Measuring Sperm Movement within the Female Reproductive Tract using Fourier Analysis

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Abstract: The adaptive significance of variation in sperm phenotype is still largely unknown, in part due to the difficulties of observing and measuring sperm movement in its natural, selective environment (i.e., within the female reproductive tract). Computer-assisted sperm analysis systems allow objective and accurate measurement of sperm velocity, but rely on being able to track individual sperm, and are therefore unable to measure sperm movement in species where sperm move in trains or bundles. Here we describe a newly developed computational method for measuring sperm movement using Fourier analysis to estimate sperm tail beat frequency. High-speed time-lapse videos of sperm movement within the female tract of the neriid fly Telostylinus angusticollis were recorded, and a map of beat frequencies generated by converting the periodic signal of an intensity versus time trace at each pixel to the frequency domain using the Fourier transform. We were able to detect small decreases in sperm tail beat frequency over time, indicating the method is sensitive enough to identify consistent differences in sperm movement. Fourier analysis can be applied to a wide range of species and contexts, and should therefore facilitate novel exploration of the causes and consequences of variation in sperm movement.

Key words: sperm motility, sperm bundle, Fourier analysis, CASA, diptera

INTRODUCTION

The extraordinary amount of variation in sperm phenotype observed across the animal kingdom has intrigued and confounded biologists for over 300 years, yet despite intensive research we still have little understanding of the causes and consequences of this variation (Birkhead & Montgomerie, 2009). It is widely accepted that sperm competition selects for increased sperm numbers (Parker, 1970), and that males strategically allocate sperm numbers to each mating event (Gage & Morrow, 2003; Pizzari et al., 2003; Kelly & Jennions, 2011). However, variability in sperm phenotype also functions in postcopulatory sexual selection, and can therefore influence the relative paternity success of males (Snook, 2005; Pizzari & Parker, 2009). Both sperm morphology (e.g., head size and shape, flagellum length) and sperm physiology (e.g., velocity, longevity) have been shown to influence competitive fertilization success, but the strength and direction of selection on sperm traits are inconsistent among studies (Humphries et al., 2008; Pizzari & Parker, 2009). We know relatively little about the adaptive significance of sperm motility (Werner & Simmons, 2008; Cummins, 2009), potentially because it is difficult to get an accurate measure of sperm movement. In addition, the limited data we do have is restricted to a few model species. Hence, to understand sperm diversity we need more empirical data, across a wide range of species, investigating how different selection pressures shape sperm form and function.

The development of computer-assisted sperm analysis (CASA) systems has enabled objective and accurate tracking of sperm movement. Modern CASA systems can capture images of 20 to >1,000 sperm at 50–60 frames per second, and provide morphological measurements in addition to comprehensive motion analyses (reviewed in Amann & Waberski, 2014). These systems measure sperm movement by establishing a centroid for each spermatozoon and evaluate cell motion based on centroid trajectory (Amann & Waberski, 2014). Thus, they can only be used when individual sperm cells are able to be identified by the computer software. In several species, sperm move in trains or bundles (reviewed in Immler, 2008; Pizzari & Foster, 2008), and thus individual cells may not be able to be tracked. In these cases, a new method of measuring sperm movement is required.

A second issue with current methods of measuring sperm movement is that, for logistical reasons, sperm are usually tracked in vitro using artificial environments outside the animal’s body. However, in vitro measures may not reflect how the sperm moves within the female tract (Werner et al., 2007; Humphries et al., 2008). Within the female tract, sperm are spatially restricted and are therefore likely to be
influenced by physical constraints such as wall effects (Humphries et al., 2008). Furthermore, natural fluids within the female tract may alter sperm movement (Curtis & Benner, 1991). Hence, selection pressures on sperm movement may only become apparent when measuring sperm movement in vivo. For example, by measuring sperm competition within the female tract of Drosophila melanogaster, Lupold et al. (2012) were able to demonstrate that males with relatively slow and/or long sperm had a competitive advantage as they were better at displacing rival sperm and resisting ejection by the female (Lupold et al., 2012).

Both of these problems were encountered when trying to measure sperm movement in the nerid fly Telostylinus angusticollis. When T. angusticollis sperm are dissected straight out of the testes, the sperm within a bundle remain associated through adhesion of the sperm heads (Fig. 1). The tails beat rapidly, but the bundles do not achieve any forward motion (see Supplementary Video 1). If a male and female are mated and then the female reproductive tract is dissected out, the sperm display a very different movement – similar to a flowing stream (see Supplementary Video 2). We were unable to track sperm within the female tract using CASA (or by manual frame-by-frame tracking), as even though individual sperm heads can be identified, the sperm heads go in and out of focus as the entire bundle slowly turns in a forward spiral movement.

**Supplementary Videos 1 and 2**
Supplementary Videos 1 and 2 can be found online. Please visit journals.cambridge.org/jid_MAM.

Even when individual sperm or tails cannot be visualized and tracked, the collective motion of the tails is apparent as a periodic change in local contrast in transmission or dark-field microscopy time-lapse images. As the sperm tail sweeps past a specific point, the light intensity at that point will vary in time, peaking with each tail crossover event. Thus, beating tails cause a periodic signal in the intensity versus time trace at a frequency equal to the beat frequency of the tails. The Fourier transform can be used to transform this signal from the time domain into the frequency domain; the maximum of the resulting power spectrum will fall at the fundamental frequency of the periodic signal, here taken as the tail beat frequency (Bloomfield, 2000). A similar approach has been used successfully to analyze rotational speeds in bacteria (Kobayasi et al., 1977) and the influence of dynein structure and calcium flux on flagellum beating frequencies in Chlamydomonas (Kobayasi et al., 1977; Smyth & Berg, 1982; Kamiya & Hasegawa, 1987; Sakakibara & Kamiya, 1989; Takada & Kamiya, 1997; Wakabayashi et al., 2009). These methods are only capable of measuring a signal from a single cell at any one time. Time-lapse image acquisitions capture many locations in a field of view in parallel and each pixel in the resulting video yields an independent intensity versus time trace. Repeating the Fourier analysis at each pixel in the image area generates a map of beat frequencies as a function of position (Fig. 2). Hence, sperm movement within a female tract can be estimated by calculating the average beat frequencies of pixels within regions of interest (ROIs) from a time-lapse video. Here we describe both this method and the necessary computational tools to perform this analysis on similar video data sets of sperm tail motion in vivo.

**Materials and Methods**

**Source and Rearing of Flies**

Nerid flies (T. angusticollis) were sourced from outbred lab stocks, originally captured from and annually supplemented with wild-collected individuals from Fred Hollows Reserve in Sydney, Australia. To obtain a range of sperm phenotypes, male flies were raised on either a high-quality (rich) or low-quality (poor) larval diet (see Bonduriansky, 2007 for details) and housed as adults in either single sex or mixed sex groups. The effects of larval diet and adult social environment on sperm phenotype will be investigated in a separate manuscript. All females were raised on a rich larval diet and

![Figure 1. Sperm dissected from the testes (a) and swimming within the female reproductive tract (b) of Telostylinus angusticollis.](image-url)
Sperm Imaging

To observe sperm movement within the female reproductive tract, a single male was placed in a glass scintillation vial with a single female until mating was observed. When mating ceased, females were sacrificed and their reproductive tract was immediately dissected. With the aid of a dissecting microscope, the female ovipositor was severed from the abdomen and placed in a drop of phosphate-buffered saline solution on a microscope slide. The saline solution was maintained at a concentration of 6,450 mg/L, as pilot studies showed that sperm movement stopped when the solution varied significantly above or below this concentration (E.L. Macartney, unpublished data). The bursa and spermathecal ducts and glands were gently teased apart from the surrounding tissue using micropin probes and fine forceps. The separated female reproductive tract was then transferred to a new slide with a fresh drop of saline, and covered with a coverslip. To observe sperm directly from the testes, males were sacrificed and their testes immediately dissected with the aid of a dissecting microscope. A single testis was placed in a drop of saline solution on a microscope slide, and the tip of the proximate end (close to the ejaculatory duct) severed using micropin probes to release mature sperm.

Videos of sperm movement were captured using a Zeiss AxioCam HSc high speed camera mounted on a Zeiss AxioScope A1 compound microscope, and time-lapse image stacks recorded using Axiovision software (Carl Zeiss Microscopy, Germany) creating time-stamped ZVI files. The time from female sacrifice to recording the first video was ~5 min. Sperm were viewed under darkfield illumination, using the maximum amount of transmitted light that was possible without causing overexposure (average exposure time 5–8 ms). Videos within reproductive tracts were recorded for 60 s at a capture rate of 45–55 frames per second, limited by the camera data transfer speed. Data for method validation (Fig. 3) were recorded for 12.1 s at a frame rate of 50 frames per second (606 frames). Sperm movement within both sperm ducts and spermathecae (storage organs) was recorded to test for differences in sperm movement within different sections of the reproductive tract. The majority of videos were captured at 40× magnification, but a subsample of videos was captured at 20× magnification to allow the measurement of multiple sections of the reproductive tract within a single video. A second video of a different area of the reproductive tract was captured when possible.

Tail Position Tracking

In a video of sperm dissected straight from the testes, regions where a single beating sperm tail was clearly visible were identified. Within one of these ROIs, a line was drawn perpendicular to the tail axis (Fig. 3a, red and blue lines), and the position of the maximum intensity pixel along that line was determined for each frame (Fig. 3b, cyan marker). We plotted the position along this line as a function of time (Fig. 3c, black circles and dashed connecting line), applied a moving average filter of length equal to the number of data points to remove large-scale motion, and subtracted the smoothed data from the original trace to yield only the high-frequency periodic signal. We then fit sections in time of the filtered trace to a cosine function with amplitude, period, and phase as free parameters, and re-added the smoothed trace to yield the plotted fits (Fig. 3c, colored lines).

Fourier Analysis of Sperm Tail Beat Frequency

Fourier analysis was performed on a pixel-by-pixel basis on the time-lapse image stack. The described process was performed sequentially for a set of experimental data and was automated with the SpermTailMotilityProcessing GUI, custom-written in MATLAB and described below.

The Fourier analysis method was as follows: at a given pixel in a single image stack, the intensity versus time trace was extracted with the timestamps recorded in the parent ZVI file utilized to generate the time axis. This data were interpolated to a regular time axis before filtering. For sperm motion quantification, the interpolated intensity versus time trace was high-pass filtered at 2.5 Hz to suppress slow motion (such as muscle contraction or stage drift), notch filtered to suppress peaks at 13.22 and 16.3 Hz, and comb filtered to remove harmonics at $F/3$ and $F/4$, where $F$ is the imaging frame rate. The high-pass filtering with a 10th-order Butterworth filter was generated with the butter function, the notch filters with the iirnotch function, and comb filters with the iircomb function in MATLAB.

The frequency position of the maximum of the Fourier transform of the filtered trace was taken as the average beat frequency at that pixel. In addition, the beat intensity can be taken as the value of the maximum of the Fourier transform.
Fourier transforms were performed with the `fft` function in MATLAB following zero-padding of the intensity versus time trace to a length of $2^{1+N}$, where $N$ is the binary logarithm of the number of frames in the image stack, rounded down to the next lower integer value. Repeating this process at every pixel and mapping the beat frequency back to the 2D pixel position resulted in a map of beat frequency with respect to position. To represent both frequency and intensity, an image was generated where image hue represents the pseudocolored map of beat frequency and image intensity the beat intensity from the Fourier transform amplitudes (see Fig. 2), defined as the logarithm of the square of the peak power in the calculated power spectrum.

Saved MATLAB data files of the calculated average beat frequency for each pixel in the composite image were loaded into a second MATLAB-based GUI program named `SpermTailMotilityVisualization` that calculated the mean, median, minimum, maximum, and standard deviation of all

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**Figure 3.** Benchmarking sperm-beating frequency. Lines drawn perpendicular to active and isolated sperm tails in a sample of extracted sperm acquired as video data at 50 frames per second (a). Magenta line with yellow bounding box shows region of representative data for (b–f). The position of maximum intensity (b, cyan x) along the drawn line (b, magenta) shows reciprocal motion as the sperm tail beats in time. The position of maximum intensity in time manifests this motion as a periodic signal (c, black) that can be fit to a cosine function (c, solid colors). Phase shifts in this signal preclude fitting to a single function, though separate segments can be fit individually with frequencies of 4.61–4.68 Hz (weighted mean of 4.65 Hz). The Fourier transform (d) of this signal shows a peak at 4.64 Hz. The intensity versus time trace (e) at the mid-point of the drawn line (b, green squares) shows a similar periodic signal with a peak in the Fourier transform (f) at 4.69 Hz. Line in (b) is 5.15 µm long in image scale and times are relative to the acquisition start. The first 500 of 606 acquired frames shown in (c) and (e).
beat frequencies within a user-defined ROI. ROIs were selected by tracing around the internal perimeter of spermathecae and straight sections of ducts, leaving a slight gap from the edge to avoid wall effects. Estimates of tail beat frequency were calculated from each separate duct and spermathecae visible within each video.

Fourier analysis on benchmarking data (Fig. 3) was first performed by taking the Fourier transform of the raw position versus time trace as determined above (Fig. 3d). The intensity versus time trace for this same ROI was determined by segmenting the signal at a single pixel located at the midpoint of the drawn line (position indicated by a green square in Fig. 3b; extracted signal Fig. 3e). The Fourier transform of this intensity versus time trace was taken in the same manner as the position versus time trace (Fig. 3f). The peak of this trace was defined as the position of the largest amplitude at a position past a threshold of the first five data points in order to avoid the influence of large and slow motions.

**Results**

The Fourier transform method was able to successfully identify areas of sperm movement within each video, with areas of time-lapse images showing the greatest beat intensity corresponding to spermathecae and ducts containing active sperm tails (Fig. 2). Mean tail beating frequencies calculated from each ROI were consistently higher than median values (mean of means ± SE = 3.787 ± 0.051 Hz, mean of medians ± SE = 3.465 ± 0.036 Hz, paired \( t_{136} = 12.012, p < 0.001 \), as mean values appeared to be heavily influenced by rare high value pixels (as evident from max values of up to 29.53 Hz) that were presumably not reflective of actual sperm movement. Thus, median values for each ROI were used in all further analyses. Estimated sperm tail beating frequency (calculated as the median value from a ROI) ranged from 2.97 to 5.5 Hz, suggesting there is variance in sperm movement among males that can be detected using this method.

**Measurement Error of ROIs**

In 15 haphazardly chosen videos, a single ROI was measured on 5 separate days. Median tail beat frequency calculated from these ROIs was highly repeatable across days [\( \text{MS}_{\text{video}} = 1.550, \text{MS}_{\text{error}} = 0.001, \text{repeatability} > 0.99 \) (Lessells & Boag, 1987)]. Thus, measures of sperm movement are not influenced by slight differences in the positioning of ROIs when tracing around the duct and/or spermathecae.

**Spermathecae Versus Ducts**

The easiest section of the female reproductive tract to visualize sperm movement is within the spermathecae. However, as the sperm are spiraling around and on top of each other in a circular motion in these storage organs (see Supplementary Video 2), we were concerned that the Fourier analyses would pick up multiple frequencies and give inaccurate measurements in the spermathecae. To test this, we compared tail beat frequency calculated from spermathecae versus ducts in videos where both areas of the female tract were visible simultaneously (\( n = 10 \)). No difference was detected between measures from spermathecae versus ducts in these videos (mean of medians ± SE: spermathecae = 3.570 ± 0.218 Hz, ducts = 3.551 ± 0.092 Hz; paired \( t_{9} = 0.114; p = 0.912 \)). Thus, either area of the female reproductive tract may be used to estimate sperm tail beat frequency in *T. angusticollis*. This result was not caused by a lack of variation among individuals in sperm tail beat frequency, as estimates from ducts were positively correlated with estimates from spermathecae across individuals (\( \beta = 1.578, F_{1,9} = 6.322, p = 0.036 \)).

**Changes in Sperm Speed Over Time**

For 22 males we recorded two replicate videos. Due to the time taken for the computer to process each video file, the time between these consecutive videos ranged from 4 to 12 min (mean = 7 min). Because the second video was always filmed later than the first video, we were able to use the replicate videos to look for changes in sperm tail beat frequency over time. To increase the precision of estimates of sperm movement, we used the average of median values from all ROIs within each video. Sperm tail beat frequency estimated from the first video was consistently higher than from the second video (mean of medians ± SE: video 1 = 3.501 ± 0.061 Hz; video 2 = 3.323 ± 0.031 Hz; paired \( t_{21} = 3.213, p = 0.004 \)). This not only tells us that sperm tail beat frequency slows over time, but that the method is accurate and precise enough to detect consistent changes among videos. Estimates from video 1 significantly predicted estimates from video 2 (\( \beta = 0.466, F_{1,19} = 2.419, p = 0.026 \)), indicating there are consistent differences among males in sperm tail beating frequency. However, estimates from video 1 and time between videos only explained 29% of the variance in estimates from video 2 (\( R^2 = 0.294, F_{2,19} = 3.964, p < 0.036 \)), suggesting that males differ in the rate of decline of their sperm tail beat frequency.

**Validating the Fourier Analysis Method**

To benchmark the Fourier analysis technique, sperm tail beat frequency was calculated by tracking an individual sperm tail in a video of sperm extracted directly from the testes (Fig. 3). The position of maximum intensity along the lines indicated in Figure 3a show periodic motion in time. It was not possible to fit the entire time series to a single cosine function as there are several phase shifts through the time captured (Fig. 3c). However, the frequency of the tail beat is nearly constant throughout the imaged timeframe, varying from 4.61 to 4.68 Hz across the analyzed section (first 500 frames). A Fourier transform of the full maximum intensity position versus time trace shows a peak corresponding to 4.64 Hz, which is qualitatively equivalent to the time-weighted average
of the cosine fits (4.65 Hz). Furthermore, a Fourier transform of the single-position intensity versus time representative data shows a single well-defined peak corresponding to 4.69 Hz, which closely matches estimates obtained from the maximum intensity position versus time fits and Fourier transform. Tracks of other individual sperm tails show respective periods of 3.94, 4.22, and 3.94 Hz for cosine fitting, 3.76, 4.14, and 3.81 Hz for Fourier analysis of the respective position versus time traces, and 3.76, 4.10, and 3.80 Hz for Fourier analysis of the respective intensity versus time traces.

**DISCUSSION**

We developed a new method of quantifying sperm movement using Fourier analysis of single-position intensity versus time traces in video microscopy data to generate maps of estimated sperm tail beat frequency. Unlike current CASA systems (Amann & Waberski, 2014), this method does not rely on tracking individual sperm heads or tails. Hence, the Fourier analysis method can be used to measure sperm movement in species where sperm move in bundles or trains. Estimates of sperm tail beat frequency calculated using the new method were qualitatively equivalent to measures of individual sperm tail beat frequencies calculated with computer-assisted tracking of sperm tail positions. Thus, we are confident that sperm tail beat frequencies estimated using the Fourier analysis technique accurately represent the true tail beat frequency of sperm.

This technique is made available to researchers through a group of MATLAB GUIs for performing the Fourier analysis and visualizing and exporting the generated results. Processing can be optimized with the SpermTailMotilityProcessingSingle function with the ability to interactively view data, add filters, and quickly investigate the Fourier analysis at a single point before processing an entire data stack. This analysis can then be replicated across a group of stacks in a batch-wise fashion with the corresponding SpermTailMotilityProcessingGUI routine. Visualization and ROI selection is accomplished with SpermTailMotility Visualization. The visualization GUI allows the user to select regions with rapid feedback of the summary statistics for the ROI. Additional analysis is accomplished by exporting results from the selected ROI to a CSV or Excel file. Source codes are available by request from the corresponding author.

Our new method was able to detect small changes in sperm tail beat frequency over 5–10 min intervals, and is therefore sensitive enough to detect consistent differences in sperm movement. Furthermore, measures of sperm tail beat frequency estimated from different sections of the female reproductive tract were correlated across males, indicating that sperm from different males show consistent variation in their tail beat frequency. This suggests that the method can be used to test for environmental effects on sperm phenotype, which was the main motivation for developing the method.

In addition to being able to measure sperm bundle movement, the greatest advantage of the Fourier analysis method is that it can be used to measure sperm movement within the female tract, allowing measurement of functional differences in sperm movement among males (Humphries et al., 2008). Even in species where individual sperm can be tracked *in vitro*, it is much more difficult for tracking software to identify and follow individual sperm in the confines of a female tract. The approach we developed avoids the need for tracking individual sperm, and can thus potentially be used in all species. Sampling sperm within the female tract rather than sperm stripped from males ensures that only ejaculated sperm are included in the measurement. However, males may strategically alter ejaculates depending on female quality (Cornwallis & Birkhead, 2007; Kelly & Jennions, 2011). Therefore, the condition and mating history of females used in assays should be standardized (Lupold et al., 2012). Regardless, female variation is likely to increase uncertainty in the data set, and thus these female effects should be examined in future studies by mating a given male to multiple females and factoring the resulting variation into analyses.

There was no difference in sperm tail beat frequency measured from sperm movement within ducts or spermathecae (storage organs). Hence, either region of the female tract may be used to measure sperm movement in *T. angusticollis*. However, the fastest measurements were observed around bends in ducts, which is likely to be caused by the physics of the flow dynamics around the bend rather than sperm movement itself (Hawthorne, 1951). In addition, the lining of the female tract may influence sperm movement via wall effects (Woolley, 2003; Humphries et al., 2008). Thus, we suggest the most precise estimates of sperm movement for each male will be obtained by averaging the median tail beat frequency from several ROIs within a video, avoiding bends and edges of ducts.

Previous Fourier analysis methods for quantifying beat frequency (Kobayasi et al., 1977; Smyth & Berg, 1982; Kamiya & Hasegawa, 1987; Sakakibara & Kamiya, 1989; Takada & Kamiya, 1997; Wakabayashi et al., 2009) were performed as single-point measurements. The extension to video data with the parallel acquisition of many thousands of points allows for maps of sperm activity through an imaging region. These maps provide a unique visualization for the motion within a sample and allow for analysis of sperm motion variations through different structures in a field of view without having to control for asynchronicity artefacts. Sources of fluctuations in the intensity versus time trace not caused by sperm motion (such as slow motions due to muscle contractions of the female reproductive tract or stage drift) appear in the frequency domain as very low frequency signals and are straightforward to filter out to avoid contaminating the analysis. In addition, Fourier analysis methods average out noise such that a low signal-to-noise periodic signal can still be recovered given a signal of sufficient length. At the same time, the upper limit to the frame rates that can be measured is half of the imaging frame rate. To capture high-speed motion requires fast
imaging rates, which often produces lower-quality images, but this can be compensated for by taking more images rather than exposing each image for a longer time and sacrificing frequency range.

The Fourier analysis method was developed to measure male variation in sperm movement in species where individual tracking of sperm is not possible. However, we do not know how our measure of sperm movement relates to current measures of sperm velocity from CASA systems (e.g., average path velocity, straight line velocity, curvilinear velocity; Amann & Waberski, 2014). Thus, while qualitative results of experiments can be compared with previous studies, quantitative measures such as effect sizes and absolute measures of sperm movement will not be directly comparable. This may be addressed in the future by measuring sperm movement concurrently with a traditional CASA system and the newly developed Fourier analysis method. It should be noted that sperm measures from different CASA systems may not be directly comparable (Jasko et al., 1990; Boryshpolets et al., 2013), so this may be a more general problem that needs to be addressed in the study of sperm quality. It will also be important to assess how sperm tail beat frequency relates to progressive motility and sperm swimming performance in general before comparing results of experiments using the Fourier analysis method to other studies of sperm movement.

**Conclusions**

Despite over 300 years of research into the diversity of sperm form and function, we still only have a superficial understanding of the adaptive significance of sperm motility, particularly in species with internal fertilization (Cummins, 2009). Sperm motility has obvious implications for male reproductive tactics, sperm competition, and fertilization success. In addition, intriguing links found between sperm longevity and offspring survival suggest that sperm motility may have transgenerational consequences for offspring fitness (Crean et al., 2012). Advances in our understanding of sperm motility rely on the development of suitable technologies in microscopy, recording and analysis (Birkhead & Montgomerie, 2009). We have developed a method of measuring sperm movement in species where individual sperm cannot be tracked using CASA systems. The general nature of the method means it can be applied to a wide range of species and contexts, and therefore we believe this method will facilitate novel exploration of the causes and consequences of variation in sperm movement.

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**References**


