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Probing human sperm metabolism using ^{13}C-magnetic resonance spectroscopy

Running Title: ^{13}C-MRS of human sperm metabolism

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Abstract

STUDY QUESTION: Can ^{13}C-Magnetic Resonance Spectroscopy (MRS) of selected metabolites provide useful information about human sperm metabolism and how glycolysis or oxidative phosphorylation are used by different sperm populations?

SUMMARY ANSWER: Sperm populations, prepared by density gradient centrifugation (DGC) and incubated with either $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate, showed consistent evidence of metabolism generating principally lactate and more intermittently bicarbonate, and significantly more lactate was produced from $^{13}C_u$-glucose by vital or motile sperm recovered from the 40/80% interface compared to those from the pellet, which could not be accounted for by differences in the non-sperm cells present.

WHAT IS KNOWN ALREADY: Previous studies have focused on CO_2 or other specific metabolite production by human sperm and there remains considerable debate about whether glycolysis and/or oxidative phosphorylation is the more important pathway for ATP production in sperm.

STUDY DESIGN, SIZE, DURATION: Sperm populations were prepared by DGC and subjected to ^{13}C-MRS to answer the following questions. (i) Is it possible to detect human sperm metabolism of ^{13}C substrates implicated in energy generation? (ii) What are the kinetics of such reactions? (iii) Do different sperm populations (e.g. '80%' pellet sperm and '40%' interface sperm) utilise substrates in the same way? Semen samples from 97 men were used in these experiments; 52 were used in parallel for aims (i) and (ii) and 45 were used for aim (iii).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Sperm populations were prepared from ejaculates of healthy men using a Percoll/Phosphate Buffered Saline (PBS) DGC and then incubated with a range of ^{13}C-labelled substrates ($^{13}C_u$-glucose, $^{13}C_u$-fructose, $^{13}C_1$-pyruvate, $^{13}C_1$-butyrate, $^{13}C_3$-lactate, $^{13}C_{2,4}$-D-3-hydroxybutyrate, $^{13}C_5$-L-glutamate, $^{13}C_{1,2}$-glycine or $^{13}C_u$-galactose) along with penicillin/streptomycin antibiotic at 37°C for 4 hours, 24 hours or over 48 hours for an estimated rate constant. Sperm concentration, vitality and motility were measured and, for a subset of experiments, non-sperm cell concentration was determined. A 9.4T magnetic resonance spectrometer was used to acquire 1D ^{13}C, inverse gated ^{1}H decoupled, MRS spectra. Spectrum processing was carried out using spectrometer software and Matlab scripts to determine peak integrals for each spectrum.

MAIN RESULTS AND THE ROLE OF CHANCE: $^{13}C_u$-glucose, $^{13}C_u$-fructose and $^{13}C_1$-pyruvate were consistently converted into lactate and, to a lesser extent, bicarbonate. There was a significant correlation between sperm concentration and lactate peak size for $^{13}C_u$-glucose and $^{13}C_u$-fructose, which was not observed for $^{13}C_1$-pyruvate. The lactate peak did not correlate with the non-sperm cell concentration up to 6.9 x 10^6/ml. The concentration of $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate (1.8, 3.6, 7.2 or 14.4 mM) had no influence on the size of the observed lactate peak over a 4 hour incubation. The rate of conversion of $^{13}C_1$-pyruvate to lactate was approximately three times faster than for $^{13}C_u$-glucose or $^{13}C_u$-fructose which were not significantly different from each other. After incubating for 4 hours, the utilisation of $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate by sperm from the

'40%' interface of the DGC was no different from those from the pellet when normalised to total sperm concentration. However, after normalising by either the vital or motile sperm concentration, there was a significant increase in conversion of $^{13}C_u$-glucose to lactate by '40%' interface sperm compared to pellet sperm (Vital = 3.3 ± 0.30 x10^6 vs 2.0 ± 0.21 x10^6; p = 0.0049; Motile = 7.0 ± 0.75 x10^6 vs 4.8 ± 0.13 x10^6; p = 0.0032. Mann-Whitney test p<0.0055 taken as statistically significant). No significant differences were observed for $^{13}C_u$-fructose or $^{13}C_1$-pyruvate.

LARGE SCALE DATA: Not applicable.

LIMITATIONS, REASONS FOR CAUTION: Only ^{13}C labelled metabolites that accumulate to a sufficiently high concentration can be observed by ^{13}C MRS. For this reason, intermediary molecules in the metabolic chain are difficult to observe without trapping the molecule at a particular step using inhibitors. Non-sperm cell concentration was typical of the general population and no link was found between these cells and the magnitude of the ^{13}C-lactate peak. However, it is possible that higher concentrations than the maximum observed (6.9 x 10^6/ml) may contribute to exogenous substrate metabolism in other experiments.

WIDER IMPLICATIONS OF THE FINDINGS: ^{13}C-MRS can provide information on the underlying metabolism of multiple pathways in live sperm. Dysfunction in sperm metabolism, as a result of either impaired enzymes of lack of metabolisable substrate, could be detected in sperm by a non-destructive assay, potentially offering new treatment options to improve overall sperm quality and outcomes for reproduction.

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KEYWORDS: magnetic resonance spectroscopy, metabolism, glycolysis, Krebs cycle, human sperm

Introduction

Poor sperm quality significantly contributes to cases of infertility within couples (Pacey, 2009), yet many basic aspects of sperm physiology remain unknown (Barratt *et al.*, 2018). One important and unanswered question is how human sperm generate the ATP (adenosine triphosphate) necessary to sustain motility and undergo the metabolically demanding processes of capacitation and hyperactivation (Suarez and Pacey, 2006). Furthermore, such information may be useful both to help understand the molecular causes of poor sperm motility (e.g. asthenozoospermia) and to provide insights into targets for novel agents to enhance sperm motility.

After more than 50 years of research, it is now clear that human sperm can produce ATP through the metabolic processes of glycolysis and/or oxidative phosphorylation (du Plessis *et al.*, 2015). This has been examined using a variety of experimental approaches over the years, including: (i) the measurement of oxygen consumption of washed sperm either in the presence (Ford and Harrison, 1981) or absence (Peterson and Freund, 1970) of metabolic inhibitors; (ii) incubation with ^{14}C radiolabelled substrates (Ford and Harrison, 1981, Murdoch and White, 1968); (iii) the measurement of ADP and ATP in semen samples with different phenotypes (Vigue *et al.*, 1992); and (iv) the use of proteomics to identify new metabolic enzymes and pathways (Amaral *et al.*, 2014). However, there remains considerable debate about whether glycolysis and/or oxidative phosphorylation is more important for the various aspects of human sperm function during their post-ejaculatory life (du Plessis *et al.*, 2015, Ruiz-Pesini *et al.*, 2007).

In a recent paper, we used ^{1}H Magnetic Resonance Spectroscopy (MRS) to examine the endogenous metabolome of live human sperm isolated from semen using 40/80% Density Gradient Centrifugation (DGC) (Reynolds *et al.*, 2017a). This showed that several metabolite peaks, including those associated with lactate, could be used to discriminate sperm recovered from the pellet ('80%' sperm) from those recovered from the '40%' interface. As '80%' sperm typically have better motility, this suggested that there may be important metabolic differences between these two sperm populations with respect to their utilisation of the pathways of glycolysis and oxidative phosphorylation.

^{13}C-MRS has been used to examine metabolic pathways in other cell types (Buescher *et al.*, 2015) including metabolic regulation in cancer cells (Shestov *et al.*, 2016). Using ^{13}C labelled substrates provides three advantages. Firstly, they are metabolised the same as those found within human physiology and their ^{13}C-MRS spectra are greatly simplified compared to ^{1}H MRS, displaying known peaks for the source substrate and those peaks having arisen from cellular metabolism. Secondly, particular metabolic pathways can be identified through strategic placement of the ^{13}C label (Buescher *et al.*, 2015). Alternative or multiple pathways can be assayed through varying ^{13}C labelling patterns, even if the end product is the same (Bruntz *et al.*, 2017). Finally, the cells under study remain viable throughout the experiment and therefore can be measured at multiple time points (Reynolds *et al.*, 2017b).

As many aspects of sperm metabolism remain unknown and ^{13}C-MRS can provide insights into metabolism in live cells, we reasoned that this combination would be able to further elucidate the metabolic pathways used by live human sperm. In this

paper, we use ^{13}C-MRS to investigate three questions. (i) Is it possible to detect human sperm metabolism of ^{13}C substrates implicated in energy generation? (ii) What are the kinetics of such reactions? (iii) Do different sperm populations (e.g. '80%' and '40%' sperm) utilise substrates in the same way?

Materials and methods

Semen donation and analysis

Semen samples were obtained from men attending the Andrology Laboratory (Jessop Wing, Sheffield, UK) for semen analysis (approved by the North of Scotland Research Ethics Committee (16/NS/0009) on 17/02/16). Informed consent was obtained from each man to use their ejaculates in this project and semen samples were produced after at least two days of sexual abstinence. Each ejaculate was collected into a sterile plastic container (Sarstedt, Leicester, UK) and examined according to World Health Organisation (2010) methods within one hour of production. Samples selected for experiments contained at least a total of $25x10^6$ sperm and 40% progressively motile sperm, as these contain sufficient sperm of normal motility to complete sample preparation.

Sperm preparation techniques

Sperm were isolated from seminal plasma using DGC based on the methods outlined in Reynolds *et al.* (2017a) and summarised in Figure 1. Briefly, this involved placing approximately 1 ml of liquefied semen on either 40% (v/v) (Process A for aims i and ii) or layered 40% and 80% (v/v) (Process B for aims iii) Percoll/PBS solution (Percoll, GE Healthcare Life Sciences, Little Chalfont, UK) in a 13 ml polypropylene tube with ventilation cap (Sarstedt, Leicester, UK). These were then

centrifuged for 20 minutes at 300 *g* to produce an unfractionated pellet (Process A) or a population of sperm trapped at the 40-80% interface (termed '40%' sperm) and those found at the bottom of the tube (termed '80%' sperm) (Process B). In both cases, these sperm were re-suspended in PBS to at least three times their recovered volume before being centrifuged again for 10 minutes at 500 *g*. At each stage, the supernatant was removed, and the sperm was suspended in fresh PBS to a minimum volume of 600 µl.

Baseline measurements

From each prepared sample, a 2.5 µl aliquot was placed in a 10 µm depth Leja chamber (Leja Products, Nieuw Vennep, the Netherlands) which was then placed on a heated plate at 37℃ for 5 minutes before measuring concentration and motility using Sperm Class Analyzer, version 6 (Microptic SL, Barcelona, Spain) attached to a Microtec LM-2 Microscope (Mazurek Optical Services Ltd, Southam, UK) via a Basler acA1300-200uc camera (Basler AG, Ahrensburg, Germany). Since PBS does not contain any metabolites, sperm suspended in it generally swim poorly and so this process was repeated with a 20 µl aliquot of prepared sperm diluted 1 in 2 in PureSperm Wash (Nidacon, Gothenburg, Sweden) to assess the ability of the prepared sperm to swim when placed in a conventional medium. In addition, sperm vitality of each prepared sample was assessed using the LIVE/DEAD™ sperm viability kit (Fisher Scientific, Loughborough, UK), counting two replicates of at least 200 sperm as either alive (green) or dead (red) in order to establish the percentage of viable sperm.

Sperm incubation with ^{13}C substrates

209 In order to identify which ^{13}C labelled substrates could be metabolised by sperm (aim
210 i), 400 µl of unfractionated sperm (prepared by Process A in Figure 1) was added to
211 a 5 ml snap cap polystyrene round-bottom tube (Corning Falcon, Fisher Scientific)
212 along with 15 µl antibiotics (10000 units/ml penicillin and 10 mg/ml streptomycin
213 diluted to 1/3 with PBS so that in tube concentrations were 90 units/ml penicillin and
214 90 µg/ml streptomycin, Sigma Aldrich) and 40 µl of 100 mM ^{13}C labelled substrate (to
215 give a final concentration of 8.8 mM). The substrates (obtained from either Sigma
216 Aldrich or Cambridge Isotopes Laboratories, Tewksbury, MA, USA) tested were:
217 $^{13}C_u$-glucose, $^{13}C_u$-fructose, $^{13}C_1$-pyruvate, $^{13}C_1$-butyrate, $^{13}C_3$-lactate, $^{13}C_{2,4}$-D-3-
218 hydroxybutyrate, $^{13}C_5$-L-glutamate (prepared from glutamic acid), $^{13}C_{1,2}$-glycine and
219 $^{13}C_u$-galactose. Each substrate was incubated for 24 hours at 37℃ with 8 samples of
220 prepared sperm from individual men and where possible more than one substrate
221 incubation was performed in parallel (in these cases the sperm were always shown
222 to metabolise at least one substrate). After each incubation the sample was frozen at
223 -80℃ until MRS analysis (see below).
224
225 For each substrate found in aim (i) to be consistently metabolised by washed sperm,
226 the rate constant was estimated for sperm from 9 ejaculates to determine substrate
227 kinetics (aim ii). Briefly, from each ejaculate, a 380 µl aliquot of unfractionated sperm
228 (Figure 1, Process A) was placed in a 5 mm MRS tube along with 40 µl of 100mM
229 ^{13}C labelled substrate, 10 µl ^{13}C-urea (concentration and frequency reference), 20 µl
230 D_2O and 12 µl of antibiotics (as above). The tube was inserted into the MRS scanner
231 which had been preheated to 37℃ and a series of sequential ^{13}C-spectra were then
232 acquired approximately every 3 hours (see below for details) until the change in the
233 magnitude of the MRS peaks began to plateau, typically over a 18- 48 hour period.

To assess the effect of substrate concentration on sperm metabolism (aim ii), incubations were performed with unfractionated sperm (Figure 1, Process A) and ^{13}C labelled substrates consistently metabolised by sperm, identified in aim (i), (n=3). From each ejaculate, 460 µl of unfractionated sperm, 15 µl of antibiotics and 80 µl of ^{13}C labelled substrate diluted to a final concentration of 0, 1.8, 3.6, 7.2 or 14.4 mM was incubated for 4 hours at 37°C in a 5 ml snap cap polystyrene round-bottom tube (Corning Falcon, Fisher Scientific). At the end of the incubation, each sample was frozen at -80°C until MRS analysis.

Metabolism differences between '40%' and '80%' sperm (aim iii), were examined by incubating 500 µl of each (prepared from individual samples using Process B shown in Figure 1) with 15 µl of antibiotics and 40 µl of the 100 mM ^{13}C labelled substrates, identified in aim (i) and confirmed in aim (ii), for 4 hours at 37°C (n=15). Samples were then frozen at -80°C until MRS analysis.

To assess the potential impact of any non-sperm cells present in the sperm fractions obtained for aim (iii), the concentration of non-sperm cells was determined according to the method outlined in WHO (2010). Briefly, 10 µl from each sperm preparation was smeared onto two polysine slides (Thermo Scientific, Saarbrücken Germany), and after air-drying stained with Diff-Kwik kit (Thermo Scientific) and imaged on a Microtec LM-2 microscope at 40 x magnification. At least 400 sperm were counted along with any non-sperm cells observed in these fields of view; sperm heads without tails were excluded from the analysis. The concentration of non-sperm cells was determined using the formula in WHO (2010).

259

260 Magnetic Resonance Spectroscopy (MRS)

261 All samples were scanned using a 9.4 T Bruker Avance III MRS spectrometer
262 (Bruker BioSpin GmbH, Karlsruhe, Germany), with a 5 mm broadband observe
263 probe operating at either room temperature (21℃ ± 0.5) for the frozen-thawed
264 samples or at (37℃ ± 0.5) for the experiments carried out for the rate constant
265 experiments in aim (ii). Samples that were frozen at -80℃ were thawed and 380 µl
266 was placed in a 5 mm MRS tube (Norell, Morganton, NC, USA) with 20 µl D_2O
267 (Sigma Aldrich) and 10 µl of 200 mM ^{13}C-urea (chemical shift and concentration
268 reference, Sigma Aldrich) for MRS analysis. Spectra were acquired using a $^{13}C\{^1H\}$
269 inverse-gated pulse sequence (Spectral Width = 239 ppm, Number of acquisitions =
270 4096, Acquisition Time = 0.5 s, Delay Time = 2 s, Time domain points = 24036, flip
271 angle = 16°). Each acquired spectrum was apodised with a 5 Hz exponential line
272 broadening function, phase and baseline corrected using Bruker Topspin v2.1
273 software and referenced to the urea signal at a frequency offset δ = 165.5 ppm.

274

275 Data analysis

276 For aim (i), all ^{13}C-MRS spectra were first inspected visually by an expert in MRS
277 (SR) for evidence of substrate metabolism, which could be identified by the
278 appearance of new metabolite peaks and a visual reduction in the peak height of the
279 ^{13}C labelled substrate added. Identification of unknown peaks present in the spectra
280 was assisted by reference to relevant metabolic pathways known to utilise the
281 substrate and chemical shift values obtained from the human metabolome database
282 version 4.0 (Wishart *et al.*, 2018).

283

All ^{13}C-spectra peaks were integrated using the 'trapz' function in a custom Matlab script (R2017b, Mathworks, Natick, MA, USA) and predefined chemical shift integral ranges as appropriate for the substrate molecule (185.8-184.8 ppm, $^{13}C_1$-lactate; 173.5-172.5 ppm, $^{13}C_1$-pyruvate; 166.0-165.0 ppm, ^{13}C-urea; 163.5-162.7 ppm, ^{13}C-bicarbonate; 127.9-127.3 ppm, $^{13}CO_2$; 99.2-98.2 ppm, $^{13}C_u$-glucose; 102-99.6 ppm, $^{13}C_u$-fructose; 71.5-70.5 ppm, $^{13}C_2$-lactate; 23.4-22.4 ppm, $^{13}C_3$-lactate). The integrals for peaks assigned to bicarbonate and carbon dioxide were summed to account for the biological equilibrium in which these molecules exist.

To examine rates of metabolism in aim (ii), each set of sequentially acquired ^{13}C-spectra obtained from a single experiment were imported into Matlab and collated into sets of peak integral time courses obtained from each spectrum. Integrals versus time for each peak were plotted and fitted to either a mono-exponential growth (lactate and bicarbonate/CO_2 peaks) or mono-exponential decay (glucose, fructose and pyruvate). Only fits to the data that had a Pearson correlation of $r > 0.5$ and a $p < 0.01$ were retained in order to avoid misestimation of rate values due to poor signal to noise (principally arising from the bicarbonate/CO_2 integrals). The mean ± standard error (S.E.) was determined for each peak from each source substrate. Differences between metabolic rates were tested using a one-way ANOVA with Bonferroni post-hoc multi-comparison test $p < 0.05$ taken as significant.

The effect of concentration of supplied substrate on sperm metabolism (aim ii) was analyzed by measuring lactate integrals for the integral from the $^{13}C_1$ position (normalised by total sperm concentration) across the concentration ranges and comparing them using a Kruskal-Wallis test with $p < 0.05$ taken as significant.

The correlation between $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate derived lactate integrals and total sperm concentration was determined by Pearson linear regression using GraphPad Prism (version 7.03, La Jolla, USA). The value of r^2 and significance of the correlation are reported for the fit. A similar regression fit was also performed between lactate integral and non-sperm cell concentration.

In the comparison of metabolism by '40%' and '80%' sperm (aim iii), the spectra from co-incubation of '40%' or '80%' sperm with ^{13}C substrates were initially phase- and baseline-corrected and referenced to the urea peak as above. Custom MatLab code was then used to integrate the lactate peak between 185.8-184.8ppm and the bicarbonate peak between 163.5-162.7ppm. These integrals were then normalised according to: (a) sperm concentration; (b) concentration of vital sperm; and (c) concentration of motile sperm (where the motility was determined for sperm in PureSperm wash at time zero – see above). In all of these normalisations, the concentration of sperm in PBS was used. Normalised substrate integrals were compared between '40%' and '80%' sperm by Mann-Whitney with $p < 0.0055$ taken as significant (0.05/9 as 9 comparisons were done).

Results

Semen samples from 97 men, recruited as part of a larger study, were used in these experiments; 52 were used in parallel for aims (i) and (ii) and 45 were used for aim (iii).

Aim (i): Assessment of metabolically active substrates

334 The ability of ^{13}C-MRS to detect human sperm metabolism was tested eight times,
335 for each of the 9 substrates associated with energy generation, over a 24 hour
336 incubation period. This showed that the metabolism of ^{13}C substrates directly
337 involved in the glycolytic pathway, glucose, fructose and pyruvate, were easily
338 detected through conversion to lactate (Table 1). Whilst conversion to lactate was
339 always observed for these molecules, bicarbonate/CO_2 was not (however,
340 bicarbonate/CO_2 peaks were observed in a subset of experiments from aim (ii)
341 (Figure 2) and aim (iii), see below). Incubation with $^{13}C_3$-lactate showed shuttling to
342 pyruvate in most samples (7 of 8) and this was often accompanied by production of
343 peak associated with an acetyl methyl group (6 of 8), which could be from
344 dissociated acetyl-coA. Two of these eight experiments also showed $^{13}C_3$-lactate
345 metabolism to bicarbonate/CO_2. Of the other ^{13}C substrates, small quantities of
346 acetoacetate were metabolised from $^{13}C_{2,4}$-D-3-hydroxybutyrate, but further
347 metabolism (including entry into the Krebs cycle) was not detected. There was no
348 evidence of regular metabolism of $^{13}C_1$-butyrate, $^{13}C_5$-glutamate and $^{13}C_{1,2}$-glycine by
349 sperm, however, there was occasional production of bicarbonate by these substrates
350 (Table 1). Finally, in these 8 samples, incubation with $^{13}C_u$-galactose analysis by ^{13}C-
351 MRS showed only the original $^{13}C_u$-galactose peaks. Example ^{13}C-spectra from other
352 24h incubations are shown in Supplementary Figures S1-S6.
353
354 <u>Aim (ii): Substrate kinetics of human sperm</u>
355 In order to establish the appropriate conditions for sperm/substrate incubations in
356 MRS experiments $^{13}C_u$-glucose, $^{13}C_u$-fructose, $^{13}C_1$-pyruvate and $^{13}C_3$-lactate were
357 selected for further analysis to examine their kinetics and optimum concentration for
358 metabolism. Sperm from 31 ejaculates were used to determine rate constants: n = 9

each for $^{13}C_u$-glucose, $^{13}C_u$-fructose, and $^{13}C_1$-pyruvate; n = 4 for $^{13}C_3$-lactate. Repeated sequential acquisition of spectra (n = 9 for $^{13}C_u$-glucose, $^{13}C_u$-fructose, $^{13}C_1$-pyruvate; n = 2 for $^{13}C_3$-lactate) showed mono-exponential growth and decay in product and source substrate respectively (see supplementary Figures S7-S9). The rates of metabolite production and substrate consumption can be seen in Table 2, excluding $^{13}C_3$-lactate which did not provide reliable measures of metabolism.

Universally isotopically labelled $^{13}C_u$-glucose and $^{13}C_u$-fructose will label all three carbons of lactate whereas $^{13}C_1$-pyruvate will only be converted to $^{13}C_1$-lactate. An ANOVA test with Bonferroni post-hoc test showed that the choice of lactate peak (1, 2 or 3) had no significant effect on the estimated rate constant derived from $^{13}C_u$-glucose ($p = 0.99$) or $^{13}C_u$-fructose ($p = 0.69$). Given that a fructose MRS peak obscures the C2 labelled position of lactate and the pyruvate labels only C1, therefore, only the $^{13}C_1$ peak of lactate was used for subsequent analysis. This showed that there was no significant difference (ANOVA with Bonferroni post-hoc test) in the rate of $^{13}C_u$-glucose ($1.7 \pm 0.4 \times 10^{-5}$ s^{-1}) or $^{13}C_u$-fructose ($1.4 \pm 0.2 \times 10^{-5}$ s^{-1}) conversion to lactate. However, the single enzymatic step of $^{13}C_1$-pyruvate to lactate ($5.0 \pm 0.8 \times 10^{-5}$ s^{-1}) was approximately three times faster than that of $^{13}C_u$-glucose and $^{13}C_u$-fructose ($p = 0.0011$ and $p = 0.00042$, respectively). Visually inspecting the rate data showed that typically $^{13}C_1$-pyruvate incubations reached over half the maximum lactate production by the second time point (an average of 4.5 hours into the experiment), whereas from $^{13}C_u$-glucose and $^{13}C_u$-fructose, over half the maximum lactate production was often reached at the sixth time point (an average of 19.5 hours into the incubation).

In contrast to 24 h incubations (aim i), bicarbonate was produced during some rate experiments (aim ii). It appeared more frequently from $^{13}C_1$-pyruvate (6 of 8) than $^{13}C_u$-glucose (3 of 8), although the rate constants for these were not significantly different. This was probably due to the smaller peak intensity causing large standard error in the rate constant (see Table 2). $^{13}C_u$-fructose did not produce bicarbonate under these conditions. Sequential spectra were also acquired where $^{13}C_3$-lactate was the source substrate, however, in these experiments no consistent build-up of pyruvate or bicarbonate/CO_2 was observed (n=4), most likely due to the low signal to noise of the pyruvate peak that had been observed for $^{13}C_3$-lacate from aim (i). A long incubation allows time for MRS peaks to increase leading to a reduction in integration errors due to signal noise. However, sperm quality also degrades with time, therefore, 4 hours was chosen for subsequent experiments.

The optimum metabolite concentrations were assessed during a 4 hour incubation for the principal metabolic substrates of $^{13}C_u$-glucose, $^{13}C_u$-fructose and $^{13}C_1$-pyruvate (three washed sperm samples per substrate). Overall, the amount of lactate produced by sperm (normalised to total sperm concentration) was not significantly influenced by the supplied substrate concentration (1.8 - 14.4 mM) (see Figure 3 and supplementary Figure S10). However, after 4 hours of incubation the remaining substrate peaks for the lowest concentration, 1.8 mM, were almost absent from the MRS spectrum. Consequently, to ensure substrate metabolism was not limited by its availability and to allow for higher sperm concentration than used here in subsequent experiments, a concentration of 7.2 mM was selected for the experiments in aim (iii).

<u>Aim (iii): Substrate Metabolism by '40%' and '80%' sperm</u>

409 Sperm metabolism was assessed using sperm from 45 individual ejaculates that had
410 been separated by DGC (Process B in Figure 1). This method yields two sperm
411 populations (termed '40%' and '80%' sperm in Reynolds *et al.*, 2017a) with
412 significant differences in both motility and vitality (see Table 3). The percentage
413 sperm motility were not similar to the viability (i.e. sperm motility was not entirely due
414 to a lack of viable sperm) and, importantly for our study, there was no significant
415 difference in sperm concentration between the '40%' and '80%' groups.
416
417 The '40%' and '80%' sperm samples also contained small numbers of other cell
418 types (e.g. leukocyte, germ and epithelial cells) that may have been metabolically
419 active. Therefore, it was important to determine their prevalence in the sperm
420 preparations assessed in aim (iii). Briefly, there were more non-sperm cells in the
421 '40%' compared to the '80%' sperm population (median: 1.1, range: 0.1 – 6.9 vs.
422 median: 0.4, range: 0.0 – 4.5 x 10^6/ml, $p < 0.0001$, respectively). Both fractions
423 showed a correlation between the concentration of non-sperm cells and sperm
424 concentration but the r^2 was higher for the '40%' sperm fraction ($p = 0.0018$, $r^2 =$
425 0.21), than the '80%' fraction ($p = 0.046$, $r^2 = 0.09$), see supplementary Figure S11.
426
427 The '40%' and '80%' sperm were separated into cohorts incubated with different
428 substrates (n = 15 samples per sperm population) with sperm concentration, vitality
429 and motility measured at the start of incubation (Table 4). A Kruskal-Wallis test with
430 a Dunn's multiple comparison correction showed that none of these parameters were
431 significantly different for the '80%' or the '40%' sperm for any substrate.
432

433 Lactate peaks in the ^{13}C-MRS spectra were plotted against total sperm concentration
434 to determine whether the observed lactate arose from sperm metabolism. When
435 sperm were incubated with either $^{13}C_u$-glucose or $^{13}C_u$-fructose, there was a
436 significant strong linear correlation with total sperm concentration (see Figures 4a
437 and 4b). However, the lactate integrals obtained from sperm incubated with $^{13}C_1$-
438 pyruvate showed no correlation ($p > 0.05$) with concentration for either '40%' or
439 '80%' sperm populations (Figure 4c). Importantly, no correlations were found when
440 lactate peak integrals were plotted against non-sperm cell concentrations (Figures
441 4d, 4e, 4f), strongly suggesting that non-sperm cells did not make a significant
442 contribution to the metabolism observed ($p > 0.05$).
443
444 After a 4 hour incubation at 37℃, lactate peaks could be identified from all
445 substrates metabolised by '40%' and '80%' sperm ($n = 15$). The peak integral
446 measured from its MRS spectrum represents an absolute concentration of the
447 metabolite present in solution, which is a function of the sperm concentration as
448 seen in Figure 4. We reasoned vital and motile sperm would have larger impacts on
449 metabolism than immotile or dead sperm and therefore normalised lactate integrals
450 by three different methods: total sperm concentration (Figure 5a), concentration of
451 vital sperm (Figure 5b) and concentration of motile sperm (Figure 5c). Briefly, the
452 lactate integral was only significantly higher for $^{13}C_u$-glucose incubations with '40%'
453 compared to '80%' sperm when normalised to vital concentration ($3.3 \pm 0.3 \times 10^6$ vs
454 $2.0 \pm 0.21 \times 10^6$; $p = 0.0049$) or motile concentration ($7.0 \pm 0.75 \times 10^6$ vs 4.8 ± 1.3
455 $\times 10^6$; $p = 0.0032$), Mann-Whitney test ($p < 0.0055$ taken as statistically significant).
456 No significant differences were found for these sperm populations incubated with

457 either $^{13}C_u$-fructose or $^{13}C_1$-pyruvate or in any incubation when normalised to total
458 sperm concentration.
459
460 After a 4 hour incubation at 37℃, bicarbonate, a marker of oxidative
461 phosphorylation, was occasionally observed. Bicarbonate was produced from $^{13}C_1$-
462 pyruvate metabolised by '40%' sperm (5 occurrences) and '80%' sperm (3
463 occurrences), $^{13}C_u$-glucose metabolised by '40%' sperm (2 occurrences) and '80%'
464 sperm (2 occurrences) and $^{13}C_u$-fructose metabolised by '40%' sperm (2
465 occurrences). The appearance of bicarbonate was associated across a range of
466 sperm concentrations (mean 59.2 x 10^6/ml, range 17.9 - 115.4 x 10^6/ml) and also
467 typical non-sperm cell concentrations (mean 1.2 x 10^6/ml range 0 – 2.8 x 10^6/ml) so
468 bicarbonate appearance was not associated with high sperm or non-sperm cell
469 concentrations. The sporadic nature of bicarbonate production and low signal to
470 noise for this peak meant that no significant differences were observed for
471 bicarbonate integrals from '40%' and '80%' sperm.
472
473 **Discussion**
474 This paper continues our previous work that used ^{1}H-MRS to investigate the
475 endogenous metabolites present in human sperm (Reynolds *et al.*, 2017a). Here we
476 examine human sperm metabolism of a range of exogenous ^{13}C labelled substrates
477 by ^{13}C-MRS and define the experimental conditions necessary to observe them. The
478 chosen metabolites feed into glycolysis and oxidative phosphorylation at various
479 locations in the metabolic pathways and they included sugars, ketone bodies, fatty
480 acids and amino acids. Glucose, fructose, pyruvate and lactate were chosen for their
481 role in glycolysis and their predominance in the female reproductive tract and

seminal fluid (Andrade-Rocha, 1999, Ford and Rees, 1990, Weed and Carrera, 1970), with galactose also included as its enzyme, galactose-1-phosphate uridylyltransferase, has been predicted to be important for sperm motility (Asghari *et al.*, 2017). Ketone body D-3-hydroxybutyrate was selected due to its ability to maintain and restore motility of mouse sperm (Tanaka *et al.*, 2004) and the fatty acid analogue butyrate was chosen as it had previously been demonstrated to be metabolised by ram, bull, dog and fowl spermatozoa (Scott *et al.*, 1962). Finally, amino acids glycine and glutamate feed into different aspects of oxidative phosphorylation and are common amino acids found in seminal fluid which may have a role in protecting sperm motility in bovine and ram spermatozoa (Kondo, 1975, Setchell *et al.*, 1967, Tyler and Tanabe, 1952).

In these experiments, we found that $^{13}C_u$-glucose and $^{13}C_u$-fructose were predominately metabolised to lactate which is indicative of glycolysis in a process that also produces two net units of ATP per carbohydrate molecule. The end point of glycolysis (i.e. $^{13}C_1$-pyruvate) was also mainly converted to lactate. The enzyme lactate dehydrogenase catalyzes a shuttling reaction between pyruvate and lactate to maintain cofactors NADH/NAD^+ to support further glycolysis and the electron transport chain as required for cellular function.

The relative magnitude of the lactate and pyruvate ^{13}C-MRS peaks demonstrate an important feature of these experiments. The degree of ^{13}C-lactate or ^{13}C-pyruvate observed by ^{13}C-MRS is dependent not only on enzyme activity and substrate preference but also the endogenous concentration of unlabeled lactate/pyruvate. This is because any ^{13}C-lactate generated from ^{13}C-pyruvate can, in principle, be

converted back to ^{13}C-pyruvate. However, where there is a large concentration of unlabeled ^{12}C-lactate present then there is a great probability of those molecules being converted back to pyruvate, leading to ^{13}C-lactate being retained within the lactate pool. Conversely, for $^{13}C_3$-lactate sperm incubations, the lower endogenous ^{12}C-pyruvate concentration resulted in fewer ^{13}C-labelled pyruvate molecules being retained leading to smaller MRS pyruvate signal. Hence, how metabolites are enzymatically exchanged and their endogenous concentration affects the ^{13}C MRS observability of intermediate metabolites within a metabolic pathway.

In addition to pyruvate, $^{13}C_3$-lactate often produced a small acetate peak. In mammalian cells, pyruvate can be metabolised to acetyl-CoA by pyruvate dehydrogenase and then to acetate by acetyl-CoA hydrolase but this will not generate ATP (Knowles *et al.*, 1974). Unlike for $^{13}C_u$-glucose and $^{13}C_u$-fructose, ^{13}C-MRS was not able to detect human sperm metabolism of $^{13}C_u$-galactose. ^{14}C galactose studies have reported that this molecule is not metabolised by human sperm (MacLeod, 1941, Rogers and Perreault, 1990), however, it has been reported to undergo slow glycolysis in human semen (Mann, 1946). It is likely that if galactose is metabolised by human sperm then its concentration falls below the sensitivity of conventional MRS.

$^{13}C_{2,4}$-D-3-hydroxybutyrate was converted to acetoacetate catalysed by β-hydroxybutyrate dehydrogenase, an enzyme that in other tissues (Smith *et al.*, 1969), including rat testis (Niemi and Ikonen, 1962), can be switched on by environmental cues, such as starvation. The reversible conversion of hydroxybutyrate to acetoacetate is linked to NADH generation and calcium uptake in

532 bovine epididymal sperm (Vijayaraghavan *et al.*, 1989) and to supporting motility in
533 mouse sperm (Tanaka *et al.*, 2004). Although acetoacetate can feed into the Krebs
534 cycle, no evidence for this was observed. Finally, there were two instances of $^{13}C_1$-
535 butyrate conversion to glutamate. It is possible that glutamate production from $^{13}C_1$-
536 butyrate was through Krebs cycle metabolism, however, as there were no
537 anaplerotic markers in the MRS spectrum, the exact mechanism for generation of
538 glutamate remains unknown.
539
540 Bicarbonate was occasionally produced from incubations with $^{13}C_1$-butyrate, $^{13}C_3$-
541 lactate, $^{13}C_{2,4}$-D-3-hydroxybutyrate, $^{13}C_5$-L-glutamate or $^{13}C_{1,2}$-glycine after 24 hours,
542 but under the same conditions $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate did not
543 show bicarbonate production. In aims (ii) and (iii), bicarbonate and carbon dioxide
544 production was most often observed from $^{13}C_1$-pyruvate and, to a lesser extent, $^{13}C_u$-
545 glucose, and least often from $^{13}C_u$-fructose. Generally, the production of carbon
546 dioxide/bicarbonate by sperm samples was intermittent and why some samples
547 produced measurable ^{13}C-bicarbonate and others did not remains unclear as there
548 was no obvious visible bacteria, fungi, high sperm concentration or a high proportion
549 of non-sperm cells in these samples. Bicarbonate plays an important role in sperm
550 capacitation (Miraglia *et al.*, 2010) and its role in relationship to these substrates and
551 capacitation warrants further investigation.
552
553 These experiments were conducted under atmospheric oxygen and carbon dioxide
554 levels which is likely to have influenced which metabolic pathways were selected.
555 Atmospheric oxygen and carbon dioxide levels were chosen for practical reasons
556 while using the 9.4T scanner. These conditions would provide more oxygen and less

carbon dioxide than would be expected in vivo (Ng et al. 2018), which should promote the aerobic use of OxPhos. Regardless of these conditions, OxPhos was still only recorded at low levels, which does seem to suggest that human sperm inherently prefer anaerobic glycolysis to meet their energy needs. This is supported by Hereng et al. (2011) who also did not observe OxPhos metabolism in human sperm and commented that they used glycolysis.

Rate constants for $^{13}C_u$-glucose and $^{13}C_u$-fructose conversion to lactate were similar, suggesting that transport across the cellular membrane was not a limiting factor. The estimated rate constant for $^{13}C_1$-pyruvate to lactate was much faster than $^{13}C_u$-glucose /$^{13}C_u$-fructose conversion to lactate. This is to be expected as the rate limiting step for glycolysis is catalyzed by phosphofructokinase which will affect metabolism of both $^{13}C_u$-glucose and $^{13}C_u$-fructose, but not $^{13}C_1$-pyruvate. No significant differences were measured for bicarbonate rate constants estimated for $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate, due to large errors probably resulting from the low signal to noise observed for this molecule. It was not possible to obtain rates measurements for $^{13}C_3$-lactate incubations, due to low concentrations of pyruvate being produced. Whilst incubating sperm with ^{13}C labelled substrate for longer would increase the magnitude of the peaks in MRS spectrum, there was a concern that, over extended times, sperm death would confound the results. An incubation time of 4 hours was chosen for subsequent experiments where the build-up of metabolic product from the rates constant experiments in aim (ii) was approximately linear.

581 The concentration of ^{13}C labelled substrates chosen for further experiments was 7.2
582 mM for each incubation. This is within a similar concentration range to that used by
583 Williams and Ford (2001) who found that the optimal in-vitro concentration of glucose
584 was 5.56 mM for supporting human sperm motility. ^{13}Cu-glucose, $^{13}C_u$-fructose and
585 $^{13}C_3$-lactate were within physiological levels experienced by sperm either within
586 seminal plasma or the female reproductive tract (Ford and Rees, 1990) (Gardner *et*
587 *al.*, 1996). Pyruvate is found at slightly lower concentrations in seminal plasma (1 - 6
588 mM)(Mann and Lutwak-Mann, 1981) and in the female reproductive (0.1 - 0.2
589 mM)(Gardner *et al.*, 1996, Tay *et al.*, 1997). Therefore, our examinations were within
590 optimal concentration ranges to support motility for some substrates, but were super-
591 physiological for pyruvate. Our experiments used each substrate in isolation and
592 interaction between sperm and multiple substrates is likely to be different (Hereng *et*
593 *al.*, 2011) both in vitro and in the female reproductive tract.
594
595 During preparation for assisted conception, the '40%' sperm are normally discarded
596 as they tend to be of poorer quality and also are co-localised with a higher proportion
597 of non-sperm cells (Henkel and Schill, 2003). However, these sperm are still
598 biologically relevant, as in vivo all sperm are deposited in the female reproductive
599 tract and '40%' sperm may represent a substantial fraction of the sperm population in
600 men with male factor infertility. As in our previous work using ^{1}H-MRS (Reynolds *et*
601 *al.*, 2017a), we exploited the difference between these two sub-populations to test
602 the ability of ^{13}C-MRS to detect metabolism differences. First it was important to
603 consider the metabolic role of any non-sperm cells but, unlike that for sperm, the
604 concentration of non-sperm cells did not correlate with the lactate integral,
605 suggesting that they have a minimal effect on recorded lactate production at the

606 concentrations we observed for them. Therefore, the highest non-sperm cell
607 concentration in this analysis, of 6.9 x10^6/ml, was taken as the limit for non-sperm
608 cell concentrations known not to affect sample metabolism measurably. Metabolism
609 of ^{13}C-labelled substrates by the differing non-sperm cells types found in seminal
610 plasma could be done in future studies.
611
612 Both '40%' and '80%' sperm produced similar amounts of ^{13}C-lactate when
613 normalised to total sperm concentration, however, unlike ^{1}H-MRS where sperm
614 concentration will affect metabolite detection regardless of vitality, ^{13}C-MRS will only
615 detect the ^{13}C-products of metabolising sperm. Therefore, normalising the lactate
616 signal by the vital sperm concentration was considered more appropriate.
617 Additionally, as sperm motility is estimated to account for 70% of the sperm's total
618 ATP production (Rikmenspoel, 1965), normalisation of the lactate signal by motile
619 sperm concentration was also performed. For either of these normalisations, it was
620 found that '40%' sperm incubated with $^{13}C_u$-glucose produced more lactate, i.e. a
621 larger metabolic output, than the equivalent '80%' sperm, whereas fructose or
622 pyruvate incubations showed no significant differences between '40%' and '80%'
623 sperm.
624
625 It is interesting that only measurements after incubation with $^{13}C_u$-glucose showed a
626 difference between motile or vital '40%' and '80%' sperm. Kinetic experiments
627 presented here suggest that $^{13}C_u$-fructose and $^{13}C_u$-glucose were metabolised by
628 glycolysis to similar levels and should have similar effects on sperm function. Sperm
629 are highly polarised cells and one reason for a difference between the ability of
630 glucose and fructose to support sperm motility could be related to the roles of

differing hexose transporter and their distribution within sperm (Angulo *et al.*, 1998, Bucci *et al.*, 2011). In turn these molecules may support different phases of sperm life (du Plessis *et al.*, 2015).

There are many reasons why '80%' sperm might show lower lactate production than '40%' sperm. The '40%' sperm are more likely to have abnormal morphology with increased cytoplasm (Aitken and West, 1990). As glycolysis is an uncontrolled reaction that takes place in the cytoplasm, simply having more cytoplasm may produce more lactate. Conversely, tightly controlled energy production in '80%' sperm may limit ROS production and subsequent DNA damage (Aitken *et al.*, 1996).

In conclusion, we have examined sperm metabolism by ^{13}C-MRS and found metabolic differences in sub-populations from the same individuals ('80%' vs. '40%' sperm). Human sperm, from either sub-population, seem to predominantly use the glycolytic pathway to meet their energy needs when supplied with $^{13}C_u$-glucose or $^{13}C_u$-fructose. The level of glycolysis was increased for '40%' sperm incubated with $^{13}C_u$-glucose compared to equivalent '80%' sperm, which perhaps suggests that poor quality sperm are metabolically noisy. By implication, this may also be true of poor quality sperm from sub-fertile men and further work to examine this using MRS technology may help to better unravel the metabolic characteristics of poor quality sperm.

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Authors' roles

AAP, SR and MNP proposed the study. AAP and MNP obtained the MRC grant funding. All authors designed the study and contributed to the writing of the manuscript. SJC and SR jointly carried out experimental protocols and data analysis. SJW advised on statistical analyses.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1: Sperm washing methods used to remove seminal plasma. Process (A) was used to yield higher concentration of sperm for aims (i) and (ii). Process (B) was used fractionate sperm into higher average motility, '80%', sperm and lower average motility, '40%', sperm. See main text for further details.

Figure 2: Example ^{13}C MRS spectra for sperm incubated with: (a) $^{13}C_u$-glucose; (b) $^{13}C_u$-fructose; or (c) $^{13}C_1$-pyruvate. Integral locations and widths are highlighted in red. Key: sub – peaks from respective incubated substrate; L1, L2, L3 – lactate peaks, where the number indicates the carbon position, B – bicarbonate, C – carbon dioxide, U – urea.

Figure 3: Normalising the lactate integral for sperm concentration and plotting against substrate concentration showed that sperm metabolism was not limited by substrate availability as tested by Kruskal-Wallis. Sperm were tested over a 4 hour incubation (n=3) with $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate (shown in blue, red and green respectively) at 0 mM, 1.8 mM, 3.6 mM, 7.2 mM and 14.4 mM concentrations.

Figure 4: Comparing lactate integral correlation with sperm and non-sperm cell concentrations. Sperm concentrations (n = 15) correlated with lactate integrals for (a) $^{13}C_u$-glucose, (b) $^{13}C_u$-fructose and (c) $^{13}C_1$-pyruvate incubations. Non-sperm cell concentrations did not correlate with lactate integrals for (d) $^{13}C_u$-glucose and (e) $^{13}C_u$-fructose or (f) $^{13}C_1$-pyruvate incubations. Lines of best fit are shown for correlations where $p < 0.05$ for slope being non-zero.

Figure 5: Comparing $^{13}C_u$-glucose, $^{13}C_u$-fructose or $^{13}C_1$-pyruvate metabolism (n = 15 per substrate) between '40%' and '80%' sperm. Integrals were measured for $^{13}C_1$-lactate and normalised against either (a) sperm concentration, (b) concentration of vital sperm or (c) concentration of motile sperm.

Legends to supplementary figures:

Supplementary Figure S1: Example ^{13}C-MRS spectrum of $^{13}C_3$-lactate after a 24h incubation with sperm at 37°C. Peak assignment: S - 22.8 ppm is $^{13}C_3$-lactate (source substrate); P - 29.2 ppm is $^{13}C_3$-pyruvate; A - 26.0 ppm is $^{13}C_2$-acetate; U - 165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S2: Example ^{13}C-MRS spectrum of $^{13}C_{2,4}$-D-3-hydroxybutyrate after a 24h incubation with sperm at 37°C. Peak assignment: S_2 - 49.2 ppm & S_4 - 24.5 ppm is $^{13}C_{2,4}$-D-3-hydroxybutyrate (source substrate); B_2 - 55.9 ppm & B_4 - 32.3 ppm is $^{13}C_{2,4}$-acetoacetate; U - 165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S3: Example ^{13}C-MRS spectrum of $^{13}C_1$-butyrate after a 24h incubation with sperm at 37°C. Peak assignment: S - 186.8 ppm is $^{13}C_1$-butyrate (source substrate); G - 184.1 ppm is $^{13}C_5$-glutamate; U - 165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S4: Example ^{13}C-MRS spectrum of $^{13}C_{1,2}$-glycine after a 24h incubation with sperm at 37°C. Peak assignment: S_1 - 175.1 ppm & S_2 - 44.1 ppm is $^{13}C_{1,2}$-glycine (source substrate); U - 165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S5: Example ^{13}C-MRS spectrum of $^{13}C_5$-glutamate after a 24h incubation with sperm at 37°C. Peak assignment: S - 184.0 ppm is $^{13}C_5$-glutamate (source substrate); U -165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S6: Example ^{13}C-MRS spectrum of $^{13}C_u$-galactose after a 24h incubation with sperm at 37°C. Peak assignment: S between 104 - 62 ppm is $^{13}C_u$-galactose (source substrate); U - 165.5 ppm is ^{13}C-urea (added reference compound post incubation).

Supplementary Figure S7: (a) Example ^{13}C-MRS spectrum of $^{13}C_u$-fructose after at least 24h incubation with sperm at 37°C in the 9.4 T MRS spectrometer. Peak assignment: S is $^{13}C_u$-fructose (source substrate between 104 - 62 ppm); L_1 - 185.3 ppm, L_2 - 71.2 ppm and L_3 – 22.8 ppm is for ^{13}C-lactate labelled in the 1, 2 or 3 position respectively; B – 162.0 ppm is bicarbonate; C – 128.0 ppm is carbon dioxide; U - 165.5 ppm is ^{13}C-urea (added reference compound). Peak integral location and range are shown as red markers. (b) Repeated spectra, acquired every 3 hours, are shown transitioning from blue to red (linearly right shifted to aid visualisation with time axis shown above a peak). Live sperm metabolism can be seen in these spectra as lactate peaks increase in size and fructose peaks decrease. (c) Integrals from $^{13}C_1$-lactate, bicarbonate and carbon dioxide (summed over both integrals), $^{13}C_u$-fructose and $^{13}C_3$-lactate were repeatedly measured over time and fitted to an exponential growth curve or decay curve, as appropriate. These show how rate values were calculated from $^{13}C_u$-fructose incubations for lactate increase and $^{13}C_u$-fructose decline.

Supplementary Figure S8: (a) Example ^{13}C-MRS spectrum of $^{13}C_u$-glucose after at least 24h incubation with sperm at 37°C in the 9.4 T MRS spectrometer. Peak assignment: S between 104 - 67.0 ppm & at 65 ppm is $^{13}C_u$-glucose (source substrate); L_1 - 185.3 ppm, L_2 - 71.2 ppm and L_3 – 22.8 ppm is ^{13}C-lactate; B – 162.0 ppm is bicarbonate; C – 128.0 ppm is carbon dioxide; U - 165.5 ppm is ^{13}C-urea (added reference compound). Peak integral location and range are shown as red markers. (b) Repeated spectra, acquired every 3 hours, are shown transitioning from blue to red (linearly right shifted to aid visualisation with time axis shown above a peak). Live sperm metabolism can be seen in these spectra as lactate peaks increase in size and glucose peaks decrease. (c) Integrals from $^{13}C_1$-lactate, bicarbonate and carbon dioxide (summed over both integrals), $^{13}C_u$-glucose $^{13}C_2$-lactate and $^{13}C_3$-lactate were repeatedly measured over time and fitted to an exponential growth curve or decay curve, as appropriate. These show how rate values were calculated from $^{13}C_u$-glucose incubations for lactate and carbon dioxide increase and $^{13}C_u$-glucose decline.

Supplementary Figure S9: (a) Example ^{13}C-MRS spectrum of $^{13}C_u$-pyruvate after at least 24h incubation with sperm at 37°C in the 9.4 T MRS spectrometer. Peak assignment: S 172.9 ppm is $^{13}C_1$-pyruvate (source substrate); L_1 - 185.3 ppm is ^{13}C-lactate; B – 162.0 ppm is bicarbonate; C – 128.0 ppm is carbon dioxide; U - 165.5 ppm is ^{13}C-urea (added reference compound) (a). Peak integral location and range are shown as red markers. (b) Repeated spectra, acquired every 3 hours, are shown transitioning from blue to red (linearly right shifted to aid visualisation with time axis shown above a peak). Live sperm metabolism can be seen in these spectra as lactate peaks increase in size and pyruvate peaks decrease. (c) Integrals from $^{13}C_1$-lactate, bicarbonate and carbon dioxide (summed over both integrals), $^{13}C_1$-pyruvate were repeatedly measured over time and fitted to an exponential growth curve or decay curve, if appropriate. These show how rate values were calculated from $^{13}C_1$-pyruvate incubations for lactate and carbon dioxide increase and $^{13}C_1$-pyruvate decline.

Supplementary Figure S10: Example spectra of substrates incubated with sperm at different concentrations for 4 hours. (a) $^{13}C_u$-glucose, (b) $^{13}C_u$-fructose and (c) $^{13}C_1$-pyruvate spectra are shown with higher concentrations of ^{13}C labelled substrate (linearly right shifted to aide visualisation, 0 mM – black spectra, 1.8 mM – orange spectra, 3.6 mM – blue spectra, 7.2 mM – green spectra and 14.4 mM – yellow spectra). The 0 mM spectra showed no substrate present or lactate created. All other concentrations produced lactate (L_1 & L_3) at similar level. However, levels of added substrate (S) depended upon the amount of substrate added and were sometimes harder to observe at the lower concentrations. ; U - 165.5 ppm is ^{13}C-urea (added reference compound). ; H – 181.1 ppm is $^{13}C_1$-hydroxypyruvate (non-metabolically active compound in aqueous equilibrium with pyruvate)

Supplementary Figure S11: Plotting the concentration of non-sperm cells against sperm concentration. Lines of best fit and r values are shown for correlations with $p < 0.05$ for slope being non-zero, grey for '40%' sperm values and black for '80%' sperm.

References

Aitken RJ, Buckingham DW, Carreras A, Irvine DS. Superoxide dismutase in human sperm suspensions: relationship with cellular composition, oxidative stress, and sperm function. *Free Radical Bio Med* 1996; **4** :495-504.

Aitken RJ, West KM. Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. *Int J Androl* 1990; **6** : 433-451.

Amaral A, Paiva C, Attardo Parrinello C, Estanyol JM, Ballesca JL, Ramalho-Santos J, Oliva R. Identification of proteins involved in human sperm motility using high-throughput differential proteomics. *J Proteome Res* 2014; **12** :5670-5684.

Andrade-Rocha FT. Seminal fructose levels in male infertility: relationship with sperm characteristics. *Int Urol Nephrol* 1999; **1** :107-111.

Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-Lopez F, Guaiquil VH, Vera JC, Concha, II. Hexose transporter expression and function in mammalian spermatozoa: cellular localization and transport of hexoses and vitamin C. *J Cell Biochem* 1998; **2** :189-203.

Asghari A, Marashi SA, Ansari-Pour N. A sperm-specific proteome-scale metabolic network model identifies non-glycolytic genes for energy deficiency in asthenozoospermia. *Syst Biol Reprod Med* 2017; **2** :100-112.

Barratt CLR, De Jonge CJ, Sharpe RM. 'Man Up': the importance and strategy for placing male reproductive health centre stage in the political and research agenda. *Hum Reprod* 2018; **4** :541-545.

Bruntz RC, Lane AN, Higashi RM, Fan TW. Exploring cancer metabolism using stable isotope-resolved metabolomics (SIRM). *J Biol Chem* 2017; **28** :11601-11609.

Bucci D, Rodriguez-Gil JE, Vallorani C, Spinaci M, Galeati G, Tamanini C. GLUTs and mammalian sperm metabolism. *J Androl* 2011; **4** :348-355.

Buescher JM, Antoniewicz MR, Boros LG, Burgess SC, Brunengraber H, Clish CB, DeBerardinis RJ, Feron O, Frezza C, Ghesquiere B et al. A roadmap for interpreting 13C metabolite labeling patterns from cells. *Curr Opin Biotechnol* 2015; **34** :189-201.

du Plessis SS, Agarwal A, Mohanty G, van der Linde M. Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? *Asian J Androl* 2015; **2** :230-235.

Ford WC, Harrison A. The role of oxidative phosphorylation in the generation of ATP in human spermatozoa. *J Reprod Fertil* 1981; **1** :271-278.

Ford WC, Rees JM. The bioenergetics of mammalian sperm motility. In Gagnon C (ed) *Controls of Sperm Motility: Biological and Clinical Aspects*. 1990. CRC Press, Boca Raton, USA pp.175-202.

Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996; **2** :349-353.

Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol* 2003; **1** :108.

Hereng TH, Elgstoen KB, Cederkvist FH, Eide L, Jahnsen T, Skalhegg BS, Rosendal KR. Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. *Hum Reprod* 2011; **12** :3249-3263.

Knowles SE, Jarrett IG, Filsell OH, Ballard FJ. Production and utilization of acetate in mammals. *Biochem J* 1974; **2** :401-411.

Kondo T. Studies of free amino acids in normal and sterile human semen. *J Showa Med Assoc* 1975; **1** :1-10.

MacLeod J. The metabolism of human spermatozoa. *Am J Physiol* 1941; **1** :193-201.
Mann T. Studies on the metabolism of semen: 3. Fructose as a normal constituent of seminal plasma. Site of formation and function of fructose in semen. *Biochem J* 1946; **4** :481-491.
Mann T, Lutwak-Mann C. *Male reproductive function and semen: themes and trends in physiology, biochemistry and investigative andrology.* 1st edn, 1981. Springer-Verlag, Berlin, Germany.
Miraglia E, Lussiana C, Viarisio D, Racca C, Cipriani A, Gazzano E, Bosia A, Revelli A, Ghigo D. The pentose phosphate pathway plays an essential role in supporting human sperm capacitation. *Fertil Steril* 2010; **7** :2437-2440.
Murdoch RN, White IG. Studies of the metabolism of human spermatozoa. *J Reprod Fertil* 1968; **3** :351-361.
Niemi M, Ikonen M. Cytochemistry of oxidative enzyme systems in leydig cells of rat testis and their functional significance. *Endocrinology* 1962; **2** :167-174.
Pacey AA. Sperm, human fertility and society. In Birkhead TR, Hosken DJ and Pitnick SS (eds) *Sperm biology: an evolutionary perspective*. 1st edn, 2009. Academic Press, Oxford, UK, pp.565-597.
Peterson RN, Freund M. ATP synthesis and oxidative metabolism in human spermatozoa. *Biol Reprod* 1970; **1** :47-54.
Reynolds S, Calvert SJ, Paley MN, Pacey AA. 1H Magnetic Resonance Spectroscopy of live human sperm. *Mol Hum Reprod* 2017a; **1** :441-451.
Reynolds S, Ismail NFB, Calvert SJ, Pacey AA, Paley MNJ. Evidence for rapid oxidative phosphorylation and lactate fermentation in motile human sperm by hyperpolarized 13C Magnetic Resonance Spectroscopy. *Sci Rep* 2017b; **1** :4322.
Rikmenspoel R. The tail movement of bull spermatozoa: observations and model calculations. *Biophys J* 1965; **4** :365-392.
Rogers BJ, Perreault SD. Importance of glycolysable substrates for in vitro capacitation of human spermatozoa. *Biol Reprod* 1990; **6** :1064-1069.
Ruiz-Pesini E, Diez-Sanchez C, Lopez-Perez MJ, Enriquez JA. The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? *Curr Top Dev Biol* 2007; **77** :3-19.
Scott TW, White IG, Annison EF. Oxidation of shortchain fatty acids (C1-C8) by ram, bull, dog and fowl spermatozoa. *Biochem J* 1962; **2** :392-398.
Setchell BP, Hinks NT, Voglmayr JK, Scott TW. Amino acids in ram testicular fluid and semen and their metabolism by spermatozoa. *Biochem J* 1967; **3** :1061-1065.
Shestov AA, Lee SC, Nath K, Guo L, Nelson DS, Roman JC, Leeper DB, Wasik MA, Blair IA, Glickson JD. ^{13}C MRS and LC-MS flux analysis of tumor intermediary metabolism. *Front Oncol* 2016; **6** :135.
Smith AL, Satterthwaite HS, Sokoloff L. Induction of brain D(-)-beta-hydroxybutyrate dehydrogenase activity by fasting. *Science* 1969; **3862** :79-81.
Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. *Hum Reprod Update* 2006; **1** :23-37.
Tanaka H, Takahashi T, Iguchi N, Kitamura K, Miyagawa Y, Tsujimura A, Matsumiya K, Okuyama A, Nishimune Y. Ketone bodies could support the motility but not the acrosome reaction of mouse sperm. *Int J Androl* 2004; **3** :172-177.

902 Tay JI, Rutherford AJ, Killick SR, Maguiness SD, Partridge RJ, Leese HJ. Human tubal fluid:
903 production, nutrient composition and response to adrenergic agents. *Hum Reprod* 1997; **11**
904 :2451-2456.
905 Tyler A, Tanabe TY. Motile life of bovine spermatozoa in glycine and yolkcitrate diluents at
906 high and low temperatures. *Proc Soc Exp Biol Med* 1952; **2** :367-371.
907 Vigue C, Vigue L, Huszar G. Adenosine triphosphate (ATP) concentrations and
908 ATP/adenosine diphosphate ratios in human sperm of normospermic, oligospermic, and
909 asthenospermic specimens and in their swim-up fractions: lack of correlation between ATP
910 parameters and sperm creatine kinase concentrations. *J androl* 1992; **4** :305-311.
911 Vijayaraghavan S, Bhattacharyya A, Hoskins DD. Calcium uptake by bovine epididymal
912 spermatozoa is regulated by the redox state of the mitochondrial pyridine nucleotides. *Biol*
913 *Reprod* 1989; **4** :744-751.
914 Weed JC, Carrera AE. Glucose content of cervical mucus. *Fertil Steril* 1970; **12** :866-872.
915 Williams AC, Ford WC. The role of glucose in supporting motility and capacitation in human
916 spermatozoa. *J androl* 2001; **4** :680-695.
917 Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vazquez-Fresno R, Sajed T, Johnson D, Li
918 C, Karu N et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic acids res*
919 2018; **D1** :D608-D617.
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921

922 Table 1: Summary of observed metabolic products of human sperm after incubating
923 with ^{13}C labelled substrates. Eight incubations were performed for each substrate at
924 37℃ for 24h with samples subsequently stored at -80℃ prior to MRS analysis.
925

926

	Number of spectra the metabolic product was observed in		
Incubated substrate	Lactate produced	Bicarbonate produced	Other metabolic products
$^{13}C_u$-glucose	8	0	-
$^{13}C_u$-fructose	8	0	-
$^{13}C_1$-pyruvate	8	0	-
$^{13}C_3$-lactate	N/A	2	pyruvate (7 of 8), acetate (6 of 8)
$^{13}C_{2,4}$-D-3-hydroxybutyrate	0	2	acetoacetate (8)
$^{13}C_1$-butyrate	0	1	glutamate (2)
$^{13}C_5$-glutamate	0	2	-
$^{13}C_2$-glycine	0	1	-
$^{13}C_u$-galactose	0	0	-

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929 Table 2: Rate constants for ^{13}C labelled substrate consumption by human sperm (n =
930 9 per substrate) and conversion to ^{13}C-lactate and ^{13}C-bicarbonate/CO_2 were
931 estimated from sequential ^{13}C MRS spectra acquired at 37°C. Peak integrals were
932 fitted to a mono-exponential, where a negative rate constant indicates consumption
933 of substrate. Mean ± S.E. values shown where only fits with a Pearson correlation, r
934 > 0.5 and significance p < 0.01 were retained (number of retained fits shown in
935 parenthesis). See method for details.
936

937

Rate measured per peak x10^5, s^{-1}	Glucose (nf)	Fructose (nf)	Pyruvate (nf)
Incubated substrate	-1.2 ± 0.3 (8)	-0.9 ± 0.2 (9)	-10.8 ± 6.3 (7)
$^{13}C_1$-lactate	1.7 ± 0.4 (9)	1.4 ± 0.2 (9)	5.0 ± 0.8 (8)
Bicarbonate	2.4 ± 0.2 (2)	N/A	5.9 ± 0.9 (7)

938 nf: number of retained fits of 9

939

940

941 Table 3: Initial characteristics of sperm separated by DGC into two pellets '40%' and
942 '80%' sperm (mean ± standard deviation, except non-sperm cells which show
943 median and range) measured. A two independent samples t-test with Welch's
944 correction was used for data that passed a Gaussian distribution (concentration,
945 motility and vitality) else Mann-Whitney test (non-sperm cell concentration) was used
946 to determine differences between '40%' and '80%' sperm populations, *** $p < 0.001$,
947 **** $p < 0.0001$.
948

949

	80% sperm (n=45)	40% sperm (n=45)	Difference
Concentration, 10^6/ml			
Sperm	43.8 ±23.4	42.1 ±25.0	1.7
NS cell	0.4**** (0.0 – 4.5)	1.1**** (0.1 – 6.9)	0.7
Total vitality,%			
	65.0 ±13.8***	55.0 ±10.0***	10.0
Total motility, %			
	42.9 ±20.9***	29.5 ±13.8***	13.4

950

951

952 Table 4: Characteristics of '40%' and '80%' sperm populations, separated by
953 incubated ^{13}C substrate (mean ± standard deviation). Concentration, vitality and
954 motility were measured the start of incubations. Differences between '40%' and
955 '80%' sperm populations were test using a Kruskal-Wallis with Dunn's multiple
956 comparison test. No significant differences were found.
957

958

	$^{13}C_u$-glucose		$^{13}C_u$-fructose		$^{13}C_1$-pyruvate	
	80% sperm (n=15)	40% sperm (n=15)	80% sperm (n=15)	40% sperm (n=15)	80% sperm (n=15)	40% sperm (n=15)
Concentration, 10^6/ml						
Sperm	48.7±25.8	50.3±28.3	43.4±28.0	34.2±20.5	39.1±15.1	41.6±24.6
Total vitality,%						
0h	63.6±15.1	52.8±8.4	66.0±13.7	59.3±9.1	65.4±13.2	52.9±11.5
Total motility, %						
0h	40.9±23.1	28.0±12.7	42.3±21.2	33.4±14.4	45.5±19.5	27.3±14.2

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