UNIVERSITY OF BIRMINGHAM
University of Birmingham Research at Birmingham

Continuous behavioural 'switching' in human spermatozoa and its regulation by Ca2+-mobilising stimuli

Achikanu, Cosmas; Correia, Joao; Guidobaldi, HA; Giojalas, LC; Barratt, Christopher L. R.; Da Silva, S M; Publicover, Steve

DOI:
10.1093/molehr/gaz034

License:
Creative Commons: Attribution (CC BY)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Achikanu, C, Correia, J, Guidobaldi, HA, Giojalas, LC, Barratt, CLR, Da Silva, SM & Publicover, S 2019, 'Continuous behavioural 'switching' in human spermatozoa and its regulation by Ca2+-mobilising stimuli', *Molecular Human Reproduction*, vol. 25, no. 8, gaz034, pp. 423-432. https://doi.org/10.1093/molehr/gaz034

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
Checked for eligibility: 21/06/2019
Cosmas Achikanu, Joao Correia, Hector Guidobaldi, Laura Giojalas, Christopher L R Barratt, Sarah Martins Da Silva, Stephen Publicover, Continuous behavioural 'switching' in human spermatozoa and its regulation by Ca2+-mobilising stimuli, MHR: Basic science of reproductive medicine, , gaz034, https://doi.org/10.1093/molehr/gaz034

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 17. Apr. 2024

1

2

3

4 # Continuous behavioural 'switching' in human spermatozoa and its
5 # regulation by Ca^{2+}-mobilising stimuli

6

7 Cosmas Achikanu[1,2], Joao Correia[1], Hector Guidobaldi[3,4] Laura Giojalas[3,4] Christopher L.R.
8 Barratt[5,6], Sarah Martins Da Silva[5,6] & Stephen Publicover[1,7*,]

9 [1]School of Biosciences, University of Birmingham, Birmingham, UK.

10 [2]Current address: Department of Applied Biochemistry, Faculty of Applied Natural Sciences,
11 Enugu State University of Science and Technology, Ebeano City, PMB 01660, Enugu, Enugu
12 State, Nigeria.

13 [3]Centro de Biologia Celular y Molecular, Facultad de Ciencias Exactas, Fisicas y Naturales
14 (FCEFN), Universidad Nacional de Córdoba (UNC), Córdoba, Argentina

15 [4]Instituto de Investigaciones Biologicas y Tecnologicas, Consejo de Investigaciones
16 Científicas y Técnicas (CONICET), UNC-FCEFN, Córdoba, Argentina

17 [5]Reproductive and Developmental Biology, School of Medicine, Ninewells Hospital and
18 Medical School,

19 [6]University of Dundee, Dundee DD19SY, UK 4Assisted Conception Unit, Ninewells
20 Hospital Dundee, Dundee DD19SY, UK

21 [7]Centre for Human Reproductive Science, University of Birmingham, UK

22 *Correspondence address: s.j.publicover.ac.uk

23

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

24 **Running title**: Long-term patterns of sperm behaviour

25

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

26 **Abstract**

27

28 Human sperm show a variety of different behaviours (types of motility) that have different
29 functional roles. Previous reports suggest that sperm may reversibly switch between these
30 behaviours. We have recorded and analysed the behaviour of individual human sperm (180
31 cells in total), each cell monitored continuously for 3-3.5 min either under control conditions
32 or in the presence of Ca^{2+}-mobilising stimuli. Switching between different behaviours was
33 assessed visually (1 s bins using 4 behaviour categories), and was verified by fractal
34 dimension analysis of sperm head tracks. In the absence of stimuli, approximately 90% of
35 cells showed at least one behavioural transition (mean rate under control conditions=6.4 ±0.8
36 transitions.min^{-1}). Type 1 behaviour (progressive, activated-like motility) was most common
37 but the majority of cells (>70%) displayed at least three behaviour types. Treatment of sperm
38 with Ca^{2+}-mobilising agonists had negligible effects on the rate of switching but increased the
39 time spent in type 2 and type 3 (hyperactivation-like) behaviours ($P<2*10^{-8}$; chi square).
40 Treatment with 4-aminopyridine under alkaline conditions (pH_o =8.5), a highly-potent Ca^{2+}-
41 mobilising stimulus, was the most effective in increasing the proportion of type 3 behaviour,
42 biasing switching away from type 1 (P<0.005) and dramatically extending the duration of
43 type 3 events ($P<10^{-16}$). Other stimuli, including 300 nM progesterone and 1% human
44 follicular fluid, had qualitatively similar effects but were less potent. We conclude that
45 human sperm observed in vitro constitutively display a range of behaviours and regulation of
46 motility by $[Ca^{2+}]_i$, at the level of the single cell, is achieved not by causing cells to adopt a
47 'new' behaviour but by changing the relative contributions of those behaviours.

48 **Key words**: spermatozoa – behaviour – motility – calcium - pH

49

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

50 **Introduction**

51

52 The ability of mammalian sperm to change behaviour is essential, as different types of
53 motility are suited to overcoming the different barriers that the sperm encounters as it ascends
54 the female tract. For instance, sperm from mice that are null for CatSper (the primary Ca^{2+}
55 channel of sperm) fail to hyperactivate and consequently cannot fully ascend the female tract
56 (as they cannot escape after binding the oviduct wall) and are unable to penetrate the zona
57 pellucida (Carlson, et al., 2003, Ho, et al., 2009). During semen analysis, the motility of a
58 sperm population is typically assessed by estimating quantitative characteristics of the
59 population (% motile, % progressively motile). Use of computer-assisted sperm analysis
60 (CASA) provides more detailed information by tracking the movement of each sperm head,
61 typically for a fraction of a second. With these data, the kinematics of each sperm (usually
62 several hundred cells) are calculated, providing an overview of the nature and variation of
63 motility in the population (Mortimer, 2000). In both cases, the quantitative data are
64 calculated/estimated from a 'snapshot' sample, effectively assuming that each cell has a
65 motility type and that the distribution of these types reflects the nature of the population
66 (Pacey, et al., 1997).

67 However, when mammalian (including human) sperm are monitored for a longer periods (1-
68 10 s), it becomes apparent that the behaviour of individual cells may change rapidly. For
69 instance, hyperactivated-like motility may occur as intermittent 'bursts' interspersed with
70 activated (progressive) swimming (Cooper, et al., 1979; Katz and Yanagimachi, 1980;
71 Johnson, 1981; Burkman, 1984; Mortimer and Swan, 1995; Pacey, et al., 1995). This
72 alternation of behaviour may well be functionally significant, possibly enhancing penetration
73 of the zona pellucida (Bedford, 1998; Katz et al., 1989) or facilitating detachment from the
74 oviduct wall.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

75 Thus, although it is likely that a primary ‘aim’ of stimuli that affect sperm motility is simply
76 to increase the fraction of the population in which a required behaviour occurs, more subtle
77 effects might be achieved at the level of individual cells. For instance, by biasing the
78 probability of switching towards (or away from) a particular behaviour, or by selectively
79 changing the duration of a specific behaviour, the relative amounts of time spent in each
80 behaviour may be regulated. It is also possible that the absolute duration of an individual
81 period of a specific behaviour is functionally important, such as during interaction with the
82 female tract or oocyte vestments.

83 We hypothesised that behavioural switching and expression of multiple behaviour types is a
84 common feature of human sperm, even under unstimulated conditions. To better understand
85 both the occurrence of multiple patterns of behaviour in individual human sperm and the
86 way in which behavioural switching is regulated, we have: (i) recorded and analysed the
87 activity of individual cells over a prolonged period (>3 minutes) and (ii) investigated whether
88 this behaviour is modified through elevation of $[Ca^{2+}]_i$.

89

90

91

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

92 **Materials and methods**

93

94 **Materials**

95 All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless stated otherwise.
96 Progesterone (P4) and 4-aminopyridine (4-AP) were prepared as stocks in
97 dimethylsulphoxide (DMSO) at 10 mM and 500 mM respectively. Human follicular fluid
98 (FF) was thawed and diluted in saline on the day of use. Thimerosal was prepared as 100 mM
99 stock in deionised water. Working concentrations were made by diluting in supplemented
100 Earle's balanced salt solution (sEBSS) at the appropriate pH prior to use. DMSO in P4 and 4-
101 AP experiments was 0.003% and 0.4% respectively. 1% DMSO had no detectable effect on
102 kinematic properties of sperm motility (Achikanu et al., 2018).

103 **Salines**

104 sEBSS contained (mM) 90 NaCl, 1.017 NaH_2PO4, 5.4 KCl, 0.81 MgSO4, 5.5 $C_6H_{12}O_6$, 2.5
105 $C_3H_3NaO_3$, 19 $CH_3CH(OH)COONa$, 25 $NaHCO_3$, 1.8 $CaCl_2$, 25 mM 4-(2-hydroxyethyl)-1-
106 piperazineethanesulfonic acid (HEPES; pH 7.4), 0.3% BSA. Osmolality was than adjusted to
107 291-294 mOsm as necessary by adding NaCl. For buffering at pH 8.5, HEPES was replaced
108 with [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS).

109 **Selection and preparation of spermatozoa**

110 Semen samples were from donors with normal sperm concentration and motility (WHO
111 2010). Samples were obtained by masturbation after 2-3 days sexual abstinence. After
112 liquefaction (30 min), sperm were swum up into sEBSS (60 min), adjusted to ≈6 million/ml
113 and incubated under capacitating conditions (sEBSS at pH 7.4; 37°C, 6% CO_2) for 5 hours
114 (Alasmari et al., 2013a).

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

115 **Human follicular fluid**

116 Follicular fluid (FF) was prepared as described in Brown et al. (2017). Briefly, oocytes
117 (metaphase II) were retrieved by transvaginal aspiration. FF without blood contamination
118 was centrifuged (2500 g for 10 min) to separate cellular components The supernatant (0.22
119 μm filtered) was stored (at −20°C) until use.

120 **Ethical approval**

121 Written consent was obtained in accordance with the Human Fertilisation and Embryology
122 Authority (HFEA) Code of Practice (version 8) under local ethical approval (University of
123 Birmingham ERC 07-009 and ERN-12-0570) and (13/ES/0091) from the East of Scotland
124 Research Ethics Service REC1.

125 **Long-duration sperm tracking**

126 Observation chambers, 20 μm-deep, were constructed by mounting a 22x32 mm coverslip
127 (0.13-0.16 mm thickness; Academy, Beckenham, UK) onto a 22x50 mm coverslip (thickness
128 no. 1.5; VWR International). Soda lime glass beads (18-22 μm; Cospheric, Santa Barbara,
129 USA) were dispersed in vacuum grease (Dow-Corning, USA) and small dabs were placed at
130 each corner of the 22 mm x 32 mm coverslip, providing a ≈20 mm space when the two
131 coverslips were pressed together.

132 Capacitated cells were pelleted by centrifugation and re-suspended in 100 ml aliquots (cell
133 density adjusted to 1.2 million/ml) in sEBSS pH 7.4 or 8.5 as required. The stimulus (1%
134 DMSO control, 300 nM progesterone, 1% FF (containing ≈300 nM P4 (Brown et al. 2017)),
135 1 μM thimerosal or 2 mM 4-AP) was added to the cell suspension and incubation (37°C) was
136 continued for a further 1-2 min. A 5μl aliquot of cell suspension was carefully loaded into the
137 coverslip chamber, which was then placed on the platform of a thermostatic controller (TC-

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

138 324B, Warner, USA) set at 36.5°C on the stage of an inverted microscope (Nikon Eclipse
139 TE200) equipped with a motorised stage (Prior Scientific, UK). Images were acquired with a
140 sCMOS camera (Zyla 5.5, ANDOR TM Technology, Belfast, UK) through a 10x phase
141 contrast objective.

142 Imaging was started approximately 5 min after stimulus application. Images were acquired at
143 50 Hz, 15 ms exposure for a period of 180-216 s using Micro Manager software v1.4.22
144 (image 1392 x 1040 pixels, 16 bit dynamic range, global camera shuttering). Illumination
145 intensity was reduced as far as possible and applied via a red filter to prevent direct effects of
146 illumination on sperm motility (Shahar, et al., 2011; Gabel, et al., 2018). For each recording,
147 a randomly selected sperm was followed. When the cell approached the edge of the field of
148 view, it was moved back using the motorised stage. Using playback of videos, sperm
149 behaviour was analysed in 1 s blocks, allocating each block to one of four different
150 behaviours (motility types 1, 2, 3 and 4; Table 1; Fig. S1; see results). Observation of videos
151 gave no indication that stage movements induced or modified behavioural switching (Fig.
152 S2). We analysed the relationship between the number of stage movements during a 3 min
153 recording (x) and number of behavioural switches in that 3 min (y). There was no significant
154 relationship (y=0.08x+18; P=0.9; R^2=0.001). Furthermore, in many cells in which switching
155 was frequent, no stage movements were required. We also assessed whether the behaviour
156 changes over the period of the recording might occur if evaporation of saline affected
157 osmolality or pH of the droplet. Neither the rate of switching nor the behaviour score showed
158 a significant trend over the duration of the recording (P=0.83; P=0.58 respectively; Fig. S3).

159 In a subset of experiments, where the head centroid could be reliably detected throughout the
160 recording, the multidimensional motion analysis application in MetaMorph® (Molecular
161 Devices, USA) was used to generate positional information for the sperm head for the entire

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

162 recording period. Separate sections of the sperm track (between stage re-centring) were
163 ‘stitched’ together by compensating the offset of ’x’ and ‘y’ co-ordinates in Microsoft Excel.

164 **CASA**

165 Hyperactivation was assessed as described previously (Achikanu et al.; 2018). After addition
166 of the stimulus, cells were loaded into pre-warmed 20 µm CASA chamber (36°C). Motility
167 was assessed (approximately 300 s after stimulus application) with a Hamilton Thorn CEROS
168 CASA system (version 14.0). Criteria for hyperactivation were VCL (curvilinear velocity)
169 ≥150µm/s, LIN (linearity) <50% and ALH (amplitude of lateral head movement) ≥7µm
170 (Mortimer, 2000).

171 **Data analysis**

172 Fractal dimension (FD) was determined from x, y coordinates over a period of 1 s (50 points)
173 for each track point along the trajectory. FD was calculated using the equation

174 $$FD = log(n)/[log (n) + log (ld/cd)]$$

175 where n is the number of points in the interval analysed (50 points), ld is the linear distance
176 between the first and the last point and cd is the length of the trajectory (Mortimer et al.
177 1996). FD was calculated with a homemade macro (paNoel 1.0; Universidad Nacional de
178 Córdoba; http://www.iibyt.conicet.unc.edu.ar/software/) using Fiji software (Schindelin et al.,
179 2012). Maximum FD was set at 2.0.

180 Behaviour score (average of all the recorded scores for each 1 s period over the entire
181 recording), % time (% of total recording period spent in each behaviour type), switching rate
182 (transitions.min^{-1}), behaviour dwell time (period during which a single behaviour type
183 occurred) and transition type (behaviour type entered upon a transition) were calculated in
184 Microsoft Excel and statistical analysis was carried out using Minitab18 and Excel.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

185 Behaviour score, % time and switching rate were calculated separately for each cell and a
186 mean was calculated from these values (n=number of cells analysed). Dwell times and
187 transition types were analysed using all individual behaviour events of the specified type
188 (n=total number of events). All summary values given in the text are mean ± SEM unless
189 stated otherwise. Before analysis, data were tested for normality (Anderson-Darling) and data
190 sets were then compared using Chi-square, Student's t-test, Mann-Whitney, 2-way ANOVA
191 and Kruskal-Wallis (with post-hoc Holm-Bonferroni sequential correction; Gaetano, 2013) as
192 appropriate. Autocorrelation assessment of periodicity was carried out in Minitab 18.

193

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

194 **Results**

195

196 Using five conditions (control and four different stimuli), each at two different values of pH_o
197 (7.4 and 8.5), we collected video recordings from a total of 180 cells. By visual analysis of
198 video playback, we categorised behaviour into four types. Types 1, 2 and 3 resembled the
199 patterns of motility normally referred to as activated, transitional and hyperactivated (Figure
200 S1, Table 1; Videos1 and 2; Mortimer, 2000). An additional type of behaviour was
201 occasionally observed, in which the mid-piece and anterior flagellum became tightly curved
202 (forming a 'J' shape or coil) and arrested or 'twitched' (Video 3, Table 1). Since behaviour of
203 this type is seen in cells treated with Ca^{2+}-ionophore, where it apparently reflects excess Ca^{2+}-
204 loading which takes the cells 'beyond' hyperactivated motility (Sanchez-Cardenas et al.,
205 2018), this was designated type 4 behaviour. This behaviour was typically interspersed with
206 very brief (100-500 ms) bursts of flagellar activity such that the cell became virtually
207 immobilised for up to 20 s (Figure S1d, Video 3).

208 **Free-swimming sperm cells repeatedly 'switch' behaviour**

209 Examination of the behaviour of cells incubated under control conditions (sEBSS; pH 7.4)
210 showed that the majority (16/18 cells) changed their behaviour at least once during the
211 recording. Type 1 behaviour was seen in 18/18 cells, type 3 occurred in 16/18 (no significant
212 difference in incidence; P=0.15; Chi square) and 13 of these cells showed three or (in one
213 case, Figure 1a) all four types of behaviour. Among the 18 control cells analysed, the number
214 of behavioural transitions varied widely (from 0 to 45), with the mean rate being 6.4 ±0.8
215 transitions.min^{-1}. Dwell-times (length of period during which a single behaviour type
216 occurred) for types 1, 2 and 3 events all formed negative exponential distributions (Figure
217 S4a), with the median duration of type 1 events being significantly greater than those for
218 types 2, 3 and 4 ($P<5*10^{-5}$; Kruskal-Wallis; Figure 2a). Analysis of transition types showed

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

219 that switching between behaviours was not random ($P<10^{-10}$; Chi square), with transitions
220 into type 4 behaviour being very rare (4.3%) and into type 1 behaviour occurring most
221 frequently (44%; Figure 2c column 1). Consequently, cells spent most time (65±6%) in type
222 1 behaviour, more than double that of any of the other behaviour types (Figure 2b column 1;
223 P<0.0005; Kruskal-Wallis with post-hoc comparison). Occurrence of type 4 behaviour was
224 very rare, making up less than 3% of behaviour events and of total time (Figure 2b). Neither
225 the rate of switching nor the type of behaviour expressed showed a significant trend over the
226 recording period (P=0.83; P=0.58 respectively; Figure S3).

227 In most cells where behavioural switching occurred there was no clear pattern, as type 1
228 behaviour was interrupted by 'bursts' of type 2 and type 3 activity (Figure 1a). In a small
229 number of cells (4/18), behaviour appeared to oscillate but analysis by autocorrelation
230 showed only one instance where an oscillatory frequency emerged (Figure S5, compared to
231 Figure 1) and even in this case, the autocorrelation failed to reach significance (P>0.05;
232 Figure S5b). For 3 of the 18 control cells, where video quality was adequate for reliable
233 detection of the sperm head throughout the 3 min recording, we were able to reconstruct
234 entire tracks lasting over 3 min, which clearly showed the observed transitions in behaviour
235 (Figure 1c). To confirm that visual analysis identified genuine behavioural transitions, we
236 used the track coordinates to assess temporal variation of fractal dimension (FD; a numerical
237 value between 1 and 2, which reflects the complexity of the sperm track; Mortimer, et al.,
238 1996). Colour coding of track points for FD confirmed that high values occur at periods of
239 complex motility and overlays of FD and visual analyses confirmed that the same
240 behavioural transitions were identified (Figure1b,c, Figure S5c,d, Figure S6). Autocorrelation
241 of the FD data in Figure S5 produced near identical results to those obtained through visual
242 analysis (Figure S5b,e). When data from all tracked cells were analysed, the mean FD and

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

243 mean behavioural score (average score over the entire recording) for each cell were clearly
244 correlated (R^2=0.56; P=5*10^{-6}; n=28; Figure S7).

245 Manipulation of $[Ca^{2+}]_i$ modifies the pattern of behavioural switching

246 A pivotal mechanism for regulation of behaviour in human sperm is $[Ca^{2+}]_i$ (Publicover, et
247 al., 2007; Suarez, 2008). To investigate the effect of $[Ca^{2+}]_i$ elevation, we monitored the
248 activity of cells exposed to four different stimuli (300 nM P4, 1% FF [containing
249 approximately 300 nM P4], 2 mM 4-AP and 1 mM thimerosal), all of which are known to
250 increase $[Ca^{2+}]_i$ and stimulate hyperactivation in human and other sperm, but with varying
251 potency (Ho and Suarez, 2001; Alasmari, et al., 2013b; Brown, et al., 2017; Achikanu et al.,
252 2018). CASA assessments, carried out during the period of behavioural data collection, using
253 cells prepared under identical conditions, confirmed the efficacy and relative potency of these
254 stimuli (Figure S8a). The proportion of cells in which we observed switching between
255 behaviours was not changed by any of these manipulations (Table 2), but the relative
256 incidence (% time) of the different behaviour types was significantly modified (P<2*10^{-8}; chi
257 square). Incidence of type 1 behaviour was reduced by all four stimuli (P<0.05) whereas 4-
258 AP significantly increased the proportion of type 3 behaviour (P=2*10^{-6}) and thimerosal
259 significantly increased the occurrence of both types 2 and 3 behaviours (P<0.05; Kruskal-
260 Wallis with post-hoc comparison; Figure 2b). Examination of switching showed that this
261 change was partly due to biasing of switching towards types 2 and 3 behaviours in stimulated
262 cells (P4 P=0.1; FF P<0.05; 4-AP and thimerosal P<5*10^{-5}; Chi-square; Figure 2c), but there
263 were also marked effects on dwell times of the different behaviours. Type 1 events were
264 significantly briefer in stimulated cells (except for those exposed to thimerosal; Figure 2d)
265 whereas type 2 and type 3 events were markedly prolonged in cells exposed to 4-AP and to
266 thimerosal (Figure 2e,f; Figure S6), such that the overall frequency of behaviour switching

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

267 was significantly lower than in controls (P=0.02 and P=0.0004 for 4-AP and thimerosal
268 respectively; Kruskal-Wallis).

269 **Effect on behaviour of elevated pH_o**

270 To investigate the effect on behavioural switching of elevated pH_o, sperm were prepared
271 using the standard protocol but then re-suspended in saline buffered at pH 8.5, which causes
272 stable alkalinisation of the cytoplasm (increase from 6.9 to 7.2) within 200-300 s and
273 increases levels of hyperactivated motility (Achikanu et al., 2018; Figure S8a). The
274 proportion of sperm in which switching occurred was not significantly changed at elevated
275 pH except in cells exposed to 4-AP, where switching was observed in only 10/20 cells
276 (P=0.005 compared to 4-AP at pH 7.4; Table 2). The frequency of switching at pH_o=8.5,
277 compared to cells incubated at pH_o=7.4, was consistently lower ($P<2*10^{-7}$, 2-way ANOVA;
278 Figure 3a), but when the five conditions were analysed separately, this effect of pH was
279 significant only in cell exposed to FF (P<0.01) and 4-AP (P<0.001; Mann-Whitney).
280 Similarly to our observations at pH_o=7.4, at pH_o=8.5 all of the stimuli significantly changed
281 the relative abundance (% time) of the four behaviours (Figure 3b; $P<10^{-14}$; Chi-square), with
282 the most striking effect being in 4-AP-treated cells, where sperm spent 94±2% of their time in
283 type 3 behaviour (Figure 3b; $P=3*10^{-9}$ compared to control; Kruskal-Wallis with post-hoc
284 comparison). Examination of the effect of stimuli on the characteristics of transitions between
285 behaviours also showed that, as at pH 7.4, Ca^{2+}-mobilising treatments biased switching
286 events towards types 2 and 3 behaviour (Figure 3c; P<0.005; Chi-square). Dwell times for
287 type 1 behaviour were not significantly altered by Ca^{2+}-mobilising stimuli at pH 8.5, but the
288 effects of agonists on type 3 dwell times were more consistent and greater than at pH_o=7.4,
289 with all four stimuli significantly extending the duration of type 3 behaviour compared to
290 control cells (Figure 3f; P<0.001; Kruskal-Wallis with post-hoc comparison). Most strikingly,
291 in the presence of 4-AP, type 3 behaviour median dwell time was 56 s (compared to 3 s in

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

292 controls; $P=10^{-16}$, Kruskal-Wallis with post-hoc comparison; Figure 3f). Only thimerosal

293 significantly increased type 2 dwell times at both $pH_o=7.4$ and $pH_o=8.5$ ($P=1*10^{-10}$ and

294 $P=1*10^{-5}$ respectively; Kruskal-Wallis with post-hoc comparison). Comparison of mean

295 behaviour scores and % hyperactivated cells (CASA) for the 10 conditions assessed (control

296 and four agonists, each at pH 7.4 and pH 8.5) showed a positive correlation between the two

297 assessments of motility ($R^2=0.5$; $P=0.022$; Figure S8b).

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

298 **Discussion**

299

300 By continuously observing 'capacitated', free-swimming human sperm, for a period of longer
301 than 3 min, we have shown, for the first time, the occurrence of continuous switching
302 between different types of motility, with the mean incidence being more than six transitions
303 per minute. Even in control (unstimulated) cells, multiple behaviours, including
304 hyperactivated-like motility, were seen in ≈90% of cells. For analysis of this switching, the
305 intervening periods, where behaviour appeared consistent, were classified using four
306 categories. Although tracks from behaviour types 1-3 resemble those referred to as activated,
307 transitional and hyperactivated motility (Mortimer, 2000), these behaviours were derived by
308 visual analysis and cannot be considered equivalent to categories defined by kinematic
309 criteria. Type 4 behaviour where the flagellum arrested, typically in a 'J' shape, appears to be
310 equivalent to the freeze and freeze-flex behaviours, which were observed in ≈7% of cells
311 from normal semen (Burkman, 1984). In cells where analysis of FD was possible, it was clear
312 that (i) visual analysis and FD identified the same behavioural transitions and (ii) for each
313 recording, the mean FD was clearly and positively correlated with mean behaviour score
314 (R^2=0.56). We conclude that our analysis reliably identifies sperm behaviours and switching
315 between them and that continuous behavioural switching is a normal activity of human sperm
316 (at least for cells observed in vitro). Though some behaviour-time plots appeared to show
317 oscillatory behaviour, autocorrelation analysis did not reveal significant periodicity.

318 The occurrence of repeated behavioural switching might be brought about by either, or both,
319 of two different mechanisms. Changes in behaviour might be completely reliant on
320 endogenous signalling activity, for instance, $[Ca^{2+}]_i$ spikes and oscillations that have been
321 described in human sperm (Harper et al, 2004; Aitken & McLaughlin, 2007; Sanchez-
322 Cardenas et al, 2014; Mata-Martinez et al, 2018). These changes were observed in

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

323 immobilised cells and were correlated with changes in flagellar beating (Harper et al, 2004;
324 Bedu-Addo et al, 2007). However, it is yet to be established whether these $[Ca^{2+}]_i$ signals
325 cause or are caused by the changes in flagellar beating. Alternatively, if mechanisms
326 controlling sperm behaviour are sensitive to changes in the environment, such as pH, ionic
327 environment, temperature and mechanical stimulation (including fluid flow), heterogeneity in
328 the sperm's environment might present such 'stimuli' as it swims. Our data indicate that
329 potential methodological artefacts related to movements of the stage and/or evaporation of
330 the saline did not cause such effects (see Methods; Long Duration Sperm Tracking).
331 However, the environment through which the sperm swims, even in the simple system used
332 for our recordings, might be sufficiently spatially heterogenous that the sperm encounters a
333 series of physico-chemical 'stimuli' due to its own movement. The complex environment
334 encountered by the sperm in the female tract will almost certainly present such 'stimuli'. If
335 behavioural switching is induced in this way, our observation that $[Ca^{2+}]_i$ elevating stimuli
336 prolong periods of type 3 behaviour (Figures 2f, 3f; see below) might indicate that Ca^{2+}-
337 signalling resets the stimulus-sensitivity of transition into this type of motility. In either case,
338 it appears likely that conditions/stimuli that result in behavioural switching in vitro will also
339 occur in vivo.

340 Analysis of the effects of manoeuvres that elevate $[Ca^{2+}]_i$ in human sperm indicate that
341 motility kinematics (assessed by CASA) are directly related to the absolute level of $[Ca^{2+}]_i$,
342 irrespective of the Ca^{2+} source mobilised, with the percentage of hyperactivated cells
343 increasing as a function of $[Ca^{2+}]$i (Achikanu et al, 2018). Exposure of cells to a range of
344 stimuli which elevate $[Ca^{2+}]_i$ significantly altered the relative abundance of the different
345 behaviours. Consistent with a shift toward hyperactivated motility, the proportion of time
346 spent in type 2 and type 3 behaviours markedly increased in cells exposed to Ca^{2+}-mobilising
347 stimuli. Thimerosal and 4-AP were particularly potent in this regard. Thimerosal modifies

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

348 sperm behaviour by mobilising stored Ca^{2+} (Ho and Suarez, 2001; Alasmari et al., 2013b).
349 Although 4-AP is often used as a K^+ channel blocker, its effects on the K^+-permeable
350 channels expressed in sperm are negligible (Tang et al, 2010; Mansell et al, 2014) and a
351 significant aspect of its action on sperm $[Ca^{2+}]_i$ and behaviour is likely to be its ability to
352 mobilise stored Ca^{2+} (Gobet 995; Grimaldi 2001; Baskhar 2008; Alasmari et al., 2013b
353 Kasatkina, 2016), The potent effects of Ca^{2+}-store-mobilising stimuli on switching are
354 consistent with their ability to persistently elevate $[Ca^{2+}]_i$, inducing prolonged hyperactivation
355 in human sperm (Achikanu, et al., 2018; Alasmari, et al., 2013a,b). When cells were
356 suspended in medium buffered at pH 8.5, some effects of Ca^{2+}-mobilising stimuli were
357 greatly enhanced, particularly the prolongation of type 3 behaviour dwell-time by 4-AP. The
358 effect of 4-AP on $[Ca^{2+}]_i$ is strongly potentiated at pH=8.5 (Achikanu et al., 2018) and it is
359 likely that this underlies the striking effect of the drug on behavioural switching under these
360 conditions. Significantly, these potent hyperactivating stimuli had negligible effect on the
361 proportion of cells in which type 3 (hyperactivated-like) behaviour was observed. Even under
362 control conditions, type 3 behaviour (and behavioural switching) occurred in 90% of cells.
363 Thus it appears that the well-characterised ability of these $[Ca^{2+}]_i$-mobilising compounds to
364 increase the level of hyperactivation detected in population motility assays occurs not by
365 recruitment of cells into a hyperactivated population, but by increasing the proportion of time
366 that continuously-switching cells spend in type 3 (hyperactivated-like) motility, achieved
367 primarily by extending the dwell-time of this behaviour.

368 If behavioural switching occurs in vivo, does it have adaptive value? Switching induced
369 externally (by the sperm's sensitivity to heterogeneity in its environment; see discussion
370 above) may simply be behavioural 'noise' that can be modulated by the cells signalling
371 activity (such as elevation of $[Ca^{2+}]_i$ as described here) but may not be functionally
372 significant. However, observations of sperm interacting with the female tract and cumulus-

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

373 oocyte-complex suggest that switching of motility types may be of value. Pacey et al (1995)
374 described an attach-detach cycle in the interaction of human sperm with epithelial cells
375 isolated from the isthmic and ampullary sections of human oviducts, that may play an
376 important role in migration to the fertilisation site. Detachment was associated with
377 hyperactivated-type motility whereas cells that were attaching showed far more linear
378 motility. Similarly, observation of sperm during the passage through the zona reveals
379 alternation of low and high amplitude flagellar beats, which may facilitate penetration by
380 alternation of 'cutting' and 'thrusting' forces (Bedford, 1998). Clearly further work is
381 required to determine the nature and complexity of sperm behaviour in vivo and its
382 significance.

383

384

385 **Authors' roles:** CA, JC and SMDS obtained samples and carried out the laboratory work.
386 CA, SP and HG analysed the data. CA, JC, HG, LG, CLRB, SMDS and SJP contributed to
387 writing of the manuscript.

388

389 **Funding:** This study was support by the Medical Research Council (MR/M012492/1). CA
390 was in receipt of a scholarship from the Nigerian government (Tertiary Education Trust
391 (TET) Fund).

392

393 **Conflict of interest:** C.L.R.B. was the editor-in-chief of Molecular Human Reproduction,
394 has received lecturing fees from Merck and Ferring and is on the Scientific Advisory Panel
395 for Ohana BioSciences. C.L.R.B. was also chair of the World Health Organization Expert
396 Synthesis Group on Diagnosis of Male infertility (2012-2016). He is an editor of RBMO.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

397 CLRB reports grants from the MRC, Gates Foundation and the Scottish Office during the
398 conduct of the study; personal fees from Pharmasure, Ferring, Ohana, and RBMO outside the
399 submitted work. CA, JC, HG, LG, SMDS and SJP declare no conflict of interest.

400

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

401

402 **References**

403 Achikanu C, Pendekanti V, Teague R, Publicover S. Effects of pH manipulation, CatSper

404 stimulation and Ca^{2+}-store mobilization on $[Ca^{2+}]_i$ and behaviour of human sperm. *Hum*

405 *Reprod* 2018;33: 1802-1811.

406 Aitken RJ, McLaughlin EA. Molecular mechanisms of sperm capacitation: progesterone-

407 induced secondary calcium oscillations reflect the attainment of a capacitated state. *Society of*

408 *Reproduction and Fertility supplement* 2007;63: 273-293.

409 Alasmari W, Barratt C, Publicover S, Whalley K, Foster E, Kay V, Martins da Silva S,

410 Oxenham S. The incidence and clinical significance of defects in calcium signalling

411 pathways mediating human sperm hyperactivation in donors and sub fertile patients. *Human*

412 *Reproduction* 2013a;28: 866-876.

413 Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, Ramalho-Santos J,

414 Kirkman-Brown J, Michelangeli F, Publicover S, Barratt C. Ca^{2+} signals generated by

415 CatSper and Ca^{2+} regulate different behaviours in human sperm. *J Biol Chem* 2013b;288:

416 6248-6258.

417 Bhaskar A, Subbanna PK, Arasan S, Rajapathy J, Rao JP, Subramani S. 4-aminopyridine-

418 induced contracture in frog ventricle is due to calcium released from intracellular stores.

419 *Indian J Physiol Pharmacol* 2008;52: 366-374.

420 Alvarez L, Dai L, Friedrich BM, Kashikar ND, Gregor I, Pascal R, Kaupp UB. The rate of

421 change in Ca^{2+} concentration controls sperm chemotaxis. *J Cell Biol* 2012; 5: 653-663

422 Bedford JM. Mammalian fertilization misread? Sperm penetration of the eutherian zona

423 pellucida is unlikely to be a lytic event. *Biol Reprod* 1998;59: 1275-1287.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

424 Bedu-Addo K, Barratt CL, Kirkman-Brown JC, Publicover SJ. Patterns of $[Ca^{2+}]_i$
425 mobilization and cell response in human spermatozoa exposed to progesterone. *Dev Biol*
426 2007;302: 324-332.
427 Brown SG, Costello S, Kelly MC, Ramalingam M, Drew E, Publicover SJ, Barratt CLR, Da
428 Silva SM. Complex CatSper-dependent and independent $[Ca^{2+}]_i$ signalling in human
429 spermatozoa induced by follicular fluid. *Hum Reprod* 2017;32: 1995-2006.
430 Burkman LJ. Characterisation of hyperactivated motility by human spermatozoa during
431 capacitation: comparison of fertile and oligozoospermic sperm populations. *Arch Androl*
432 1984;13: 153.
433 Burkman LJ. Characterization of hyperactivated motility by human spermatozoa during
434 capacitation: comparison of fertile and oligozoospermic sperm populations. *Arch Androl*
435 1984;13: 153-165.
436 Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock
437 DF. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. *Proc*
438 *Natl Acad Sci U S A* 2003;100: 14864-14868.
439 Cooper G, Overstreet J, Katz D. The motility of rabbit spermatozoa recovered from the
440 female genital tract. *Gam Res* 1979;2: 35-42.
441 Gabel CP, Carroll J, Harrison K. Sperm motility is enhanced by Low Level Laser and Light
442 Emitting Diode photobiomodulation with a dose-dependent response and differential effects
443 in fresh and frozen samples. *Laser Ther* 2018;27: 131-136.
444 Gaetano J. Holm-Bonferroni Sequential Correction: An EXCEL calculator (1.2).
445 *https://wwwresearchgatenet/publication/242331583_* 2013.
446 Gobet I, Lippai M, Tomkowiak M, Durocher Y, Leclerc C, Moreau M, Guerrier P. 4-
447 aminopyridine acts as a weak base and a Ca2+ mobilizing agent in triggering oocyte meiosis

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

448 reinitiation and activation in the Japanese clam Ruditapes philippinarum. *Int J Dev Biol*
449 1995;39: 485-491.
450 Grimaldi M, Atzori M, Ray P, Alkon DL. Mobilization of calcium from intracellular stores,
451 potentiation of neurotransmitter-induced calcium transients, and capacitative calcium entry
452 by 4-aminopyridine. *J Neurosci* 2001;21: 3135-3143.
453 Harper CV, Barratt CL, Publicover SJ. Stimulation of human spermatozoa with progesterone
454 gradients to simulate approach to the oocyte. Induction of $[Ca^{2+}]_i$ oscillations and cyclical
455 transitions in flagellar beating. *J Biol Chem* 2004;279: 46315-46325.
456 Ho HC, Suarez SS. An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca^{2+} store is
457 involved in regulating sperm hyperactivated motility. *Biol Reprod* 2001;65: 1606-1615.
458 Ho K, Wolff CA, Suarez SS. CatSper-null mutant spermatozoa are unable to ascend beyond
459 the oviductal reservoir. *Reprod Fertil Dev* 2009;21: 345-350.
460 Johnson LL, Katz, D.F., Overstreet. J.W. The movement characteristics of rabbit
461 spermatozoa before and after activation. *Gamete Res* 1981;4: 275-282.
462 Kasatkina LA. 4-Aminopyridine sequesters intracellular Ca^{2+} which triggers exocytosis in
463 excitable and non-excitable cells. *Scientific reports* 2016;6: 34749.
464 Katz DF, Yanagimachi R. Movement characteristics of hamster spermatozoa within the
465 oviduct. *Biol Reprod* 1980;22: 759-764.
466 Mansell SA, Publicover SJ, Barratt CL, Wilson SM. Patch clamp studies of human sperm
467 under physiological ionic conditions reveal three functionally and pharmacologically distinct
468 cation channels. *Mol Hum Reprod* 2014;PMID: 24442342: 392-408.
469 Mata-Martinez E, Darszon A, Trevino CL. pH-dependent Ca^{2+} oscillations prevent untimely
470 acrosome reaction in human sperm. *Biochem Biophys Res Commun* 2018;497: 146-152.
471 Mortimer ST. CASA - practical aspects. *J Androl* 2000;21: 515-524.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

472 Mortimer ST, Swan MA. Kinematics of capacitating human spermatozoa analysed at 60 Hz.
473 *Hum Reprod* 1995;10: 873-879.
474 Mortimer ST, Swan MA, Mortimer D. Fractal analysis of capacitating human spermatozoa.
475 *Hum Reprod* 1996;11: 1049-1054.
476 Pacey AA, Davies N, Warren MA, Barratt CL, Cooke ID. Hyperactivation may assist human
477 spermatozoa to detach from intimate association with the endosalpinx. *Hum Reprod* 1995;10:
478 2603-2609.
479 Pacey AA, Ladbrook MB, Barratt CL, Cooke ID. The potential shortcomings of measuring
480 hyperactivated motility by computer-aided sperm analysis when sperm motion is multiphasic.
481 *Hum Reprod Update* 1997;3: 185-193.
482 Publicover S, Harper CV, Barratt C. $[Ca^{2+}]_i$ signalling in sperm--making the most of what
483 you've got. *Nat Cell Biol* 2007;9: 235-242.
484 Sanchez-Cardenas C, Montoya F, Navarrete, FA, Hernandez-Cruz A, Corkidi G, Visconti PE,
485 Darszon A (2018) Intracellular Ca^{2+} threshold reversibly switches flagellar beat off and on.
486 *Biol Reprod* doi: 10.1093/biolre/ioy132.
487 Sanchez-Cardenas C, Servin-Vences MR, Jose O, Trevino CL, Hernandez-Cruz A, Darszon
488 A. Acrosome reaction and Ca^{2+} imaging in single human spermatozoa: new regulatory roles
489 of $[Ca^{2+}]_i$. *Biol Reprod* 2014;91: 67.
490 Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S.
491 Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K.
492 Eliceiri, P. Tomancak and A. Cardona (2012). Fiji: an open-source platform for biological-
493 image analysis. *Nat Methods* 2012; 9: 676-682.
494 Shahar S, Wiser A, Ickowicz D, Lubart R, Shulman A, Breitbart H. Light-mediated activation
495 reveals a key role for protein kinase A and sarcoma protein kinase in the development of
496 sperm hyper-activated motility. *Hum Reprod* 2011;26: 2274-2282.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

497 Suarez SS. Control of hyperactivation in sperm. *Hum Reprod Update* 2008;14: 647-657.

498 Tang QY, Zhang Z, Xia XM, Lingle CJ. Block of mouse Slo1 and Slo3 K+ channels by

499 CTX, IbTX, TEA, 4-AP and quinidine. *Channels (Austin)* 2010;4: 22-41.

500

501 **Figure 1. Behavioural switching in a free-swimming human sperm under control**

502 **conditions (pH_o=7.4).** (a) Variation in behaviour type (categorised visually as type1, type 2,

503 type 3 or type 4; see Figure S1) of a single sperm over a period of 190 s. (b) Variation in

504 fractal dimension (FD) over time (black trace) overlaid with the visually categorised

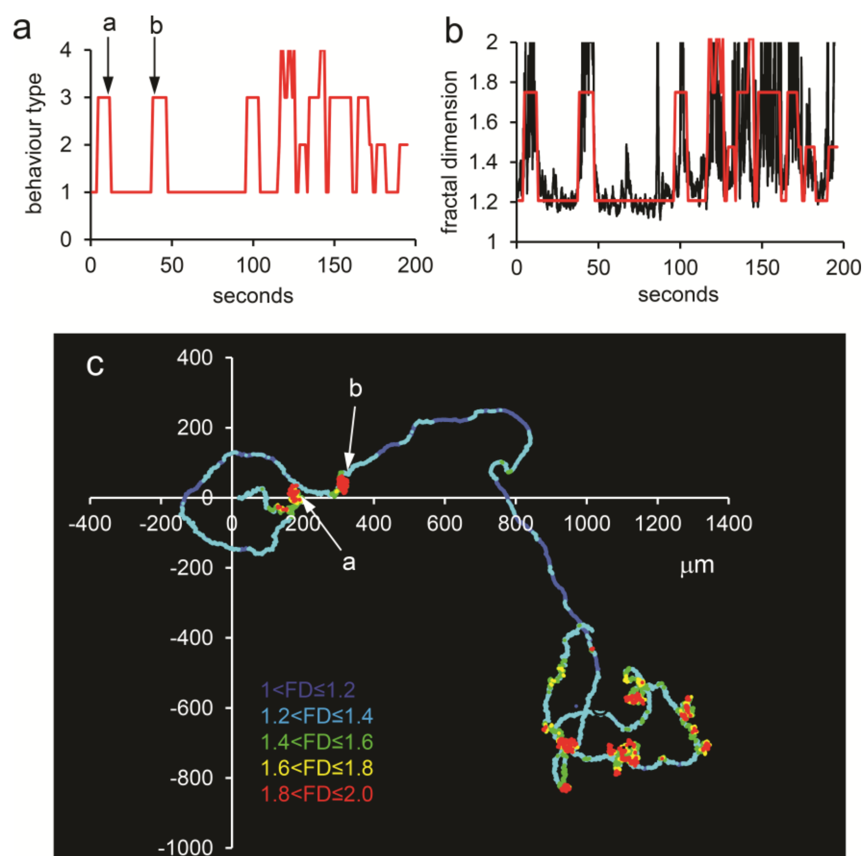
505 behaviour types (red trace). Visual analysis and FD show good agreement, with no visually

506 identified behavioural transitions that are not confirmed by FD. (c) Track of the same cell,

507 colour coded to display variation in the fractal dimension (FD); (1<FD≤1.2 (dark blue);

508 1.2<FD≤1.4 (light blue); 1.4<FD≤1.6 (green); 1.6<FD≤1.8 (yellow); 1.8<FD≤2.0 (red). Axes

509 show distance in mm.



510

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

511 **Figure 2. Characteristics of behavioural switching.** (a) Dwell times (period during which a
512 single behaviour type occurred) for types 1 (light blue, n=164 events), 2 (green, n=79), 3
513 (red, n=107) and 4 (black, n=15) behaviours in cells control cells (pH 7.4). Plots show
514 median and interquartile range (box) and maximum/minimum values (whiskers). (b) Mean %
515 time spent in each behaviour; type 1 (light blue), type 2 (green), type 3 (red) and type 4
516 (black) under control conditions (n=18 cells) and in the presence of 300 nM P4 (n=18 cells),
517 1% FF (n=17 cells), 2 mM 4-AP (n=21 cells) and 1 mM thimerosal (n=17 cells). (c) Relative
518 frequencies of transitions into type 1 (light blue), type 2 (green), type 3 (red) and type 4
519 (black) behaviours under control conditions (n=347 transitions) and in the presence of
520 progesterone (P4; 300 nM; n=420), human follicular fluid (FF nM, 1%; n=353), 4-
521 aminopyridine (4-AP, 2 mM, n=211) and thimerosal (1 mM, n=115). Asterisks indicate
522 significant difference from control; ***=P<0.005. (d-f) Dwell times for type 1 (panel 'd';
523 blue), type 2 (panel 'e'; green) and type 3 (panel 'f'; red) behaviours under control conditions
524 and in the presence of 300 nM progesterone (P4), 1% human follicular fluid (FF), 2 mM 4-
525 aminopyridine (4-AP) and 1 mM thimerosal. Plots show median and interquartile range (box)
526 and maximum/minimum values (whiskers) of 51-167 events. Asterisks indicate significant
527 difference from control; **=P<0.01; ***=P<0.001; ****=P<0.0001.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

528

529

530

531

532

533

534

535

536

537

538

539

540

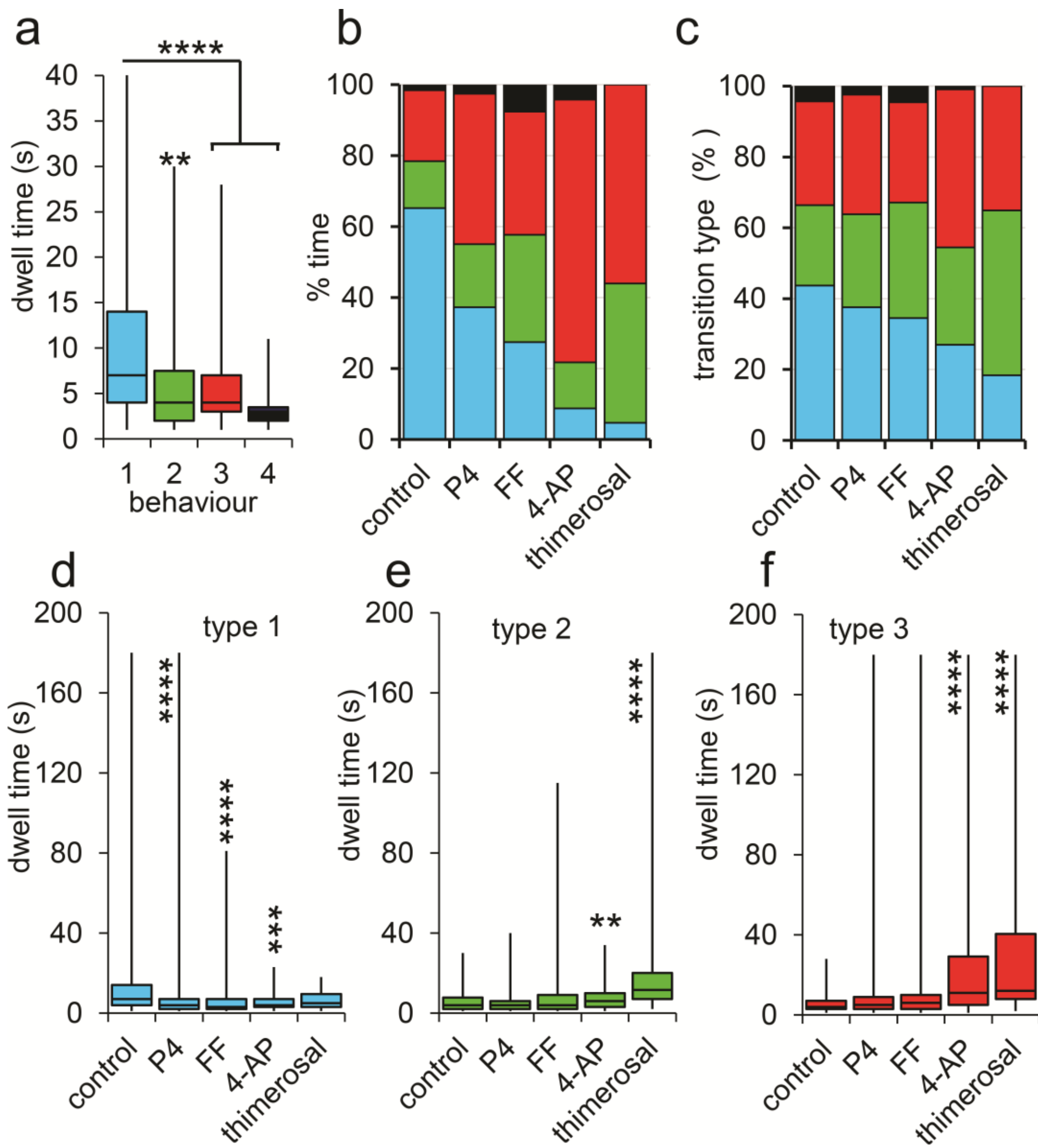
541

542

543

544

545



Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

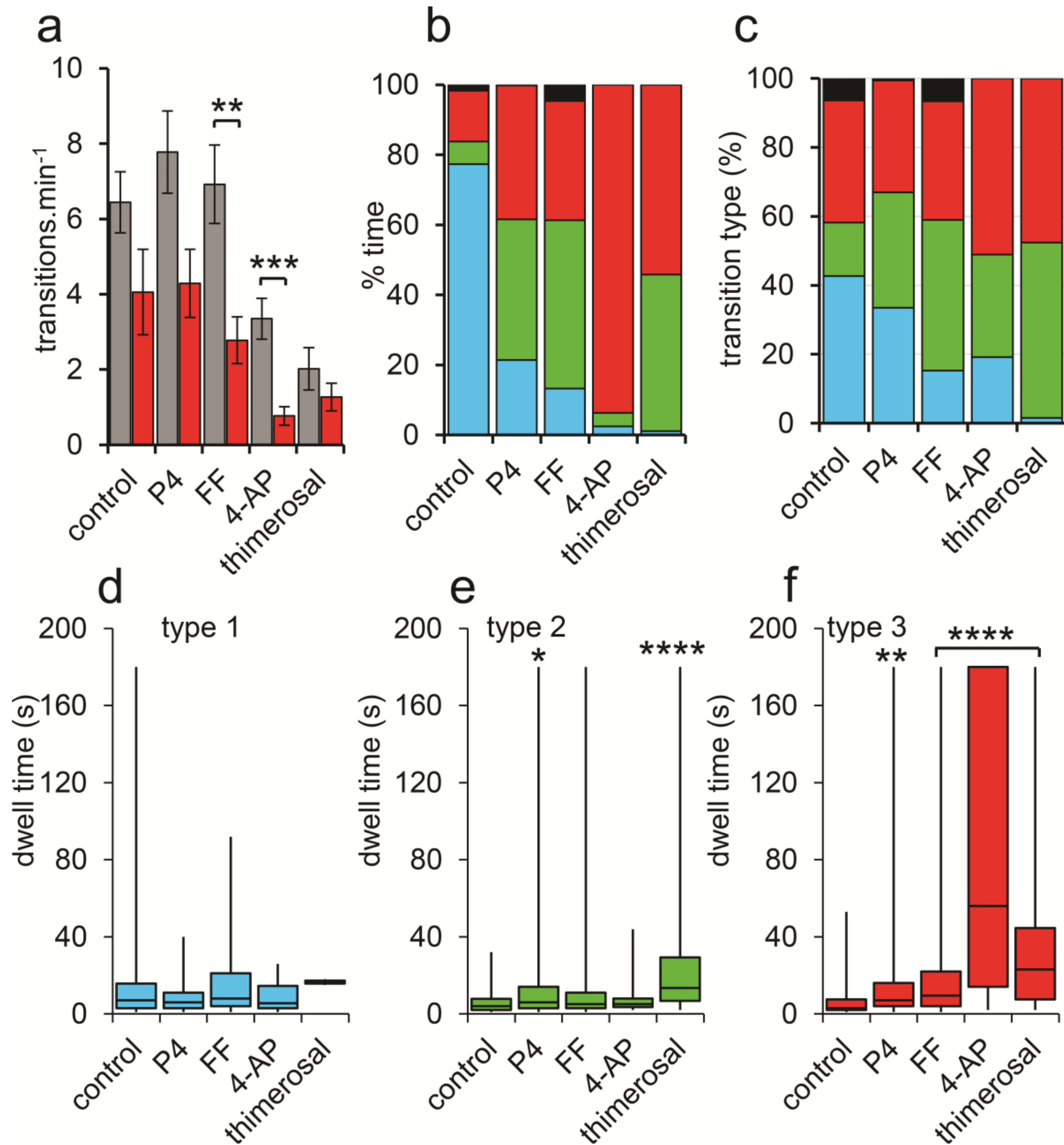
546 **Figure 3. Characteristics of behavioural switching at pH_o=8.5.** (a) Mean switching rate
547 (±s.e.m.) for cells incubated at pH_o=7.4 (grey bars) and pH_o=8.5 (red bars) under control
548 conditions (n=18 cells) and in the presence of 300 nM P4 (n=18 cells), 1% FF (n=17 cells), 2
549 mM 4-AP (n=21 cells) and 1 mM thimerosal (n=17 cells). (b) Mean % time spent in each
550 behaviour at pH_o=8.5; type 1 (light blue), type 2 (green), type 3 (red) and type 4 (black) under
551 control conditions (n=18 cells) and in the presence of 300 nM P4 (n=15 cells), 1% FF (n=18
552 cells), 2 mM 4-AP (n=20 cells) and 1 mM thimerosal (n=16 cells). (c) Relative frequencies of
553 transitions into type 1 (light blue), type 2 (green), type 3 (red) and type 4 (black) behaviours
554 at pH_o=8.5 under control conditions (n=220 transitions) and in the presence of progesterone
555 (P4; 300 nM; n=194), human follicular fluid (FF nM, 1%; n=151), 4-aminopyridine (4-AP, 2
556 mM, n=47) and thimerosal (1 mM, n=63). (d-f) Dwell times at pH_o=8.5 for type 1 (panel 'd';
557 blue), type 2 (panel 'e'; green) and type 3 (panel 'f'; red) behaviours under control conditions
558 and in the presence of 300 nM progesterone (P4), 1% human follicular fluid (FF), 2 mM 4-
559 aminopyridine (4-AP) and 1 mM thimerosal. Plots show median and interquartile range (box)
560 and maximum/minimum values (whiskers) of 10-110 events (except thimerosal type 1 where
561 n=2). Asterisks indicate significant difference from control; *=P<0.05; **=P<0.01;
562 ***=P<0.001; ****=P<0.0001.

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

563

564

565



566

567

568

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

569 **Supplementary video legends**

570 **Video 1. Example of repeated behavioural switching.** In this cell type, 1 behaviour occurs
571 at the start of the video and also for the periods 3-5.5 s, 11.5-14 s and 17.5-20 s as shown by
572 the time stamp (seconds) at the top left of the image. Periods of type 3 behaviour occur at
573 approximately 2-3 s, 10-11.5 s and 14-17.5 s. Between 5.5 and 10 s both type 3 and (briefly)
574 type 2 behaviours occur. Total duration= 20 s. Frame dimensions = 230 mm * 225 mm

575 **Video 2. Example of a cell showing type 2 behaviour.** Total duration= 6 s. Frame
576 dimensions = 230 mm * 240 mm

577 **Video 3. Example of a cell showing type 4 (arrested) motility.** This cell shows periods of
578 type 4 motility (where the flagellum arrests in a J shape) interspersed by brief periods (< 1 s)
579 of flagellar beating. Total duration= 5.9 s. Frame dimensions = 205 mm * 180 mm.

580

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019

581 **Table 1. Characteristics of the motility types identified by visual analysis of videos.** Values
582 shown for typical ALH (amplitude of lateral head displacement) show range of values obtained from
583 examination of 15-20 tracks of each type and are descriptive, not definitive. n/a indicates not
584 applicable.

motility type	characteristics	progressive/non-progressive	typical ALH
1	low amplitude flagellar beat, symmetric or occasionally weakly asymmetric causing curved or circular path	progressive	≈2-4 μm
2	intermediate amplitude beat, symmetric or slightly asymmetric (causing circling)	progressive	≈3-8 μm
3	high-amplitude highly asymmetric beat	non-progressive, continuous turning or tumbling	≥6 μm
4	tight bending of the midpiece producing a ‘J’-shape or coil	non-progressive arrested	n/a

585
586
587
588
589
590

591 **Table 2. Incidence of behavioural switching.** Data show proportion of cells in which behavioural
592 switching was observed (number of cells showing switching/total number of cells analysed) at
593 pH_o=7.4 and pH_o=8.5 under control conditions and in the presence of 300 nM progesterone (P4), 1%
594 human follicular fluid (FF), 2 mM 4-aminopyridine (4-AP) and 1 μM thimerosal. The bottom row of
595 the table (P) shows results of chi-square test to compare between pH values (corrected for multiple
596 comparisons) under each condition.

	treatment				
pH_o	control	P4	FF	4-AP	thimerosal
7.4	16/18	16/18	16/17	20/21	14/19
8.5	16/18	13/15	14/18	10/20	11/16
P	1	1	0.67	0.01	1

597
598
599
600

Downloaded from https://academic.oup.com/molehr/advance-article-abstract/doi/10.1093/molehr/gaz034/5514444 by University of Birmingham user on 21 June 2019