ВОДНІ БІОРЕСУРСИ

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SWIMMING FISH SPERMATOZOA – ANALYSIS OF FLAGELLAR MOVEMENT[1]

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In most animal species, spermatozoa motility is dependent on a long appendage called flagellum. The flagellum behavior during the motility period can provide more deeper understanding of basis of spermatozoa motility in a more detailed way and complementary to the methods used so far which consist mainly in the analysis of fish sperm head behavior (velocity, linearity of tracks, etc) obtained by regular CASA. In the present review, a description of the different mechanisms accounting for sperm flagellum movement is presented, with special emphasis regarding to fish spermatozoa. Features and difficulties encountered when studying fish sperm movement are described in detail.

Key words: flagellum, spermatozoa motility, sperm flagellum movement, fish spermatozoa.

Formulation of the problem. Spermatozoa are highly specialized cells, crucially involved in animal reproduction. Efficiency of subsequent fertilization success depends mainly on their movement ability. Fish spermatozoa swim for very short periods right after being shed in the surrounding medium where fertilization occurs (in most fish species, with external fertilization), but during this brief period, their motility characteristics change in many respects [4].

The structure of sperm flagellum is quite ubiquitous and well in described but nowadays the achievements in this field are still inconspicuous in respect to flagella movement [6]

Understanding of the mechanisms involved in movement of undulipodia (a general term which appoint for both flagella and cilia) can be investigated at several levels of resolution: 1- by observation of the translating motion of the

[1] The present review is dedicated to memory of Yurij Pilipenko.

cell body (head in case of spermatozoon), 2- by description of the variations of the flagellar shape during its active movement, 3- at a macro-molecular level, by dissecting the arrangement of its main components as mainly EM magnification allows to describe them, 4- at the detailed level involved in the description of the operation of each specific element such as the mono-molecular motor part. A global view of the flagellar mechanics need the integration between these 4 levels but for concision and description of our aims, it is more convenient to expose below in details mostly the biophysical approach pointed out in the above 2-description, while the other aspects (1-, 3- or 4-) will be approached as complementary to the targeted hydro-dynamical aspects of quantitative description.

In the present review, we intend to investigate in detail the fish sperm motility characteristics and technics for movement analysis in order to evaluation of sperm flagellum behavio .

1. Specificities and difficulties in the studies of fish sperm moveme

Just after activation by the surrounding medium, sperm flagella of many fish species beat at a high frequency, 50 to 100 Hz but for a short period of time, i.e. thirty seconds to several minutes depending on fish species [11]. The initial velocity of fish sperm cells is very high (150 to 300 μm sec-1), which makes observations and records especially difficult. To study the motility characteristic (velocity of spermatozoa, percentage of motile cells) of these very fast moving cells and to visualize their flagella, several specific methods have been developed allowing the obtainment of images of the tracks followed by sperm head, or alternatively images of flagella obtained in vivo while moving. Nowadays, these methods use video techniques, and in some cases, allow to record sharp images thank to stroboscopic illumination at a frequency up to 800 Hz. The computer assisted processing of the frames or more simply of the head tracks (CASA) allows to compare parameters of movement of fish spermatozoa exposed to various physiological situations, leading to comparative information describing their potency for swimming, in relation to their ability to fertilize and/or to be cryopreserved, but also the sensitivity of their swimming traits to traces of potent chemical pollutants [11].

1.1. Activation of motility.

As fish spermatozoa are usually immotile in the seminal fluid, one must transfer them in a specific solution where motility is activated. The activating solution contains solutes (ions or non-ionizable compounds) which greatly affect the osmotic pressure of the fluid [7]; depending on origin of the fish species, marine or fresh water, the osmotic pressure of activation solution must be significantly higher or lower than that of the seminal fluid, respectively [11]. In some particular species, the osmotic pressure is not the only factor and the concentration of some specific ions needs to be adjusted; this is the case of the K^+ concentration which must be greatly decreased in some species such as trout

[13], sturgeon [1-2], or paddlefish [19] while in others such as turbot [14] and species [17], it is the CO2 concentration which must be highly reduced to very low values. These constrains will dictate the composition of the solution used as optimal swimming medium for motility or, in contrast, optimal diluents preventing motility [25].

1.2. Period of motility.

Usually in many fish species, sperm motility lasts for short periods (several minutes), or even shorter (30 seconds) [2,10]. This means that one should avoid any pre-activation of motility by any fluid eventually contaminating the semen during stripping [15, 21] or by external water [22] or even by a solution used to transiently store sperm (diluent), otherwise, when tested for its motility sperm would be classified as immotile even though its initial potent motility was possibly correct. A second implication is that any appreciation of motility parameters needs microscopical observation, very early in order to get access to values characterizing the most efficient part of the motility period, (earliest one). As this earliest period may be lasting for only few seconds after contact with the activating solution, usually one needs to mix sperm directly in a drop of activating solution (AS) previously set up on the glass slide and to video record this very short period in order to measure motility parameters [8] during this crucial time window. Thus, a "blind" period of several seconds, where no video recording can occur, is inescapable during which mixing in the drop and correct focusing of the microscope is achieved.

1.3. Morphology of fish spermatozoa

The structure of the axoneme, as seen on Fig. 1 is canonically composed of 9 double microtubules (doublets) approximately 20 nm diameter constituting

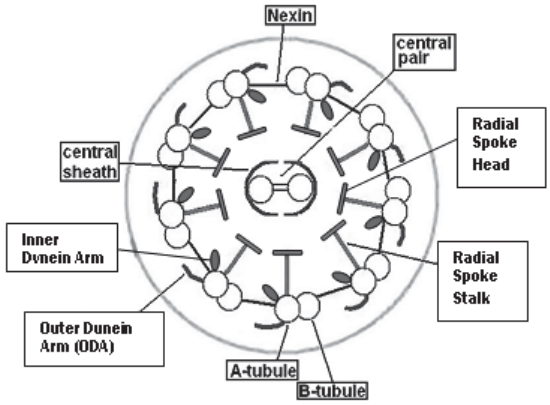

Fig. 1. The "9+2" axoneme of flagella and cilia. The whole structure is wrap in the flagella membrane which is in continuity with the cell membrane

the periphery of a cylinder which centre is occupied by two central microtubules (singlets), the whole scaffold being succinctly referred as "9+2". Conventionally, the 9 outer doublets are numbered starting from number one located in the plane orthogonal to the plane including the two central singlets; importantly, the former plane allows to define the curvature directions during beating as left or right relatively to that plane; by convention, counting is clockwise when looking from the tip of the flagellum. These microtubules are continuous all the way long of flagella, continuity is a condition of absolute necessity for the motile function of any axonemal doublet microtubule.

The head of fish spermatozoa usually ranges 3-4 μm width (frequently spherically shaped) and this makes them quite easy to observe with a microscope at low magnification. In contrast, the diameter of the sperm tail so called flagellum, which usually ranges less than 1 μm, makes it difficul or even impossible to be observed with a regular microscope, whatever the magnification used (even 40×, 60× or 100×), because of their vigorous movement which blurs the corresponding images. Therefore, observation of moving flagella usually requires two specific adaptations: (a) a dark fiel microscope making flagella appearing as a thin bright white filament ranging 40-60 μm length, on a fully black background, giving rise to a high optical contrast between the flagellum and its surrounding environment; (b) a stroboscopic illumination in order to obtain sharp images of the moving fl - gellum because during each very brief flash of light (ranging one to several micro-second duration, the image of the tail appears without any apparent movement) in successive video frames. Such combination of these observation technologies allows to obtain a detailed morphological analysis of the integrity of the flagellar length [5, 26]. Another feature of fish sperm flagell which turn out to be an advantage for observation resides also in their fla shape: in many fish species, the entire length of flagellum is ribbon shaped instead of cylindrical [23] as in the case of "simple" flagella such as those of sea urchin sperm [9], which makes its ability to diffuse light very efficien when oriented parallel to the direction of the light beam. The consequence is that flagella appear, relatively brighter when observed by dark field microscopy according to specific angles, therefore giving indications regarding its orientation relative to the light beam.

1.4. Sperm motility parameters.

Values of sperm motility parameters rapidly decreased after induction of sperm motility by discharge into activating solution. This general rule applies to most fish species, with variation in the motility duration between species but in most cases leads to full stop within a short period of time (several minutes or less) as already mentioned. This implies that any measured parameter must refer to a precise time point at which it was measured after movement

initiation, when these parameters are used to compare conditions in an intra- or inter-species way [8-10].

Flagella's beat frequency (number of beat cycles in one second) measurements are possible by changing of the frequency of stroboscopic illumination, or by counting the records with a high-speed camera. Velocity is speed of movement sperm head.

Types of velocity with are usually measured:

Velocity curvilinear (VCL) Point to point velocity (total distance traveled) per second.

Velocity average path (VAP) Point to point velocity on a path constructed using a roaming average. The number of points in the roaming average is 1/6th of the frame rate of video used.

Velocity straight line (VSL) Velocity measured using the first point and the average path and the point reached that is furthest from this origin during the measured time period.

1.5. High initial velocity and beat frequency.

Difficulty is that the initial velocity (during the 2-3 seconds following movement initiation) of fish spermatozoa is very high (up to 300 μm /sec), which is a consequence of their high beat frequency (up to 70-100 Hz). Evaluation of these two parameters is very important to obtain, because they occur at a period of the motility phase, which is the most efficient for a spermatozoon to approach and eventually reach the surface of the egg for fertilization. In contrast, this period is very difficult technically to get access to for two main reasons: (a) it follows immediately the blind period of mixing of sperm in the AS on the glass slide and (b) the very fast movement of individual spermatozoa allow them to stay only for very short periods both in focus and inside the observation field of the microscope. Fish sperm flagellum is clearly visible when using high microscopical magnification such as 20× or 40× objective lenses [8-9]

1.6. Density of spermatozoa.

In the seminal fluid, the density of spermatozoa appears very variable depending on fish species. As examples, the following values of sperm density (same units) were observed: 1 to 5.10^9 in carp [24], 50×10^9 in sea bass [15], 5 to 15×10^9 in trout [13] and 0.1 to 4×10^9 in sturgeon [26]. Therefore, the density is usually much too high to clearly distinguish individual cells without dilution of milt in a non-swimming solution. In contrast, the sperm density in milt is high enough to allow a high dilution rate in the AS leading to correct observation of individual swimming cells. An appropriate method to solve the problem of too high density in the seminal fluid is to use a two-step procedure [3]: sperm (including seminal fluid) is diluted at first in a non-swimming solution (a 1:50 dilution ratio is a good starting point) where it can stay without risk of motility activation, then this firstly diluted sperm is secondly diluted in a AS

previously located as a drop on the microscope glass slide. This method needs some knowledge of the conditions where to activate vs inactivate spermatozoa of the investigated fish species. It is frequently necessary to get access at least to two major characteristics of seminal plasma of the given fish species, i.e., concentration of K+ and osmolality [25].

1.7. Energetics.

As already mentioned, motility of fish spermatozoa is lasting for a short period. A main reason appears to be due to limitation of energy by the lack of ATP that occurs very rapidly right after motility activation. Actually, ATP consumption by axonemal dyneins ATPase (acting as motors for generating movement) is much faster than ATP production by the mitochondrion. This phenomenon has been observed in sea bass [15], in turbot [14] and in carp [22] sperm. A consequence is that a sperm cell which have exhausted its ATP store during a first round of motility is potentially able to reinitiate motility after a resting period during which ATP is restored to a normal level in a solution non permissive for motility [4]. It means that there are common examples where spermatozoa, apparently unable to move because of an « accidental » activation (contact with external medium at collection or with urine), could be revived to potential motility after such a resting period [13, 24].

1.8. Computer Assisted Sperm Analysis (CASA).

The method of CASA used for the rapid and objective assessment of sperm motility using the sophisticated image analysis methodology. This method used frequently in human fertility clinics. CASA (computer assisted sperm analysis) frequently used for the analysis of mammalian sperm motility and the human, it is very difficult to used for fish sperm which is generally motile for less than 2 min compared to several hours for mammalian sperm. In the CASA system may automatically measure many parameters, such as: Mean lateral head displacement (ALH); Straightness (STR) – a measure of VCL side to side movement determined by dividing VAP by VCL; Wobble (WOB) VAP/VCL, describes side to side movement of the sperm head. Mean velocity (VAP); Linear velocity (VSL); Curvilinear velocity (VCL); Frequency of head displacement (BCF); Linearity coefficient (LIN)

2. Technik's for fish sperm flagella observatio

2.1. Regular video camera.

A work with regular video camera usually uses stroboscopic illumination is shown below. In each shot, separate positions of a spermatozoon are thus recorded (frequency regular video camera 50 Hz) as observed at 50 or 100 Hz, but more detailed records are possible provided an augmentation of the frequency of stroboscopic illumination is applied, but in this case, one can see an overlapping of the flagellar images (150-300 Hz) making difficult the individualization of each position (Fig. 2).

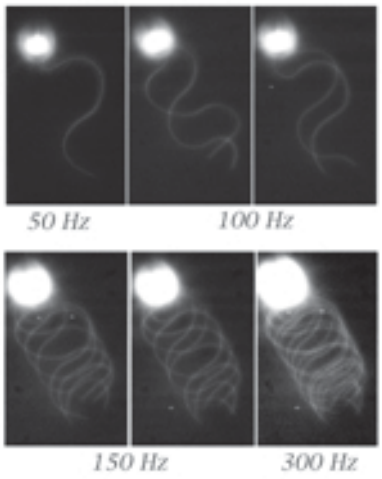

Fig. 2. Example of overlapping: resulting from the augmentation of frequency of stroboscopic illumination to 150 Hz, the image analysis becomes complicated, and at 300 Hz, it becomes impossible

2.2. High-speed video camera.

For sperm observations and for obtained detailed images of moving spermatozoa can be used next technics: Olympus BX50, 500×, with 100× Phase contrast optics (Zeiss Ph 3 NeoFluar 100×Oil immersion) and recorded with a high-speed video camera (Olympus i-speed TR) providing 848x688 pixels spatial resolution, 1000 frames/sec (Fig. 3 and Fig 4.). For regular sperm observations, an Olympus BX50 microscope, with dark-field optics (objective 20×) illuminated by pulsed light from a stroboscopic lamp (Chadwick-Helmut, 9630, USA) was combined with video recording by 3CCD video camera (SONY DXC-970MD, Japan).

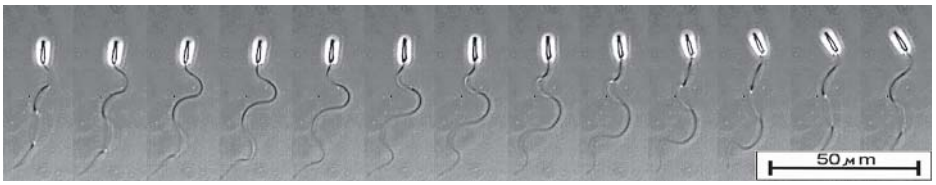

Fig. 3 Example of high resolution video-images (1000 frames per second, the time interval between two images 2 μsec time duration of one full cycle of beating 26 μsec which corresponds to 13 images in this example, for prepare this image rate taken each second frame)

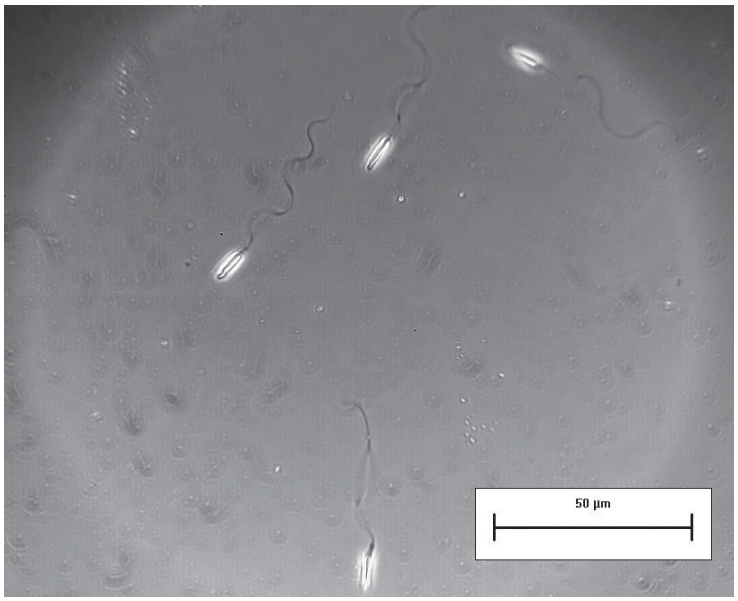

Fig. 4. An example of one individual video frame shot with a high-speed camera at 1000 frames per second, image size 848×688 px., resulting in record of individual positions of flagellum without overlap at high frame rate

Analysis of Fig. 3 and 4 makes it possible to obtain information about the sperm movement such as: direction of movement of the head, rotation of spermatozoon's, wave length, wave amplitude, the number of waves and beat frequency of waves, defects occur in the membrane. The use of high-speed camera allows as to get information about the movement of sperm, not previously been used for the analysis of their mobility for example records collected from high-speed camera we obtained sperm parameters.

Conclusion and perspectives of further research. One main characteristic of fish spermatozoa is that they remain immotile in the fish seminal tract and testes due to the high osmolality and ionic composition of the seminal plasma which prevent sperm motility in fish sperm ducts [18] but a second characteristics is that fish sperm becomes motile after discharge into the aqueous environment due to external signals, e.g. low K+ concentrations in acipenserids or hypo-osmotic shock in freshwater teleost fishes [20] or opposite, for marine fish. During microscopic observation, spermatozoa move at water-glass or water-air interfaces which contrasts with natural situation occurring during fish reproduction where spermatozoa moves in the water column. Fish spermatozoa are also characterized by short and very rapid motility (from 40 sec and up to few minutes) with 50-90 Hz initial frequency of flagella beating [1].

ПЛАВАЮЩИЕ РЫБНЫЕ СПЕРМАТОЗОИДЫ – АНАЛИЗ ДВИЖЕНИЯ ЖГУТИКА

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У большинства видов животных подвижность сперматозоидов зависит от длинного придатка, называемого жгутиком. Поведение жгутика в течение периода подвижности может более детально осмыслить основы подвижности сперматозоидов и дополнить методы, используемые до сих пор, которые состоят в основном из анализа поведения головки рыбной спермы (скорость, линейность треков и т. д.), полученных CASA. В статье представлено описание различных механизмов учета движения жгутика спермы с особым акцентом на рыбные сперматозоиды. Подробно описаны особенности и трудности, возникающие при изучении движения спермы рыбы.

Ключевые слова: жгутик, подвижность сперматозоидов, движение жгутика спермы, сперматозоиды рыб.

ПЛАВАЮЧІ РИБНІ СПЕРМАТОЗОЇДИ – АНАЛІЗ РУХУ ДЖГУТИКА

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У більшості видів тварин рухливість сперматозоїдів залежить від довгого придатка, названого жгутиком. Поведінка джгутика протягом періоду рухливості може більш детально осмислити основи рухливості сперматозоїдів і доповнити методи, що використовуються до цих пір, які складаються в основному з аналізу поведінки головки рибної сперми (швидкість, лінійність треків і т. д.), отриманих CASA. У статті представлено опис різних механізмів обліку руху джгутика сперми з особливим акцентом на рибні сперматозоїди. Детально описано особливості і труднощі, що виникають при вивченні руху сперми риби.

Ключові слова: джгутик, рухливість сперматозоїдів, рух джгутика сперми, сперматозоїди риб.

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