

Review article

# The measurement of sperm motility and factors affecting sperm quality in cultured fish

E. Rurangwa<sup>a,\*</sup>, D.E. Kime<sup>b</sup>, F. Ollevier<sup>a</sup>, J.P. Nash<sup>a</sup>

<sup>a</sup>*Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Ch. De Beriotstraat 32, B-3000 Louvain, Belgium*

<sup>b</sup>*Department of Animal and Plant Sciences, The University of Sheffield, Sheffield S10 2TN, UK*

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## Abstract

The fish farming industry has been more focused on the quality of eggs or larvae rather than that of sperm, even though the quality of both gametes may affect fertilisation success and larval survival. In some species, poor sperm quality can be a limiting factor in their culture, however, even when fertilisation success is high, differences in sperm quality between males when mixed sperm from multiple males is used may severely reduce the apparent population size and may affect the future genetic integrity of the stock. Sperm quality in farmed fish may be affected by different components of broodstock husbandry, during collection and storage of sperm prior to fertilisation or the fertilisation procedure. Although other approaches for quantification of sperm quality have been suggested, motility is most commonly used since high motility is a prerequisite for fertilisation and correlates strongly with fertilisation success. The assessment of sperm motility has historically relied on subjective estimates of motility characteristics, the value of which is questionable in predicting fertility. Computer-assisted sperm analysis (CASA) systems that were initially developed to examine sperm quality in mammals and birds have only recently been applied to fish sperm. CASA can play an important role in aquaculture since it can rapidly and objectively quantify the effects of husbandry conditions and sperm handling on sperm motility and hence, fertilising capacity in farmed fish. This paper reviews existing methods of sperm quality assessment in fish, surveys the factors affecting quality and shows how the application of computer-calculated motility analysis may achieve a better understanding and quantification of the impact of aquaculture practices on sperm quality and fertilisation success. The review focuses primarily on teleost fish which predominate in both

\* Corresponding author. Tel.: +32-16-32-39-66; fax: +32-16-32-45-75.

E-mail address: [eugene.rurangwa@bio.kuleuven.ac.be](mailto:eugene.rurangwa@bio.kuleuven.ac.be) (E. Rurangwa).

aquaculture and research, but also includes some of the studies on sturgeon which are of increasing interest in aquaculture.

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## 1. Introduction

### 1.1. Why do we need good quality sperm?

The use of high quality gametes from captive fish broodstock is of great importance for ensuring the production of valuable offspring for aquaculture (Kjørsvik et al., 1990; Bromage and Roberts, 1995). The fish farming industry has been more focused towards the quality of eggs and larvae rather than that of sperm, even though the sperm quality of male broodstock also affects the production of healthy larvae. Nevertheless, in commercial hatcheries, milt is often inadequate both in terms of quantity and quality and does not always give successful fertilisation in the artificial insemination procedures commonly used for aquaculture species. This is particularly the case in species that do not readily release sperm by hand stripping like the African catfish (*Clarias gariepinus*) in which intratesticular sperm has to be taken out of the testes, species that require hormonal induction of spermiation or for which sperm is exposed to urine contamination during hand stripping. Moreover, in some fish species, a limited amount of sperm is released; turbot (*Psetta maxima*) and yellowtail flounder (*Pleuronectes ferrugineus*) produce less than 1 ml of milt per fish (Suquet et al., 1992a; Clearwater and Crim, 1998). Moreover, milt production can be insufficient to support artificial fertilisation because multiple batches of eggs produced during artificially extended spawning seasons mean that the males are repeatedly handled and are stripped of all their milt.

To overcome some of the problems mentioned above, fish semen preservation has become an indispensable alternative in fish selection and synchronisation of gamete availability. However, the treatment of sperm prior to storage and the cryopreservation conditions are known to reduce its ability to fertilise the eggs (Billard, 1983; Saad et al., 1988; Dreanno et al., 1997).

Mixing of sperm from different males is a very common practice in the culture of many commercial species. So while the fertilisation success may seem high, it is possible that not all the males are equally contributing to the gene pool due to sperm competition (see Bekkevold et al., 2002; Gage et al., 1998; Mjølnerod et al., 1998; Vladic et al., 2002). If the pool is too small, there is a risk of severe genetic bottlenecks and inbreeding that would ultimately produce homozygous strains of low fitness. Moreover, there are practical reasons why in some aquaculture situations, it is advantageous to use only a low ratio of male to female broodstock and this lowers limit of the number of males used is fixed by genetic considerations. As an example, it is especially costly to maintain males in some species which have late maturity such as the Siberian sturgeon *Acipenser baeri*, which matures at up to 5–6 and 6–8 years for males and females, respectively (Williot et al., 2000). When a low number of male broodstock is used, it is therefore especially important

to ensure that the sperm quality is good between all males and that they are all contributing substantially to the gene pool. Understanding differences in sperm quality between males may help to understand the relative success of each male and how these differences could be reduced to maintain the genetic integrity of the broodstock used.

As in the process of broodstock selection, artificial reproduction in hatcheries as well as in the management of cryo sperm banks requires adequate, rapid and sensitive tools to assess sperm quality during the different steps of artificial insemination. While spermatocrit, viability and motility are scored relatively easily, the usefulness of motility measurements has long been questioned since *subjective* scoring methods are used and have produced variable results. In spite of the recent introduction of computer-assisted sperm analysis (CASA) systems which have provided an *objective* assessment of sperm quality in fish, their application to improve the culture conditions of male broodstock in fish farms and the success of artificial fertilisation in hatcheries is still in its infancy. The assessment of the quality of fish spermatozoa using *computer-assisted techniques* is a *novel field* and little practical progress has been achieved in aquaculture science.

## 1.2. Objectives

The objectives of this paper are three-fold: (a) to define sperm quality in fish (Section 2); (b) to give an overview of some of the more traditional methods used for the assessment of sperm quality (Section 3) and to highlight the advantages of using computer-aided analysis of sperm motility as a measure of sperm quality (Section 4); and (c) to review the factors that affect sperm quality (Section 5).

## 2. Definition and characteristics of sperm quality in fish

### 2.1. Definition of sperm quality

Ultimately, sperm quality is a measure of the ability of sperm to successfully fertilise an egg. Any quantifiable physical parameter that directly correlates with the fertilisation capacity of sperm could be potentially used as a measure of sperm quality. Through the process of natural selection, the number and characteristics of sperm will be optimised so as to maximise the fitness of the individual male in relation with the particular reproductive strategy of each species. In natural circumstances, males are in competition and depend on the reproductive strategy and physical environment of their species. The result is that the teleost species produce different types and quantities of sperm (Taborsky, 1998). In some cases, processes such as sperm competition may drive individuals to produce an excess of sperm to ensure fertilisation (see Mjølnerod et al., 1998; Peterson and Warner, 1998). In aquaculture, however, different specific characteristics may be required for successful *in vitro* fertilisation. Such methodologies rely overwhelmingly on using an excess of sperm mixed with eggs in a small volume of fluid. Even though this is wasteful, if quality falls below a minimal threshold then fertilisation rates may rapidly decrease even under those conditions. At present, there is little general information on how much the quality can deteriorate before fertilisation rate is affected. Furthermore, if mixed batches of sperm are

used, differences in sperm quality may be important in reducing the apparent population size and genotype diversity and it is difficult to determine the relative success of each male. Perhaps the more important driving force behind finding useful measures of sperm quality other than fertilising ability is to have a measure that can be applied to a batch of sperm without the need for eggs. This would enable the sperm to be analysed prior to fertilisation and circumvents the practical difficulties of using eggs in fertilisation tests.

There has therefore been a drive towards measuring other parameters or biomarkers of sperm quality that directly relate to the fertilisation ability of sperm. Biomarkers of sperm quality so far documented include spermatocrit, sperm density, osmolarity and pH of seminal plasma, chemical composition of seminal plasma, enzymatic activity, adenosine triphosphate (ATP) concentration, motility, morphology and ultrastructure, fertilising capacity, and several others (Billard and Cosson, 1992; Billard et al., 1995a,b; Ciereszko and Dabrowski, 1993, 1994; Lahnsteiner et al., 1996a, 1998; Fauvel et al., 1998; Geffen and Evans, 2000; Chowdhury and Joy, 2001). Since studies describing milt characteristics, which can influence fertilisation ability, have shown large individual variations in the different parameters investigated (Dreanno et al., 1998) this rendered difficult the use of a single sperm characteristic to define good sperm quality. Other works show that non-motile spermatozoa can fertilise eggs (Truscott and Idler, 1969). All these examples highlight again the limitations of using a single trait to define the quality of the sperm. Indeed, some of the parameters are relatively easily scored and commonly used (spermatocrit, viability and subjective motility) while others need sophisticated laboratory analyses (biochemical analyses), expensive equipment (objective and quantitative motility) or availability of eggs (fertilisation success).

## 2.2. General characteristics of fish sperm

Spermatozoa of are stored in seminal plasma fluid in the genital tract and in contrast with mammals, most externally fertilising teleosts have sperm that are immotile on ejaculation. Spermatozoa only become motile and metabolically active after release into the water. In most freshwater species, spermatozoa usually moves for less than 2 min and in many cases is only highly active for less than 30 s (Morisawa and Suzuki, 1980; Perchec et al., 1993; Billard et al., 1995a; Kime et al., 2001). Some fish species such as the spotted wolffish (*Anarhichas minor*) and the 3- and the 15-spined sticklebacks (*Gasterosteus aculeatus*, *Spinachia spinachia*), which are characterised by release of eggs in a sticky gelatinous mass, have sperm which remains motile for a far longer period after release (Elofsson et al., 2003a,b; Kime and Tveiten, 2002). In these species, the sperm has characteristics which differ markedly from that of the majority of other teleost species: the sperm is motile on stripping, remains so for 1–2 days and becomes immotile in contact with seawater. Similar characteristics have also been found in some marine sculpins (Koya et al., 1993), and the ocean pout *Macrozoarces americanus*, a species which has internal fertilisation (Yao and Crim, 1995). The spermatozoa of the ocean pout remain motile in seminal fluid or in specially designed milt diluent for up to 5 days at 4 °C without losing much activity (Yao et al., 1999). The gametes of chondrosteian fish such as the sturgeon and paddlefish differ from the general teleost model. Sperm with an acrosome and eggs with numerous micropyles coexist in sturgeons (Linhart and Kudo, 1997; Linhart et al.,

1995). Methods of assessment of sperm quality must take these factors into consideration (Cosson et al., 2000).

### 3. Assessing sperm quality in fish

Historically interest in developing tools to assess the sperm quality in fish has been motivated by several goals, including the need to improve methods of artificial fertilisation, the preservation of male gametes, and more recently, to study the impact of exposure to environmental pollutants on fish reproductive success. Although several simple and complex techniques have been used, these did not always reflect the fertilising capacity of the sperm, the ultimate measure of sperm quality.

#### 3.1. Fertilising capacity

Indirect measure of sperm quality, using its fertilising capacity, may not be reliable since the quality of ova may be variable and affect fertilisation success. In some species, the availability of eggs at the same time as the sperm may limit the period during which tests of quality can be conducted. Other factors such as the number of spermatozoa per egg, the duration of contact between gametes or the fertilisation protocol employed, may also influence the fertilisation success (Suquet et al., 1995; Chereguini et al., 1999). In commercial production, an optimal sperm to egg ratio has been recommended, while in experimental work, using fertilisation success as an endpoint of sperm quality a minimum sperm/egg ratio was suggested (Suquet et al., 1995; Rurangwa et al., 1998).

One of the most important uses of fertilisation tests is to check the validity of other measures of sperm quality. For example, sperm motility and fertilisation capacity of either fresh or frozen–thawed milt are correlated in rainbow trout (Lahnsteiner et al., 1996b), carp (Magyary et al., 1996), African catfish (Rurangwa et al., 2001) and Atlantic halibut (Tvedt et al., 2001). At some stage, all other measures of sperm quality should be validated against successful egg fertilisation, even though this may be time consuming.

#### 3.2. Whole milt characteristics

##### 3.2.1. Spermatocrit and sperm density

The concentration of sperm in the seminal fluid has been traditionally used for the assessment of sperm quality in fish. The standard method for determining sperm density (sperm cells/ml milt) in fish milt is to count spermatozoa generally using a haemocytometer or a similar counting chamber (Buyukhatipoglu and Holtz, 1984). Since the method is time-consuming, both centrifugation to determine spermatocrit (%; the ratio of volume of white packed material to total volume of milt  $\times 100$ ) and spectrophotometry to determine optical density have been used to rapidly determine sperm density. A direct relationship between sperm density and optical density or spermatocrit of fish sperm has been established in coho salmon, *Oncorhynchus kisutch* (Bouck and Jacobson, 1976), Atlantic salmon, *Salmo salar* (Piironen, 1985), rainbow trout, *Oncorhynchus mykiss*, whitefish, *Coregonus clupeaformis*, yellow perch, *Perca flavescens* (Ciereszko and Dabrowski, 1993), Atlantic halibut,

*Hippoglossus hippoglossus* (Tvedt et al., 2001) and other species. In the turbot (*P. maxima*), however, sperm density was correlated with optical density but not with spermatocrit (Suquet et al., 1992b).

Accuracy and precision in sperm counts depend on the ease of sperm pipetting (Tvedt et al., 2001). Aggregations of spermatozoa are sometimes observed under the microscope during counts and small volumes of high-density milt are subject to pipetting errors. While in most fish species, after centrifugation, a clearly defined interface exists between the packed sperm cells and the clear seminal fluid, there was no sharp separation between the phases in yellow perch (*P. flavescens*) (Ciereszko and Dabrowski, 1993), which could induce a false estimate of the spermatocrit. Since both spermatocrit and optical density are easy to measure, the choice is generally based on access to equipment but the type of apparatus used to count the sperm cells may have an impact on the results obtained. Using a Coulter counter as a replacement to a haemocytometer proved to be faster, and a positive correlation between the spermatocrit and spermatozoa density was obtained with the Coulter Counter ( $r^2=0.75$ ) but not with the haemocytometer ( $r^2=0.13$ ) in Atlantic cod *Gadus morhua* (Rakitin age coefficient of variation was high with the haemocytometer (27.7%, range 5.1–80.0%) versus 9.0% (range 2.4–25.7%) for the Coulter Multisizer).

The spermatocrit and the viscosity of the milt also vary between conspecific males, between species and across the reproductive season (Piironen, 1985; Munkittrick and Moccia, 1987; Methven and Crim, 1991; Christ et al., 1996; Rakitin et al., 1999). The spermatozoa concentration declines as the spawning season advances in rainbow trout, *O. mykiss* (Buyukhatipoglu and Holtz, 1984) and carp, *Cyprinus carpio* (Christ et al., 1996; Lubzens et al., 1997), and in lake sturgeon (*Acipenser fluvescens*) spermatocrit changes from year to year (Toth et al., 1997).

Until now, a noticeable lack of standardisation in units in which the sperm density is expressed persists. The sperm concentration is calculated either in number of sperm cells collected per kilogram body weight (cell/kg), per gram of testis or per fish (cells/fish). Although the number of spermatozoa per ml of milt is most appropriate, it must be complemented by the total volume of milt. Highly concentrated sperm does not always give the highest motility or the highest fertilisation rate (Geffen and Evans, 2000; Williot et al., 2000). Sperm concentration, therefore, is not a particularly sensitive and specific measure of sperm fertilising capacity and may vary greatly within a given fish species. It is however important to note that the sperm concentration becomes a relatively important characteristic when fertilising eggs with a constant volume of milt to analyse the fertilising capacity of different milt samples. Even though widely used in the past, such an approach is not scientifically correct since the results are biased by different numbers of spermatozoa available per egg for fertilisation.

### 3.2.2. Constituents of the seminal plasma

The composition of teleost fish milt has been investigated since decades (Piironen and Hyvärinen, 1983; Billard and Menezo, 1984; Linhart et al., 1991). Seminal plasma and enzymatic profile of sperm have received considerable attention in the past decades, mostly in salmonids (Lahnsteiner et al., 1998) and in cyprinids (Billard et al., 1995b). Plasma analysis includes: inorganic constituents ( $K^+$ ,  $Na^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ) involved in the



process of inhibition or activation of sperm motility (Morisawa, 1985; Morisawa et al., 1983a,b,c), organic compounds indicative for energy metabolism (triglycerides, glycerol, fatty acids, glucose, lactate; Lahnsteiner et al., 1993) and several enzymes (acid phosphatase, alkaline phosphatase,  $\beta$ -D-glucuronidase, protease, malate dehydrogenase, lactate dehydrogenase, adenosine triphosphatase, aspartate aminotransferase; Lahnsteiner et al., 1996c; Babiak et al., 2001). The most important substrates and metabolites of the spermatozoal metabolic pathways studied are ATP, ADP, AMP, NADH, lipids, fatty acids, glucose, lactate, pyruvate, creatine phosphate (Lahnsteiner et al., 1998). The enzymes creatine kinase, malic enzyme, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase and lactate dehydrogenase are also measured and used to assess the sperm quality in fish species such as the herring (Gronczewska et al., 2003), catfish and carp (Rurangwa et al., 2002). The whole milt characteristics described above give a global view of sperm quality but do not describe the potential of individual spermatozoa in achieving fertilisation. The logic for extending sperm analysis to considerations of individual sperm parameters derives thus from the conspicuous heterogeneity in spermatozoa populations. This is discussed in the sections below.

### 3.3. Individual spermatozoa-based measurements

#### 3.3.1. Introduction

Methods such as the measurement of the concentration of spermatozoa in the milt allow an estimation of the whole spermatozoa population without taking into account the individual spermatozoon status. This may have particular implications when using mixed sperm from multiple males since the single sperm that ultimately fertilises the egg may have had competitive advantages over other sperm. Thus, techniques that focus on features of individual sperm cells, such as motility and morphology, should be fundamentally more discriminatory than bulk measurements of the whole milt.

#### 3.3.2. Sperm viability and membrane integrity

Although the true viability of spermatozoa is ultimately defined by their capacity to move and fertilise an egg, fish spermatozoa has also been investigated using live/dead sperm viability kits. In this case, viability refers really only to the viability or integrity of their membranes. Such tests are based on dual-staining protocols using fluorescent dyes, flow cytometry protocols and observations under a confocal microscope. The commonly used stains are, a membrane-permeate nucleic acid stain (SYBR 14 dye) and a conventional dead cell stain (propidium iodide) or rhodamine 123 (Segovia et al., 2000; Grzyb et al., 2003). When the milt is incubated with these two stains, live sperm cells with intact membranes fluoresce green and cells with damaged cell membranes fluoresce red. SYBR can penetrate the cell membrane of the sperm head and stain the nucleic acids of viable cells, while propidium is not able to pass through the membrane of living cells but is able to penetrate and stain the nuclear DNA of degenerated or dead sperm. Rhodamine 123 is able to stain functional mitochondria of viable cells (Segovia et al., 2000). Sperm can also be stained with trypan blue and counted under the microscope for the percentage intact (viable, unstained) and damaged (dead, red-stained) spermatozoa (Lubzens et al., 1997; Rurangwa et al., 2002). Such measurements are best used to simply indicate the integrity

of the sperm membrane rather than true viability since membrane intact sperm may also have limited viability due to other factors as are discussed elsewhere in this paper.

### 3.3.3. Sperm morphology and ultrastructure

Teleost sperm is characterised by the absence of an acrosome, which contrasts with mammalian sperm, although an active acrosome is present in Acipenseriform fish (Linhart and Kudo, 1997). The shape of the head and the nucleus is highly variable between species (Billard and Cosson, 1992; Billard et al., 1982; Billard, 1986; Jamieson, 1991) and the mid-piece containing a few mitochondria is either well developed (guppy) or reduced (salmonid, cyprinid) (Billard et al., 1995a,b). Some internally fertilising fish like the ocean pout have biflagellate spermatozoa (Mattei, 1988; Yao et al., 1995) while in general fish with external fertilisation have a simple flagellum, although some biflagellate spermatozoa have been reported in channel catfish (*Ictalurus punctatus*) (Jaspers et al., 1976).

So far there is no evidence that longer sperm achieve faster swimming velocities (Gage et al., 2002). However, sperm deformities are associated with functional deficiencies and cause reduced motility and fertilisation ability. As an example, fish sperm directly exposed to  $\text{Hg}^{2+}$  ions is characterised by broken tails (Van Look, 2001; Van Look and Kime, in press), and reduced motility and fertilising capacity (Rurangwa et al., 1998). Exposure of zebrafish (*Danio rerio*) to tributyltin (TBT) during a critical period in early life resulted in the production of sperm lacking flagella at sexual maturity (McAllister and Kime, 2003).

### 3.3.4. Sperm motility characteristics

Fish spermatozoa show species differences in the initiation (Morisawa, 1985; Cosson et al., 1995), duration (Billard, 1978; Billard and Cosson, 1992) and pattern of motility (Boitano and Omoto, 1992; Ravinder et al., 1997). The difference in  $\text{K}^+$  ion concentration (in salmonids) or osmotic pressure (in cyprinids, clariids and other families) between the seminal plasma and water, trigger the initiation of movement (Morisawa et al., 1983a; Billard, 1986). Osmotic pressure seems to be the major controlling factor in cyprinids (Morisawa et al., 1983c; Billard et al., 1995b; Redondo-Müller et al., 1991) and partly controls the motility in paddlefish (*Polyodon spathula*) spermatozoa (Linhart et al., 1995). The circular trajectories of trout spermatozoa, which become tighter with time elapsed after activation, is induced by the influx of  $\text{Ca}^{2+}$  ions (Cosson et al., 1989). The very short window of sperm motility (Kime et al., 2001) found most teleost fish (e.g. <30 s in salmonids) has a critical influence on successful fertilisation, since the spermatozoa must find and enter the micropyle during this limited period. For large eggs with diameters around 5 mm such as those of salmonids, the time of motility (<30 s) allows spermatozoa to swim less than halfway (3 to 4.9 mm) round the egg (Perchec et al., 1993). Clearly evolution has selected mating strategies which maximise the chances of sperm egg contact such that the gametes of both sexes are released in close proximity. Nevertheless, it is clear that spermatozoa which are highly motile will have a greater chance of fertilisation. In most farmed fish species, the motility lifespan is brief (approx. 1 min) and accurate evaluation of the motility characteristics can only be made using rapid and sensitive methods. The methods used to evaluate sperm motility are discussed in Section 4.



## 4. Sperm motility analysis in fish

### 4.1. Introduction

In commercial fish production, it is necessary to evaluate sperm quality in order to increase the efficiency of artificial fertilisation. A methodology which can rapidly assess the quality of sperm and is not dependent upon the availability of eggs from a female would greatly facilitate the optimisation of the production, collection and storage methodologies employed. Although motility is of major importance as a measure of quality, it is of our opinion that its evaluation has most often been of low accuracy in the past.

### 4.2. Subjective methods

The assessment of motility of fish sperm has essentially relied for a long time on subjective estimates of motility characteristics (Guest et al., 1976; Billard et al., 1977; McMaster et al., 1992), by the percentage of motile sperm cells (Levandusky and Cloud, 1988), by the total duration of movement (Duplinsky, 1982) or by a combination of both parameters (Baynes et al., 1981). The percentage of motile spermatozoa and the swimming vigor have usually been given a motility score corresponding to an arbitrary scale of criteria from 0 (immotile) to 5 (all spermatozoa vigorously motile) (Guest et al., 1976).

Some less descriptive subjective scales have defined the motility rating in terms of percent moving spermatozoa in the field of view (McMaster et al., 1992). This was used to evaluate the percentage of moving cells as: 0, when no movement was observed; 1, when up to 25% cells were moving; 2, when up to 50% cells were moving; 3, when up to 75% cells were moving and 4, when more than 75% cells were moving (Viveiros et al., 2003). In sea bass, motility classes were also used according to the percentage of rapid, vigorous and forward-moving motile spermatozoa (Sansone et al., 2002). Since all of these use arbitrary, nonlinear scales, they cannot be used for any statistical analysis.

As expected such measurements have, in general, led to variable results between laboratories or observers and the value of such measurements of motility in predicting fertility is still questionable due to the subjectivity of the technique. Subjective estimation of the sperm quality is affected by various factors but in particular variation in the observers' experience, the endpoints that are chosen and how these measures are interpreted. For example, in freshwater teleosts, the duration of spermatozoa motility is generally believed to be shorter than in marine fish species. The data are however patchy and this belief may in some cases be an artefact of subjective approach to the assessment of motility. The claim that cod sperm is motile for around 60 min (Trippel and Morgan, 1994), for example, was based on a scoring that counts vibration as motility even though actual progressive movement had ceased within a few min.

### 4.3. Semi-quantitative methods

To increase accuracy of sperm motility measurements, numerous attempts were made to standardize the analysis. Several techniques were proposed to overcome subjectivity and

to make motility analysis more reliable. Semi-quantitative measurements of sperm motility were analysed on videotape recordings viewed by two or more independent observers. Only forward-moving sperm were judged motile, those simply vibrating or turning on their axes was considered immotile (Aas et al., 1991). The video recordings were viewed at low speed using a video cassette player with a 'og and shuttle' video function. A grid was fixed to a television screen, and the percentage of initially motile sperm was counted. This allowed individual sperm to be counted on the basis of whether they were motile or not. This method provided a less subjective measure of sperm motility than most scoring techniques. The total duration of motility was timed by stopwatch, as well as the time at which 50%, and 95% of the sperm ceased moving. For individual sperm evaluation and velocity measurements, these techniques were time consuming and observer dependent. Such techniques can also be used to measure velocity from distance moved and frame frequency of the videotape. Although this is quantitatively accurate, there is subjectivity in choice of sperm tracked and the methodology is extremely time consuming.

#### *4.4. Quantitative computer-assisted methods*

Computer-Aided Sperm Analysis (CASA) systems are the evolution of multiple photomicrography exposure and video-micrography techniques for spermatozoa track, using a computer equipped with imaging software. A CASA system refers to the physical equipment used to visualize and digitize static and dynamic sperm images, and to the methods used to process and analyse them (Boyer et al., 1989).

Although the development of techniques to assess the motility of spermatozoa has recently progressed much, most advances have been registered in mammals, including humans, compared to fish and other aquatic organisms. The systems were initially developed to examine male fertility in clinical andrology laboratories, and were later applied to other mammalian species (Vantman et al., 1989; Farrell et al., 1998; Moore and Akhondi, 1996; Hirano et al., 2001). The objective measurement of fish sperm motility was first reported by Cosson et al. (1985) using stroboscopic illumination and video recording of activated trout sperm movement. Only during the last few years have modern CASA systems been adapted to fish spermatozoa studies (Toth et al., 1995, 1997; Christ et al., 1996; Kime et al., 1996, 2001; Ravinder et al., 1997). The differences in the biology of spermatozoa between fish and mammals may explain the delay in release of adequate tools for the measurement of the motility of spermatozoa in fish. Introduction of CASA to fish reproduction studies had first to overcome the particular problems related to their spermatozoa such as the short period of motility following activation and the high frequency of flagellar beat after activation (Billard and Cosson, 1992).

With the subjective estimations of fish sperm motility used until recently, the different components of sperm motility and general patterns could not be identified and determined. Computer digitization of the sperm head after videotaping under phase contrast microscopy allows accurate and reliable measurements of the physical parameters of sperm motion. The technique has permitted the study of the motility components in carp in which three distinguishable patterns could be observed (linear, circular and haphazard) (Ravinder et al., 1997). A rolling motion of spermatozoa was observed in turbot, paddlefish and shovelnose sturgeon (Dreanno et al., 1996; Cosson et al., 2000).

Computer-Assisted Sperm Analysis (CASA) systems quantify different sperm motility parameters of interest, including those which are not observable manually. Assessment of sperm motility using CASA methodology, apart from being rapid, allows elaborated analysis of the components of that motility. However, temperature, the size and the concentration of sperm as well as the speed, may all affect the quality of results and must be taken into consideration during the calibration process. Computer tracking of sperm movement using CASA employs expensive equipment and expertise beyond the capabilities of many laboratories and farms. However, the procedures used to videotape the sperm movement are relatively simple and the equipment, which is not sophisticated, can be accessible to any laboratory or farm with a minimally trained employee. With a centralised computer unit and expertise, the information on sperm movement is extracted in a time little more than that required for playing the videotape, provided that the recordings are of good quality. Agreement within and between laboratories on the assessment of sperm quality could be achieved and videotapes distributed together with data on the threshold velocities acceptable for use in fertilisations. For hatcheries not yet equipped with a CASA system, technicians could adjust their subjective assessments to CASA data. CASA has high repeatability and provides a more discriminating estimate of the sperm motility than does the visual/microscopic subjective procedure.

#### 4.4.1. Sperm trackers

Computer-assisted sperm trackers comprise essentially a microscope coupled to a CCD camera which conveys a signal to a monitor, VCR recorder and computer (Boyer et al., 1989). Sperm movement is usually recorded onto videotape which is later analysed by the computer software. The parameters measured are similar for all instruments since development has been dictated by the needs of human and veterinary fertility applications. From studies using such sperm tracking systems conducted on African catfish, carp, goldfish, roach, Eurasian perch, trout, lake sturgeon, the most useful parameters of velocity are the curvilinear velocity (VCL, the actual velocity along the trajectory) and the straight line velocity (VSL, the straight line distance between the start and end points of the track divided by the time of the track) (Ciereszko et al., 1996b; Kime et al., 2001; Rurangwa et al., 2001, 2002; Jobling et al., 2002; Rurangwa and Kime, unpublished data). If the trajectory is a straight line, then VCL and VSL are identical. The angular path velocity (VAP, the velocity along a derived smoothed path) is generally of little use in most fish since, unlike mammalian spermatozoa, the tracks are general smooth curves, so that VAP and VCL are identical. However, in both stickleback (*G. aculeatus*) and wolffish (*A. minor*) in which fertilisation takes place within a gelatinous egg mass, spermatozoa follow a much more erratic path and VCL and VAP are both useful measurements (Kime and Tveiten, 2002; Elofsson et al., 2003a). For such species, the VCL/VAP ratio gives a good estimate of the “wiggliness” of the trajectory. Immediately after activation, teleost spermatozoa generally move in a straight or slightly curved trajectory. The various velocity patterns are schematically represented in Fig. 1. Towards the end of the activation period, during exposure to pollutants or in an inappropriate extender, the trajectory can become increasingly curved and eventually become tight concentric circles. Under such conditions, the linearity (LIN, the ratio of net distance moved to total path distance (VSL/VCL)) or straightness (STR, the ratio of net distance moved to smoothed path distance (VSL/VAP)) can be very useful indicators of

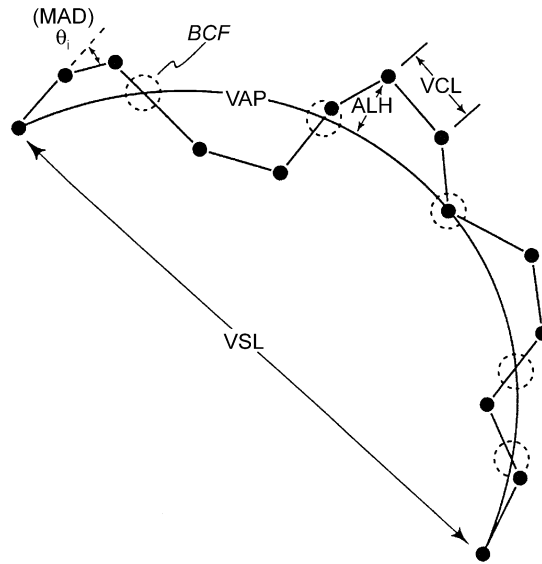


Fig. 1. Schematic representation of some of the motility patterns measured by the CASA system (Boyer et al., 1989). Black circles represent successive images of the head of a motile sperm and are joined by straight path lines. Curved line indicates a smoothed path fitted through sperm track. MAD: mean angular displacement, BCF: beating cross frequency, VAP: average path velocity, ALH: amplitude of lateral head displacement, VCL: curvilinear velocity, VSL: straight line velocity.

curvature of the trajectory. Percent motility (MOT, percentage of motile sperm) and motile concentration (MOC) are good indicators of the numbers of sperm. Fertility may be dependent on both the number of motile sperm and their velocity. From our experience, other parameters of movement that are automatically generated by the tracker have not so far provided any additional useful information on sperm quality in fish.

#### 4.4.2. Achievements with CASA

When the parameter setting of the CASA system and the handling of the sperm sample are defined, the reproducibility of the CASA value is better than that obtained by visual estimation of motility (Kime et al., 2001). The quality of the CASA results comprises the precision, the reliability and reproducibility of measurement as well as the significance of values with respect to their biological relevance. Computer-assisted sperm analysis (CASA) permits a determination of sperm motility characteristics, improves reproducibility and facilitates the documentation of the results.

CASA assessment is of significant value in predicting the ability of sperm to achieve fertilisation since percent motile sperm and sperm progressive velocity are the sole predictors of fertility (Rurangwa et al., 2001). With the CASA system, we have established that the progressive sperm motility velocities (VCL, VSL and VAP) correlate better with the fertilisation rates than other parameters of movement in African catfish (Rurangwa et al., 2001). Similar correlations between sperm motility and fertilisation capacity were also reported in turbot (Dreanno et al., 1999), carp (Linhart et al., 2000) and rainbow trout

(Lahnsteiner, 2000). If sperm quality can be related to fertilising ability, then it is possible to use CASA to routinely assess a very wide range of freezing protocols without the requirement of females and without the variability inherent in the use of eggs from different females. We have already shown that CASA can be useful for determining the most suitable extender for cryopreservation of catfish sperm (Rurangwa et al., 2001). It should therefore provide an extremely valuable tool in aquaculture for improving existing cryopreservation protocols and for modifying them for new species. The system has also been used to study the effects of toxicants on the motility of fish spermatozoa (Kime et al., 1996; Rurangwa et al., 1998).

Choice of extenders and conditions of activation has been very empirical and based simply on what seems to work since it is extremely tedious to test a wide range of extenders or activators with different osmolalities or ionic compositions. A rapid methodology in which sperm quality can be tested for a wide range of solutions could rapidly produce improved solutions. CASA has recently been employed to optimise extender composition for the wolffish, a marine species with aquaculture potential but an unusual reproductive strategy (Kime and Tveiten, 2002).

#### 4.4.3. Possible future applications of CASA

Rapid assessment of sperm quality could facilitate choice of broodstock which in turn could select improved sperm quality in the succeeding generations. There is at present the possibility that selection of broodstock for characteristics which either lead to more rapid growth, or are favourable to the consumer could unwittingly also select males which have poorer fertility and lead over several generations to poor quality broodstock. As a first indication, certain strains of Atlantic salmon *S. salar* selected genetically for fast growth, produce less than 0.1 ml of milt per kg body weight (Zohar, 1996) compared with 2 ml per kg (20-fold) in natural fish (Aas et al., 1991; Mylonas et al., 1995). CASA may assist in the determination of the effects of such selection on the milt quality. The creation of fish sperm and whenever possible also embryo cryobanks is justified by the development of genetic selection programmes in fish farming together with the need for the protection of biodiversity in wild fish populations (Maisee et al., 1998). Although cryopreservation of fish spermatozoa has increased the number of offspring from genetically superior males, aided in the transport of milt, and provided a year-round supply of male gametes, sperm cryopreservation can furthermore increase the economic utilization of males and is a prerequisite for the establishment of gene banks (Munkittrick and Moccia, 1984; Lubzens et al., 1997).

A recent study has shown that increased commercialization of frozen sperm is expected to occur as research protocols for fish sperm cryopreservation are being applied not only in the public sector, but increasingly in the private sector and as markets of cryopreserved sperm are established (Caffey and Tiersch, 2000). This offers an opportunity for the large-scale application of CASA in the rapid control of the quality of frozen sperm samples for aquaculture. CASA already has similar application for assessing sperm quality of land animal breeding stocks in agriculture and horse racing (Gravance et al., 1997; Suzuki et al., 1997; Farrell et al., 1998).

The use of efficient and objective methods such as CASA for the quantification of sperm quality should enable researchers to gain urgently required basic knowledge on the

factors that control or affect sperm quality in fish. The potential problems that could be investigated by sperm quality analysis are detailed in Section 5.

## 5. Factors affecting sperm quality in fish

### 5.1. Introduction

In fish farms and hatcheries, the biotic and abiotic factors that affect sperm quality are diverse and are dependent on complex interactions between genetic, physiological and environmental factors. The objective evaluation of sperm quality in a hatchery can thus provide novel and informative data that could be used to devise the best rearing conditions for male broodstock and the optimal handling and storage of spermatozoa prior to fertilisation. In aquaculture, fish are commonly held in artificial tanks or cages in unnaturally crowded condition, fed an artificial diet and in some cases exposed to water containing pollutants of industrial and agrochemical origin, or to effluent from sewage works or intensive aquaculture. Overcrowding may well lead to the rapid spread of disease, which may need to be treated with antibiotics. In fact stress caused by inappropriate holding conditions, overcrowding or by transport between farms, may well suppress the immune system and lead to increased susceptibility to diseases. In addition, farmed fish are specifically selected to be appealing to the consumer and to grow at maximal rates for commercial profit. Such selection is very different from natural selection and may not necessarily select for best sperm quality and quantity. For successful fertilisation, it is essential that the sperm produced in a farm is of good quality. It might be reasonable to expect that sperm from a farmed fish species should have the characteristics comparable to the best found in the wild. Sperm quality in farmed fish, however, may be affected by a number of factors either at different levels of the aquaculture production process or during collection and storage of sperm ex-vivo prior to fertilisation and at activation after spawning. Factors which affect sperm quality in farmed fish are briefly reviewed in the following sections. Understanding of the factors that affect sperm quality is paramount in perfecting the methods and manipulations needed for successful in vitro fertilisation. If we can objectively measure sperm quality in these complex situations, then we might be able to unravel the myriad of factors that determine fertilisation success and moreover understand the factors that lower fertilisation success and motility in important cultured species. These factors have been split into effects on the broodstock, effects that occur at fertilisation and impacts that are due to storage or cryopreservation.

### 5.2. Effects on broodstock

#### 5.2.1. Rearing photoperiod and temperature

Photoperiod manipulation is employed in aquaculture to accelerate or delay gonadal recrudescence so that fish spawn at a convenient time of the year for the aquaculturist (Nash, 1999). Although such an artificial breeding cycle could affect sperm development few data are available. In sunshine bass (*Morone chrysops* × *M. saxatilis*) exposed to shifted photothermal cycles (6–9–12 months), sperm concentration, duration of motility



and seminal fluid pH differed among males on the different cycles, but these differences produced no changes in fertilities (Tate and Helfrich, 1998). In wolffish (*A. minor*), however, there was no difference in volume of ejaculate or sperm concentration between males kept under two different light cycles (18D/6L and 6D/18L) (Pavlov et al., 1997) and in goldfish, photoperiod manipulation did not affect sperm production (Iigo and Aida, 1995). Although recent results have strengthened the evidence for temperature-dependent sex determination in some reared fish, no data exist on how rearing temperature can affect sperm quality. However, in a study of Labbé and Maisse (1996), the ability of rainbow trout (*O. mykiss*) spermatozoa to withstand cryopreservation was improved by rearing at high temperature during gametogenesis followed by transfer to colder water.

### 5.2.2. Adult nutrition

Improvement in broodstock nutrition and feeding greatly improves gamete quality and seed production (Izquierdo et al., 2001). Polyunsaturated fatty acid (PUFAs)-enrichment of commercial diets enhances reproductive performance of male sea bass (*Dicentrarchus labrax*) (Astuarino et al., 2001). Sea bass fed commercial pelleted diet enriched with fish oil had a longer spermiation period, higher milt volumes and spermatozoa concentration and higher survival of embryos and larvae after fertilisation when compared to those fed a non-enriched wet diet. In rainbow trout, dietary lipids alter the composition but not the fluidity of the sperm plasma membrane and increase their fertilisation capacity (Labbé et al., 1995).

The importance of dietary ascorbic acid (Vitamin C) on male fish fertility has been demonstrated in rainbow trout (Ciereszko et al., 1996a; Dabrowski and Ciereszko, 1996). The antioxidant function of vitamin C provides a protection for the sperm cells by reducing the risk of lipid peroxidation and ascorbic acid deficiency reduces both sperm concentration and motility and consequently the fertility (Ciereszko and Dabrowski, 1995).

Feeding rainbow trout with gossypol, a naturally occurring compound in cotton seeds, did not affect sperm motility and fertilising ability although testosterone (T) and 11-ketotestosterone (11-KT) levels were elevated in some experimental groups (Dabrowski et al., 2000, 2001). However, in male lamprey (*Petromyzon marinus*) injected with gossypol acetic acid, sperm motility was reduced (Rinchard et al., 2000) and in vitro sperm assays confirmed its toxicity to perch spermatozoa (Ciereszko and Dabrowski, 2000). More importantly, gossypol was recently found to be transferred to the eggs in trout which may lead to reduced reproductive performance in female rainbow trout although it had no effect on fish growth and mortality (Blom et al., 2001).

### 5.2.3. Water and food contamination

Exposure to environmental toxicants or hormones can affect reproduction in general, leading to decreased sperm quality. Juvenile black porgy (*Acanthopagrus schlegeli*) fed a diet containing 4 mg/kg estradiol-17 $\beta$  had suppressed spermiation after 7 months of exposure (Chang et al., 1995). Estrogenic substances such as 17 $\alpha$ -ethynylestradiol and genistein are sufficiently potent to produce sex-reversed male fish and masculinisation (Kwon et al., 2000). In genistein-fed rainbow trout, sperm motility and concentration were decreased in a dose-dependent manner at spawning (Bennetau-Pelissero et al., 2001). Dietary mercury at levels that are found in North American lakes impaired gonadal development in male juvenile walleye (*Stizostedion vitreum*) (Friedmann et al., 1996). It is

probable that in some, at least, of these cases, there may also have been an effect on sperm quality since a recent study has clearly shown a correlation between sperm quality assessed by CASA, fertilisation rate, incidence of intersex and plasma vitellogenin levels in wild roach (*Rutilus rutilus*) exposed to sewage effluents (Jobling et al., 2002).

#### 5.2.4. Stress to broodstock

The quality of fish gametes depends on the appropriate hormonal environment during development but this may be disturbed by stress (Kime and Nash, 1999). During the breeding season, male sockeye salmon (*Oncorhynchus nerka*) respond to confinement stress with elevated levels of cortisol and glucose and decreased levels of reproductive steroids (T and 11-KT) (Kubokawa et al., 1999). Stress may also act by inducing changes in plasma osmolality which in turn can affect sperm quality in fish. As an example, white bass *M. chrysops* transported for 5 h in freshwater had reduced seminal fluid osmolalities and motility at activation (10–25% motile cells in 38% of sperm samples) (Allyn et al., 2001). In striped and white bass, male broodstock captured from the wild during the spawning season and moved to captivity produce milt with non-motile sperm (Berlinsky et al., 1997). In rainbow trout, repeated acute stress during reproductive development prior to spawning significantly delayed ovulation and reduced egg size, and significantly decreased sperm counts and most importantly significantly decreased survival rates for progeny from stressed fish compared to that from unstressed controls (Campbell et al., 1992). Since many of the handling and transportation procedures used in aquaculture can be potentially stressful, quantitative evaluation of the effects of such procedures on sperm quality could facilitate changes in the conditions employed so that stress is minimised and sperm quality is not affected.

#### 5.2.5. Age of broodstock and breeding season

The age of broodstock has a significant influence on the sperm quality and may affect the success of storing sperm (Vuthiphandchai and Zohar, 1999). In captive-reared striped bass (*Morone saxatilis*), 3-year-old fish had higher sperm quality than the 1- or 12-year-old fish, based on higher sperm production and increased sperm longevity during short-term storage. However, the fertilising capacity of virgin and repeat spawners was comparable in Atlantic cod, *G. morhua* (Trippel and Neilson, 1992). In fish species with an annual reproductive cycle, the quality of sperm varies across the spawning season and the mating frequency. In the three-spined stickleback *G. aculeatus*, the amount of sperm in the testes and the size of the ejaculate were reduced in males that had mated several times (Zbinden et al., 2001). In the common carp (*C. carpio*), CASA has shown that sperm production and quality can be lower at the beginning and end of the breeding season (Christ et al., 1996). Similar decreases in sperm quality were also observed in turbot, *P. maxima* (Suquet et al., 1998). Sperm analysis can therefore be used to determine the optimal period in which sperm should be collected.

#### 5.2.6. Diseases of broodstock

The cestode *Ligula intestinalis* (L.), a common parasite of cyprinid fishes, may affect fish gamete production by preventing gonad development. Infectious Pancreatic Necrosis (IPN) virus has been reported to attach to sperm cells of farmed rainbow trout (Rodriguez

et al., 1993) which could affect sperm quality, although no confirmatory or experimental data is available yet.

#### 5.2.7. Holding temperature of male broodstock

The holding temperature of the breeders affects the quality of sperm. In the Siberian sturgeon *A. baeri*, spermatozoa motilities were significantly higher at 10 °C (culture temperature) and the lowest at high temperature (17.5 °C) (Williot et al., 2000).

#### 5.2.8. Hormonal induction of spermiation

Many farmed fish species do not spawn readily in captivity and hormonal treatments are necessary to either induce ovulation/spermiation or to synchronise gamete release of the two sexes at a time convenient for the fish farm (Zohar and Mylonas, 2001). However, this practice is known to increase the fluidity of the milt (low concentration of spermatozoa) in plaice *Pleuronectes platessa* (Vermeirssen et al., 1998), winter flounder *P. americanus* (Shangguan and Crim, 1999) and Atlantic halibut *H. hippoglossus* (Vermeirssen et al., 2000a,b). Artificial induction of spermiation can also affect the responsiveness of male fish. In European catfish (*Silurus glanis*), the total number of spermatozoa collected was significantly higher when carp pituitary extract was injected than when GnRH analogue implants were used to artificially induce spermiation (Linhart and Billard, 1994). In common carp (*C. carpio*), oral and intraperitoneal administration of salmon gonadotropin hormone-releasing hormone analogue (sGnRH-a) and Pimozide (Pim) induced gonadotropin II (GtH II) release and milt production significantly (Roelants et al., 2000). Sperm production, milt volume, sperm motility and seminal plasma pH were increased by GnRHa treatment in yellowtail flounder *P. ferrugineus* (Clearwater and Crim, 1998). In captive white bass (*M. chrysops*) treated with GnRHa during the spermiation period, GtH II levels and milt production increased (Mylonas et al., 1997). Increased milt volume and prolonged spermiation were also observed in sea bass (*D. labrax*) administered GnRHa (Sorbera et al., 1996). GnRHa-microspheres increased significantly sperm production in Atlantic salmon, *S. salar* and striped bass, *M. saxatilis* (Mylonas et al., 1995).

In male goldfish, the oocyte maturation-inducing steroid 17,20 $\beta$ -dihydroxy-4-pregnen-3-one (17,20 $\beta$ P) also functions by release into the water as a pheromone that increases male serum GtH-II concentration, milt volume, duration of sperm motility, proportion of motile sperm and sexual activity and paternity in multi-male spawnings (Zheng et al., 1997). Mature male goldfish placed with either a receptive female or stimulus pairs of spawning goldfish had sperm volumes greater than those of males kept in all-male groups (Kyle et al., 1985). Similar stimulation of spermiation in males by ovulating females was noticed in carp in earthen ponds (Billard et al., 1989). The increase in milt production in pair-spawners may be due to both neurally and hormonally mediated events that ensure milt availability for imminent spawning activity. In natural populations of a coral reef fish, the bluehead wrasse (*Thalassoma bifasciatum*), males with the higher spawning frequency produced fewer sperm per mating indicating a trade off between spawning frequency and sperm volume, and an ability to vary the amount of sperm produced (Warner et al., 1995). In carp, the osmolality of seminal plasma and the capacity of spermatozoa to move are highly variable after hormonal injection (Redondo-Müller et al., 1991). The time lapse between hypophysation and the moment at which the initial quality of sperm begins to

decline may vary according to species. As an example in Siberian sturgeon *A. baeri*, a delay of 36 h after stimulation before milt collection clearly provided the most motile spermatozoa as compared with shorter (24 h) or longer (48, 60 h) delays (Williot et al., 2000). Time schedules for hormonal injection should therefore take this into account. Assessment of sperm quality could therefore be used to optimise the hormonal dosage, and its timing, or the proportions of males in the holding tanks.

### 5.3. Direct effects at fertilisation

#### 5.3.1. Anaesthetics use

Other factors that may affect the sperm quality during handling are the type and the concentrations of anaesthetics used to reduce stress when handling the fish. In rainbow trout, the percentage of motile sperm was unaffected by anaesthetic treatment of male broodstock, but the duration of motility decreased as anaesthetic concentration increased (Wagner et al., 2002).

#### 5.3.2. Urine contamination of milt

Freshwater fish are hyperosmotic regulators and control the ionic composition of their blood in part through the volume of urine excreted to balance the quantity of water entering the body from the low salt content environment. However, in some fish species, contamination of sperm by urine during stripping and probably ejaculation is unavoidable, due to the close proximity of sperm duct and ureter, or the presence of a single urogenital pore through which both milt and urine are released. In some farmed fish species, urine initiates spontaneous sperm movement: in European catfish *S. glanis* (Linhart and Billard, 1994), in common carp *C. carpio* (Billard, 1998; Billard et al., 1995b; Perchee et al., 1995, 1998), in turbot *P. maxima* (Dreanno et al., 1998), in tilapia *Oreochromis mossambicus* (Linhart et al., 1999), in tench *Tinca tinca* L. (Linhart and Kvasnicka, 1992), in paddlefish *P. spathula* (Linhart et al., 1995). Since such contamination by urine prematurely induces motility, sperm may have become immotile before fertilisation can occur if contact with eggs is delayed. Contaminated carp milt had decreased endogenous stores of energy and motility (Perchee et al., 1995, 1998) which characterise such premature induction of motility. In Atlantic salmon (*S. salar*), urine contamination increased the variability of the seminal plasma composition and reduced the osmolality as well as the concentration of potassium (Rana, 1995). Extensive contamination of milt with urine could modify the spermatozoan environment and decrease the potential for movement. The squeezing of sperm out of testis after decapitation or surgery is applied when the fish species is not stripable (e.g. African catfish, *C. gariepinus*) but this may in some cases result in collection of immature intratesticular sperm. In some cases, sperm is collected by catheterization via the urogenital papilla in order to obtain sperm free of blood, urine or faeces contamination (Cabrita et al., 2001). Assessment of sperm quality after collection and before fertilisation is therefore of major importance in most farmed fish species.

#### 5.3.3. Initiation of sperm motility or activation

The seminal fluid is rich in many nutrients and ions, some of which are important in maintaining sperm quality when stored in an immotile state in the genital tract. External

environmental factors may affect the quality and motility during the activation process. Factors, such as the pH or ions present, may polarize the cell membrane and stimulate motility of fish spermatozoa (Morisawa et al., 1999). In carp, osmolality-dependent permeabilization and structural changes are induced in the sperm membrane by hyposmolality, and reorganization of lipid structure has been proposed as a possible mechanism (Marian et al., 1993). While osmolalities isotonic to seminal plasma suppresses sperm motility in marine and freshwater teleosts, exposure of sperm to hypertonicity of seawater or hypotonicity of freshwater, respectively, induces the initiation of sperm motility at spawning (Takai and Morisawa, 1995). In trout, the inhibition of sperm motility is mainly due to  $K^+$  ion concentration (Gatti et al., 1990; Billard and Cosson, 1992). In turbot, anaerobiosis and high  $CO_2$  content within the genital tract contribute to the inhibition of spermatozoa motility (Dreanno et al., 1995). However, although spermatozoa of fish are naturally spawned in either fresh- or seawater, results obtained in laboratory conditions of most studies suggest that water is not a suitable activation medium (Billard and Cosson, 1992; Cosson et al., 2000). The sperm motility of paddlefish *P. spathula* and shovelnose sturgeon *Scaphirhynchus platyrhynchus* was significantly increased by activation in a buffered media instead of activation in distilled water (Cosson et al., 2000). The time of motility was prolonged and there were fewer damaged sperm cells than in distilled water. At activation, sperm cells are exposed to a hostile environment, i.e. low or high osmolality compared with that of the seminal fluid. To increase the viability of spermatozoa during handling sperm extenders or sperm activator media have been developed for some fish species to mimic either the seminal composition, the ovarian fluid substitute or to have appropriate activation properties. Simple physiological solutions and various complicated media are currently used in hatcheries, although no thorough study has yet defined the best medium per species. Accurate quantitative assessment of motility requires that all sperm are simultaneously activated. Since a high dilution (more than 1000-fold) is required for induction of synchronous motility in 100% spermatozoa, a two-step procedure is necessary, with an initial dilution of 1 to 100 in a medium that keeps the spermatozoa immotile and allows good mixing of the viscous milt (Billard and Cosson, 1992). The second dilution (1 to 20) in the activating solution can be made directly under the microscope.

#### 5.3.4. Temperature during fertilisation

In the sperm of salmonid and cyprinid fish, temperature affects the sperm beat frequency (Cosson et al., 1985). In trout, higher temperature increased the beat frequency and decreased the duration of forward movement (Billard and Cosson, 1992) while the lower temperature that trout experience during natural spawning (4–10 °C) increases the duration of sperm movement (Van Look, 2001). In African catfish, low temperature (4 °C) also prolonged motility and viability of spermatozoa compared to the culture temperature (25 °C) (Mansour et al., 2002).

#### 5.4. Effects during storage

##### 5.4.1. Cold room storage of sperm

Some fish farming practices use cold room stored sperm for the fertilisation of different batches of eggs. When they are kept at low temperature (around 4 °C),

spermatozoa have a low metabolism and can be kept for a few days in appropriate sperm extenders without significant changes in the quality (Kime et al., 1996). However, prolonged cold room storage conditions can greatly affect the quality of sperm since anaerobic conditions and associated microbial contaminations may reduce sperm motility and viability. Channel catfish sperm stored at 4 °C kept its motility for 10 days in sterile solutions which coincided with the time at which increased bacterial infection, mostly of the genus *Pseudomonas* (65%), occurred that contributes to the decrease in sperm quality by production of extracellular enzymes and consumption of oxygen (Jenkins and Tiersch, 1997). In non-sterile solution, the motility was completely lost after 3 days. Addition of antibiotics at appropriate concentrations can lengthen storage time of refrigerated spermatozoa by preventing the sperm cells from bacterial infection. However, high concentrations (gentamicin: 750 µg/ml; ampicillin >250 µg/ml) have significantly reduced sperm viability and mitochondrial function in tilapia, *Oreochromis niloticus* (Segovia et al., 2000). Oxygen has also been added to increase the survival of refrigerated sperm cells (Geffen and Evans, 2000).

#### 5.4.2. Cryopreservation

Fish sperm is now routinely held frozen in liquid nitrogen for long periods (McAndrew et al., 1993). Although there has been considerable research on sperm preservation in at least a hundred fish species, the techniques for cryopreservation, however, are rarely optimised and are frequently based simply on what works in one species. The reader is referred to recent reviews for further details (Leung and Jamieson, 1991; McAndrew et al., 1993; Tiersch and Mazik, 2000). The major problem in controlling the quality of preserved sperm is that variables such as freezing and thawing regimes and composition of cryoprotectants each have to be tested by measurement of the fertilisation ability of the sperm in the absence of a direct measure of sperm quality. This is a tedious process.

## 6. Conclusions

At several stages of the fish farming and hatchery activities, there is a need for a reliable measure of sperm quality. However, although computer-assisted semen analysis in fish has developed over the last 10 years, it is limited to a few institutions and mostly in research laboratories. The main reason may be related to the cost of the equipment, which hampers its access to fish breeders although good collaboration between institutes, laboratories and hatcheries could allow its broader use. Presently, the system can provide precise and accurate information on sperm motion characteristics and positive correlations have been found between fertilising capacity and defined sperm motility characteristics in the fish species in which the system has been tested so far. Four central fields for CASA application in aquaculture can be proposed: (1) general evaluation of suitability of male broodstock for reproduction, (2) improvement of artificial fertilisation, (3) optimization of dilution/extension media and cryopreservation protocols, (4) management of sperm cryobanks.



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