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

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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 common method in supervised machine learning. This multiclass SVM model is based on more than 2000 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 nonvigorous categories. Additional equations classify vigorous tracks as progressive, intermediate, or hyperactivated and nonvigorous 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.

computer-assisted sperm analysis, hyperactivation, sperm capacitation, sperm motility and transport, support vector machines

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 symmetrical, low-amplitude flagellar bends [4]. Sperm obtained from the oviduct display motility characterized by more asymmetrical, higher-amplitude 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 (for review, see [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 (for review, see [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 frames are captured at 60 Hz (0.5 sec), although extended tracking intervals have proven to be 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, asymmetrical 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 (VCL) and straightness (STR) [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 [14], 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 approximately 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 affect 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 a software program, which we named CASAnova, 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. except sodium chloride and glucose (Fisher Scientific); sodium pyruvate (Invitrogen); sodium bicarbonate (EM Science); potassium chloride, magnesium sulfate heptahydrate, and potassium phosphate (Mallinckrodt Chemical); and penicillin/streptomycin 100× stock solution containing 10 000 U/ml of penicillin G and 10 mg/ml of streptomycin (Gemini Bio-Products).

Human tubal fluid (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 [28, 29]. HTF complete medium consists of 101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 2.78 mM glucose, 0.33 mM pyruvate, 21.4 mM lactate, 5 mg/ml of bovine serum albumin, 100 U/ml of penicillin G, and 0.1 mg/ml of 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 approximately 315 mOsm/kg with 5 M NaCl using a Model 3300 micro-osmometer (Advanced Instruments).

Animals and Sperm Collection

Adult CD1 male mice were purchased from Charles River Laboratories and allowed to acclimatize before use. C57BL/6J (BL6), 129S1/SvImJ (129), and PWK/PhJ (PWK) male mice were kindly provided by Fernando Pardo-Manuel de Villena (Department of Genetics, University of North Carolina at Chapel Hill). $Gapdhs^{-/-}$ and wild-type 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 (age, >8 wk) mice. Each cauda was carefully trimmed to remove adipose and other tissue, rinsed in PBS (140 mM NaCl, 3 mM KCl, 4 mM $NaH_2PO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4; pH 7.4), and placed in 1 ml of HTF medium 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°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 approximately $2–4 \times 10^5$ 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 noncapacitating 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°C under 5% CO_2 in air, and motility was assessed at 30-min intervals. Initial time points were completed within 2 min 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). 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°C with a 4× 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. The default settings include the following: 60 frames per second, 90 frames acquired, minimum contrast = 30, minimum size = 4 pixels, default cell size = 13 pixels, default cell intensity = 75, cells counted as progressive if average path velocity (VAP) > 50 μm/sec and STR > 50%, slow cells counted as motile, low VAP cutoff = 10 μm/sec , low straight-line velocity (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 pipette tip into one chamber (depth, 100 μm) of a prewarmed Leja slide. To minimize drift in the medium, 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 were generated for every sperm population analyzed at every time point, providing field, track, VAP, VSL, VCL, amplitude of lateral head displacement (ALH), beat cross frequency (BCF), 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°C 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 because 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 a Microsoft Excel worksheet along with their visual classification to create the training data set. All classified tracks and parameters were loaded into Matlab software (release 2009b; The Mathworks). 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, and hyperactivated) or nonvigorous (slow and weakly motile). The function then generated an equation that best separated the two groups of data in multidimensional space (SVM1; see Results). This process was repeated within the vigorous and nonvigorous groups to generate four equations used to classify motility patterns. We have submitted a patent application for the use of SVMs to classify sperm motility patterns based on CASA parameters [32].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). All data are shown as the mean ± SEM. Statistical significance was determined using either two-tailed unpaired t-tests or one-way ANOVA after arcsine transformation of percentages. Differences were considered to be significant if $P < 0.05$. The percentage agreement of the model was evaluated by calculating the Cohen 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
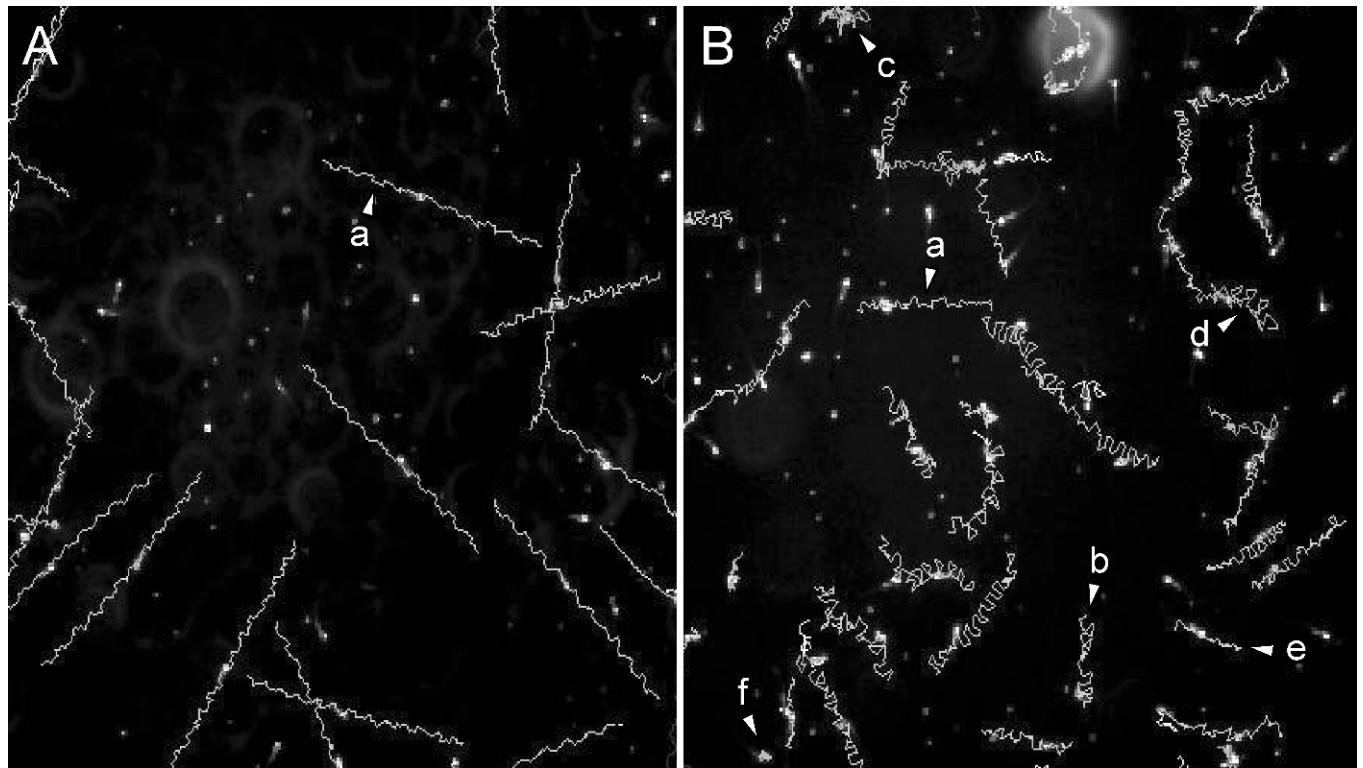
FIG. 1. Changes in sperm motility patterns during in vitro capacitation. Representative CASA fields showing sperm tracks immediately after isolation (0 min; **A**) and after 90 min (**B**) of incubation in HTF complete medium. Representative tracks corresponding to five distinct motility groups used to generate the multiclass SVM model are shown: progressive (a), intermediate (b), hyperactivated (c and d), slow (e), and weakly motile (f).
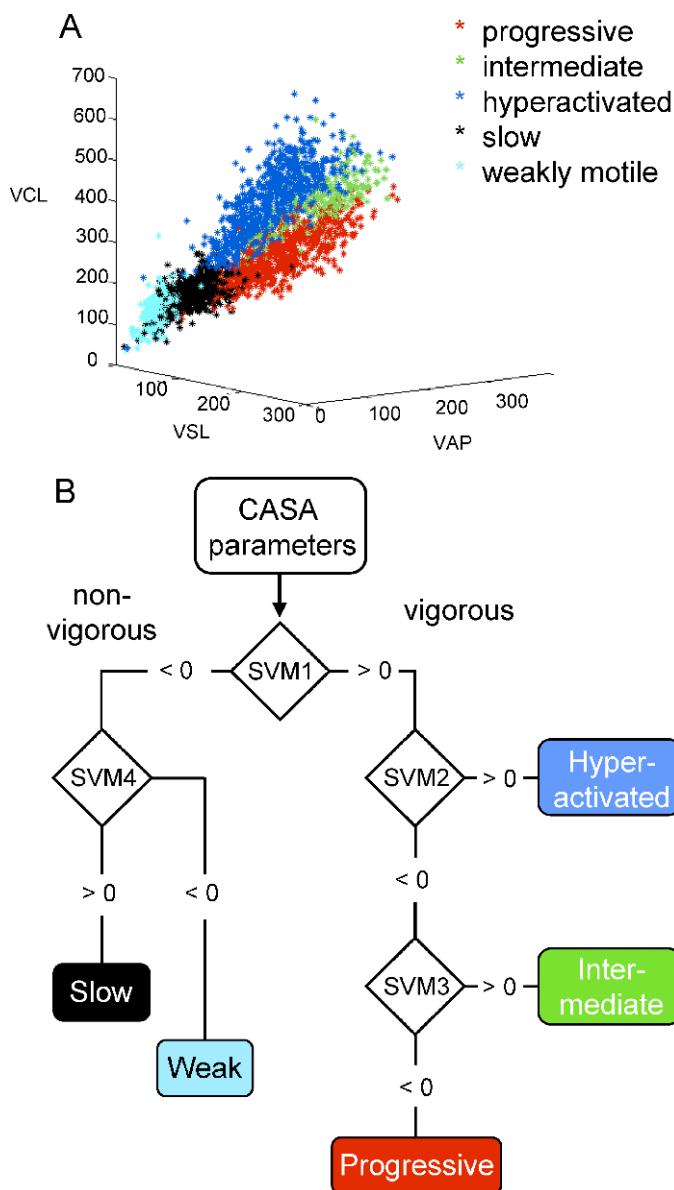
90 min (Fig. 1B) indicates that motility changes during this interval from predominantly progressive to more varied profiles that are less linear. The first step in developing a quantitative model that distinguishes these different types of motility was to generate a training set of sperm tracks for analysis. We collected CASA tracks of sperm incubated for 90 min 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, and hyperactivated) in our training set had mean VCL values of greater than 279 μm/sec, whereas the nonvigorous groups (slow and weakly motile) had mean VCL values of less than 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° along the majority of the track (track a in Fig. 1, A and B; see also Supplemental Fig. S1A). Intermediate sperm tracks showed more vigorous motion, with larger deviations from the net direction of movement and angles of approximately 90° along most of the track length (track b in Fig. 1B; see also Supplemental Fig. S1B). Sperm were classified as hyperactivated if they displayed highly vigorous motility accompanied by turns of greater than 90° 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; see also 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; see also 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; see also Supplemental Fig. S1D). Tracks that were motile but not vigorous and did not have significant forward motion were characterized as weakly motile (track f in Fig. 1B; see also Supplemental Fig. S1E). To maintain very strict criteria for defining the five motility patterns, we excluded tracks that were

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 ± SD. 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.

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, and 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.

derived from sperm with abnormalities such as flagellar bending at the annulus or adherence to other sperm (~400 tracks), that were the result of sperm collisions (~300 tracks), or that could not be identified confidently (~400 tracks). A

total of 2043 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. Because 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 zero were incorporated to determine their effect on the SVM equations. Because these time-zero 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 SVMs. We developed a set of equations that mathematically define the boundaries that 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, and hyperactivated) and nonvigorous (slow and weakly motile). The program generated a binary equation that separates these two groups in the training set (SVM1 in Table 2). If CASA parameters from an unclassified track are applied to this equation and the result is greater than zero, the track is

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

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

* 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

classified as vigorous. Otherwise, the track is classified as nonvigorous. 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 zero and then removes them from further examination. Vigorous sperm tracks that have an SVM2 value of less than zero are further analyzed by SVM3. Tracks are classified as intermediate if their SVM3 value is greater than zero or as progressive if less than zero.

Tracks with SVM1 values of less than zero are classified as nonvigorous. This nonvigorous 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 zero, whereas SVM4 values of less than zero are categorized as weakly motile.

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 nonprofit 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 1068 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 percentage 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, whereas CASAnova classified the remaining tracks in this group as progressive or hyperactivated in approximately equal proportions. We also employed Cohen 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 Noncapacitating 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 noncapacitated sperm, we generated motility profiles of sperm from six CD1 mice incubated in HTF complete medium with or without 25 mM bicarbonate over a 2-h time course (Fig. 3). Whereas the percentage of motile sperm in both media remained above 50% throughout the time course (Supplemental Fig. S2A), marked differences were found 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 in Fig. 3A). In this experiment, progressive tracks declined to
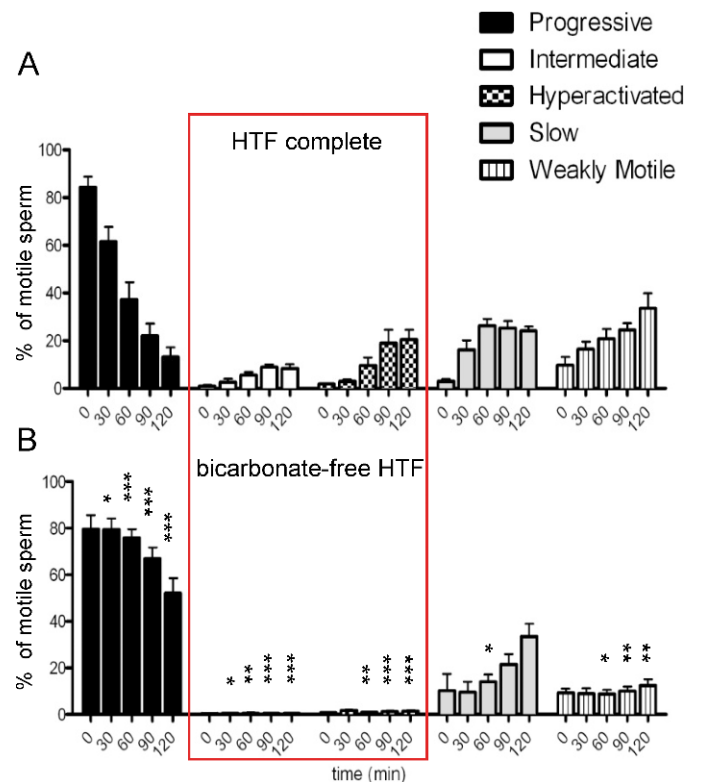

FIG. 3.   Time-dependent changes in the distribution of sperm motility patterns during in vitro capacitation. Motility of sperm from six CD1 mice was assessed at 30-min intervals during incubation in HTF complete medium with (**A**) or without (**B**) 25 mM sodium bicarbonate, and CASA parameters were 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 percentage (mean ± 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. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
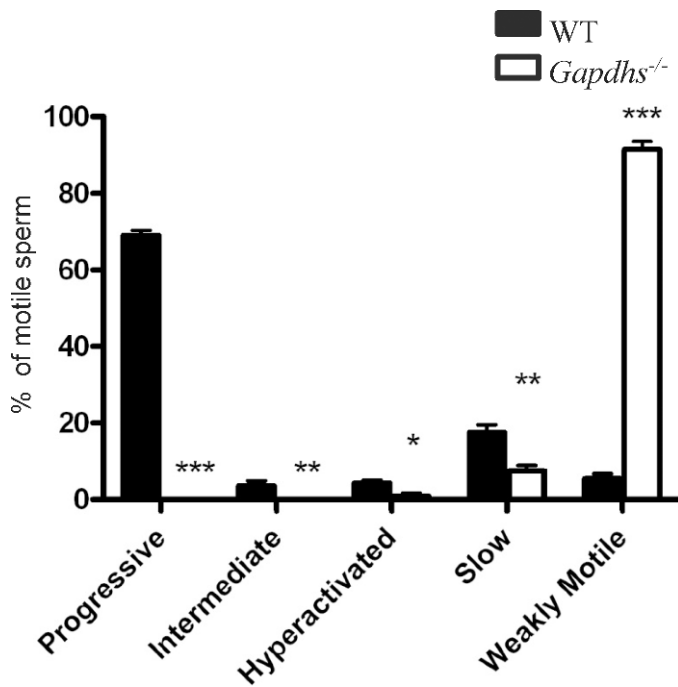
FIG. 4. Distribution of sperm motility patterns in wild-type (WT) and *Gapdhs*$^{-/-}$ mice. CASAnova analysis of CASA parameters was used to compare motility patterns of sperm from wild-type (black bars, n = 3) and *Gapdhs*$^{-/-}$ mice (white bars, n = 3). Bars represent the percentage (mean ± SEM) of motile tracks identified in each group immediately after isolation in HTF complete medium (time zero). Significance was determined using two-tailed unpaired *t*-test. *P < 0.05, **P < 0.01, ***P < 0.001.

[30]. CASAnova classified 99% of GAPDHS-null sperm as weakly motile or slow (open bars in Fig. 4). Sperm from wild-type mice with the same genetic background (mixed 129S6/SvEvTac and C57BL/6NCrl) displayed predominantly progressive motility (black bars in Fig. 4). 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 BL6, 129, and PWK inbred mouse strains over a 90-min incubation in HTF complete medium (assayed in parallel, n = 4 mice/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 those of CD1 sperm immediately after isolation, except that a higher percentage of 129 sperm were classified as slow (time, 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 (mean, 79.2% progressive and 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%), whereas PWK hyperactivation was comparable to CD1 hyperactivation. To determine whether reduced hyperactivation resulted from 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. No statistically significant differences were found between hyperactivation levels determined visually or by using CASAnova (Supplemental Fig. S4). BL6 sperm did display more asymmetrical 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 percentage 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 × 10^6) than from CD1 mice (mean, 31 × 10^6), but we analyzed more than 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 min) 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.

22.2% of all motile tracks by 90 min, whereas intermediate tracks increased to 9% and hyperactivated tracks to 18.9%. The proportion of sperm with nonvigorous motility (slow or weakly motile) also increased during in vitro capacitation, reaching 49.9% by 90 min. Further increases in nonvigorous 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 nonvigorous 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 in 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

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 no consensus exists regarding the parameters that best define hyperactivation. We used CASA parameters from 2043 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 approximately 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 is greater than 50 μm/sec and STR is greater than 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 50 μm/sec. In contrast, the progressive tracks in our training set were linear and had 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, whereas sperm that have linear tracks with substantially lower velocities (VAP, 85.2 ± 19.0 in our training set) are classified as slow. Inclusion of the nonvigorous 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% or more 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
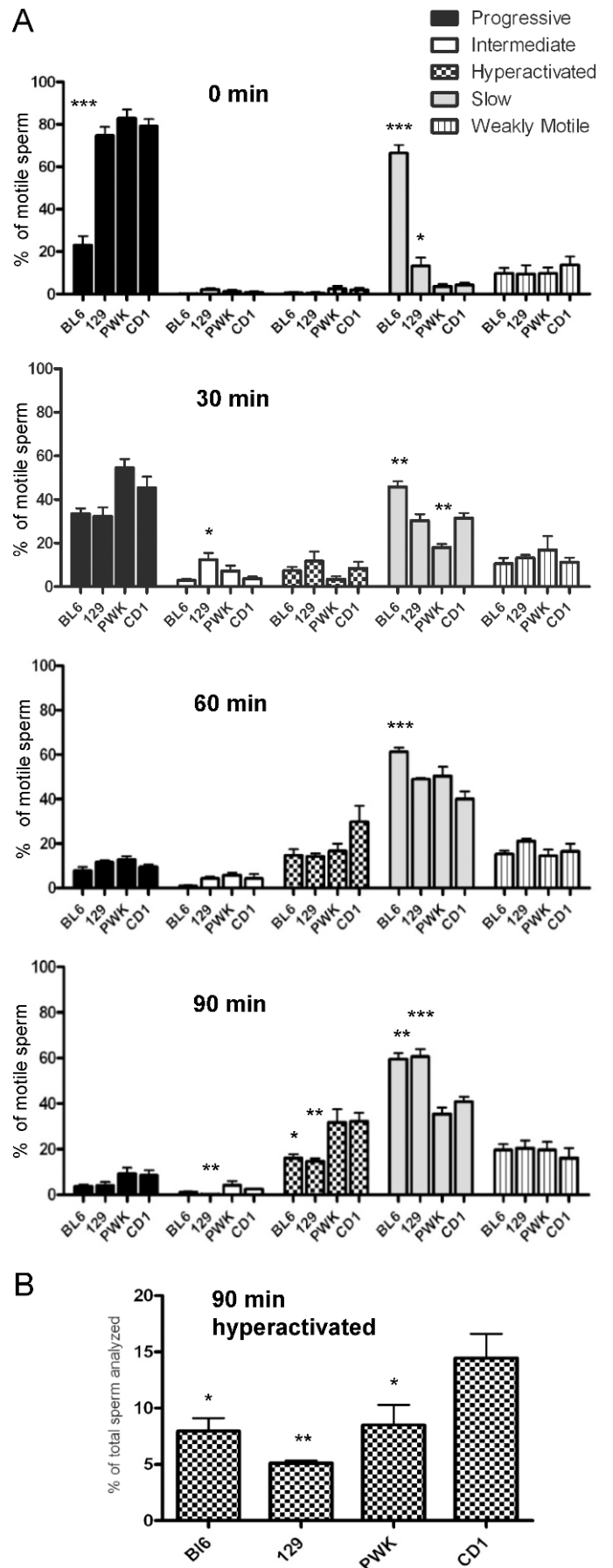

FIG. 5. Differences in motility profiles between inbred and outbred mouse strains. **A**) Sperm from BL6, 129, PWK, and CD1 mice (n = 4 mice/strain) were incubated in HTF complete medium and assayed by CASA over a 90-min time course, followed by analysis by CASAnova. Bars represent the percentage (mean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

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 approximately 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 approximately 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 asymmetrical flagellar beats, and it highlights the ability of CASAnova to accurately identify physiological alterations in motility that occur during capacitation.

The ability 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 BL6 mice are substantially less vigorous at initial time points than sperm from two other inbred strains (129 and PWK). This difference is interesting, because both BL6 and 129 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 40 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]. For cases in which 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 in assessing genetic, biomolecular, and pharmaceutical effects on sperm motility.

ACKNOWLEDGMENTS

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