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Classification of Mouse Sperm Motility Patterns Using an Automated Multiclass Support Vector Machines Model[1]

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Short title: A Support Vector Machines Model for Sperm Motility

Summary sentence: The application of multiclass support vector machines to CASA parameters from mouse sperm tracks generates a quantitative model that rapidly and accurately identifies physiologically relevant patterns of motility occurring in heterogeneous populations.

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ABSTRACT

Vigorous sperm motility, including the transition from progressive to hyperactivated motility that occurs in the female reproductive tract, is required for normal fertilization in mammals. We developed an automated, quantitative method that objectively classifies five distinct motility patterns of mouse sperm using Support Vector Machines (SVM), a commonly used method in supervised machine learning. This multiclass SVM model is based on more than 2,000 sperm tracks that were captured by computer-assisted sperm analysis (CASA) during in vitro capacitation and visually classified as progressive, intermediate, hyperactivated, slow or weakly motile. Parameters associated with the classified tracks were incorporated into established SVM algorithms to generate a series of equations. These equations were integrated into a binary decision tree that sequentially sorts uncharacterized tracks into distinct categories. The first equation sorts CASA tracks into vigorous and non-vigorous categories. Additional equations classify vigorous tracks as progressive, intermediate or hyperactivated, and non-vigorous tracks as slow or weakly motile. Our CASAnova software uses these SVM

equations to classify individual sperm motility patterns automatically. Comparisons of motility profiles from sperm incubated with and without bicarbonate confirmed the ability of the model to distinguish hyperactivated patterns of motility that develop during in vitro capacitation. The model accurately classifies motility profiles of sperm from a mutant mouse model with severe motility defects. Application of the model to sperm from multiple inbred strains reveals strain-dependent differences in sperm motility profiles. CASAnova provides a rapid and reproducible platform for quantitative comparisons of motility in large, heterogeneous populations of mouse sperm.

INTRODUCTION

During transit through the epididymis sperm develop the capacity for progressive motility and binding to the zona pellucida, two maturational changes required for fertilization [1]. Sperm undergo further changes that are essential for fertilization in the female reproductive tract or in appropriate media in vitro. These changes, collectively known as capacitation, include changes in sperm motility [2, 3]. Sperm isolated from the cauda epididymis display progressive motility, which is characterized by high velocities and low amplitude, symmetrical flagellar bends [4]. Sperm obtained from the oviduct display motility characterized by higher amplitude, more asymmetric flagellar bends leading to more vigorous and less progressive 'whiplash' motility [5]. This change in motility, termed hyperactivation [1, 6], is required for fertilization and facilitates several processes including detachment of sperm from oviductal epithelia, navigation within the viscoelastic oviduct environment and penetration of the zona pellucida (reviewed in [7]). In both mouse and human, several infertile phenotypes are correlated with genetic perturbations that affect either the onset of motility or the development of hyperactivated motility [8, 9].

Multiple methods have been developed to analyze physiological changes in sperm motility (reviewed in [10]). Techniques that examine flagellar movement provide detailed analyses of motion dynamics at the single sperm level, and are especially useful for analyzing changes in flagellar beat associated with hyperactivation [11-13]. Computer-assisted sperm analysis (CASA) facilitates the assessment of motility in larger populations of sperm, generating tracks for each sperm in a microscopic field based on the position of the sperm head in successive frames. Typically, at least 30 framed are captured at 60 Hz (0.5 sec), although extended tracking intervals have proven useful for evaluating hyperactivation in rat sperm [14]. Objective and quantitative measurements are provided for each track, including velocities and other kinematic parameters, along with population measures such as percentages of motile and progressive sperm [15]. When analyzed by CASA, hyperactivated sperm display vigorous, asymmetric tracks characterized by directional changes. The percentage of hyperactivated sperm has commonly been assessed by setting thresholds based on specific combinations of CASA parameters, such as curvilinear velocity and straightness [9, 16-20]. Thresholds were developed for human sperm by first analyzing flagellar movement to identify hyperactivated sperm, followed by the derivation of kinematic parameters applicable to CASA [14, 21]. Hyperactivation thresholds based on CASA parameters from tracks captured at 60 Hz have also been proposed for other species, including mouse, rat [14], macaque [22], and stallion [23]. In the mouse, this gating approach has been reported to underestimate the percentage of hyperactivated sperm at relevant time points [20] or falsely detect hyperactivation at early time points before capacitation takes place [19, 24]. Our preliminary studies agreed with these conclusions. We tested multiple threshold gates and found underestimates of hyperactivated mouse sperm with percentile gates [20] and overestimates with other proposed thresholds [9, 16, 19], including ~5-12% of sperm classified as hyperactivated immediately after isolation.

Other gating-independent methods have been developed to simultaneously identify progressive and hyperactivated CASA tracks, including Minimum Bounding Square Ratio algorithms for stallion sperm and lineal equations for ram [25, 26]. To our knowledge, similar approaches have not yet been validated for mouse sperm. Therefore, an automated, objective, and reproducible method that distinguishes distinct patterns of sperm motility would significantly enhance our understanding of perturbations that effect mouse sperm function. To that end, we utilized Support Vector Machines (SVM), a common method of supervised machine learning [27], to develop a multiclass SVM model and CASAnova software to classify hyperactivated sperm and four other distinct patterns of mouse sperm motility based on standard CASA parameters.

MATERIALS AND METHODS

Reagents and Media

All reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) except sodium chloride and glucose (Fisher Scientific), sodium pyruvate (Invitrogen, Carlsbad, CA), sodium bicarbonate (EM Science, Gibbstown, NJ), potassium chloride, magnesium sulfate heptrahydrate, and potassium phosphate (Mallinckrodt Chemical, Phillipsburg, NJ), and penicillin/streptomycin 100x stock solution containing 10,000 U/ml penicillin G and 10 mg/ml streptomycin (Gemini Bioproducts, West Sacramento, CA).

HTF, the medium used for all sperm motility assays, is based on the composition of human oviductal fluid and has been used extensively for both mouse and human in vitro fertilization (IVF) [28, 29]. HTF complete medium consists of 101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7\ H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.78 mM glucose, 0.33 mM pyruvate, 21.4 mM lactate, 5mg/ml BSA , 100 U/ml penicillin G and 0.1mg/ml streptomycin. HTF medium without energy substrates did not include glucose, lactate, or pyruvate. Bicarbonate-free HTF replaced 25 mM sodium bicarbonate with 21 mM HEPES. For all media, the osmolality was adjusted to ~315 mOsm/kg with 5M NaCl using a Model 3300 micro-osmometer (Advanced Instruments, Norwood, MA).

Animals and Sperm Collection

Adult CD1 male mice were purchased from Charles River Laboratories (Raleigh, NC) and allowed to acclimatize before use. C57BL/6J, 129S1/SvImJ, and PWK/PhJ male mice were kindly provided by Fernando Pardo-Manuel de Villena (University of North Carolina). *Gapdhs*$^{-/-}$ and wildtype mice were obtained from an established breeding colony [30]. At least three mice of each strain or genotype were used for each experiment. All procedures involving mice were approved in advance by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Sperm were collected from the cauda epididymides of sexually mature (>8 weeks) mice. Each cauda was carefully trimmed to remove adipose and other tissue, rinsed in PBS (140 mM NaCl, 3mM KCl, 4 mM $NaH_2PO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4), and placed in 1 ml HTF media lacking both bicarbonate and energy substrates. Four to six cuts were made in each cauda using iris scissors, and sperm were released into the media by incubation for 10 min at 37^{o} C under 5% CO_2 and air. After the incubation, the tissue was removed and the suspension was mixed gently by swirling. This suspension was then diluted 1:20 to 1:60 in HTF complete medium to a concentration of ~2-4 x 10^5 sperm/ml, equivalent to 50-120 sperm per microscope field for CASA when using the following equipment and settings. For the analysis of motility under non-capacitating conditions, sperm were diluted at the same ratio into bicarbonate-free HTF.

Analysis of Sperm Motility

After dilution in HTF complete medium or bicarbonate-free HTF, sperm were incubated for 2 h at 37^{o} C under 5% CO_2 in air, and motility was assessed at 30 min

intervals. Initial time points were completed within two minutes of dilution into HTF. Quantitative parameters of sperm motility were recorded by CASA using the CEROS sperm analysis system (software version 12.3, Hamilton Thorne Biosciences, Beverly, MA). The CEROS system includes an Olympus CX41 microscope equipped with a MiniTherm stage warmer and a Sony model XC-ST50 CCD camera. Sperm tracks (1.5 sec) were captured at 37^{o}C with a 4x negative phase contrast objective and a frame acquisition rate of 60 Hz. The default Mouse 2 analysis settings provided by Hamilton Thorne were used, except that 90 frames were recorded and slow cells were counted as motile. These settings include: 60 frames per second, 90 frames acquired, minimum contrast = 30, minimum size = 4 pixels, default cell size = 13 pixels, default cell intensity = 75, cells progressive if VAP >50 μm/sec and STR > 50%, slow cells counted as motile, low VAP cut off = 10 μm/sec , low VSL cutoff = 0 μm/sec , minimum intensity gate = 0.10, maximum intensity gate = 1.52, minimum size gate = 0.13 pixels, maximum size gate = 2.43 pixels, minimum elongation gate = 5 pixels, and maximum elongation gate = 100 pixels.

Sperm suspensions were gently mixed before measuring motility. For each motility measurement, a 25 μl aliquot of sperm suspension was loaded by capillary action using a large bore pipet tip into one chamber of a pre-warmed Leja slide (100 μm-deep, Leja, The Netherlands). To minimize drift in the media, excess liquid was removed from the outside of the slide by blotting with a laboratory tissue as recommended by the manufacturer, and loading was examined to ensure the absence of air pockets in the chamber. At least 10 fields were recorded for each sample analyzed, covering the entire viewable area of the chamber without overlapping successive fields. Tracks and kinematic parameters were recorded for individual sperm. Tracks included in subsequent analyses were required to have a minimum of 45 points which represents half the number of total frames, as in previous studies using extended tracking intervals [9, 14]. Individual database text (DBT) files with track details were generated for every sperm population analyzed at every time point, providing Field #, Track #, average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), and linearity (LIN) values for every track.

SVM Model Training

A training set was created from sperm analyzed after 90 min of incubation in HTF complete medium at 37^{o} in an atmosphere of 5% CO_2 and air. This time point was selected because high levels of vigorous motility were maintained consistently at 90 min and five motility patterns (progressive, intermediate, hyperactivated, slow, and weakly motile) were well represented. Individual sperm tracks were assessed visually and assigned to one of these five motility patterns (see results for details of the criteria for each group). The kinematic parameters for these tracks were identified in the CASA-generated DBT files and copied into an Excel worksheet, along with their visual classification to create the training data set. All classified tracks and parameters were loaded into Matlab (software version 2009b, The Mathworks, Natick, MA). The "svmtrain" LibSVM function [31] was used to generate the SVM equations that we incorporated into the CASAnova software program for automated sperm motility analyses. First, classified tracks were labeled as "vigorous" (progressive, intermediate, hyperactivated) or "non-vigorous" (slow and weakly motile). The function then generated an equation that best separates the two groups of data in multidimensional space (SVM1, see Results). This process was repeated within the vigorous and non-vigorous groups to generate four equations used to classify motility patterns. We have submitted a patent application for the use of support vector machines to classify sperm motility patterns based on CASA parameters [32].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). All data are shown as mean ± SEM. Statistical significance was determined using either two-tailed unpaired t-tests or by one-way ANOVA after arcsine transformation of percentages. Differences were considered significant if $P < 0.05$. The percent agreement of the model was evaluated by calculating Cohen's Kappa Coefficient [33].

RESULTS

Characterization of Sperm Motility Patterns

Current CASA instruments capture multiple images and generate tracks for each sperm by marking the position of the head in successive frames (Fig. 1). Visual examination of tracks recorded immediately after isolation of sperm from the cauda epididymis (Fig. 1A) and after in vitro capacitation for 90 min (Fig. 1B) indicates that motility changes during this interval from predominantly progressive to more varied profiles that are less linear. The first step in developing a quantitative model that distinguishes these different types of motility was to generate a training set of sperm tracks for analysis. We collected CASA tracks of sperm incubated for 90 minutes in HTF complete medium and assessed both sperm motion and track pattern with the playback function in the software. Tracks of sperm from 12 CD1 mice were classified as progressive, intermediate, hyperactivated, slow, or weakly motile. Figure 1 shows examples of all motility patterns and Table 1 provides the mean values of all kinematic parameters associated with each group. Sperm with vigorous motility (progressive, intermediate or hyperactivated) in our training set had mean VCL values >279 μm/sec, while the non-vigorous groups (slow and weakly motile) had mean VCL values <176 μm/sec . Additional examples of each motility pattern are shown at higher magnification in Supplemental Figure S1 (all supplemental figures are available online at www.biolreprod.org). Tracks classified as progressive were typically very straight with little deviation of the head from the average path and angles between consecutive points of less than 90 degrees along the majority of the track (track a in Fig. 1A and B, Supplemental Fig. S1A). Intermediate sperm tracks showed more vigorous motion, with larger deviations from the net direction of movement and angles of approximately 90 degrees along most of the track length (track b in Fig. 1B, Supplemental Figure S1B). Sperm were classified as hyperactivated if they displayed highly vigorous motility accompanied by turns of greater than 90 degrees between consecutive points along the majority of the track. This category includes both the classic star-spin pattern of motility (track c in Fig. 1B, Supplemental Fig. S1C) and sperm tracks that exhibit large deviations from the average path but maintain a more defined direction of movement (track d in Fig. 1B, Supplemental Fig. S1C) [34, 35]. Slow sperm tracks covered much less distance than progressive sperm and generally did not show a high level of displacement of the head from the path of movement (track e in Fig. 1B, Supplemental Fig. S1D). Tracks that were motile but were not vigorous and did not have significant forward motion were characterized as weakly motile (track f in Fig. 1B, Supplemental Fig. S1E). To maintain very strict criteria for defining the five motility patterns, we excluded tracks that were derived from sperm with abnormalities such as flagellar bending at the annulus or adherence to other sperm (~400 tracks), were the result of sperm collisions (~300 tracks), or could not be identified confidently (~400 tracks). A total of 2,043 tracks were included in the final training set used to develop the CASAnova equations.

Development of the Multiclass SVM Model and CASAnova Software

The Hamilton Thorne CASA systems generate data files that list parameter values

for each sperm track analyzed in an experiment. Independent kinematic parameters, including VAP (μm/sec), VSL (μm/sec), VCL (μm/sec), ALH (μm) and BCF (Hz) were used to develop our multiclass SVM model. Since STR (VSL/VAP) and LIN (VSL/VCL) are ratios of other parameters, they were not used in building our prediction model. CASA parameters for visually classified tracks in our training set were grouped into separate Excel files for each motility pattern. This set of tracks from sperm incubated for 90 min under capacitating conditions included 539 progressive tracks, 236 intermediate tracks, 515 hyperactivated tracks, 556 slow tracks, and 197 weakly motile tracks. After generating the model using tracks from 90 min time points, progressive tracks recorded at time 0 were incorporated to determine their effect on the SVM equations. Since these time 0 tracks did not significantly alter the multiclass SVM model equations (data not shown), they were not included in the final model.

Three-dimensional scatter plots of CASA parameters associated with the five motility groups revealed clustering of tracks according to their visual classification (Fig. 2A). Hyperactivated sperm tracks (blue data points) clustered separately from progressive sperm tracks (red data points). Intermediate tracks (green data points) clustered between these groups, indicating that this motility pattern may constitute a phase that occurs between progressive and hyperactivated states. Tracks classified as weakly motile (cyan data points) generally had the lowest velocities and were grouped below the slow tracks (black points).

The clustering of sperm tracks based on their visual classification (Fig. 2A) suggested that sperm motility patterns could be mathematically separated into groups using support vector machines. We developed a set of equations that mathematically define the boundaries which distinguish these clusters (Table 2). Each equation includes all independent CASA parameters, and the number multiplied by each parameter reflects its relative importance in that equation. In equation SVM1, for example, VAP is the most important determinant and BCF is the least important. The decision tree shown in Figure 2B summarizes how these equations are sequentially applied to sort sperm tracks into the five motility groups. We first used established algorithms available in LibSVM to divide the tracks into two principal groups: vigorous (progressive, intermediate, hyperactivated) and non-vigorous (slow and weakly motile). The program generated a binary equation that separates these two groups in the training set (Table 2, SVM1). If CASA parameters from an unclassified track are applied to this equation and the result is greater than 0, the track is classified as vigorous. Otherwise, the track is classified as non-vigorous. After defining two groups with the initial equation, the process was repeated to further subdivide these populations into discrete motility groups.

Within the vigorous group, we developed two additional SVM equations, SVM2 and SVM3 (Table 2). SVM2 classifies tracks as hyperactivated if the value of SVM2 is greater than 0, and removes them from further examination. Vigorous sperm tracks that have a SVM2 <0 are further analyzed by SVM3. Tracks are classified as intermediate if their SVM3 >0, or progressive if SVM3 <0.

Tracks with SVM1 values less than 0 are classified as non-vigorous. This non-vigorous group can be further classified as slow or weakly motile based on SVM4 (Table 2). The SVM4 equation classifies a sperm track as slow if its value is greater than 0, while SVM4 values less than 0 are categorized as weakly motile.

A software program, which we named CASAnova, incorporates these four SVM equations for the automated classification of the motility patterns of individual sperm. This program utilizes CASA-generated DBT files with all CASA parameters for each motile track. CASAnova applies the SVM equations to individual CASA tracks, generates a summary showing the number of sperm that were classified into each motility group, and calculates the percentage of tracks in each group as a function of the motile population. This program also generates a detailed list showing each track analyzed, along with its CASA parameters and multiclass SVM classification. The CASAnova software and instructions for use can be downloaded for non-profit purposes

at http://www.csbio.unc.edu/CASAnova/.

Accuracy of Track Identification with CASAnova

To assess the ability of CASAnova to accurately classify sperm motility tracks, we visually classified the motility patterns of 1,068 sperm tracks from four additional CD1 mice after incubation for 90 min in HTF complete medium. We then determined how many of these tracks were correctly assessed by CASAnova, and calculated the percent agreement (Table 3). All tracks were included in these assessments. In agreement with our visual classification, we noted that tracks of sperm with hairpin bends at the annulus were identified almost exclusively as slow or progressive. Tracks from agglutinated sperm were typically excluded from the analysis because of size exclusions in the software or short tracks with less than 45 points. Tracks derived from sperm collisions and errors in tracking were also greatly reduced by requiring a minimum of 45 points. Agreement in all groups, except intermediate, exceeded 83% and the overall agreement was 88.2%. Just over half of the visually-identified intermediate tracks in this set (52.1%) agreed with the model classification, while CASAnova classified the remaining tracks in this group as progressive or hyperactivated in approximately equal proportions. We also employed Cohen's Kappa, a measurement of interrater reliability [33], to assess the overall level of agreement between visual and CASAnova classifications. This index indicates that the strength of agreement between these classifications is very good, with a Kappa coefficient of 0.848, which is within the 95% confidence interval.

Motility Profiles of Sperm Incubated in Capacitating and Non-capacitating Medium

Bicarbonate is required for sperm capacitation as well as the acquisition of hyperactivated motility [17, 36-38]. To assess the ability of the model to distinguish motility pattern distributions of capacitated and non-capacitated sperm, we generated motility profiles of sperm from six CD1 mice incubated in HTF complete medium ± 25 mM bicarbonate over a 2 hr time course (Fig. 3). While the percentage of motile sperm in both media remained above 50% throughout the time course (Supplemental Fig. S2A), there were marked differences in the sperm motility profiles. In complete medium containing bicarbonate (Fig. 3A), the number of progressive tracks steadily decreased over time. This decrease in progressive motility was accompanied by increases in all other motility groups. Among vigorously motile tracks, both intermediate and hyperactivated tracks increased by 60 min and reached maximum values by 90 min (red box, Fig. 3A). In this experiment, progressive tracks declined to 22.2% of all motile tracks by 90 min, while intermediate tracks increased to 9% and hyperactivated tracks increased to 18.9%. The proportion of sperm with non-vigorous motility (slow or weakly motile) also increased during in vitro capacitation, reaching 49.9% by 90 min. Further increases in non-vigorous motility were frequently observed by the 2 h time point. We confirmed that CASAnova is effective when sperm are analyzed in chambers with depths of either 100 μm (Leja) or 80 μm (2X-cel slides, Hamilton Thorne) (Supplemental Fig. S3).

Sperm incubated in medium that does not stimulate capacitation (Fig. 3B) showed a significantly different motility distribution than sperm incubated for the same period in HTF complete medium (Fig. 3A). When bicarbonate was omitted from the medium, the mean proportion of progressive sperm tracks remained above 50% over the entire time course, in agreement with visual observations. The proportion of non-vigorous tracks increased throughout the incubation, although the percentage of weakly motile sperm remained lower than observed in HTF complete medium. As expected, intermediate and hyperactivated tracks were rarely observed throughout the incubation, reaching mean values of 0.4% and 1.2%, respectively, by 90 min (red box, Fig. 3B).

Analysis of Sperm with Motility Defects

To determine how CASAnova classifies sperm with severe defects in motility, we assessed the motility distribution of mice lacking the sperm-specific glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDHS) (Fig. 4). These males are infertile and produce sperm that are motile (Supplemental Fig. 2B), but exhibit little forward progression [30]. CASAnova classified 99% of GAPDHS-null sperm as weakly motile or slow (Figure 4, open bars). Sperm from wild-type mice with the same genetic background (mixed 129S6/SvEvTac and C57BL/6NCrl) displayed predominantly progressive motility (Fig. 4, black bars). Approximately 17% of wild-type sperm were classified as slow immediately after isolation, perhaps reflecting contributions of the C57BL/6 genetic background (see next section).

Motility Profiles of Sperm from Inbred Mouse Strains

We developed CASAnova using sperm tracks from CD1 outbred mice. To determine its suitability for assessing sperm motility in other mouse strains, we assessed sperm from C57BL/6J (BL6), 129S1/SvImJ (129) and PWK/PhJ (PWK) inbred mouse strains over a 90 min incubation in HTF complete medium (assayed in parallel, n = 4 mice per strain). Motility profiles were generated for each strain and compared to CD1 profiles (Fig. 5A). Sperm from two inbred strains (129 and PWK) exhibited motility profiles that were comparable to CD1 sperm immediately after isolation, except that a higher percentage of 129 sperm were classified as slow(0 min). At this initial time point, BL6 sperm tracks contained significantly fewer progressive tracks (mean = 23%, $P < 0.001$) and significantly more slow tracks (mean = 66.4%, $P < 0.001$) compared to CD1 sperm (79.2% progressive, 4.2% slow). This proportion of slow tracks persisted in BL6 sperm throughout the assay.

Throughout the time course, sperm from the three inbred strains showed the expected decrease in the percentage of progressive motility and concomitant increase in other motility classes (Fig. 5A). After 90 min of incubation, the mean percentage of slow tracks was significantly higher for both BL6 (59.5%) and 129 (60.6%) sperm than for CD1 (40.9%) sperm. At this time point, the mean percentages of hyperactivated sperm in both BL6 (16%) and 129 (15%) mice were significantly lower than the levels observed in CD1 mice (32%), while PWK hyperactivation was comparable to CD1. To determine whether reduced hyperactivation was due to the inability of the model to identify hyperactivated sperm in BL6 and 129 mice, we visually assessed the percentage of hyperactivated sperm for each strain. There were no statistically significant differences between hyperactivation levels determined visually or by using CASAnova (Supplemental Fig. S4). BL6 sperm did display more asymmetric tracks than 129 sperm at 90 min, but many of these tracks had low velocities and were not classified as hyperactivated by the model or visual assessment.

CASAnova classifies all motile sperm in each population. When the number of sperm analyzed or the percent motility varies substantially between animals, additional considerations may be needed for a more complete assessment of motility. We typically recovered fewer sperm from these inbred strains (mean = 5.7 - 16.7 x 10^6) than from CD1 mice (mean = 31 x 10^6), but analyzed >150 tracks/mouse at each time point to provide robust assessments of sperm motility in each strain. We also found that the percentage of motile sperm at later time points was significantly lower in PWK mice (mean = 26.5% at 90 minutes) compared to the other strains (37% - 50%, Supplemental Fig. 2C). Therefore, we also calculated the percentage of hyperactivated sperm as a function of the total number of sperm analyzed by CASA at the 90 min time point (Fig. 5B). When immotile sperm are included in the calculation, the percentage of hyperactivated sperm in PWK mice falls to levels that are comparable to those observed for the other two inbred strains.

DISCUSSION

During capacitation sperm motility patterns shift from largely progressive tracks at early time points to more varied patterns of movement, including hyperactivation. CASA-based approaches for identifying sperm motility patterns in the mouse have focused predominantly on distinguishing progressive and hyperactivated sperm populations [9, 16-20, 30, 39, 40], although there is no consensus on the parameters that best define hyperactivation. We used CASA parameters from 2,043 sperm tracks (1.5 sec, 90 frames) to develop an automated model that identifies and quantitates five distinct patterns of sperm movement in large populations of mouse sperm. CASAnova is built upon a series of SVM equations (Table 2) that take into account both the relationships between CASA parameters and the relative importance of each parameter in assigning tracks to specific motility groups. This approach classifies all recorded tracks simultaneously, providing a more comprehensive analysis of the changes in motility that occur during capacitation compared to identifying only the percentage of hyperactivated sperm by visual assessment or the use of thresholds for selected CASA parameters. CASAnova was developed with mouse sperm tracks captured at 60 Hz using a Hamilton Thorne CEROS instrument. Although CASA systems typically calculate similar kinematic parameters, further validation studies will be necessary to test the applicability of this model for other CASA platforms.

Immediately after isolation from the cauda epididymis, mouse sperm display vigorous motility with ~80% of the motile population classified as progressive by CASAnova. The percentage of motile sperm is typically maintained during a 120 min in vitro capacitation period. In addition, the percentage of sperm displaying progressive motility does not change substantially during this interval when standard CASA cutoffs are used. The Mouse 2 default settings recommended by Hamilton Thorne categorize sperm as progressive if VAP >50 μm/sec and STR >50, a broad definition that includes virtually all linear tracks. The Hamilton Thorne software also identifies sperm as rapid if VAP exceeds the progressive threshold of 50μm/sec. In contrast, the progressive tracks in our training set were linear and had mean values for VAP of 146.9 ± 31.5 μm/sec. CASAnova classifies sperm as progressive only if they have motility that is both linear and vigorous, while sperm that have linear tracks with substantially lower velocities (mean VAP = 85.2 ± 19.0 in our training set) are classified as slow. Inclusion of the non-vigorous classifications in this model provides better discrimination of sperm velocities and clearly shows that the motility of many sperm becomes less vigorous as capacitation proceeds, with ≥40% of the sperm classified as slow or weakly motile by 60 min (Fig. 3).

CASAnova also identifies intermediate and hyperactivated tracks, the vigorous patterns of sperm motility that develop during capacitation. In our training set both intermediate and hyperactivated sperm had higher mean values for VCL and ALH than progressive sperm (Table 1), reflecting the increased vigor expected during hyperactivation [14]. Hyperactivated motility patterns, including both star-spin tracks and tracks that show some directional movement, were classified with 94% accuracy. At initial time points, hyperactivation is essentially absent by visual inspection of sperm tracks (Fig. 1) and multiclass analysis reflects this observation (Fig. 3). Consequently, CASAnova eliminates the need for the subtraction of noise detected at time zero from the levels of hyperactivation detected at later time points[24]. As expected, this model detects an increase in the proportion of hyperactivated sperm over the course of a 2 h period of in vitro capacitation. The percentage of hyperactivated sperm reaches ~15%-35% by 90 min, consistent with levels reported in mouse and other species using validated approaches [14, 25, 34, 41, 42].

The percentage of intermediate tracks increases to ~9% of the motile population over the same time course (Fig. 3). Analyses of multidimensional scatter plots show that the intermediate tracks cluster between the progressive and hyperactivated groups (Fig. 2), suggesting that the intermediate category may represent sperm shifting from progressive to hyperactivated motility. Similar transitional patterns of sperm motility

have been described in other species [14, 35, 43]. The loss of both intermediate and hyperactivated tracks when bicarbonate is omitted from the medium (Fig. 3) provides support for considering intermediate sperm as a subset of the population developing asymmetric flagellar beats, and highlights the ability of CASAnova to accurately identify physiological alterations in motility that occur during capacitation.

The design of CASAnova to quickly distinguish and quantitate five patterns of motility, along with its applicability to sperm from multiple mouse strains, suggests that this model will be useful for more detailed analyses of sperm motility under a variety of experimental conditions. Inclusion of the slow category in CASAnova revealed that sperm from C57BL/6J mice are substantially less vigorous at initial time points than sperm from two other inbred strains (129S1/SvImJ and PWK/PhJ). This difference is interesting since both C57BL/6J and 129S1/SvImJ strains are used extensively in the production of knockout and transgenic mouse lines. In addition, all three of these inbred strains are founder strains of the Collaborative Cross, a project that is producing a large number of recombinant inbred lines with extensive genetic diversity [44]. Given the phenotypic variation that we observed in these inbred lines, it will be interesting to complete a similar analysis of sperm motility with fully inbred Collaborative Cross lines. We anticipate that use of CASAnova will contribute to more quantitative analyses of genetic factors that influence the complex traits of sperm motility and male fertility.

Gene targeting strategies have produced more than forty mutant mouse models with defects in sperm motility [8]. These include a broad range of structural, metabolic and signaling defects that reduce the proportion of motile sperm and/or impair required aspects of sperm movement such as forward progression [30, 45] or hyperactivation [46]. The inclusion of multiple categories in CASAnova will facilitate rapid assessment of motility deficits, as demonstrated for GAPDHS-null sperm. Although further validation will be required for sperm with severe structural defects or unusual motility patterns, CASAnova could be useful for establishing a standardized assessment of sperm motility that automatically discriminates between motility patterns based on the mathematical relationship of CASA parameters. Standardization would facilitate more meaningful comparisons among mutant mouse lines that may significantly enhance our understanding of the regulation of motility transitions required for fertilization.

Toxicological studies are being conducted to investigate the effects of various compounds on reproductive function in mice [40, 47, 48]. In cases where male reproductive toxicity is observed, use of CASAnova could improve discrimination of effects on physiologically relevant patterns of motility that are not easily discerned by comparison of individual CASA parameters. Similarly, the model could serve as an important screening tool for evaluating compounds as potential contraceptives that impair sperm motility, particularly when mechanisms that regulate hyperactivation are targeted.

Motility classification by CASAnova is rapid, reproducible and quantitative. This model provides an objective assessment of the entire motile population of sperm analyzed by CASA that is consistent between experiments, thereby facilitating standardization of motility pattern analyses. The CASAnova program is easy to use and quickly calculates both the number and percentage of motile sperm in each of the five categories. It also generates a list showing the CASA parameters and multiclass SVM classification for each track. Classification of sperm motility patterns using CASAnova shows good agreement with visual classifications by observers with extensive training (Table 3), eliminating the need for visual verification of results or complex training to achieve accurate, detailed analyses. By combining CASA and standard machine learning tools to identify physiologically relevant patterns of sperm movement, CASAnova has the potential for being a valuable tool for assessing genetic, biomolecular, and pharmaceutical effects on sperm motility.

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REFERENCES

1. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill J (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 189-317.
2. Fraser LR. The "switching on" of mammalian spermatozoa: molecular events involved in promotion and regulation of capacitation. Mol Reprod Dev 2010; 77:197-208.
3. Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol 2002; 53:133-150.
4. Fraser L. Motility patterns in mouse spermatozoa before and after capacitation. Journal of Experimental Zoology 1977; 202:439-444.
5. Suarez SS, Osman RA. Initiation of hyperactivated flagellar bending in mouse sperm within the female reproductive tract. Biol Reprod 1987; 36:1191-1198.
6. Yanagimachi R. The movement of golden hamster spermatozoa before and after capacitation. J Reprod Fertil 1970; 23:193-196.
7. Suarez SS. Control of hyperactivation in sperm. Hum Reprod Update 2008; 14:647-657.
8. Matzuk MM, Lamb DJ. The biology of infertility: research advances and clinical challenges. Nat Med 2008; 14:1197-1213.
9. Si Y, Olds-Clarke P. Evidence for the involvement of calmodulin in mouse sperm capacitation. Biol Reprod 2000; 62:1231-1239.
10. Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. Hum Reprod Update 1997; 3:403-439.
11. Ho HC, Granish KA, Suarez SS. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca2+ and not cAMP. Dev Biol 2002; 250:208-217.
12. Ishijima S, Baba SA, Mohri H, Suarez SS. Quantitative analysis of flagellar movement in hyperactivated and acrosome-reacted golden hamster spermatozoa. Mol Reprod Dev 2002; 61:376-384.
13. Wennemuth G, Carlson AE, Harper AJ, Babcock DF. Bicarbonate actions on flagellar and Ca2+ -channel responses: initial events in sperm activation. Development 2003; 130:1317-1326.
14. Cancel AM, Lobdell D, Mendola P, Perreault SD. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis. Hum Reprod 2000; 15:1322-1328.
15. Mortimer ST. CASA--practical aspects. J Androl 2000; 21:515-524.
16. Bray C, Son JH, Kumar P, Meizel S. Mice deficient in CHRNA7, a subunit of the nicotinic acetylcholine receptor, produce sperm with impaired motility. Biol Reprod 2005; 73:807-814.
17. Neill JM, Olds-Clarke P. A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. Gamete Res 1987; 18:121-140.
18. Neill JM, Olds-Clarke P. Incubation of mouse sperm with lactate delays capacitation and hyperactivation and lowers fertilization levels in vitro. Gamete Res 1988; 20:459-473.
19. Odet F, Duan C, Willis WD, Goulding EH, Kung A, Eddy EM, Goldberg E. Expression of the gene for mouse lactate dehydrogenase C (Ldhc) is required for male fertility. Biol Reprod 2008; 79:26-34.
20. Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, Garbers DL. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. Proc Natl Acad Sci U S A 2003; 100:14869-14874.
21. Mortimer ST, Swan MA. Kinematics of capacitating human spermatozoa analysed at 60 Hz. Hum Reprod 1995; 10:873-879.
22. Baumber J, Meyers SA. Hyperactivated motility in rhesus macaque (Macaca mulatta) spermatozoa. J Androl 2006; 27:459-468.
23. McPartlin LA, Suarez SS, Czaya CA, Hinrichs K, Bedford-Guaus SJ. Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. Biol Reprod 2009; 81:199-206.
24. Cao W, Aghajanian HK, Haig-Ladewig LA, Gerton GL. Sorbitol can fuel mouse sperm motility and protein tyrosine phosphorylation via sorbitol dehydrogenase. Biol Reprod 2009;

80:124-133.
25. Kaula N, Andrews A, Durso C, Dixon C, Graham JK. Classification of hyperactivated spermatozoa using a robust Minimum Bounding Square Ratio algorithm. Conf Proc IEEE Eng Med Biol Soc 2009; 2009:4941-4944.
26. Vulcano GJ, Moses DF, Valcarcel A, de las Heras MA. A lineal equation for the classification of progressive and hyperactive spermatozoa. Math Biosci 1998; 149:77-93.
27. Wang L. Support vector machines: theory and applications. Berlin: Springer; 2005.
28. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985; 44:493-498.
29. Byers SL, Payson SJ, Taft RA. Performance of ten inbred mouse strains following assisted reproductive technologies (ARTs). Theriogenology 2006; 65:1716-1726.
30. Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci U S A 2004; 101:16501-16506.
31. Chih-Chung Chang and Chih-Jen Lin Lalfsvm, 2001. Software available at http://www.csie.ntu.edu.tw/~cjlin/libsvm
32. Goodson SG, O'Brien, DA, Zhang, Z, Tsuruta, JK, Wang, W, (inventors) The United States of American as represented by the University of North Carolina at Chapel Hill, assignee. Sperm Motility Analyzer and Related Methods. U.S. Patent Application 61/409,688; 2010.
33. Cohen J. A coefficient of agreement for nominal scales. Educational and Psychological Measurement 1960; 20:37-46.
34. Robertson L, Wolf DP, Tash JS. Temporal changes in motility parameters related to acrosomal status: identification and characterization of populations of hyperactivated human sperm. Biol Reprod 1988; 39:797-805.
35. Mortimer ST, Mortimer D. Kinematics of human spermatozoa incubated under capacitating conditions. J Androl 1990; 11:195-203.
36. Lee MA, Storey BT. Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. Biol Reprod 1986; 34:349-356.
37. Boatman DE, Robbins RS. Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. Biol Reprod 1991; 44:806-813.
38. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995a; 121:1129-1137.
39. Shao M, Ghosh A, Cooke VG, Naik UP, Martin-DeLeon PA. JAM-A is present in mammalian spermatozoa where it is essential for normal motility. Dev Biol 2008; 313:246-255.
40. Wang RS, Ohtani K, Suda M, Kitagawa K, Nakayama K, Kawamoto T, Nakajima T. Reproductive toxicity of ethylene glycol monoethyl ether in Aldh2 knockout mice. Ind Health 2007; 45:574-578.
41. Burkman LJ. Characterization of hyperactivated motility by human spermatozoa during capacitation: comparison of fertile and oligozoospermic sperm populations. Arch Androl 1984; 13:153-165.
42. Young RJ, Bodt BA, Heitkamp DH. Action of metallic ions on the precocious development by rabbit sperm of motion patterns that are characteristic of hyperactivated motility. Mol Reprod Dev 1995; 41:239-248.
43. Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in sperm. Biol Reprod 1991; 44:375-381.
44. Chesler EJ, Miller DR, Branstetter LR, Galloway LD, Jackson BL, Philip VM, Voy BH, Culiat CT, Threadgill DW, Williams RW, Churchill GA, Johnson DK, et al. The Collaborative Cross at Oak Ridge National Laboratory: developing a powerful resource for systems genetics. Mamm Genome 2008; 19:382-389.
45. Danshina PV, Geyer CB, Dai Q, Goulding EH, Willis WD, Kitto GB, McCarrey JR, Eddy EM, O'Brien DA. Phosphoglycerate kinase 2 (PGK2) is essential for sperm function and male fertility in mice. Biol Reprod 2010; 82:136-145.
46. Carlson AE, Quill TA, Westenbroek RE, Schuh SM, Hille B, Babcock DF. Identical phenotypes of CatSper1 and CatSper2 null sperm. J Biol Chem 2005; 280:32238-32244.
47. Elangovan N, Chiou TJ, Tzeng WF, Chu ST. Cyclophosphamide treatment causes impairment

of sperm and its fertilizing ability in mice. Toxicology 2006; 222:60-70.
48. Bone W, Jones AR, Morin C, Nieschlag E, Cooper TG. Susceptibility of glycolytic enzyme activity and motility of spermatozoa from rat, mouse, and human to inhibition by proven and putative chlorinated antifertility compounds in vitro. J Androl 2001; 22:464-470.

FIGURE LEGENDS

FIG. 1. Changes in sperm motility patterns during in vitro capacitation. Representative CASA fields showing sperm tracks immediately after isolation (0 min, **A**) and after 90 min (**B**) incubation in HTF complete medium. Lower case letters in each panel denote representative tracks corresponding to five distinct motility groups used to generate the multiclass SVM model—progressive (a), intermediate (b), hyperactivated (c and d), slow (e), and weakly motile (f).

FIG. 2. Generation of a multiclass SVM model to identify sperm motility patterns. **A**) In a multidimensional scatter plot of CASA velocity parameters (VCL, VSL, VAP) associated with sperm tracks in the training set, the tracks are clustered based on their visual classification. **B**) This decision tree diagrams how the multiclass SVM equations (Table 2) are used to classify sperm motility patterns.

FIG. 3. Time-dependent changes in the distribution of sperm motility patterns during in vitro capacitation. Motility of sperm from 6 CD1 mice was assessed at 30 min intervals during incubation in HTF complete medium with (**A**) or without (**B**) 25 mM sodium bicarbonate, and CASA parameters were subjected to analysis by CASAnova. The red box highlights significant differences in the appearance of intermediate and hyperactivated tracks under capacitating conditions. Bars represent the mean percentage ± SEM of motile tracks identified in each group at each time point. Differences between motility groups in A and B at corresponding time points were analyzed using one-way ANOVA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

FIG. 4. Distribution of sperm motility patterns in wildtype (WT) and *Gapdhs*$^{-/-}$ mice. CASAnova analysis of CASA parameters was used to compare motility patterns of sperm from wild-type (black bars, n = 3) and *Gapdhs*$^{-/-}$ mice (white bars, n = 3). Bars represent the mean percentage ± SEM of motile tracks identified in each group immediately after isolation in HTF complete medium (time 0). Significance was determined using a two-tailed unpaired t-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

FIG. 5. Differences in motility profiles between inbred and outbred mouse strains. (**A**) Sperm from C57BL/6J (BL6), 129S1/SvlmJ (129), PWK/PhJ (PWK), and CD1 mice (n = 4 mice per strain) were incubated in HTF complete medium and assayed by CASA over a 90 min time course, followed by analysis by CASAnova. Bars represent the mean percentage ± SEM of motile tracks identified in each group at each time point. (**B**) The percentage of hyperactivated sperm at 90 min (mean ± SEM) was determined as a function of the total number of sperm analyzed by CASA (motile + immotile). Differences between motility groups at corresponding time points were analyzed using one-way ANOVA followed by Dunnett's posttest to determine significance relative to the outbred CD1 strain. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 1. CASA parameter means for groups identified in the multiclass training set.*

Group	VAP	VSL	VCL	ALH	BCF
Progressive	146.9 ± 31.5	119.5 ± 29.5	279.6 ± 59.1	16.7 ± 4.5	25.4 ± 5.2
Intermediate	183.3 ± 31.1	140.4 ± 28.5	406.3 ± 61.5	23.7 ± 4.1	21.1 ± 4.1
Hyperactivated	171.1 ± 37.9	73.3 ± 36.1	373.6 ± 78.4	22.7 ± 5.4	29.1 ± 11.8
Slow	85.2 ± 19.0	40.2 ± 20.5	175.6 ± 32.4	12.3 ± 4.3	33.8 ± 10.3
Weakly Motile	56.2 ± 16.5	13.6 ± 6.8	127.2 ± 36.4	9.9 ± 4.0	45.3 ± 12.6

* Values are means ± standard deviation. VAP = average path velocity in μm/sec, VSL = straight line velocity in μm/sec, VCL = curvilinear velocity in μm/sec, ALH = amplitude of lateral head displacement in μm, BCF = beat cross frequency in Hz.

TABLE 2. CASAnova Multiclass Support Vector Machine (SVM) equations.

SVM1	(0.0388 x VAP) + (0.0335 x VSL) + (0.0225 x VCL) - (0.0248 x ALH) + (0.0051 x BCF) - 10.9540
SVM2	(0.0123 x VAP) - (0.1034 x VSL) + (0.0307 x VCL) + (0.0427 x ALH) + (0.0175 x BCF) - 3.6222
SVM3	(0.0146 x VAP) - (0.0701 x VSL) + (0.0371 x VCL) + (0.0167 x ALH) - (0.0132 x BCF) - 6.4943
SVM4	(0.0418 x VAP) + (0.1115 x VSL) + (0.0163 x VCL) - (0.0243 x ALH) - (0.0471 x BCF) - 4.7717

VAP = average path velocity in μm/sec, VSL = straight line velocity in μm/sec, VCL = curvilinear velocity in μm/sec, ALH = amplitude of lateral head displacement in μm, BCF = beat cross frequency in Hz.

TABLE 3. Agreement between visual and model-assigned tracks.

Track group	agree/total	% agreement
Progressive	192/231	83.2
Intermediate	38/73	52.1
Hyperactivated	251/265	94.7
Slow	232/248	93.5
Weakly Motile	229/251	91.2
Total	**942/1068**	**88.2**

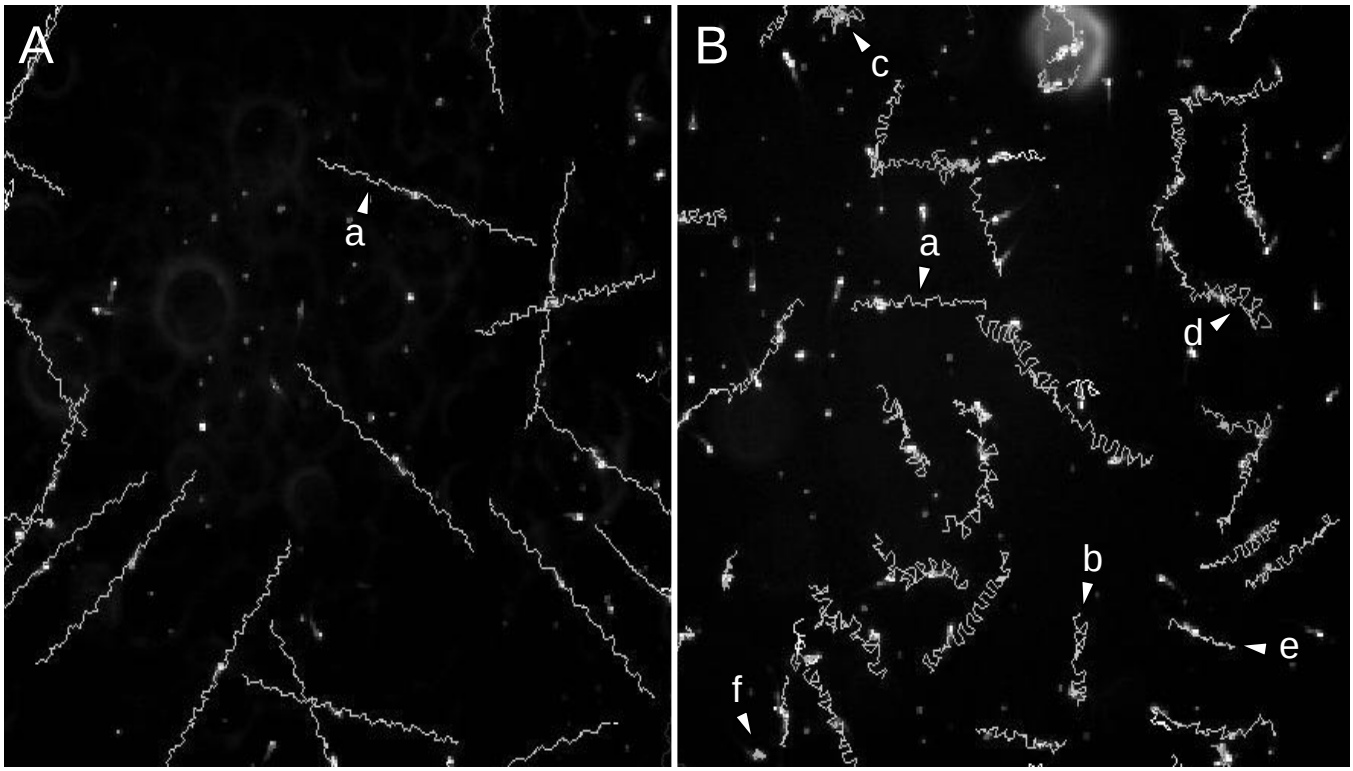

Figure 1

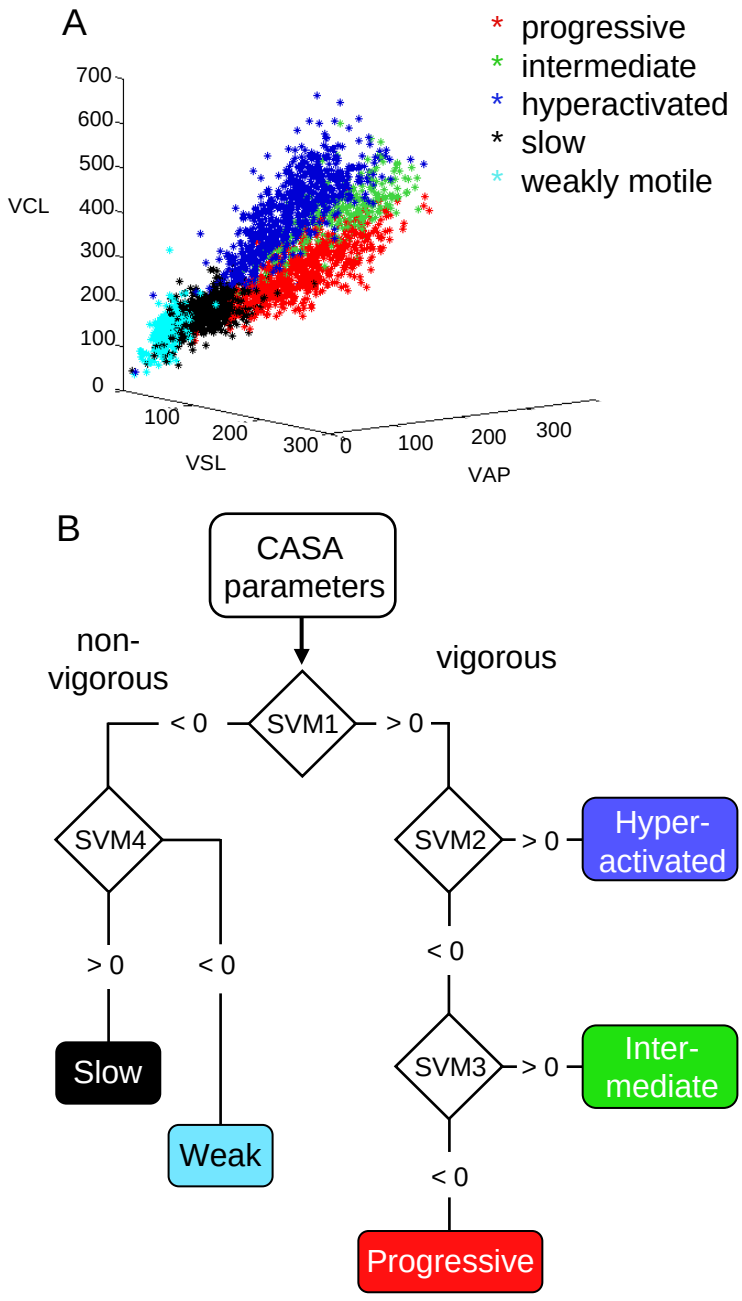
A
progressive
intermediate
hyperactivated
slow
weakly motile
VCL
VSL
VAP
B
CASA parameters
non-vigorous
vigorous
SVM1
< 0
> 0
SVM4
SVM2
Hyper-activated
> 0
< 0
Slow
Weak
SVM3
Inter-mediate
Progressive

Figure 2

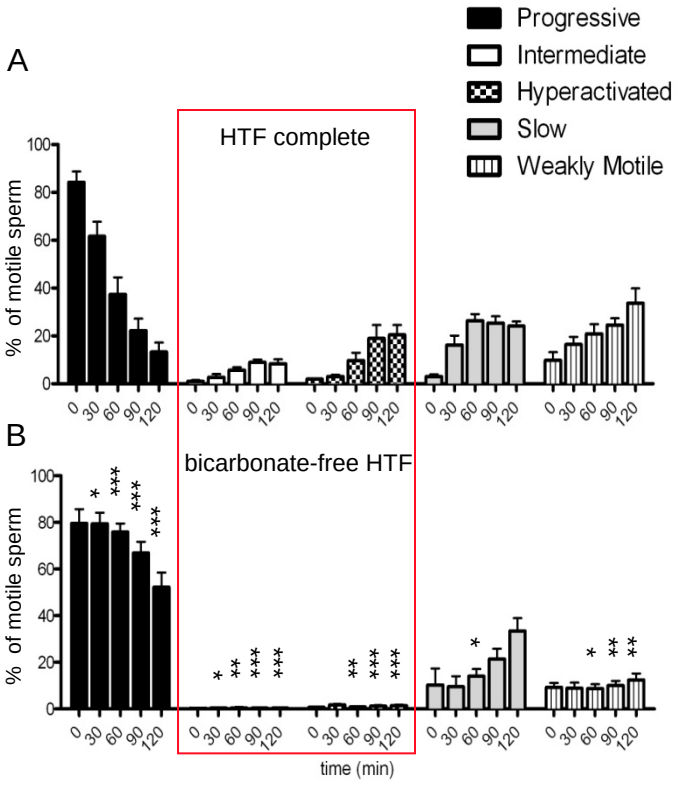
A
Progressive
Intermediate
Hyperactivated
Slow
Weakly Motile
HTF complete
% of motile sperm
100
80
60
40
20
0
0 30 60 90 120 0 30 60 90 120 0 30 60 90 120 0 30 60 90 120
B
bicarbonate-free HTF
% of motile sperm
time (min)

Figure 3

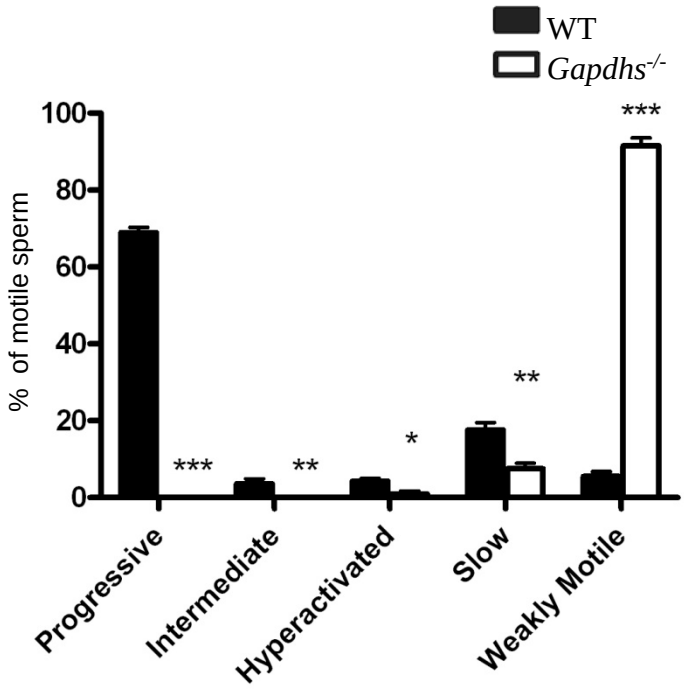
WT
Gapdhs-/-
% of motile sperm
100
80
60
40
20
0
***
**
*
**
***
Progressive
Intermediate
Hyperactivated
Slow
Weakly Motile

Figure 4

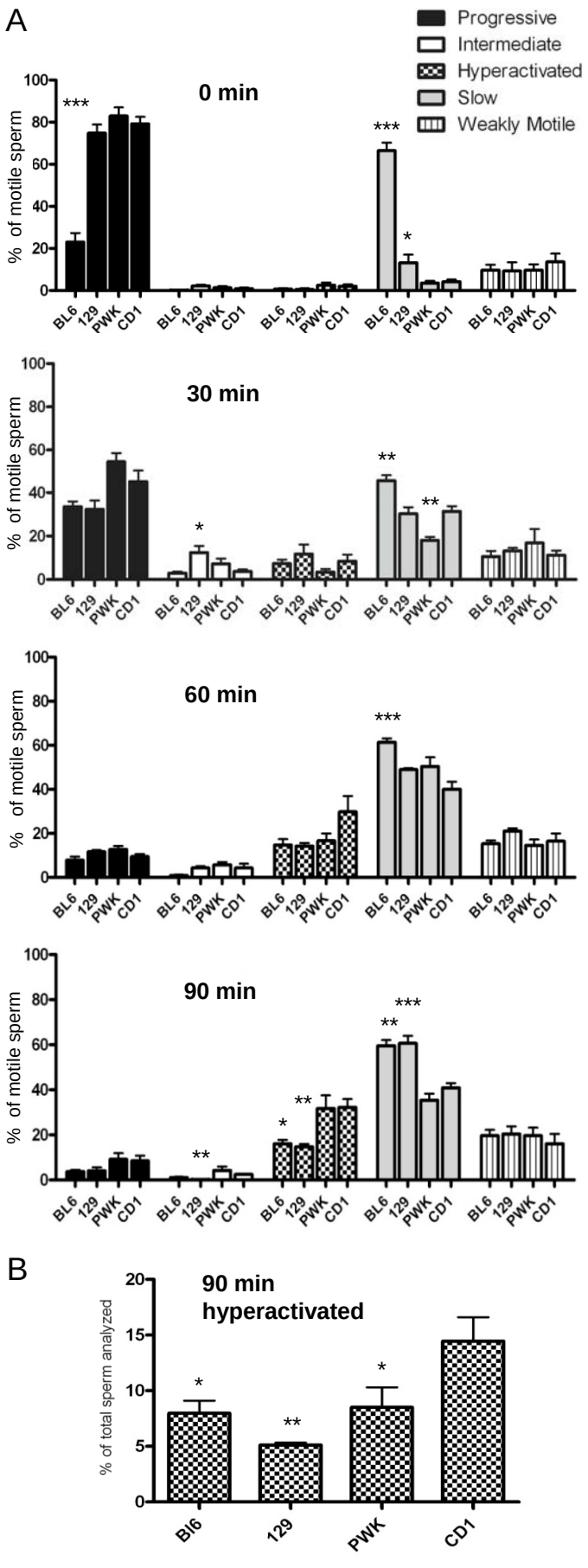
A
Progressive
Intermediate
Hyperactivated
Slow
Weakly Motile
0 min
30 min
60 min
90 min
% of motile sperm
BL6
129
PWK
CD1
B
90 min
hyperactivated
% of total sperm analyzed

Figure 5