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Functional Evidence for Differences in Sperm Competition in Humans and Chimpanzees

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ABSTRACT Sperm competition occurs when the gametes of two or more males compete for opportunities to fertilize a given set of ova. Previous studies have demonstrated that certain morphological characteristics are affected by sperm competition intensity (e.g. relative testes size and sperm midpiece volume). This study examined whether aspects of sperm energetics may also be affected by sexual selection. We compared the membrane potential of mitochondria in live sperm between *H. sapiens* (single partner mating system) and *P. troglodytes* (multiple partner mating system). Flow cytometry of sperm stained with the carbocyanine fluorescent dye JC-1 (an assay for mitochondrial membrane potential) revealed marked differences in red fluorescence intensity. *P. troglodytes* sperm showed significantly higher mitochondrial membrane potential. Mitochondria provide a substantial part of the energy required for sperm motility. A higher mitochondrial loading may therefore be associated with enhanced sperm motility and/or longevity. Additionally, examination of JC-1 red fluorescence levels before and after *in vitro* capacitation revealed further differences. Whereas chimpanzee sperm showed maintenance of membrane potential after capacitation (in some cases even an increase), sperm from humans consistently showed reduction in membrane potential. These results indicate that the sperm of human beings and chimpanzees exhibit marked differences in mitochondrial function, which are affected by selection pressures relating to sperm competition and that these pressures differ significantly between humans and chimpanzees. Am J Phys Anthropol 000:000–000, 2007.

Published studies of chimpanzee sperm biology have pointed out many similarities between chimpanzees and humans, while providing evidence for much individual variation within each species (Olson and Gould, 1981; Marson et al., 1991; Gould et al., 1993; Young et al., 1995; Gould and Young, 1996). For example, data on chimpanzee sperm motility and fertilizing capacity were directly comparable with those from humans (Bedford, 1974; Gould et al., 1993). In contrast, chimpanzees have higher sperm numbers, a reduced duration of epididymal transit (Smithwick et al., 1996), and the capacity to produce subsequent ejaculates with high sperm counts (Marson et al., 1989). All these characteristics are thought to relate to greater investment in gonadal tissue in chimpanzees and in turn reflect adaptation for sperm competition (Møller, 1989).

Sperm competition, a form of sexual selection, describes the postcopulatory rivalry, which occurs between the ejaculates of different males for the fertilization of a given set of ova (Parker, 1982). This phenomenon is widespread in nature (Birkhead, 1995). In primates, relative testes size is a prime example of a morphological trait that is affected by sexual selection acting via sperm competition. Relative testes weights are greatest in those primate species where females mate with multiple partners during a single peri-ovulatory period (Harcourt et al., 1981, 1995). These increases in testes size are due, primarily, to the greater mass of gamete-producing tissues (seminiferous tubules) in the testes (Schultz, 1938; Short, 1997; Jolly and Phillips-Conroy, 2003). Males of multiple partner species also produce sperm with higher motility and larger volumes of ejaculate than those mammals in which females typically mate with a single male during the peri-ovulatory period (Møller, 1989). Mammalian sperm morphology is characterized by three distinct parts, a head, a midpiece, and a tail. The haploid genome is contained in the head, the midpiece houses energy producing mitochondria, which are spirally arranged around the first portion of the tail. The tail makes >90% of the length of the sperm and

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provides their characteristic mobility (Bedford and Hoskins, 1990). Whether selection pressures emanating from sperm competition also affect sperm morphology has been widely debated. Cummins and Woodall (1985) found total sperm length to be negatively correlated allometrically with body mass in mammals, whereas later studies failed to find such a relationship in primates (Gomendio and Roldan, 1991, Harcourt, 1991). A study by Gage (1998) found no effect of body size on sperm length in a sample of 445 mammalian species. Gomendio and Roldan (1991) had previously examined other possible determinants of the observed variance in sperm morphology among primates. More recently, Anderson and Dixson (2002) showed that primates with multiple partner mating systems possess sperms with significantly larger midpiece volume, whilst all other measures appertaining to sperm head, midpiece, and tail (both linear and volumetric) lacked such differences. Subsequent expansion of this study to include sperm morphometrics from 123 mammalian species mirrored these findings (again, midpiece volume was found to be the only morphological trait that differed significantly in relation to sperm competition intensity (Anderson et al., 2005)).

Observed differences in sperm midpiece volume are thought to be related to the potential selection for higher mitochondrial loading (the number and/or volume of mitochondria contained) in the midpiece and resulting energy production as mitochondria are the site of oxidative phosphorylation and contribute much of the energy needed for sperm motility. The effect of sperm competition levels on functional aspects of sperm have recently been documented for different species of mice (*Mus spec.*) by Gomendio et al. (2006). These authors have shown that the fraction of sperm that capacitate is larger in species with more sperm competition among the four species of mice with different levels of sperm competition.

We undertook comparisons between chimpanzees and humans, two sister species that possess markedly different mating systems. Whereas in both species of *Pan,* females regularly mate with multiple males during a single peri-ovulatory period, humans show markedly more monogamous mating patterns (Dixson, 1997). An assay for measuring the relative strength of mitochondrial membrane potential in large populations of live sperm in humans and chimpanzees based on staining and fluorometric measurement by flow cytometry was utilized. Comparisons were also undertaken to examine changes in mitochondrial membrane potential after *in vitro* capacitation. Sperm capacitation is a characteristic process in mammalian fertilization and involves changes in sperm metabolism and sperm surfaces. These changes are thought to be triggered by factors encountered in the oviduct of the female. Such changes result in hyperactivation (specifically a dramatic change in the sperm's motility pattern). This process has been shown to be a prerequisite for fertilization *in vivo* (Yanagimachi, 1970a) and can be triggered *in vitro* (Yanagimachi, 1970b).

MATERIALS AND METHODS

We studied sperm samples from a total of ten humans, five chimpanzees, one bonobo, and one gorilla. Any given experiment had a maximum of five chimpanzees and five humans analyzed side by side. Human samples were provided by volunteers under informed consent and obtained by masturbation after a minimum of 2 days of sexual abstinence and regulated by UCSD Human Research Protections Program project no. 040613. Chimpanzee samples were collected utilizing a no-contact, noninvasive method: artificial vaginas made from 2-inch diameter 14-inch long PVC pipe with an inner water jacket formed by sterile latex surgical tubing (Penrose drain 18-inch long, 1-inch diameter, Bard Inc. Covington Georgia) lubricated with KY jelly, and animals specially trained by positive reinforcement. Chimpanzee samples were obtained under IACUC protocol reviewed and approved by the Primate Foundation of Arizona, Institutional Animal Care and Use Committee (IACUC) and supported, in part, by the National Institutes of Health, Division of Research Resources, Grant U42RR15090-01. Samples from one bonobo at the San Diego Zoo were collected off the cage floor immediately after the animal had been seen masturbating and placed into BWW buffer with penicillin/streptamycin (see below for composition). The gorilla sample was obtained at Zoo Atlanta after review and approval by the Zoo's scientific review committee. The sample was collected opportunistically (cage floor after animal masturbated) and shipped to San Diego over night in BWW medium with penicillin/streptamycin. Of the ten human volunteers, eight have fathered one or more children and two of the five chimpanzees have sired offspring and so has the bonobo sampled.

Sample processing, storage, and transport

All samples were allowed to liquefy at room temperature for 30 min and were then added to 10 ml of BWW with penicillin and streptomycin. Chimpanzee samples were transported in a cooler at 4–10°C and human samples were kept under the same conditions in the laboratory until the chimpanzee samples arrived. 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 napyruvate, 1% BSA, pH 7.5, filter-sterilized over 22 μm filter and kept at 4°C and warmed to room temperature prior to use) by spinning at 600*g* (1400 rpm in clinical centrifuge) for 6 min and capacitated *in vitro* in BWW with addition of BSA to 1%, and 4-h incubation at 37°C and 5% CO_2. The capacitated samples were compared with the uncapacitated with regard to their motility, and the characteristic lateral lashing motion was observed in a large fraction of the sperm in each of the capacitated samples. Chimpanzee samples all contained copulatory plugs of varying size. The majority of sperm is found in the uncoagulated distal portion of the ejaculate and can be clearly distinguished when cutting open the latex tubing after collection. The chimpanzee samples had sperm counts of 648, 760, 427, 510, and 364 million and 53, 71, 70, 64, 63.5% live cells as assayed by propidium iodide staining and flow cytometry. The values for human samples were sperm count: 250, 316, 225, 308 millions and percentage viability were 46, 70, 28, and 45. Gorilla and bonobo samples were from the cage floor and thus the counts represent underestimates. The gorilla sample had 72 million sperm with 17% live cells and the bonobo sample had a count of 460 million with 84% live cells. The lower viability than the 75% normative value suggested by the WHO criteria was likely because of the 20-h storage of the samples between collection and analysis.

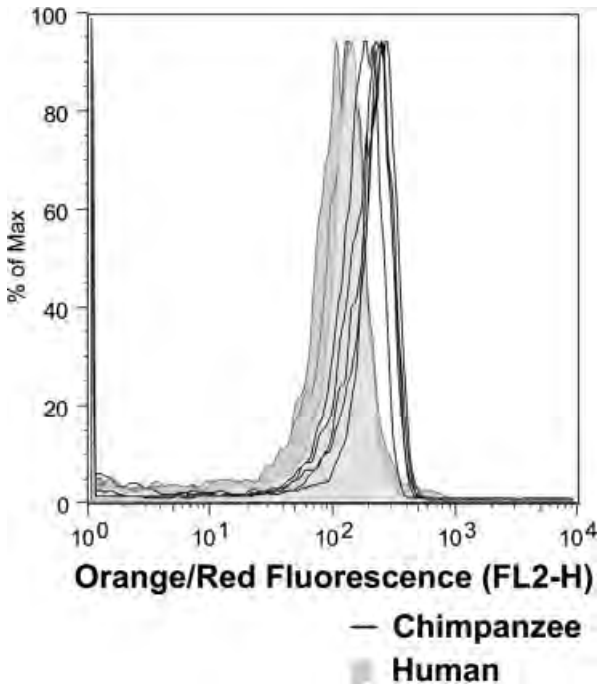

Fig. 1. Distribution of red fluorescence values for sperm populations from four humans and five chimpanzees as measured by flow cytometry. Cell numbers are standardized to the maximum observed.

Staining

Sperm cells were counted in a hemocytometer by two different observers and adjusted to a density of 20 million/ml in BWW buffer. Subsequently, 50 μl aliquots of this suspension were used for each staining in 500 μl total volume. JC-1 (5.5′, 6.6′, tetrachloro-1.1′,3.3′,tetraethyl–benzimidazolyl carbocyanine iodide) was purchased from Molecular Probes, reconstituted in DMSO and further diluted in BWW 1:50, spun at top speed in table top centrifuge for 10 min to remove the precipitate, and then 500 μl of supernatant was then used to stain sperm cells in BWW at 37°C in the dark for 30 min. Sperm were washed with 1 ml of HEPES buffered saline 1% BSA (HBS) and resuspended in 500 μl to 1 ml for flow cytometry. In control experiments, sperm samples were incubated with JC-1 in the presence of the decoupling agent, valinomycin, suspended in DMSO and adjusted to 10 μM final concentration in BWW (Molecular Probes, Eugene, OR) for 30 min at 37°C in the dark. Samples were rinsed with HBS and subjected to flow cytometry immediately thereafter.

Staining of ejaculated spermatozoa with JC-1 produces a distinct red fluorescence pattern when excited with an argon laser. Dead or metabolically inactive cells only weakly fluoresce in the red/orange spectrum (590 nm) under UV light, whereas cells with active electron transport chains across the inner mitochondrial membrane fluoresce intensely orange. We used the intensity as an assay for mitochondrial membrane potential. Figure 1 shows the distribution of gated single sperm for red/orange fluorescence of a human and chimpanzee sperm sample. The dye JC-1, a cationic lipophilic dye, easily permeates cell membranes and forms aggregates inside the negatively charged mitochondrial matrices. These aggregates cause a shift in fluorescence from green to red/orange, which can be quantified (Reers et al., 1991; Smiley et al., 1991; Cossarizza et al., 1993; Troiano et al., 1998; Marchetti et al., 2004). The red/orange fluorescence of JC-1 stained sperm was measured using quantitative methods (flow cytometry) and has been shown to correlate with sperm forward motility (Marchetti et al., 2004) We directly compared the red fluorescence of JC-1 stained samples rather than using a ratiometric approach. The intensity of green fluorescence (527 nm) does not correlate with mitochondrial membrane potential. There is a controversy about the value of including the green fluorescence as opposed to just considering the red/orange fluorescence (590 nm). In this study we followed Marchetti et al. (Marchetti et al., 2004) and used the direct measurements of red fluorescence for quantifying sperm membrane potential (for an example of the read out see Fig. 5).

To validate the use of JC-1 as a proxy for mitochondrial membrane potential, we stained sperm cells before and after treatment with the decoupling agent valinomycin. Valinomycin treatment drastically reduced the intensity of JC-1 staining, indicating that JC-1 staining is specific for mitochondrial membrane potential (Fig. 6).

Samples from up to five human subjects and five chimpanzees were stained side by side using the same reagents for sample storage and transport, staining, washing, and flow cytometry.

Flow cytometry

Samples were measured by flow cytometry using a FACScalibur machine (Beckton Dickinson) and Cell Quest Soft ware. Analysis was carried out using Flow Jo Software (Treestar Inc., Ashland, OR) on a McIntosh G4 Powerbook computer. Cells were gated for single sperm, excluding debris and highly granular (damaged and clumped) cells. The same single sperm gate was applied consistently for all samples. Instrument settings on the flow cytometer were: Detector, voltage value, AmpGain, mode, respectively: FSC, E00, 3.45, linear; SSC, 396, 557, linear; FL1,505, 1.00, log; FL2,396, 1.00, log; FL3650, 1.00, log. The setting for FL2 (red channel) was set so as to center the high intensity peak of red JC-1 fluorescence on the horizontal logarithmic scale. Excitation wave length for JC-1 is 490 nm and its red emission wave length is 590 nm. Compensation was set at FL1 − 12.7% FL2 and FL2 − 25.4% FL1.

Quantitative analysis

Only fluorescent values from the same experiments were compared. Statistical comparisons were undertaken using Statview for Apple Macintosh. Comparisons in JC-1 stain intensity between precapacitated chimpanzee and human sperm were undertaken using an analysis of variance (ANOVA). Where the ANOVA was found to be statistically significant, a Neumann–Keuls post-hoc pair test was used to ascertain where the differences occurred. Subsequent comparisons between both pre- and post-capacitated JC-1 stained sperm from chimpanzee and humans were then made using a multiple analysis of covariance (MANCOVA) with repeated measures. As above, where significance was observed, Neumann–Keuls post-hoc pair tests were undertaken to investigate possible differences between the membrane potential of pre- and post-capacitated sperm. This approach also allowed for both intra- and interspecific comparisons to be undertaken. Fluorescence intensities of metabolically

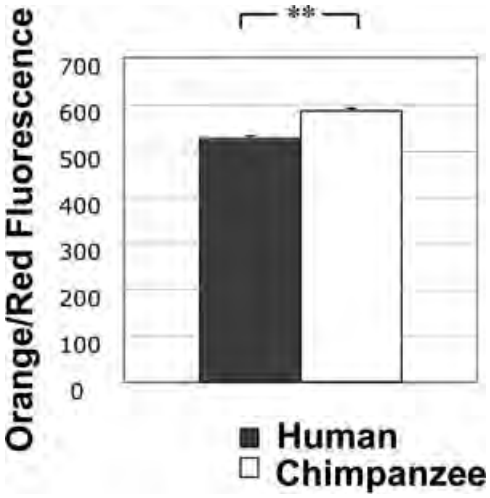

Fig. 2. Red fluorescence values for JC1 stained sperm samples from multiple human (4) and chimpanzee (5) stained side by side and measured by flow cytometry (** indicates $P < 0.01$ in ANOVA).

active (JC-1 positive) fractions from chimpanzee and human samples were compared via an unpaired *t*-test.

Fluorescence microscopy

After flow cytometry, stained samples were spun for 6 min at 600*g* at room temperature, and the pellet was smeared on microscope slides, briefly air dried for 15 min, and mounted with gel mount and dapi counter stain. Microphotographs were produced using a Zeiss Axiomat microscope and a Cannon digital Camera using the Apple Macintosh Remote Capture Software. Fluorescence microscopy allowed for the verification of specific midpiece staining.

RESULTS

A direct comparison of the mitochondrial membrane potential in live sperm was undertaken using JC-1 staining and flow cytometry. This enabled us to document several differences between humans, chimpanzees, bonobo, and gorilla.

Higher JC-1 staining in sperm mitochondria from chimpanzees than from humans

An analysis of variance (ANOVA) was undertaken and found to be significant ($F = 3.15$, $P < 0.01$). Subsequent Neumann–Keuls post-hoc pair tests showed that human sperm exhibited consistently lower red fluorescence intensity than seen in the chimpanzee samples ($P < 0.01$) after being stained with JC-1. Figure 2 shows the results from multiple human (4) and chimpanzee (5) sperm samples stained side by side. The red fluorescence values of each species showed limited overlap (Fig. 2.). All the ten humans sampled showed consistently lower red fluorescence, irrespective of their viability values (data not shown).

Larger fraction of JC-1 positive sperm in ejaculates from chimpanzee than from humans

Only sperms that are metabolically active will fluoresce at high intensity with an emission at 590 nm when stained for mitochondrial matrix potential with the dye JC-1. The fraction of such metabolically active sperm ranged from 20 to 85% and was significantly higher in the samples from chimpanzees than from humans. This was true even when comparing chimpanzee sperm 24 h after collection with fresh human sperm. The average fraction of JC-1 positive sperm cells over five different experiments was: humans 43% ± 5.2% and chimpanzees 69% ± 1.5% ($t = 11.27$, $P < 0.01$).

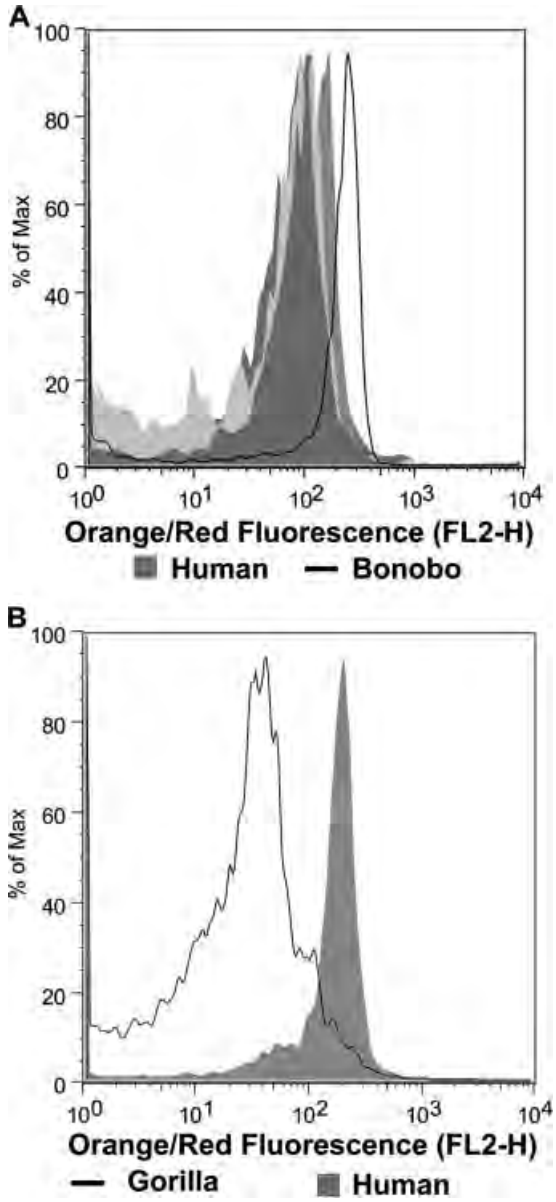

Fig. 3. Flow cytometry read out for one bonobo compared with four humans (**A**) and for one human and one gorilla sperm sample (**B**).

Differences between humans, bonobo, and gorilla

JC-1 values for one bonobo and one gorilla were obtained. The bonobo sample stained very similarly to the chimpanzee samples and the gorilla sample had by far the weakest intensity of all samples examined

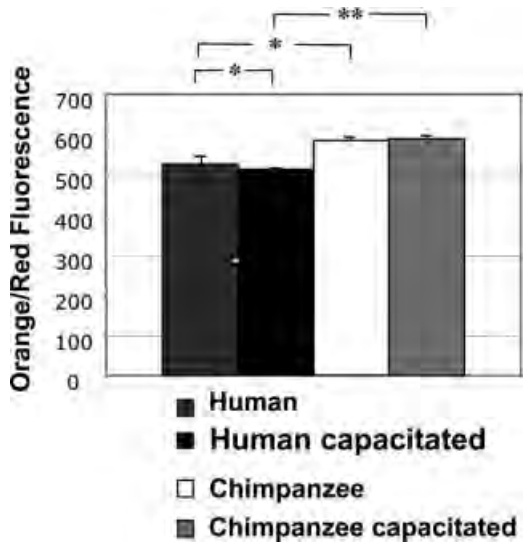

Fig. 4. Average JC1 red fluorescence of stained human (4) and chimpanzee (5) sperm prior to and after *in vitro* capacitation (** indicates $P < 0.01$, * indicates $P < 0.05$ in MANCOVA).

(Fig. 3). However, low sample sizes precluded quantitative comparisons between these species and humans and chimpanzees.

Different effects of *in vitro* capacitation on mitochondrial membrane potential of sperm from *Pan* and *Homo*

A multiple analysis of covariance (MANCOVA) with repeated measures revealed significant differences between levels of red fluorescence (following JC-1 staining) observed in human and chimpanzee sperm capacitated *in vitro* ($F = 10.26$, $P < 0.01$). Human and chimpanzee sperm were seen to differ both precapacitation ($P < 0.05$) and postcapacitation ($P < 0.01$). A further difference was observed in the intensity of fluorescence between precapacitated and postcapacitated human sperm ($P < 0.05$), exhibiting a loss of staining intensity in the capacitated human sperm. A similar comparison between precapacitated and postcapacitated chimpanzee sperm revealed no such differences, with all the capacitated samples exhibiting no decline in membrane potential. Although no significant differences were observed, a trend for a slight increase in membrane potential in three of the five chimpanzee samples was recorded. Figure 4 shows average levels of JC-1 fluorescence intensity representing four humans and five chimpanzees before and after capacitation, respectively. A part from confirming a change in movement patterns in sperm after *in vitro* capacitation, we did not carry out a functional test for capacitation and so this last result should be considered preliminary.

DISCUSSION

Comparisons of sperm mitochondrial membrane potential in large populations of live sperm from humans and their closest relatives the chimpanzees reveal clear differences in their sperm bioenergetics. The consistently higher intensity seen in chimpanzees is taken as an indication of more stringent energetic demands relating to higher sperm competition intensity in *Pan troglodytes*.

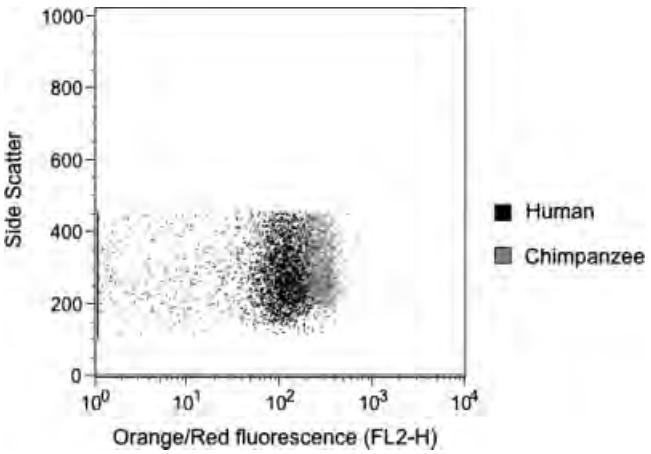

Fig. 5. Dot plot of human (black) and chimpanzee (grey) sperm samples.

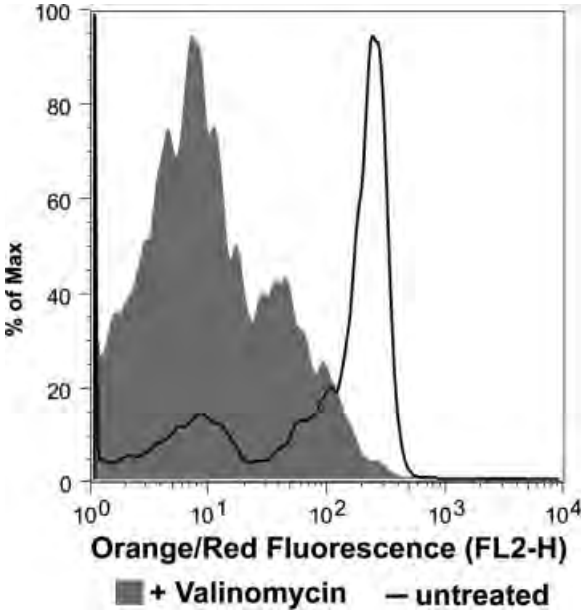

Fig. 6. Effect of valinomycin treatment on JC1 red fluorescence of stained chimpanzee sperm: close to complete loss of fluorescence intensity.

To what degree a stronger mitochondrial membrane potential in chimpanzees directly translates into differences of motility still requires investigation. However, JC-1 staining has been shown to correlate with sperm forward motility in humans (Kasai et al., 2002; Marchetti et al., 2004). Published studies of sperm motility in chimpanzees have measured motility levels comparable with those known for human sperms. However these data were on uncapacitated sperm and did not take into account the duration of motility maintenance. Clearly, both, uncapacitated motility and post-capacitated or hyperactivated motility should be compared. Although uncapacitated motility is likely to contribute to sperm transport through the lower female reproductive tract, hyperactivated motility may be crucial for the detachment of the sperm from the oviduct epithelium in the isthmus (Suarez, 2002) and for the penetration of the vestment and membrane of the egg

(Yanagimachi, 1994). Direct measurements of swimming force might reveal differences in power between human and chimpanzee sperms. Such measures have recently been undertaken using laser trapping methods and results from measurements of average curvilinear velocity and escape force in chimpanzee and human sperm correlate with the differences in JC-1 staining reported here (Nascimento et al. in review).

The existence of roughly twice the number of mitochondria in the midpiece of chimpanzee sperm has long been documented using scanning electron microscopy (Bedford, 1974) and correlates with the more recent finding that chimpanzee sperm midpiece volume is almost twice that of humans (Anderson and Dixson, 2002). This raises the question of the relationship between total mitochondrial number and intensity of JC-1 fluorescence. Mitochondria can not only differ in number, but also in the density of cristae formed by the inner membrane, the volume of inner matrix, and the density of oxidative phosphorylation (OXPHOS) complex molecules on the inner membranes (Scheffler, 1999).

We suggest that sexual selection, via sperm competition, in the chimpanzee and bonobo may have resulted in the evolution of higher mitochondrial membrane potentials in both these ape species in comparison with the values measured in humans.

Increased levels of sperm competition in chimpanzees (and closely related bonobos) is thought to be driving the selection for the higher mitochondrial membrane potential in *Panids.* This is perhaps indicative of much more stringent demands both in terms of absolute intensity as well as individual sperm cell longevity in those species with more intense sperm competition.

Recent genomic studies have documented rapid evolution of electron transport chain proteins in the mitochondria of anthropoid primates (Lomax et al., 1992; Wu et al., 1997; Uddin et al., 2004). These studies have not found changes unique to the chimpanzee lineage but rather across all anthropoid primates. In light of our findings, it would be interesting to examine the sperm-specific splice variants of enzymes involved in sperm energetics in humans and chimpanzees. A testable prediction would be that more positive selection has occurred in the lineage leading to both species of *Pan,* or alternatively, that there is less stringent negative selection acting on these same genes in the human lineage. Mitochondria are not the only determinants of sperm motility as the flagellum also contains glycolytic enzymes, which generate ATP locally. Evidence from *Gapds* knock-out mice indicates that glycolysis may even be the dominant source of ATP, especially during hyperactivation (Miki et al., 2004). Both, mitochondria and the flagellum contain sperm-specific splice variants of glycolytic enzymes (hexokinase, lactate dehydrogenase, and glyceraldehydes 3-phosphate dehydrohenase) (Scheffler, 1999; Jaiswal and Conti, 2001). It is tempting to speculate that additional differences are likely to exist between human and chimpanzee sperm at the level of flagellar glycolysis and ATP mobilization. However, it has to be kept in mind that unlike midpiece volume, no morphological differences were observed between sperm flagellae of humans and chimpanzees (Anderson and Dixson, 2002; Anderson et al., 2005).

The mating system of the common ancestor of humans and chimpanzees remains unknown. Whether energy production was upregulated in *Panids* during the course of evolution or down regulated in humans remains a mystery, but expanding the current study to include additional closely related species such as gorilla may help elucidate this subject. Are sperm energetics or morphology lagging behind recent changes in prevailing mating system in humans? We analyzed a single gorilla sample, which showed much weaker fluorescence than humans or chimpanzees. Sperm energetics in the last common ancestor of humans and chimpanzees may thus have been more similar to that seen in Gorilla. Indeed, the phylogenetic distribution of intense sperm competition in the hominoids would indicate that *Panids* are more likely to exhibit the derived condition, whilst gibbons, orangutans, and gorillas all show much lower levels of sperm competition. It should also be born in mind that mating systems and corresponding differences in levels of resulting sperm competition may be more plastic, and thus prone to much homoplasy. Additionally, levels of sperm competition are known to be low in gorilla, and the much higher mitochondrial loading observed in human sperm may be indicative of occasional sperm competition in our own species, even if relative testis size and midpiece volume group *H. sapiens* with the more monogamous primate species (Harcourt et al., 1981).

Sperm competition is only one facet, which can differ between mating systems, and it is not occurring in a passive arena within the female reproductive tract. Differences appertaining to cryptic female choice (Eberhard, 1996) could also create differing demands on sperm function. Potential differences in several female characteristics may impose different demands on sperm motility as well. These include: differences in composition of secretions such as cervical mucins and tubular fluid, differences in affinity between oviduct epithelium and sperm, overall length of the oviduct, levels of immune surveillance, composition and/or viscosity of oviductal secretions, and vestment through which sperm have to swim. The evidence for the role of sexual selection in shaping the bioenergetics of hominoid sperm presented here leads one to posit: the likely existence of additional differences in sperm biology between these closely related species (e.g. differences with regard to the composition and abundance of their sperm surface molecules involved in mediating sperm–female reproductive tract interactions).

Finally, it may be interesting to speculate to what extent sexual selection could have acted upon mitochondria through sperm competition in the chimpanzee lineage, especially with regard to creating special energetic demands for sperm function.

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