2008). The other principle is that of 'minimal interference', which leads to the expectation that the post-synchronisation frequency will be approximately the mean of the two pre-synchronisation frequencies, because the slower flagellum tends to decelerate the faster one whereas the faster one tends to accelerate the slower one (Gray, 1930; Machemer, 1974).

In the present study, flagellar synchronisation between pairs of bull spermatozoa has been recorded and analysed. We have concluded that viscous coupling, by itself, does not bring the two flagella into synchrony. Instead, synchronisation is, in virtually all cases, dependent on a tight mechanical coupling between the flagellar bases; it does not arise from mere proximity of the flagellar shafts. However, once the bends on the two flagella are initiated simultaneously, in phase, then the shafts come into very close apposition and this is probably through viscous coupling. In relation to beat frequency, we found, surprisingly, that synchronisation produced an enhancing rather than an averaging effect. This effect is concomitant with an enhancement of the conjoint wave velocity. There was also a gain in the forward swimming velocity but this was partly dependent on a subtle re-orientation of the conjoined spermatozoan heads that probably reduced the frictional resistance they encountered. Evidence for these conclusions has come from two subsidiary sets of observations. Plausible hydrodynamic reasons for the enhancement of wave velocity can be put forward. By

contrast, the mechanism that produces the enhancement of beat frequency is mysterious, unless we consider that it is determined, in some way, by the rise in the wave velocity.

Although the focus of this research is on flagellar mechanics, the observations have a wider relevance to the question of inter-ciliary coordination. The possible relevance to spermatozoan transport and fertility is also discussed.

### MATERIALS AND METHODS

Bull spermatozoa (*Bos taurus*, Linnaeus) were obtained from Genus Breeding Ltd (Ruthin, Wales, UK), a commercial supplier of bull semen for breeding purposes. Ejaculates were diluted (1+9) in 'Ruthin' extender, which is based on the 'Reading' extender (Revell and Glossop, 1989). 'Ruthin' extender is the same as the 'Reading' extender except for the substitution of sorbitol for glucose and the removal of trehalose; it promotes spermatozoan longevity at ambient temperatures. The diluted samples were sent to the University of Bristol by overnight mail and were worked on during the following 1–2 days.

The slide chambers used in microscopy were 70–90  $\mu\text{m}$  in depth (true depth, corrected for refractive index). They consisted simply of a supported cover slip beneath which was a central droplet (15  $\mu\text{l}$ ) of Hanks BSS containing 2% methyl cellulose (M0387, Sigma-Aldrich, St Louis, MO, USA), which raised the viscosity to 1.5 Pa s. (This is the measurement of apparent viscosity at 20°C provided by Sigma-Aldrich.) The diluted semen sample was then run in to form a circular interface with this viscous solution. Video-recordings were made of spermatozoa that had swum across the interface into the high viscosity saline and accumulated beneath the cover slip. All observations were made at room temperature (20–23°C).

Raising the viscosity slows and regularises the movement of spermatozoa and promotes their tendency to accumulate at the cover slip and to swim there with essentially planar waveforms (Woolley, 2003). Raising the viscosity has made the present study possible. We have chosen a macromolecule (methyl cellulose) that mimics the properties of (human) cervical mucus (Ivic et al., 2002). At 'normal' viscosity, the beat frequencies would have been approximately tenfold greater [using the data of Rikmenspoel (Rikmenspoel, 1984)]. There is, however, evidence that synchronisation between bull spermatozoa does also occur in saline of 'normal' viscosity (Machin, 1963).

The apparatus for dark field video-microscopy has been described (Woolley, 2003). To obtain instances of spermatozoan synchronisation, a  $\times 25$  objective lens (Leitz NPL 25/0.50) gave a suitable compromise between the width of field and spatial resolution. With continuous tungsten illumination, the exposure duration of the recordings was 20 ms, which was sufficiently brief, given that the beat frequencies were in the range 0.5–3.5 Hz.

Measurements of beat frequency ( $f$ ) and progressive velocity ( $U$ ) were made using the video-timer and slow motion replay of the videotape. For bull and similar mammalian spermatozoa it is difficult to measure wave velocity ( $v_w$ ), wavelength ( $\lambda$ ) and wave amplitude conventionally, with reference to the progression axis. (The progression axis is the smoothed line that represents the mean path taken by the spermatozoan head or it can mean the centre line drawn through superimposed tracings of the flagellum.) This is because all of these variables change, from proximal to distal parts of the flagellum, along the progression axis (Gray, 1958; Rikmenspoel et al., 1973); also it can be difficult to delineate the progression axis in asymmetrical waves with relatively few bends. Therefore, instead, the  $v_w$  and  $\lambda$  have been measured along the flagellar axis (i.e. along the sinuous midline of the flagellum itself):

they will be called the arc wave velocity ( $\text{arc}v_w$ ) and the arc wavelength ( $\text{arc}\lambda$ ). This type of measurement was first used by Brokaw (Brokaw, 1965). Wave amplitude has been considered to be related to the length of the waveform ( $L_w$ ), such that a waveform that is shortened or compressed proximo–distally must have a greater mean amplitude. In fact, compression also involves gains in bend angle ( $\theta$ ), i.e. the angle subtended by the full arc of a bend.

More detail is now given of these definitions and of how the measurements were made.

### Arc wave velocity

The moment when a new bend is initiated was detected visually using the fact that at this moment the spermatozoan head begins to reverse its direction of lateral motion. The actual moment was taken to be the last video-field prior to the detection of the change in head movement (i.e. 20 ms prior to it). Now, at this moment, the base of the flagellum is the boundary position between two bends – we refer to it as the inter-bend (position). It is the mid-point, of zero curvature, between two bends. We recorded the time [ $t$  (s)] for this new inter-bend to run along the flagellum and just propagate off the tip. Our measurements have given 61.6  $\mu\text{m}$  as an estimate of the mean total flagellar length ( $L$ ) from the neck–head junction to the end of the end-piece; for convenience, this was treated as invariant. Thus, the mean  $\text{arc}v_w$  (along a single flagellum) was  $L/t$  ( $\mu\text{m s}^{-1}$ ). Left and right sides of the waveform were defined and the  $\text{arc}v_w$  was always taken as the mean for the two sides of the flagellum, based on two consecutive bend initiations.

### Arc wavelength

Estimating fractions of a wavelength was impractical. Therefore, this parameter was obtained by calculation, as  $\text{arc}v_w/f$  (Lighthill, 1975). In fact, because the switching rate is the focus of interest, the arc bend length, taken to be  $\text{arc}\lambda/2$ , has been shown in the figures. As a visual check of the validity of this calculation, there was good consistency between the visually estimated number of bends on the flagellum ( $N$ ) and the number calculated from  $L/(\text{arc}\lambda/2)$ . Because  $\text{arc}v_w$  is the mean speed along the flagellum, the calculated  $\text{arc}\lambda$  is a mean for the flagellum.

### Progressive velocity

Progressive velocity ( $U$ ) is the mean path velocity of the spermatozoon as it swims through the fluid, i.e. it ignores the side-to-side motion of the head. The curvilinear velocity ( $U_c$ ), which includes the yawing motion of the spermatozoan head, was taken as  $U \times 1.08$ . This correction factor was obtained as a mean from the detailed plotting of the movement of the head–neck junction in three spermatozoa recorded with a  $\times 100$  objective lens.

### Waveform length

This is the distance, measured along the progression axis, from the head–neck junction to the intersection of a line drawn perpendicularly and touching the most distally positioned part of the flagellum. This length was estimated by eye, using a ruled acetate overlay, and standardised by choosing two images representing the times when two consecutive inter-bend loci just ran off the tip.

These methods have been applied to the study of the synchronisations. In addition, for further information,  $\text{arc}v_w$  and  $\text{arc}\lambda$  have been analysed more thoroughly for some individual spermatozoa. These were recorded using a  $\times 100$  objective lens. Video-fields were captured at 100 ms intervals and measurements were made with a map measure on large scale ( $\times 4200$ ), contrast-reversed prints. With the shallower depth of field, it was apparent

that bends on one side of the flagellum were slightly out of focus, i.e. they tilted away from the cover slip. A correction was made to obtain the true length of such bends. This involved: (1) obtaining the regression coefficient of the true  $z$ -axis displacement of the bend crests, on image width, using through-focus images of immotile flagella (Woolley, 1981); and then (2) correcting for the change from an arc-to-elliptical curve produced by the projection of the out-of-focus region of the flagellum. This latter correction factor was obtained for bends of varying angles by simple optical projection experiments rather than by calculation.

The subject of the present study, the flagellar synchronisations, resulted from chance contacts between individual spermatozoa. These events will be called 'conjunctions'. In a few instances, the two spermatozoa separated again after a period of conjunction and they resumed the swimming speeds and beat frequencies that they had shown before the conjunction. Therefore, it was decided to include in the present study conjoined pairs that happened to be conjoined when they were first seen and which were observed to separate. 'Conjunctions' and 'separations' will not be distinguished in the displayed data, because it is believed they are equally meaningful in terms of the effects of synchronisation.

The data for all the conjoined spermatozoa were recorded at times when their alignment was optimal and the synchronisation was most exact.

To assist interpretation, data were also gathered for singleton spermatozoa not involved in conjunctions. Some of these were observed on the rare occasions that they became stuck to the cover slip (by the head or by the tip of the tail) and then broke free again.

## RESULTS

### A description of spermatozoan conjunction and flagellar synchronisation

We observed spermatozoa swimming just beneath the cover slip in a viscous saline. The spermatozoa had swum there from an adjacent aliquot of diluted semen. Not infrequently, conjointly paired spermatozoa were seen, swimming faster than the singletons, with their flagella synchronised (Fig. 1A). More rarely, triple and quadruple assemblies were seen, with variable degrees of flagellar synchrony (Fig. 1B). The proportion of conjoined spermatozoa seen was probably greater than would have existed in the original semen because of their superior swimming ability. (In our unpublished work with salines of more extreme viscosity, only paired spermatozoa or multiple assemblies could penetrate.) However, some paired spermatozoa were found, by phase-contrast microscopy, in very thin preparations of the original diluted semen. No paired spermatozoa were seen in supra-vitally stained smears of diluted semen (nigrosin/eosin method). This meant that the conjunctions were impermanent. The smears also showed that no pathologically biflagellate spermatozoa were present to cause confusion.

Our present study was based on 'conjunction events' and 'separation events'. Conjunction became possible when the paths of two spermatozoa, having fairly similar velocities, intersected at a shallow angle (Fig. 2A–C). Varying degrees of head-to-head adhesion might then occur. Only when the heads became rigidly fixed together did the flagella synchronise. Bends were then initiated simultaneously on the two flagella and synchronisation spread distally to become complete with one transit of the flagellum (see Movie 1 in supplementary material). Perfect superposition of the heads gave the most exact and lasting synchrony. Often, such heads had a changed appearance (narrower and less evenly illuminated as seen in Fig. 1A) – see later sections of this paper. Rigid, side-by-side attachment of the heads also gave synchronisation, as did rigid

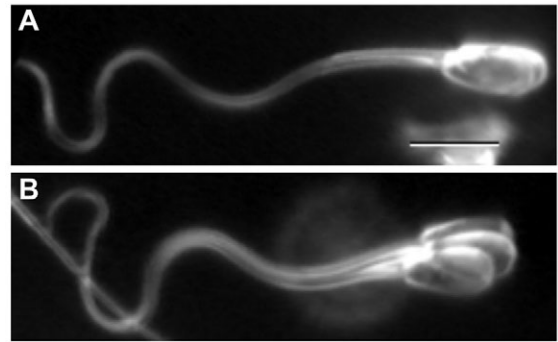


Fig. 1. Video-fields depicting conjoined spermatozoa. A  $\times 100$  objective lens was used. Scale bar,  $10\ \mu\text{m}$ . (A) A pair of spermatozoa, with flagella synchronised, swimming progressively. This example was found as such, which means that the conjunction could have occurred in the native semen or in the male reproductive tract. It was typical that the spermatozoan heads appeared unevenly illuminated. (B) An example of a triple conjunction, fully synchronised and swimming progressively. This conjunction also was already established when first observed.

attachment with some fore-and-aft displacement of the heads. However, whenever the adhesion was weak, and there was some rotatory motion between the spermatozoan heads, the two flagella failed to synchronise (Fig. 3A,B). 'Separation events' usually followed collision of the conjoint pair with other flagella or with debris.

### The effects of flagellar synchronisation

Flagellar movement and swimming behaviour were compared before and during synchronisation (in 21 conjunctions) and during and after synchronisation (in 11 separations). Thirty of the 32 events yielded complete sets of data. In all, about 24 h of video-recordings were searched.

Flagellar synchronisation immediately produced a characteristic set of changes. These were: (1) an increase in the  $f$  to above that of the mean of the two singleton spermatozoa (in 31/32 instances); (2) an increase in the  $\text{arccv}_w$ , likewise (in 30/30 instances); (3) an increase in the  $U_c$ , likewise (in 30/31 instances); (4) a tendency, only, for the calculated bend length (nominally  $\lambda/2$ ) to increase (20/32 instances). The data are shown graphically in Fig. 4A–D. The length of the wave, which was used as an indicator of wave amplitude, did not change in a consistent direction (data not presented). Statistical analyses of the data in Fig. 4 are given in tabulated form as a supplementary information file (see Table S1 in supplementary material).

In attempting to account for the enhancement of  $f$ , bearing in mind that  $f = \text{arccv}_w / \text{arcc}\lambda$ , it was shown that the change in frequency

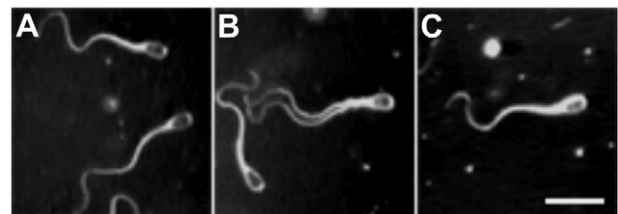


Fig. 2. (A–C) Three video-fields from a sequence to show the process of conjunction and synchronisation. A  $\times 25$  objective lens was used to provide a sufficient field of view. Scale bar,  $25\ \mu\text{m}$ .

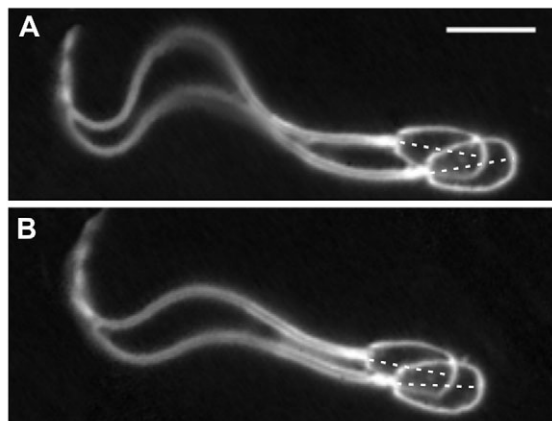


Fig. 3. (A,B) Two video-fields, 260 ms apart, of a conjoined pair of spermatozoa, swimming progressively. In this example some rotatory movement was possible between the attached spermatozoan heads, as emphasised by the superimposed axial lines (broken lines). The lack of a rigid attachment prevented the flagella from becoming synchronised. Scale bar, 10  $\mu\text{m}$ .

( $\Delta f$ ) was very highly correlated with the change in  $\Delta \text{arc} v_w$  ( $R=0.700$ ;  $P<0.0001$ ) (see Fig. 5).

Correspondingly, there was no significant correlation between  $\Delta f$  and the change in the calculated arc wavelength,  $\Delta \lambda$  ( $R=-0.180$ ;  $P=0.33$ ; graph not shown). Thus the rise in  $f$  is mainly correlated with a rise in  $v_w$ .

For both the singletons and the pairs, there was a correlation between the  $\text{arc} v_w$  and the  $U_c$ , as might be expected (Fig. 6). Also, the percentage 'wave slip', given by  $[(\text{arc} v_w - U_c)/\text{arc} v_w] \times 100$ , did not change significantly on synchronisation. (The greater the wave slip, the less effective the  $v_w$  is in producing forward motion.) Had

the wave slip changed, a difference would have been apparent between the linear regression coefficients for singleton and conjoint data sets, which are superimposed on the graph (Fig. 6); these slopes were not significantly different. There was also a clear trend for the change in swimming speed (on synchronisation) to be positively correlated with the change in  $v_w$  ( $R=0.325$ ,  $P=0.075$ ).

In an extremely rare and exceptional spermatozoon–spermatozoon interaction (seen only once), the two flagella became fully synchronised but the spermatozoan heads were adhering to each other side-by-side. Bends were initiated simultaneously at the proximal part of the midpiece (Fig. 7). Because the heads were not superposed, this conjunction and the following separation were not included in the Fig. 4 data set. It is an informative interaction, however, because although the conjoint flagella showed clear enhancement of both  $f$  and  $\text{arc} v_w$ , there was a loss of forward velocity (Table 1).

#### Related observations 1: exact description of beat plane with reference to the spermatozoan head

When observed using a  $\times 100$  objective lens, the typical spermatozoon, swimming singly, was seen to have its head flat against the cover slip. Bends on the right side of the wave axis were in focus at this same level but bends on the left side projected deeper and were maximally out-of-focus at the bend crests (Fig. 8A–C). Such bends were regarded as planar and tilted away from the plane of the right-side bends, the tilt occurring in the region of the interbends. Using the width of the out-of-focus images and correcting for perspective (see Materials and methods), the angle of tilt was measured as individual bends propagated. This angle was constant along the flagellum and was constant for successive left-side bends. However, between the three spermatozoa analysed, the angle varied, with the mean of bend-means being 0.091, 0.164 and 0.220 rad or 5.2 deg., 9.4 deg. and 12.5 deg. Fig. 9 (closed symbols) illustrates the spermatozoon with the mean value of 5.2 deg. The constancy

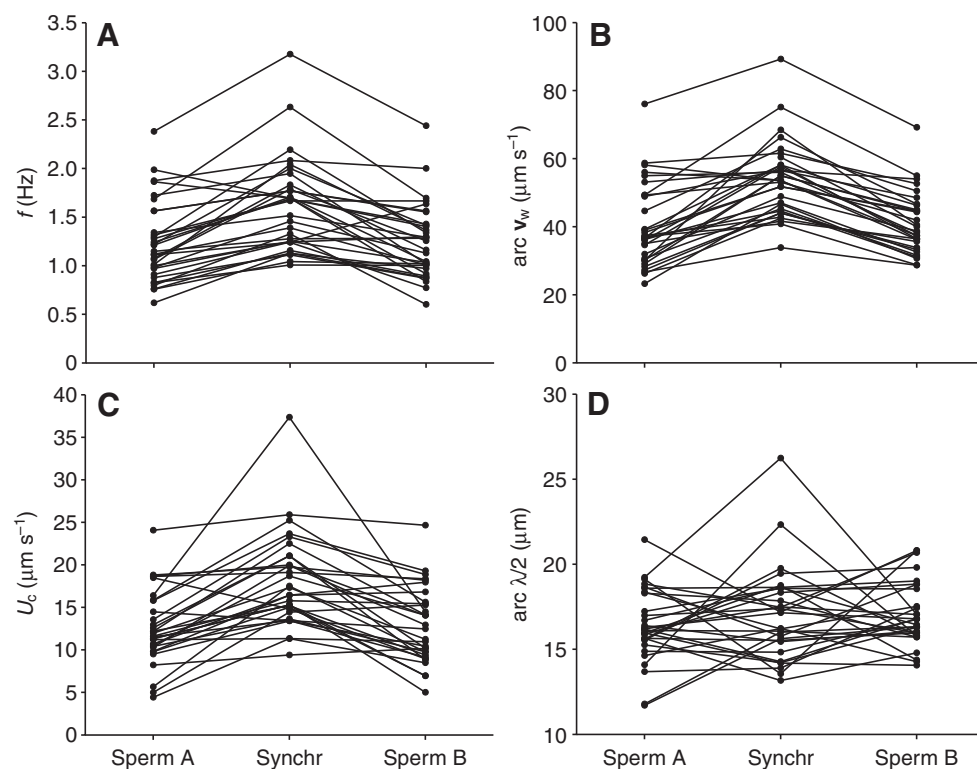


Fig. 4. (A–D) Results for the observed conjunctions and separations. In each instance, the two singleton spermatozoa were arbitrarily identified as Sperm A and Sperm B and the values for them are shown in the left and right columns. The new values attributable to full synchronisation are shown in the central columns of each panel, marked 'Synchr'. (A) The data for flagellar beat frequency ( $f$ ); (B) arc wave velocity ( $\text{arc} v_w$ ); (C) curvilinear swimming velocity ( $U_c$ ); and (D) the calculated values for arc bend length ( $\text{arc} \lambda/2$ ). The synchronised values in panels A–C have been compared with the means of the individual values and with the differences between the individual values: the correlation statistics are supplied, with an additional analysis, as a supplementary information file (see Table S1 in supplementary material). A negative correlation was found between  $\Delta \text{arc} v_w$  and the mean of the individual values, which suggests that the wave velocity is approaching some limit, perhaps a metabolic limit.

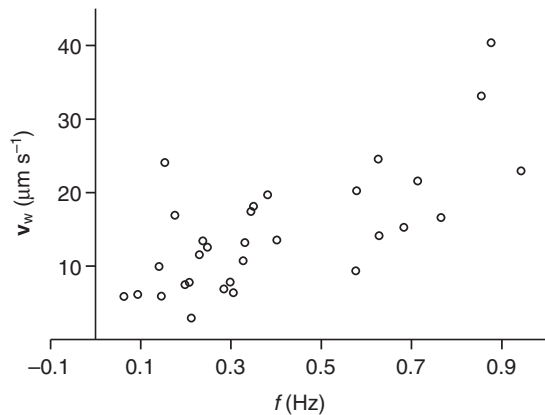


Fig. 5. Using the data displayed in Fig. 4, this graph relates the change in beat frequency ( $\Delta f$ ) to the change in arc wave velocity ( $\Delta \text{arc}v_w$ ). 'Change' is defined as the difference between the value when synchronised and the mean of the values when swimming independently. The correlation is high and very significant ( $R=0.700$ ;  $P<0.0001$ ).

of the left/right distinction is because all the spermatozoa stabilize at the cover slip with the same sidedness, for anatomical reasons (Woolley, 2003); thus, a further aspect is that the right-side bends were always the 'principal' bends in these preparations, developing greater bend angles.

A small number of individual spermatozoa had a different appearance. For these, the flagellum was entirely in focus at the level of the cover slip and the spermatozoan head appeared narrower and unevenly illuminated (Fig. 10); these spermatozoa had a higher beat frequency, a smaller wave amplitude and a greater velocity (analysis not pursued). Where there is a slight rotation (about the progression axis) between the plane-of-flattening-of-the-head and the plane-of-the-flagellar-beat, two stable orientations against the cover slip might

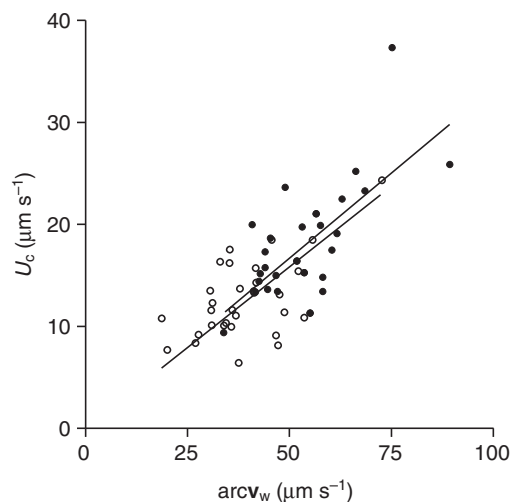


Fig. 6. Using the data displayed in Fig. 4, this graph relates the arc wave velocity ( $\text{arc}v_w$ ) to the curvilinear swimming velocity ( $U_c$ ) for the singletons (open circles) and the synchronised pairs (closed circles). Within each data set there was a significant correlation, as expected ( $R=0.573$ ,  $P=0.0008$  for the singletons and  $R=0.672$ ,  $P<0.0001$  for the synchronised pairs). Linear regression lines (forced to pass through the origin) for both data sets are superimposed; the regression coefficients were 0.32 (singletons) and 0.33 (pairs); for their interpretation, see the text.

be expected, i.e. with either the spermatozoan head or the flagellar waveform being flat against the surface. We observed that the heads of paired, synchronised spermatozoa appeared narrow and unevenly lit, suggesting that this pairing produced a tilting of the spermatozoan heads away from the cover slip.

Other conclusions are recorded from these images. The  $\theta$  increased with propagation, levelling off at approximately 3.5 rad or 200 deg. (Fig. 9, open symbols). Also, from plots of the inter-bend positions (distance from neck) against time (Fig. 11), it was found that the arc wave speed was constant along the flagellum except for a possible tendency to increase in the most distal part, with an obligatory rise in bend length. Both these results confirm those of Rikmenspoel (Rikmenspoel, 1984). The wave speed was the same for left- and right-side bends.

#### Related observations 2: effect of forward swimming on beat frequency, etc.

Some spermatozoa, that were first seen swimming freely and independently, became attached to the cover slip by their heads (Fig. 12A,B). This resulted in an immediate fall in  $f$ . This effect has previously been reported for echinoderm spermatozoa (Brokaw, 1965; Gibbons, 1975; Woolley and Vernon, 2001). Most examples were observed until they broke free again, when the former frequency was immediately restored. Detailed analysis showed that the fall in  $f$  (Fig. 13A) was accompanied by a fall in  $\text{arc}v_w$  (Fig. 13B). There was a significant positive correlation between these two changes ( $R=0.474$ ;  $P=0.040$ ). Waveform length was also reduced (Fig. 13C), reflecting increases in  $\theta$  (apparent in Fig. 13). But the effect on the  $\text{arc}\lambda/2$  was uncertain (Fig. 13D); although the correlation between the fall in frequency and a rise in calculated bend length was not significant ( $R=-0.277$ ;  $P=0.251$ ), there did seem to be a trend in that direction. Further analysis showed that the arc-wave-velocity-when-free-swimming was apparently equal to the sum of the arc-wave-velocity-when-stuck and the  $U_c$  (Fig. 14). This relationship will play a part in interpreting the effect of synchronisation on swimming velocity and  $v_w$ .

In six instances, spermatozoa were observed to break free after having been attached to the cover slip by the tip of the flagellum. In five of the six cases, breaking free produced a clear and immediate fall in  $f$ , with a rise in wave amplitude. This fact is recorded for interest, even though there are few data.

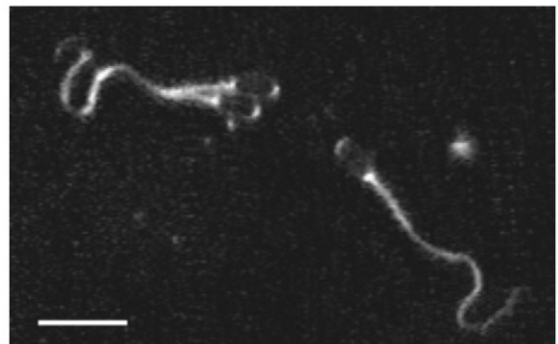


Fig. 7. An unusual case of flagellar synchronisation in which the two attached spermatozoan heads remained separately in contact with the cover slip. Because there was no superposition of the heads, the data for these two spermatozoa were not included in Fig. 4. Instead the data are presented separately in Table 1: for their interpretation, please see the Discussion. The third spermatozoon in the field would soon collide with the pair and bring about their separation. Scale bar, 20  $\mu\text{m}$ .

Table 1. Data relating to the rare (unique in our series) conjunction and separation, as illustrated in Fig. 7

	$f$ (Hz)	$\text{arc}v_w$ ( $\mu\text{m s}^{-1}$ )	$U_c$ ( $\mu\text{m s}^{-1}$ )	Wave slip (%)
Pre-sync, sperm A	2.42	75.12	17.02	77.3
Pre-sync, sperm B	1.89	66.24	15.21	77.0
Synchronised	3.00	91.94	14.58	84.1
Post-sync, sperm A	2.67	78.97	21.48	72.8
Post-sync, sperm B	2.22	64.84	17.33	73.3

The columns show beat frequency ( $f$ ), arc wave velocity ( $\text{arc}v_w$ ), curvilinear velocity ( $U_c$ ) and wave slip (see text). This event was treated separately because the spermatozoan heads failed to superpose, yet the flagella synchronised. The identities of spermatozoa A and B were not lost sight of during synchronisation and it is thereby shown that their same, relative values were regained. The absolute values regained were not exactly the same, presumably because of local variations in viscosity and surface friction.

DISCUSSION

For bull spermatozoa, we have observed that full flagellar synchronisation depended on the rigid attachment of the two spermatozoan heads, which usually took the form of an exact or nearly exact superposition of the heads. The process by which the heads attach may perhaps be analogous to that of erythrocytes forming rouleaux (see Armstrong et al., 2004; Rampling et al., 2004). The suggested explanation for synchronisation is this: with the neck regions of the two flagella bound together in parallel, when one neck region is swung to one side during bend growth, the attached neck region must swing in the same direction, experiencing initially passive bending and being triggered into active bend growth. We think the situation is equivalent to the entrainment effect shown by

Eshel and Gibbons (Eshel and Gibbons, 1989), who found that, by attaching a sinusoidally vibrating micropipette to the head of a sea urchin spermatozoon, the flagellar  $f$  could be driven away from its normal frequency in either direction. In the case of the conjoint bull spermatozoa, the mechanical influence will be mutual and will result in the new bends always growing together and in the same direction. That is, at bend initiation there is a common frequency and phase. The fact that no phase difference develops with bend propagation is probably because viscous coupling is effective over the short distances involved. Also, the fact that the conjoint bends are always both either ‘principal’ or ‘reverse’ may have contributed to this outcome.

It might be objected that flagellar synchronisation has been reported to occur between sea urchin spermatozoa without head-to-head attachment (Riedel et al., 2005). This work by Riedel and colleagues was a study of a type of ‘aggregate swimming’ [see Jahn and Bovee (Jahn and Bovee, 1967) for other examples] in which groups of about 10 spermatozoa swam in a hydrodynamically entrained fashion. Riedel et al. (Riedel et al., 2005) referred to ‘a particular form of synchronisation’ that developed even though the spermatozoa in the group were swimming at different speeds and with different beat frequencies. Their movie, however, seemed to reveal some transient side-to-side contacts between the spermatozoan heads; the importance of this is hard for us to assess.

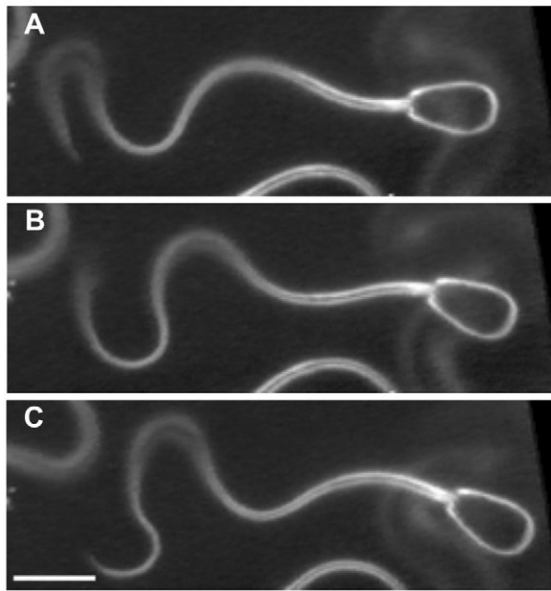


Fig. 8. (A–C) Three video-fields, successively 200 ms apart, showing bend propagation in a typical singleton spermatozoon. With the shallow depth of field given by a  $\times 100$  objective lens, it is possible to see that bends on the left side (here uppermost) of the flagellum are not in contact with the cover slip. The interpretation is that the true plane-of-beating is rotated slightly (about the progression axis and clockwise as viewed from in front of the spermatozoan head) with respect to the plane-of-flattening of the spermatozoan head (which here coincides with the plane of the glass surface). In fact, the ‘plane’ of beating is necessarily deformed and includes a downward tilt localised to the inter-bend regions. As the left-side bends propagate, their crests become progressively more out-of-focus but this does not mean that the angle between the ‘head plane’ and the ‘beat plane’ is growing. It is instead a result of the increasing bend angle on the distal flagellum (see Fig. 9 and text). Scale bar, 10  $\mu\text{m}$ .

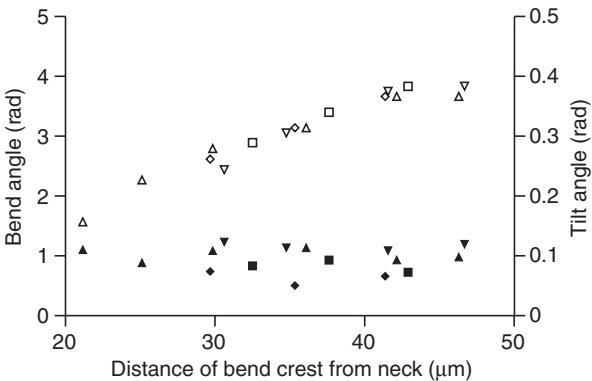


Fig. 9. Geometry of bend propagation in a typical singleton spermatozoon – similar to the one shown in Fig. 8. As the bend crests travel from the mid-piece region, there is a growth in bend angle that tends to level off at approximately 3.5 rad (open symbols). The calculated angle between the plane of the spermatozoan head/cover slip and the plane of the left-side bends (the ‘tilt’ angle) remains the same as the bends propagate, within the accuracy of this type of measurement (closed symbols). Successive bends on the flagellum are indicated by differently shaped symbols.

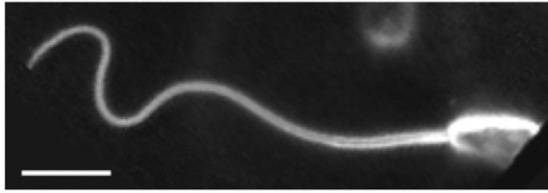


Fig. 10. A rare alternative appearance for singleton spermatozoa. This micrograph should be compared with Fig. 8. Our interpretation is that this cell has the same form as that in Fig. 9 but that it has stabilised against the cover slip with the 'beat plane' coinciding with the glass surface and the 'head plane' tilted away. The direction of its tilt should be 'right-edge-of-head downwards' but this was too difficult to confirm given the small magnitude of the displacement. Spermatozoa in this orientation swam noticeable faster than the majority – a fact used in understanding the effects of synchronisation (see Discussion). Scale bar, 10  $\mu\text{m}$ .

In our present work on bull spermatozoa, flagellar synchronisation, with head superposition, produced simultaneous 'triple' enhancement of  $f$ ,  $v_w$  and swimming velocity. An attempt will now be made to interpret these changes. Notwithstanding the inter-relatedness of the three variables, to pursue the 'problem of the oscillation', as recognised by Brokaw (Brokaw, 1989) and others, requires some consideration of whether there might be a primary change that has secondary consequences. The problem of flagellar oscillation can be resolved into the question of how a new bend is initiated with its direction of curvature opposite to that of its forerunner. And this resolves into the question of how the inter-doublet sliding direction is reversed close to the flagellar base, which further resolves to how the opposing dynein arrays are switched between active and inactive states in relation to force generation. For recent, detailed reviews of this topic, see Lindemann (Lindemann, 2007) and Brokaw (Brokaw, 2009).

In seeking the primary change brought about by synchronisation, we consider the category of low Reynolds number interactions. When two objects, each falling at its terminal velocity, come into proximity, they will fall in tandem faster than either alone. The closer

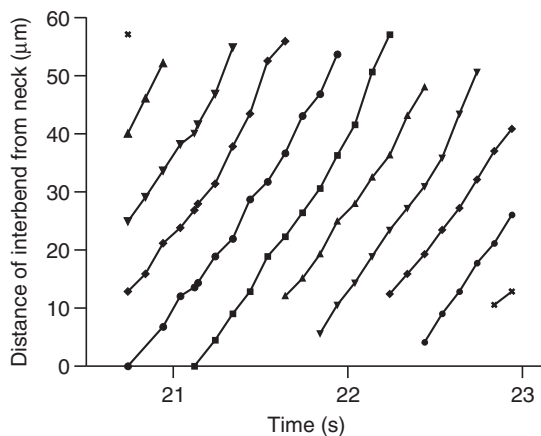


Fig. 11. Arc wave velocity ( $\text{arc}v_w$ ) in a singleton spermatozoon. The graph shows the arc distance of inter-bend loci from the head–neck junction, with the passage of time.  $\text{arc}v_w$  is given by the slope of the lines connecting the points. Arc bend length ( $\text{arc}\lambda/2$ ) is given by the vertical distance between the points at any time. There may be a slight trend towards increasing velocity in the distal region, with a necessary slight rise in  $\text{arc}\lambda/2$ . If this trend is real, it confirms that the estimates of  $\text{arc}v_w$  and  $\text{arc}\lambda/2$ , as used in our analysis, are indeed means for the transit of the flagellum.

they are, the faster they will fall (by up to 30%). This is because each is carrying fluid with it and the retarding effect of this moving fluid is less than if it were static. This explanation is based on experimental work by Happel and Brenner (Happel and Brenner, 1965), summarised by Vogel (Vogel, 1994). As an alternative explanation, the conjoint flagella may be touching and behaving as a single, thicker structure; it would then be relevant that an approximate doubling of the diameter would produce a less-than-twofold increase in the viscous drag on the cylinder. This may be seen from the formulae for the coefficients of viscous resistance in current use by hydrodynamicists (Johnson and Brokaw, 1979). Supposing that the active bending force has doubled and the elastic resistance to bending has doubled, the relatively lesser rise in viscous resistance should lead to a greater rate of bending and of bend propagation. This is consistent with the general finding that raising the viscosity of the fluid around a single flagellum slows the rate of bend propagation (Brokaw, 1966). Our first suggestion, then, is that the primary change is the enhancement of the  $\text{arc}v_w$ .

The enhancement of  $v_w$  should have two consequences. (1) An enhancement of  $U$ , provided there is no proportionate increase in the viscous resistance experienced by the spermatozoan head(s) (Lighthill, 1976). The exceptional spermatozoon–spermatozoon interaction (Fig. 7; Table 1) was included to illustrate this proviso. In fact, we think the drag on two heads when superposed is less than might be expected because they now tilt away from the glass surface (Fig. 1A; Figs 8 and 10). A further point is that a component of the observed enhancement of  $v_w$  will be accounted for by the increase in  $U$ , because  $v_w$  is compounded of rearward motion of the wave and forward motion of its point of origin (Gray, 1953) (Fig. 14). (2) An enhancement of  $f$ , provided there is no compensating reduction in wavelength ( $\text{arc}v_w = f \cdot \text{arc}\lambda$  for continuous travelling waves). We have found that the wavelength did not change in such a way (Fig. 4). We therefore suggest that the  $f$ , i.e. the timing of new bend initiation, is determined in some mechanical way by the  $v_w$ . Proposals for how mechanical feedback from the distal flagellum might operate on the proximal flagellum can be found in recent papers (Brokaw, 1985; Morita and Shingyoji, 2004; Lindemann, 2007; Woolley, 2007).

We have shown that, for bull spermatozoa, exact flagellar synchrony enhances  $f$  and  $v_w$  rather than averaging them. This is not the result predicted by Gray (Gray, 1930) nor by Machemer (Machemer, 1974), neither does it fit the prediction of Machin

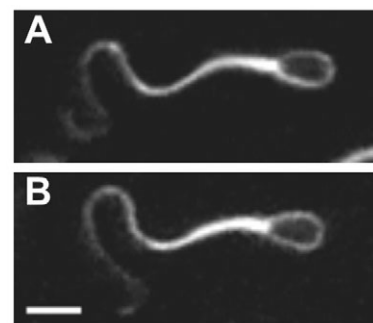


Fig. 12. An illustration of the change in wave amplitude on the distal flagellum between the free-swimming condition (A) and the arrest of the same cell by adhesion of the head to the cover slip (B). This cell later broke free again. Scale bar, 10  $\mu\text{m}$ . A full analysis of such occurrences is given in Fig. 13. The phenomenon shows how the free-swimming wave velocity contains a component equal to the forward swimming velocity (see text).

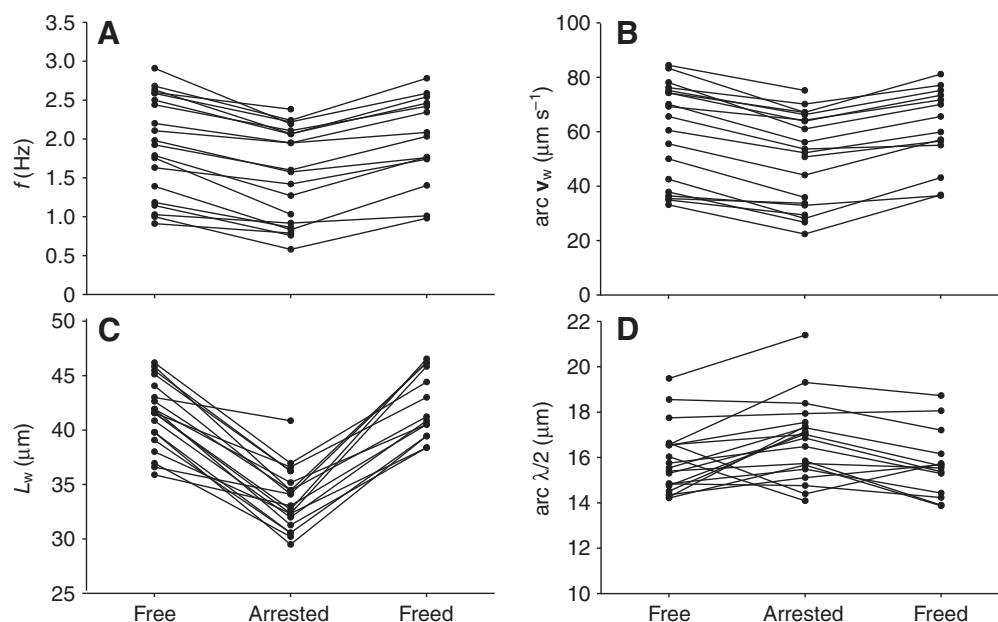


Fig. 13. Changes in flagellar attributes resulting from adhesion of the spermatozoon by the head (as illustrated in Fig. 12). In each panel, the data are shown for the free-swimming cell just before it became stuck (left column), then during the period of arrest (central column) and then again for the just-regained, free-swimming state (right column). The graph shows the effects of adhesion on (A) beat frequency ( $f$ ); (B) arc wave velocity ( $\text{arc } v_w$ ); (C) length of the waveform ( $L_w$ ); and (D) calculated arc bend length ( $\text{arc } \lambda/2$ ).

(Machin, 1963), who thought the conjoint frequency would be very close to one of the original frequencies. Theoretical studies of pairs of cilia, however, have predicted an enhancement of  $f$ , with the effect being greater as the inter-ciliary distance is reduced (Gueron et al., 1997; Gueron and Levit-Gurevich, 1998; Yang, X. et al., 2008). The theoretical model of Yang, Y. et al. (Yang, Y. et al., 2008) predicts that two spermatozoan flagella will synchronise without, necessarily, head-to-head contact whereas, in our present study, we saw no synchronisation without attachment between the sites of origin of the two waves.

If our findings on synchronisation can be applied to cilia, an enhancement of  $f$  and  $v_w$  would be expected to improve the effectiveness of water/mucus movement or locomotion. Attention is therefore drawn to the prevalence of highly synchronised groups of cilia in certain organisms, such as the 'compound' cilia of lamellibranchs and ctenophores (e.g. Baba and Hiramoto, 1970; Tamm, 1984).

For two bull spermatozoan flagella to synchronise, we have seen that the sites of origin of the two waves must be mechanically attached together. Is this conclusion applicable to other systems? The near-ubiquitous coordination between adjacent cilia (both synchrony and metachrony) may owe something to the fibrous strands that link together their basal bodies [as described by Pitelka (Pitelka, 1974)], such that, perhaps, a mechanical tilting of one basal body must be transmitted to the next, with a triggering effect. The elaborate ordering of such fibrous strands (the kinetodesmata) in ciliates certainly awaits a satisfying explanation. In the flagellates, specifically in the bi- and quadri-flagellates, there is often flagellar coordination, sometimes only in frequency, not phase (Inoue and Hori, 1991; Goldstein, 1992). Are the complex inter-basal body 'connectives' necessary for this? In fact, in one algal species, Hoops and Witman have presented evidence that they are not (Hoops and Witman, 1985). Turning to spermatozoa, synchronised beating might be expected in the biflagellate arrangement that has evolved in a few vertebrate groups (Spadella et al., 2006) but the motility has rarely been described. The only biflagellate spermatozoa to have been examined in the living state are those of the mollusc, *Corbicula* (Howard et al., 2004). Surprisingly, there was no synchrony, even though the basal bodies lie parallel, in rotational alignment, and

only about  $0.4 \mu\text{m}$  apart (Komaru and Konishi, 1996). Clearly there are limits to how far we can generalise our findings. However, if, in the bull, conjoint pairing of a proportion of the spermatozoa is established in the female tract, the elevation of the wave speed and the reduction in total head surface exposed to the fluid should mean that their swimming speed is greater than that of single spermatozoa. In some other mammals, spermatozoan conjunction occurs in the epididymis and the synchronised spermatozoan assemblies are better able to swim in viscous media, *in vitro* [guinea pig (Cody, 1925); opossum (Moore and Taggart, 1995); wood mouse (Moore et al.,

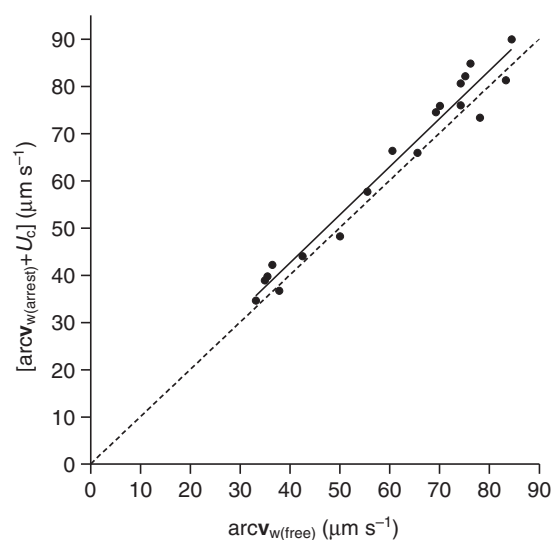


Fig. 14. In relation to the phenomenon of adhesion of the spermatozoon by the head, this graph shows that the arc wave velocity ( $\text{arc } v_w$ ) of the free-swimming cell is equal to the sum of the  $\text{arc } v_w$  of the arrested cell and the previous curvilinear forward velocity ( $U_c$ ). The data used were from the 'before-arrest' and 'during-arrest' states. The dotted line, the line of equality, lies close to the slope that represents the coefficient of linear regression ( $y=1.93+1.02x$ ). As an example of the magnitudes of these velocities, for  $\text{arc } v_w(\text{free})=55.5 \mu\text{m s}^{-1}$ , the  $\text{arc } v_w(\text{arrest})$  was  $44.0 \mu\text{m s}^{-1}$  and  $U_c$  was  $13.7 \mu\text{m s}^{-1}$ .

2002); rat (Immler et al., 2007); and echidna (Johnson et al., 2007)]. Spermatozoan conjunction is found also among invertebrate phyla, with most examples being listed in a paper by Ishijima et al. (Ishijima et al., 1999).

### LIST OF ABBREVIATIONS

$\text{arc}v_w$	arc wave velocity
$\text{arc}\lambda$	arc wavelength
$\text{arc}\lambda/2$	arc bend length
$f$	beat frequency
$L$	mean total flagellar length
$L_w$	length of waveform
$N$	number of bends on the flagellum
$t$	time
$U$	progressive velocity
$U_c$	curvilinear velocity
$v_w$	wave velocity
$\Delta f$	change in frequency
$\theta$	bend angle
$\lambda$	wavelength

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Table S1. Statistical analysis of the data displayed as Fig. 4. Correlations have been sought between the values for the changes in beat frequency ( $\Delta f$ ), arc wave velocity ( $\Delta \text{arcv}_w$ ) and swimming velocity ( $\Delta U_c$ ) and, in each case, the means of the individual values and the differences between the individual values

Comparison	Number of pairs	Correlation		
		coefficient ( $R$ )	$P$ value	Significance
$\Delta f$ relative to $(f_A + f_B)/2$	32	0.19	0.30	NS
$\Delta f$ relative to $ f_A - f_B $	32	-0.17	0.36	NS
$\Delta \text{arcv}_w$ relative to $(\text{arcv}_{wA} + \text{arcv}_{wB})/2$	32	-0.36	0.04	*
$\Delta \text{arcv}_w$ relative to $ \text{arcv}_{wA} - \text{arcv}_{wB} $	32	0.50	0.004	**
$\Delta U_c$ relative to $(U_{cA} + U_{cB})/2$	31	-0.11	0.54	NS
$\Delta U_c$ relative to $ U_{cA} - U_{cB} $	31	-0.04	0.84	NS
$\Delta \text{arcv}_w / \text{mean arcv}_w$ relative to $\Delta f / \text{mean } f$	32	0.49	0.004	**

Attention is drawn to the negative correlation between  $\Delta \text{arcv}_w$  and  $(\text{arcv}_{wA} + \text{arcv}_{wB})/2$ . This is suggestive of a metabolic limit to the rise in wave velocity. The positive correlation between the relative change in  $f$  and the relative change in  $\text{arcv}_w$  (bottom row) was perhaps to be expected. A and B refers to Sperm A and Sperm B, respectively. NS, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ .