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Technical note

Automated sperm head morphology analyzer for open-source software

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Abstract

Sperm head morphology has been identified as a characteristic that can be used to predict a male's semen quality. In the present study, we have developed an automated sperm head morphology analysis (ASMA) plug-in for open-source ImageJ software (http://rsbweb.nih.gov/ij/). We describe the plug-in's functionality, and confirm its validity for sperm head morphology analysis using fish sperm. Sperm head morphological measurements (length and width) made with the ASMA plug-in did not differ from manual measurements. Using the plug-in to measure sperm head-shaped objects of known size, the associated plug-in error rate was $< 0.5\%$. Brightness and contrast ratios influenced sperm head measurements, suggesting the need for standardized protocols. This plug-in was effective at measuring elliptical (i.e., Atlantic cod) as well as slightly irregular (i.e., Chinook salmon) shaped sperm heads. In conclusion, our ASMA plug-in represents a versatile alternative to costly sperm morphology software.

Keywords: Sperm morphology; Sperm head; Automated Sperm Morphology Analysis; Sperm competition; Atlantic cod; Reproductive biology

1. Introduction

Sperm morphology has been identified as a characteristic that can be used to predict a male's ability to fertilize an egg in competitive and non-competitive contexts [1,2]. Morphological assessment of sperm also has importance in the investigation of biotechnology to cryopreserve sperm [3–5], assessing triploidy [6], male infertility [7], the impact of pollutants on sperm quality [8], describing the effects of water and saline solutions on sperm functionality [9], evolutionary significance of sperm form and function [10], and phylogenetic classification [11].

Several microscopic techniques have been used to study sperm morphology. For instance, scanning electron microscopy, transmission electron microscopy and cryo-electron microscopy have been widely used to examine morphology and ultrastructure of sperm [12–14]. Although these systems provide greater resolving power than a traditional light-powered optical micro-

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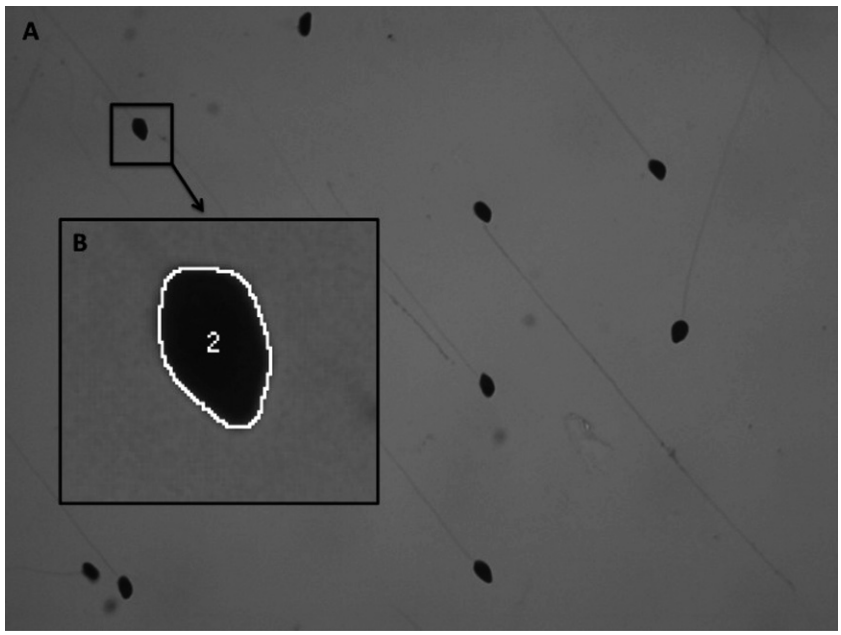

Fig. 1. Digital image of Atlantic cod *Gadus morhua* sperm before (A) and after (B) running the automated sperm morphology analysis (ASMA) plug-in. The sperm image was captured at the 50:50 brightness to contrast ratio.

scope, there are some disadvantages. For example: electron microscopes are costly; specialized equipment and numerous chemicals are required to prepare samples; selection of an appropriate fixative and methodology for sample preparation needs to be considered; interactions between artificial media and fixatives need to be known; and preparation of slides, image capture and data analysis are time consuming, especially to obtain the large samples sizes required for high statistical power. Therefore, more conventional microscopic techniques, such as dark-field microscopy in combination with stroboscopic light, and phase contrast microscopes, have been used to study sperm morphology [15]. More recently, automated sperm morphology analysis (ASMA) software has been developed to process morphology samples [16,17]. This computer-driven software has advantages over other types of microscopy, as it provides faster, automated assessment of sperm morphology [18]. However, these ASMA systems can be expensive, as well as require standardized staining techniques.

In the present study, we explored the use of open-source ImageJ software as an alternative to expensive systems for automatically measuring sperm head morphology. An open-source computer assisted sperm analysis (CASA) plug-in has already been developed for ImageJ to quantify sperm movement, and our plug-in will compliment this software [19]. Thus, the objectives of the present study were to present a freely available ASMA plug-in, describe its functionality, and confirm its validity for sperm head morphology analysis using Atlantic cod (*Gadus morhua*). Atlantic cod sperm samples were used to: (i) compare sperm head length and width from the ASMA plug-in and manual measurements; (ii) determine the accuracy and precision of the ASMA plug-in using calibrated sperm head shapes; and (iii) establish the ASMA plug-in's image detection thresholds using various brightness and contrast ratios. In addition to the above objectives, the versatility of the ASMA plug-in for measuring less elliptical sperm head shapes was tested using Chinook salmon (*Oncorhynchus tshawytscha*) sperm.

2. Materials and methods

Detailed methodology for sperm collection, staining, digital imagery and functional operation of the ASMA plug-in are provided via the Supplementary material (available in the online version only).

In the present study, for each Atlantic cod male ($n = 15$), 30 sperm were captured and measured using the ASMA plug-in (Fig. 1). The same 30 sperm, per male, were manually measured in ImageJ [20].

To determine the accuracy and precision of the ASMA plug-in, 50 calibrated sperm head shapes were

created using CorelDRAW. These artificial sperm heads were elliptical in shape and had lengths ranging from 1.3 to 3.5 μm and widths ranging from 1.0 to 2.0 μm. The ratio of sperm head length:width ranged from 1.2 to 3.0. The ASMA plug-in was then used to measure length and width of these calibrated shapes and associated error rates [ER = (plug-in value – actual value/actual value) × 100] were generated.

Furthermore, to establish the ASMA plug-ins image detection thresholds, 50 Atlantic cod sperm were captured, using a factorial design, over a series of six brightness (20, 40, 50, 60, 80 and 100) and six contrast values (20, 40, 50, 60, 80 and 100). The 50:50 brightness to contrast ratio then served as the control and associated ER deviating from these values were generated for the other brightness to contrast ratios.

To test the versatility of the ASMA plug-in a total of 50 Chinook salmon sperm, from a pooled semen sample (n = 5 males), were captured at the 50:50 brightness to contrast ratio and then measured using the ASMA plug-in. The same 50 sperm were manually measured in ImageJ.

2.1. Statistical analyses

All data were analyzed using SAS statistical analysis software [version 9.1; 21]. Residuals were tested for normality (Shapiro-Wilk test; PROC UNIVARIATE) and homogeneity of variance (plot of residuals vs predicted values; PROC GPLOT). As needed, data were $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity. Alpha was set at 0.05. To compare Atlantic cod sperm head length and width from the ASMA plug-in and manual measurements, repeated measures mixed-model ANOVAs were run (PROC MIXED) using the following model:

$$Y_{ipn} = \mu + M_i + T_p + MT_{ip} + \varepsilon_{n(ip)}$$

where μ is the population mean; M_i is the male effect (where i = male 1 to 15); T_p is the effect of treatment (where p = ASMA plug-in and manual measurement); MT_{ip} is the male × treatment interaction; and $\varepsilon_{n(ip)}$ is the residual error. Treatment was considered fixed, whereas male and the corresponding male × treatment interaction were considered random (PROC MIXED). The Satterthwaite procedure was used to approximate the denominator degrees of freedom for F-tests [22]. The repeated statement (Repeated treatment/Subject = sperm) was used to model the covariance structure within subjects [23]. Treatment means were contrasted using the least squares means method [LSMEANS/CL adjust = TUKEY, PROC MIXED]. Linear regressions were used to determine the relationship between the ASMA plug-in and manual measurement (PROC REG). Relationships were generated for both Atlantic cod sperm head length and width. For Chinook salmon a paired Student's *t*-test (PROC TTEST) was used to compare treatment means (ASMA plug-in versus manual measurement). Data are presented as mean ± SEM.

3. Results

Atlantic cod sperm head length did not differ ($F_{1,14}$ = 0.80, $P > 0.05$) when measurements were obtained from the ASMA plug-in or manually measured (3.147 ± 0.050 and 3.150 ± 0.050 μm, respectively). In addition, Atlantic cod sperm head width did not differ ($F_{1,14}$ = 4.04, $P > 0.05$) between the ASMA plug-in and manual measurement (1.905 ± 0.040 and 1.897 ± 0.040 μm, respectively). Regression analyses demonstrated highly significant relationships between the ASMA plug-in and manual measurements for Atlantic cod sperm head length ($R^2 = 0.96$, $P < 0.0001$, $y = 1.0067x - 0.0181$) and width ($R^2 = 0.95$, $P < 0.0001$, $y = 1.0198x - 0.0424$). The slopes for these relationships were not significantly different than 1.0 for both length (df = 455, t = 0.74, $P > 0.05$) and width regressions (df = 452, t = 1.83; $p > 0.05$).

Length and width of the 50 calibrated shapes were 2.712 ± 0.065 and 1.481 ± 0.034 μm, respectively. When measuring these same shapes using the ASMA plug-in, mean length was 2.715 ± 0.065 μm and width was 1.485 ± 0.034 μm for associated plug-in ER of 0.13 and 0.34%. Re-running the ASMA plug-in generated the same results for successive analyses of the same data (data not shown).

Atlantic cod sperm heads were detected and measured by the ASMA plug-in at 21 of the 36 brightness to contrast ratios tested (Table 1). To explore how brightness and contrast ratios impacted Atlantic cod sperm size assessment, the 50:50 brightness to contrast ratio was used as the control (Table 1). The ER deviating from the control ranged from −0.03 to −6.7% for length and 0.0 to 8.5% for width (Table 1). For both these indices, the 40:50 brightness to contrast ratio had the lowest ER (Table 1). In general, ER increased at the 80 and 100 brightness ratios (Table 1).

Chinook salmon sperm head length did not differ (t = 1.34, df = 49, $P > 0.05$) when measurements were obtained from the ASMA plug-in (2.851 ± 0.011 μm) or manually measured (2.849 ± 0.011 μm). In addition, salmon sperm head width did not differ (t = 1.81, df = 49, $P > 0.05$) between the plug-in and manual mea-

Table 1
Atlantic cod sperm (n = 50) were captured over a series of six brightness and contrast ratios (20, 40, 50, 60, 80 and 100). The plug-in was then used to measure length and width of these sperm. The 50:50 brightness to contrast ratio served as the control and associated error rates deviating from these values were generated for the other ratios. Mean values are reported for each ratio and associated error rates are in brackets.

	Contrast					
	20	40	50	60	80	100
Length (μm)*						
Brightness						
20	ND	ND	ND	ND	ND	ND
40	ND	3.007 (0.7)	2.986 (−0.03)	2.976 (−0.4)	ND	ND
50	ND	3.039 (1.7)	2.987 (0.0)[C]	2.967 (−0.7)	ND	ND
60	ND	3.066 (2.6)	3.011 (0.8)	2.963 (−0.8)	ND	ND
80	3.162 (5.9)	3.104 (3.9)	3.062 (2.5)	3.004 (0.6)	2.917 (−2.3)	2.788 (−6.7)
100	3.052 (2.2)[IT]	3.058 (2.4)[IT]	3.073 (2.9)[IT]	3.066 (2.6)[IT]	3.062 (2.5)[IT]	3.065 (2.6)[IT]
Width (μm)*						
Brightness						
20	ND	ND	ND	ND	ND	ND
40	ND	1.882 (0.7)	1.868 (0.0)	1.863 (−0.3)	ND	ND
50	ND	1.909 (2.2)	1.868 (0.0)[C]	1.857 (−0.6)	ND	ND
60	ND	1.937 (3.7)	1.888 (1.1)	1.849 (−1.0)	ND	ND
80	2.027 (8.5)	1.972 (5.6)	1.931 (3.4)	1.887 (1.0)	1.804 (−3.4)	1.713 (−8.3)
100	1.929 (3.3)[IT]	1.935 (3.6)[IT]	1.933 (3.5)[IT]	1.937 (3.7)[IT]	1.938 (3.7)[IT]	1.864 (−0.2)[IT]

[ND] Sperm were not detectable by the plug-in; * SEM was ± 0.02; [C] Represents the control; [IT] Sperm were incorrectly traced by the plug-in.

surement (2.292 ± 0.017 and 2.288 ± 0.017 μm, respectively).

4. Discussion

Sperm morphology is influenced by genetic and environmental components experienced by an individual during development [24,25], as well as, by numerous factors applied to sperm cells during sample preparation (i.e., staining techniques, and the quality of the image analysis system). Adding to this complexity is that sperm morphological assessment is often confounded by a researcher's individual bias and inconsistency, thus making accurate interpretations of findings between laboratories difficult [16]. Standardized staining techniques, like those employed for fish [26], bull [27], boar [28] and humans [29], can increase sample validity, especially when contrast ratios between the sperm image and background are "optimized". In addition, ASMA programs, such as the Sperm-Class Analyzer [17], Sperm Morphometry Module of ISAS [26], and the Metrix Oval Head Morphology software component of the Hamilton-Thorne CEROS system [16] pose means of generating reproducible and quantitative measurements of sperm cells.

In the present study, we have developed a freely available ASMA plug-in for open source software, described the systems functionality (via the Supplementary material), and confirmed its validity. With respect to the ASMA plug-ins' data generating capabilities, a total of nine morphological indices are automatically measured (e.g., area, perimeter, roundness) [20], and additional indices such as ellipticity and elongation can be manually calculated [26]. The majority of these indices have been proven valuable for predicting male fertility [2,30], sperm velocity [10], and/or post-thaw sperm performance [31].

Similar to species such as the ram [17], red deer [31], bull [32], and brown bear [33], after staining fish sperm, it is difficult to distinguish the head from the midpiece, unless specific staining procedures are used to differentiate the nucleus (DNA) from the mitochondria. Nevertheless, our plug-in was unable to discriminate between colors. Therefore, it should be noted that measurements obtained from the ASMA plug-in incorporated both these morphological structures. Our results showed no significant differences in length and width of sperm heads when data were generated from the plug-in or manually measured. The slope of regression analysis also demonstrated highly significant relationships between the ASMA plug-in and manual measurement treatment and were not different from 1.0, suggesting that there were no systematic biases when the two measurement approaches were compared.

To examine inter- and intra-male variability in sperm head morphology indices, data sets containing ~10,000 to 23,000 sperm cells have been easily obtained using our ASMA plug-in [25,34]. In this study,

it took ~1.25 h to measure sperm morphology (n = 15 males × 30 sperm) using our ASMA plug-in, whereas ~13 h was needed to manually measure these same sperm samples. Thus, this is a highly time-efficient system (~10x faster) for evaluation of sperm head morphology and avoids bias introduced by different people conducting morphological measurements. When testing the ASMA plug-in against calibrated shapes, it produced accurate (ER < 0.5%) and precise results. An important part of the sperm measurement procedure was standardization of image quality settings. Brightness and contrast ratios influenced sperm measurements. Therefore, when using this plug-in, or any sperm software, it is important to standardize measurement conditions, especially with respect to brightness and contrast ratios. This essentially will reduce the probability of errors associated with an algorithm's inability to discern sperm detection boundaries.

In conclusion, our freely available ASMA plug-in, for open source ImageJ software, represents an alternative method to costly ASMA software. The plug-in is effective at measuring elliptical (i.e., cod) as well as slightly irregular (i.e., Chinook salmon) sperm heads. Replication of our system should be possible by other laboratories, as should modification of the settings to fit a particular cell size.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.theriogenology.2011.06.019.

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S1. Supplementary material

S1.1. Atlantic cod sperm collection and sample preparation

Wild Atlantic cod were captured from the Bay of Fundy, Canada in 2004. Fish were fed a mixture of frozen baitfish. In February and March 2005, gametes were stripped from these fish and first filial generation (F1) families were created using IVF. Embryo incubation and larval rearing followed standard aquaculture protocols [1]. Juveniles were placed into common tanks in September 2005. Each tank was equipped with flow-through seawater and housed under a natural photoperiod regime. These fish were fed daily with a marine grower pelletized diet (EWOS Canada, Surrey, BC, Canada). During the spawning season fish were maintained between 3 and 5 °C. Semen samples were collected from 15 of these F1 cod (weight = 1374.3 ± 592.1 g, length = 46.9 ± 1.2 cm) during March 2008. Prior to sample collection, fish were anesthetized with MS-222 (76 mg/L; Syndel International, Vancouver, BC, Canada). Light pressure was applied on the abdomen of each fish and the milt was collected with 40 mL dry beakers. The initial male ejaculate was discarded and the external urogenital pore was wiped dry with a paper towel to avoid contamination. After stripping, semen samples were held in a temperature controlled (4–6 °C) cold room until processed.

Atlantic cod sperm smears were prepared in the manner described by Butts et al [2]. In brief, sperm smears were prepared by diluting 10 μL of semen in a solution composed of seawater (500 μL) and 3% citrate sodium (500 μL) [3]. It should be noted that in order to obtain a large data set, comprised of thousands of sperm cells in a relatively short time-span, a proper sperm dilution ratio should be used. The ratio chosen should be species specific or vary seasonally to coincide with changes in sperm density. Ultimately, it is best to choose a final dilution that prevents overlapping of sperm cells so as to avoid inaccuracies in the assessment of each cell's dimensions. The sperm solution, as just mentioned above, was gently shaken for 30 s. For each male, 5 μL of this sperm solution was then pipetted onto a microscope slide (prewashed with 95% ethyl alcohol) and prepared using Hemacolor [3]. The smears were allowed to air dry, then permanently sealed with Eukitt mounting medium (Kindler & Co., Freiburg, Germany) and topped with a coverslip. Digital images of sperm heads were captured using a Leica microscope (model DMLB; Leica Microsystems Inc., Richmond Hill, ON, Canada) equipped with a micropublisher 3.3 RTV digital camera (Q-imagining, Surrey, BC, Canada; Fig. 1 from the main text). Sperm heads were measured at random for each smear with a 100× oil immersion objective. A digital image was also captured of a stage micrometer, preferably equipped with a linear rather than circular measure, to be later used to calibrate the plug-in. It should be noted that the scale of the micrometer should to be appropriate for the magnification and microscope used. For instance, if sperm images are captured using a specific microscope, objective lens, and digital camera, the micrometer image should also be captured using this same system.

S1.2. Chinook salmon sperm collection and sample preparation

To test the versatility of the ASMA plug-in we used Chinook salmon as an experimental organism. In comparison to Atlantic cod, Chinook salmon sperm are not so elliptical (cells are shaped more like a "gumdrop").

Wild Chinook salmon were collected from the Credit River in Mississauga, ON, Canada (43°35'N, 79°42'W) from 3 to 6 of October 2010 using electrofishing techniques. Upon capture, fish were humanely sacrificed and semen samples were immediately placed into clear bags and then stored on crushed ice until use. During the entire sampling process special care was taken to avoid contamination of semen with water, blood, or urine. For each male, sperm smears were prepared and stained using Diff-Quik according to methods described by Tuset et al [4]. Digital images of sperm heads were captured using an Olympus BX51 microscope equipped with an Olympus DP72 digital camera.

S1.3. Installation and calibration of the ASMA plug-in

ImageJ is an open source JAVA application (National Institutes of Health, Bethesda, MD, USA) and is available for download at http://rsbweb.nih.gov/ij/. Several steps are required to properly calibrate and synchronize the ASMA plug-in with corresponding sperm head images. First, ImageJ (Mac OS X v. 1.42q or higher) was opened and the digital image of the micrometer was loaded (click *File* » *Open* » find the previously saved micrometer image). In this case the image had been captured using the system as described above. Next, the straight-line selection tool, located on the toolbar, was used to make a straight line (on the loaded micrometer image) that corresponds to a known distance (i.e., 100 μm; Fig. 2). The *Set Scale* dialog was then opened (click *Analyze* » *Set Scale*), and

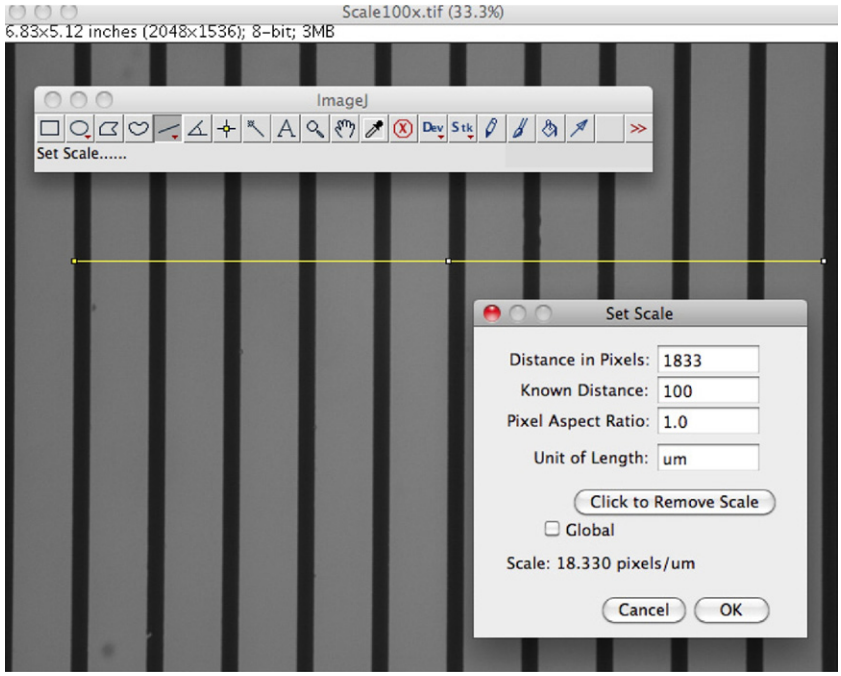

Fig. 2. Steps required for calibrating and synchronizing the ASMA plug-in with corresponding sperm head images.

the *Known Distance* and *Unit of Length* boxes were properly adjusted (Fig. 2). ImageJ automatically filled in the *Distance in Pixels* based on the length of the straight line. The ASMA plug-in file entitled (*Sperm_morphology.txt*; Appendix: A, online version only) was then placed in the ImageJ *plugins* folder. On the first line of the plug-in the *distance* and *known* statements were then modified to correspond with values previously specified in the *Set Scale* dialog [run("Set Scale . . .", "distance=1833 known=100 pixel=1 unit=μm global"); Fig. 2]. In addition, on the 15th line of the plug-in [run("Analyze Particles . . .", "size=X-X circularity=X-X bins=256], the *size* and *circularity* detection thresholds were adjusted according to the sperm morphology literature that is available for Atlantic cod [2,5,6]. For instance, we chose a size threshold between 2–7 μm and a circularity threshold between 0.70–1.0 [run("Analyze Particles . . .", "size=2-7 circularity=0.70-1.0 bins=256].

S1.4. Measurement of sperm head morphology using the ASMA plug-in

Digital images of sperm heads were opened (click *File* » *Open* » find the previously saved *image*) and then parameters were automatically measured using the ASMA plug-in (click *Plugins* » *Sperm morphology*). By checking the *Area*, *Shape Descriptors, Perimeter,* and *Fit Ellipse* boxes in the *Set Measurements* dialog (click *Analyze* » *Set Measurements*) ImageJ can automatically generate nine sperm head morphology parameters [7]. In this study we focused solely on sperm head length and width, as these are two of the most commonly measured sperm head morphology indices [8–10]. Images of sperm and their boundaries are displayed by the plug-in to enable the researcher to perform a quality control check and eliminate sperm detection errors if needed (Fig. 1 from the main text). Cells that are not properly situated in the image's two-dimensional plane (i.e., standing vertically) or are overlapping should be excluded from any further analysis. The output file, containing all the sperm head measurements, was then "cut and pasted" into Excel, at which time sperm detection errors were deleted.

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