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# The use of optical tweezers to study sperm competition and motility in primates

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Optical trapping is a non-invasive biophysical tool which has been widely applied to study physiological and biomechanical properties of cells. Using laser 'tweezers' in combination with custom-designed computer tracking algorithms, the swimming speeds and the relative swimming forces of individual sperm can be measured in real time. This combination of physical and engineering tools has been used to examine the evolutionary effect of sperm competition in primates. The results demonstrate a correlation between mating type and sperm motility: sperm from polygamous (multi-partner) primate species swim faster and with greater force than sperm from polygynous (single partner) primate species. In addition, sperm swimming force linearly increases with swimming speed for each species, yet the regression relating the two parameters is species specific. These results demonstrate the feasibility of using these tools to study rapidly moving ($\mu m\ s^{-1}$) biological cells.

**Keywords: primate sperm competition; laser tweezers; sperm swimming force**

## 1. INTRODUCTION

Single spot, gradient force laser tweezers were first used to manipulate single cells in the 1980s (Ashkin *et al.* 1987) and soon thereafter were applied to subcellular organelles, such as chromosomes on the mitotic spindle (Berns *et al.* 1989). Since then, laser tweezers have been used to study many aspects of cell behaviour and physiology (Ashkin 1991; Berns 1998; Ozkan *et al.* 2003; Shao *et al.* 2006). In particular, laser tweezers have been used to trap sperm cells to study laser–sperm interactions and quantify sperm motility by measuring sperm swimming forces (Tadir *et al.* 1989, 1990; Araujo *et al.* 1994; Dantas *et al.* 1995; Konig *et al.* 1996; Patrizio *et al.* 2000). These studies found that the minimum laser power needed to hold a sperm in a trap is directly proportional to the sperm swimming force ($F = Q \times P/c$ where $F$ is the swimming force, $P$ is the laser power, $c$ is the speed of light in the medium with a given index of refraction and $Q$ is the geometrically determined trapping efficiency parameter; Konig *et al.* 1996).

Recent studies found a correlation between sperm swimming forces and swimming speeds (Tadir *et al.* 1990; Nascimento *et al.* 2006). For domestic dog sperm, it was found that for a subset of the sperm population, swimming force linearly increased with swimming speed (Nascimento *et al.* 2006). The study showed that in addition to common measurements of sperm motility, such as swimming velocity and lateral head movement, the quantitative measurement of sperm swimming force is a useful parameter for assessing sperm motility and quality. However, in that study, a major problem was low throughput. The user had to manually trap the sperm and use an offline program to calculate the swimming speed of each sperm analysed. The challenges thus were (i) to catch a sperm and measure its swimming force and velocity all automatically and (ii) to repeat this process for many sperm (more than 150) during a period of approximately 3 hours (period during which sperm preparation was viable). These problems have been solved through a combination of laser tweezers and computer/robotic technology (Shi *et al.* 2006*b*), creating a system that can be used to assess sperm from a variety of species.

In primate species where the mating pattern is polygamous (multimale–multifemale), several different males copulate with a single female within a short time frame. As a result, strong competition for fertilization between the sperm from the rival males occurs within the female reproductive tract (Dixson 1998). Evidence

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indicates that sexual selection through sperm competition has directly affected sperm morphology, sexual behaviour and the structure of accessory sexual organs. Sexual selection has been shown to result in the evolution of larger testis relative to body weight in males where the level of sperm competition is high (Harcourt *et al.* 1981). Males from multi-partner mating mammals were shown to have high sperm production rates, large sperm reserves and large numbers of sperm per ejaculate (Moller 1989). In addition, the vas deferens is shorter and has a thicker muscular structure in males from multi-partner mating systems than in males from single partner mating systems (Anderson *et al.* 2004). Morphological analysis of individual sperm showed that sperm from males of multi-partner mating systems have larger midpiece volumes when compared with those from males of single partner mating systems (Anderson & Dixson 2002).

In this paper, we combine laser trapping with computer-tracking software and robotics to study the question of sperm evolution in relation to the mating patterns of different primates. We measure and compare sperm swimming force and swimming speed as well as the relationship between these two parameters for four primate species: chimpanzee; rhesus macaque; human; and gorilla. The results are supportive of sperm competition during the evolution of primates. Additionally, an interface between biology, physics and engineering has been made, resulting in the shedding of light on an important biological question.

## 2. MATERIAL AND METHODS

### 2.1. Specimens

Semen samples were collected from four primates: chimpanzee (*Pan troglodytes verus*, four males, fresh samples); rhesus macaque (*Macaca mulatta*, two males, fresh and frozen samples); human (*Homo sapiens*, four males, fresh and frozen samples); and western lowland gorilla (*Gorilla gorilla*, two males, fresh and frozen samples). Although we recognize that it is difficult to do a comparative study where semen samples from each species are prepared using different methods, the procedures used have undergone a great deal of optimization by the laboratories specialized in studying sperm of each respective species. In addition, since prior studies have shown that properly freezing, storing and thawing sperm has no significant effect on the escape force of human sperm (Dantas *et al.* 1995), frozen–thawed and fresh semen samples in the present study are considered comparable.

Sperm preparation protocol for fresh chimpanzee (collected using an artificial vagina), fresh human (collected by masturbation after 3 days of abstinence) and fresh gorilla (collected opportunistically after animal masturbated in cage) samples is as follows: samples were allowed to liquefy at room temperature for 30 min then added to 10 ml of Biggers, Whittens and Whittingham (BWW) with penicillin and streptomycin and transported in a cooler at 10°C. Samples were washed with BWW (21 mM HEPES, 21.5 mM lactic acid, 91.06 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl, 1.71 mM $KH_2PHO_4$, 2.44 mM $MgSO_4$, 5.55 mM glucose, 4 mM $NaHCO_3$, 0.25 mM Na pyruvate, 1% bovine serum albumin (BSA), pH 7.5, filter sterilized over 22 µm filter, osmolarity 295 m Osm $kg^{-1}$ water, kept at 4°C and warmed to room temperature prior to use) by spinning at 600*g* for 6 min. These samples were received within 24 hours of ejaculation and analysed within 48 hours. Fresh rhesus macaque semen samples were prepared for cooled (5°C) storage as follows: 4 ml of media (0.5 mg of BSA per 1 ml of BWW) was added to the semen and rotated for 5 min on a rocker. The coagulum was pulled up onto the side of the tube and left to retain for 10 min. The top 3.5 ml of sperm supernatant was transferred into a new tube (sperm motility and concentration were checked). The sample was centrifuged at 300*g* for 10 min then resuspended at $50\times10^6$ $ml^{-1}$ in a commercial non-fat dry skim milk semen diluent (Animal Reproduction Systems, Chino, CA). The sample was loaded into Whirl-Pak bags (Nasco, Fort Atkinson, WI) ensuring minimal air space, then placed in an Equitainer semen transporter (Hamilton Research, Inc., South Hamilton, MA) and shipped overnight to the laboratory. The samples arrived approximately 24 hours later and were analysed after warming the sample (37°C) for 5–10 min. Frozen rhesus macaque semen samples were prepared using a programmable freezer using a Tes–Tris egg yolk-containing medium in addition to 3% glycerol (freezing curve: 22°C–8°C at 0.2°C $min^{-1}$ followed by cooling to −110°C at −17°C $min^{-1}$ and plunging in liquid nitrogen), and were thawed in 37°C water bath for approximately 1 min before analysis. Frozen human semen samples were frozen according to published protocol (Ethics Committee of the American Fertility Society 1986; Serfini & Marrs 1986; DiMarzo *et al.* 1990) and prepared for analysis using a twice-wash protocol (Toffle *et al.* 1985; DiMarzo & Rakoff 1986). Frozen gorilla samples were frozen according to published protocol (O'Brien *et al.* 2002) and thawed for 8 s in 50°C water bath before analysis.

All primate samples, except human, were suspended in BWW+BSA (1 mg of BSA per 1 ml of BWW) for analysis. Human sperm were suspended in modified Human Tubal Fluid (mHTF) HEPES buffered (osmolarity 272–288 m Osm $kg^{-1}$ water, pH of 7.3–7.5) with 5% serum substitute supplement (SSS) filtered through 0.2 µm syringe filter, first five drops were disposed (Irvine Scientific, Santa Ana, CA). Sperm samples of 30 000 sperm $ml^{-1}$ of media dilutions were loaded into Rose chambers and mounted into a microscope stage holder and kept at 37°C using an air curtain incubator (NEVTEK, ASI 400 Air Stream Incubator, Burnsville, VA) interfaced with a thermocouple feedback system. A total of 622 chimpanzee sperm, 324 rhesus macaque sperm, 549 human sperm and 338 gorilla sperm were analysed.

### 2.2. Hardware, software and optical design

The optical design used to create the laser tweezers, hardware and sperm-tracking software, experimental procedure and analysis of sperm have been described in greater detail in Nascimento *et al.* (2006) and

Shi *et al.* (2006*a*,*b*). Briefly, an optical trap was generated using a 1064 nm wavelength laser, coupled into a Zeiss Axiovert S100 microscope with a 40× phase III, NA 1.3, oil immersion objective (Zeiss, Thornwood, NY).

Phase contrast images of swimming sperm are digitized to the computer at video rate. The real-time automated tracking and trapping system, RATTS, creates a region of interest (ROI) centred about a sperm in response to a mouse click. The contrast enhancement and multi-class image segmentation algorithms are applied to extract the tracked sperm head as it transitions in and out of focus. The nearest neighbour method is complemented with a speed-check feature to aid tracking in the presence of additional sperm or other particles. The swimming velocity (curvilinear velocity, VCL, $\mu m\,s^{-1}$) is calculated based on the pixel $(x, y)$ coordinates of the sperm's swimming trajectory

$$\mathrm{VCL} = \frac{\sum\left(\frac{\sqrt{(x_i - x_{(i-1)})^2 + (y_i - y_{(i-1)})^2}}{\Delta\mathrm{frame}} \times \frac{\mathrm{micron}}{1.39\ \mathrm{pixel}} \times \frac{30\ \mathrm{frames}}{\mathrm{second}}\right)}{\mathrm{no.\ of\ frames}},$$

where $i$ is the current frame. Since the sperm is tracked by the head, VCL accounts for both forward progression and lateral head movement. This measurement is the same as that made by conventional computer-assisted sperm analysis machines. After 3.33 s (100 frames), the average VCL is stabilized (Shi *et al.* 2006*a*). Therefore, RATTS automatically traps the sperm after 3.33 s using the laser tweezers (Shi *et al.* 2006*b*). Once trapped, the laser power is reduced by rotating a linear polarizer set in a rotating mount (Newport Corporation, Model PR50PP, Irvine, CA). RATTS monitors a square region, approximately 10 µm per side, centred about the laser $(x, y)$ coordinates. The image within this region is segmented and a size threshold is used to detect the presence or the absence of the sperm. RATTS then automatically identifies when the sperm is capable of escaping the trap and records the escape laser power in watts ($P_{esc}$, mW; Shi *et al.* 2006*b*). Escape power is then converted to escape force in newtons ($F_{esc}$, pN) using the equation $F_{esc} = Q \times P_{esc}/c$, where $P_{esc}$ is the escape laser power, $c$ is the speed of light in the medium with an index of refraction of 1.33 and $Q$ is the geometrically determined trapping efficiency parameter equal to 0.12 (Konig *et al.* 1996). (Go to www.robolase.ucsd.edu for video sequences of actual experiments using gorilla and chimpanzee sperm.)

## 3. RESULTS

Semen samples from chimpanzee, rhesus macaque, human and gorilla are analysed. The mating systems for both the chimpanzee and the rhesus macaque are multimale–multifemale (females of these species mate with more than one male within a short period of time). Gorillas are polygynous, defined here as one male–multiple females; that is, the single dominant male mates with the females in the 'harem' unit (Dixson 1998), and therefore, from the female point of view, the gorilla is strictly monogamous. Human mating patterns are variable, differing across cultures but can be considered to be predominantly polygynous (83% of societies), more rarely monogamous (16%) and only very occasionally polyandrous (less than 1%; Dixson 1998). Therefore, the sperm analysed in this study come from primates that represent a variety of mating patterns, ranging from strictly polygynous (gorilla) to

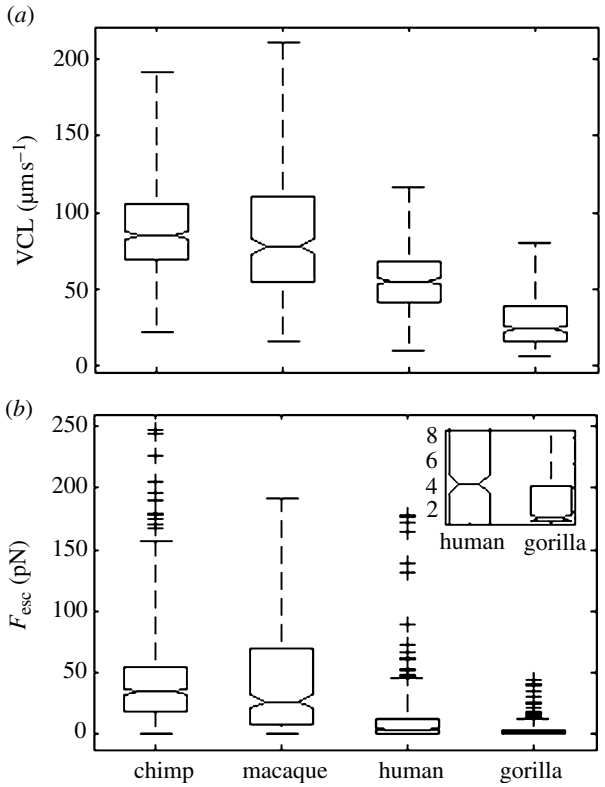


Figure 1. Swimming speed and escape force distributions. Box plots of the distributions of (*a*) swimming speed (VCL, $\mu m\,s^{-1}$) and (*b*) escape force ($F_{esc}$, pN) for all four primates. Inset in (*b*) shows an expanded view of human and gorilla distributions to emphasize the difference in median values. All distributions are found to be statistically significantly different ($p < 0.05$).

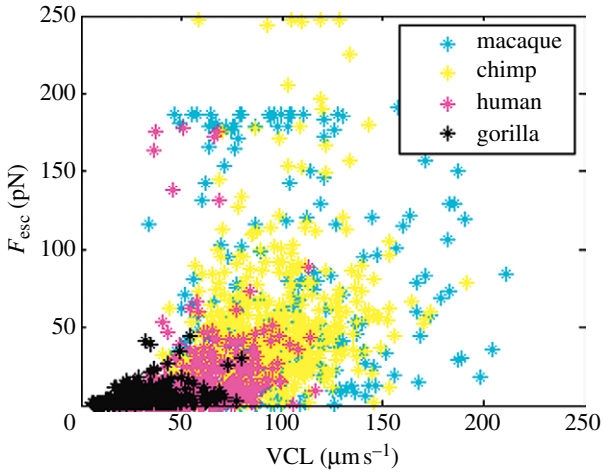


Figure 2. Escape force versus swimming speed. All four primates (chimpanzee, rhesus macaque, human and gorilla) are overlapping to show that as the level of sperm competition increases, so does the sperm swimming speed and force.

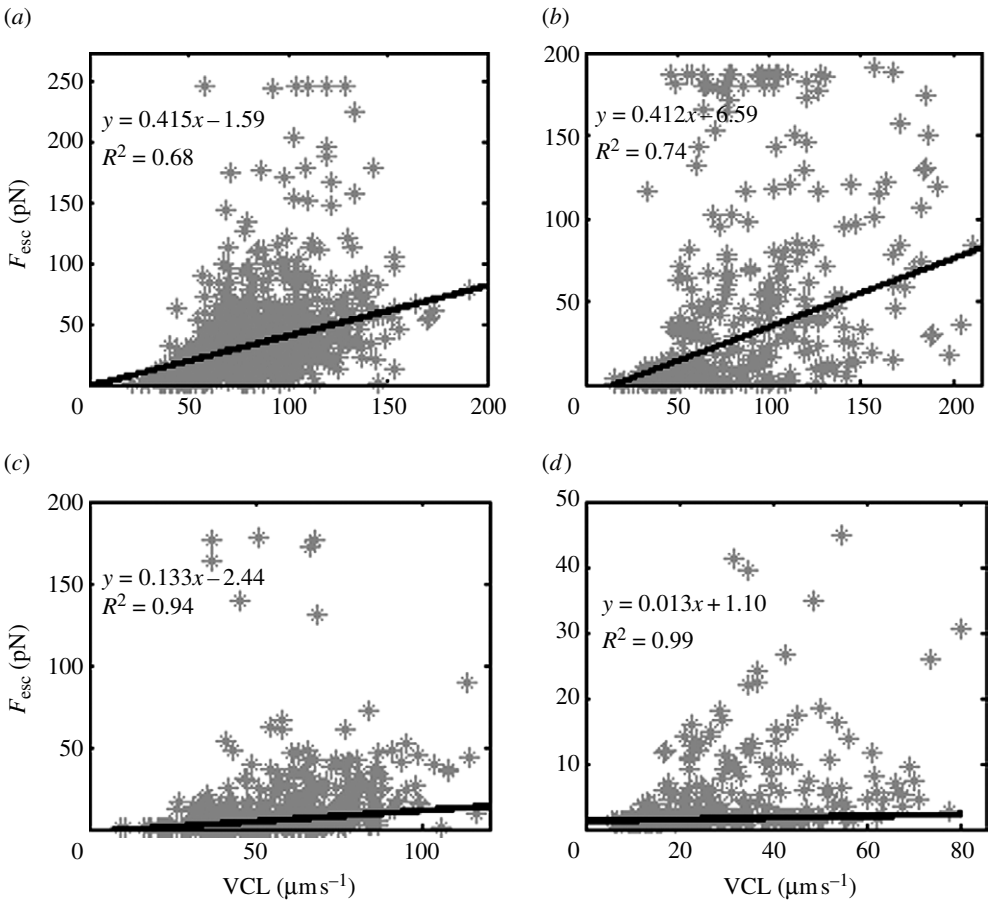


Figure 3. Escape force versus swimming speed with regressions. $F_{esc}$ (pN) versus VCL ($\mu m\ s^{-1}$) with linear regressions for four primate species: (*a*) chimpanzee, (*b*) rhesus macaque, (*c*) human and (*d*) gorilla. Inset on each graph gives the regression equation (slope and *y*-intercept) as well as the $R^2$ value to show goodness of fit. Each regression is found statistically significantly different ($p<0.05$).

multimale–multifemale (chimpanzee and rhesus macaque). The level of sperm competition, respectively, ranges from absent to very intense.

Sperm swimming forces and swimming speeds are measured for the four primate species. Slight variation in VCL and $F_{esc}$ distributions between males within a species were found (data not shown). However, in general, there is a clustering of average swimming speed and average swimming force for males within a species, thus data sets from different males per species were pooled together to represent the larger population. The box plots for distributions of sperm swimming speed (figure 1*a*; curvilinear velocity, VCL, $\mu m\ s^{-1}$) and sperm escape force (figure 1*b*; $F_{esc}$, pN) for the four primates are shown. Each box plot graphically displays the following parameters for a given distribution: (i) median (centre line of box), (ii) lower and upper quartile values (bottom and top line of box, respectively), (iii) the range of the data (dashed lines extending from the top and bottom of box), and (iv) the data points lying outside thrice the interquartile range (labelled as '+' marks). Notches in the box represent an estimate of the uncertainty about the median value. If notches on the box plots of two groups do not overlap, it can be concluded with 95% confidence that the two medians differ. Inset in figure 1*b* is an expanded view of the human and gorilla $F_{esc}$ box plots to emphasize the difference in medians between the two species. Data points lying outside thrice the interquartile range are present only in the swimming force distributions. These 'outliers' represent a small percentage of the sperm population (2.57% of chimpanzee population, 0% of rhesus macaque population, 3.46% of human population and 7.69% of gorilla population). Each species' swimming speed and escape force distributions are found to be statistically different ($p<0.05$) using the Wilcoxon rank sum test for equal medians (Zar 1984; VCL and $F_{esc}$ distributions for all primates are found not to be normally distributed using Lilliefors test (Zar 1984), thus requiring the use of the non-parametric Wilcoxon test). The medians of both measurements, VCL and $F_{esc}$, show that rhesus macaque and chimpanzee sperm swim with the fastest speeds and the strongest forces, while gorilla sperm swim with the slowest speeds and weakest forces. Human sperm swimming speeds and swimming forces lie between these two extremes.

Relationships between the two measurements, $F_{esc}$ and VCL, for each species have also been identified. Figure 2 overlaps the plots of $F_{esc}$ (pN) versus VCL ($\mu m\ s^{-1}$) for each primate species analysed. This figure reflects the range in sperm competition, showing that as the level of competition increases, the swimming speed distribution increases to faster velocities and the escape force distribution increases to stronger swimming forces. Figure 3 shows the data of $F_{esc}$ versus VCL

with robust linear regressions applied to the scatter plots for the four different primates. Inset in each graph of figure 3 are the regression equations, giving the slopes, $y$-intercepts and $R^2$ values. Each linear regression relating VCL and $F_{esc}$ was found to be statistically different, using a linear regression comparison test (Zar 1984), for all four primates ($p<0.05$).

## 4. DISCUSSION

The results shown in figure 1 compare the median escape forces and swimming speeds between primate species. The data show a relatively wide variation in the distributions of both VCL and $F_{esc}$ for all four species. This same wide variation is found for each male within a species as well (data not shown). Notwithstanding these variations, the data demonstrate that escape force and swimming speed increase as the level of sperm competition increases. These results support the theory that sperm from primates that are polygamous have experienced high competitive pressures and thus have evolved to swim both stronger and faster than sperm from primates that have not been under such high competitive pressures.

Relationships between escape force and swimming speed for each species were also identified. The results in figure 3 demonstrate that for each species, faster sperm swim with stronger forces, confirming the relationship found between the two parameters in previous studies using domestic dog semen (Nascimento *et al.* 2006). However, the slope of the linear regression becomes steeper as the level of sperm competition increases. Specifically, comparing gorilla with chimpanzee and rhesus macaque, the slope approximately increases by a factor of 30. In addition, the two multi-partner mating primates have lower $R^2$ values for the regressions, whereas the other two primates, human and gorilla, have regressions with a much higher $R^2$ value. Therefore, the regressions fit the data much better for the human and gorilla than they do for the multi-partner mating primates. The lower $R^2$ values for the chimpanzee and rhesus macaque reflect the spread in the escape force distribution, as seen in figure 1*b*, and indicate that for these polygamous species, sperm swimming speed is a poor predictor of the sperm's escape force. Explanation and speculation about the difference in the slopes and the $R^2$ values must await further studies that examine larger populations of sperm from a larger number of males for each primate species studied.

In conclusion, we have used a custom-designed real-time automated tracking and trapping system (RATTS) to measure sperm escape force and swimming speed. These results support the theory of sperm competition. The data presented here are intriguing and appear to demonstrate a correlation between sperm motility and mating type. Furthermore, this combination of physics (optics and lasers) and engineering (automated computer tracking) principles should be applicable to other problems of sperm motility as well as to the study of cell behaviour in other motile cell systems.

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