New Chapter

Application of Computer-assisted Sperm Analysis (CASA) to Aquatic Species

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Introduction

Computer-assisted sperm analysis (CASA) (also referred to as computer-assisted semen analysis) uses computer software to collect, detect, identify, and quantify attributes of motility in a sperm sample. It was first designed for use in humans and livestock, and is considered to be an objective, accurate approach for sperm motility assessment in mammals because it relies on actual counts and measurements rather than subjective observation and estimation. Basically, these systems comprise three components: 1) an optical system; 2) a method for image capture, and 3) data analysis and reporting. Currently, several manufacturers provide complete CASA systems or software (listed in Table 1). Based on the descriptions from the associated user manuals, the basic functions of these systems are similar and are typically based on quality standards developed for human semen put forth by the World Health Organization (World Health Organization 2010).

Table 1. Examples of commercially available systems for computer-assisted sperm analysis.

System name	Manufacturer	Location
Medea LAB	Medea LAB	Bruckberg, Germany
Sperm Vision	Minitube of American	Verona, WI, USA
AndroExpert	AndroExpert	Haus am See, Switzerland
Sperm Quality Analyzer (SQA-V)	Medical Electronic Systems	Los Angeles, CA, USA
Integrated Semen Analysis System (ISAS)	Projects i Serveis R+D S.L.	Valencia, Spain
Sperm Class Analyzer (SCA)	Microptic S. L.	Barcelona, Spain
IVOS sperm analyzer	Hamilton Thorne	Beverly, MA, USA
CEROS sperm analyzer	Hamilton Thorne	Beverly, MA, USA
Image J	National Institutes of Health	Bethesda, MD, USA
Hobson Sperm Tracker	Hobson Vision Ltd	Derbyshire, UK
The CellTrak/S system	Motion Analysis Corporation	Santa Rosa, CA, USA
Sperm Motility Quantifier (SMQ)	Wirson Scientific and Precision Equipment	Auckland Park, South Africa
Olympus Micro Image Analysis	Olympus C&S	Czech Republic
CASAS-QH-Q	Qinghua Tongfang	Beijing, China
Mika motion analyzer software	Medical Technologies Montreux SA	Clarens/Montreux, Switzerland
Image-Pro Plus 5.0	Media Cybernetics, Inc.	Bethesda, MD, USA
Auto sperm*	MedCalc Software bvba	Mariakerke, Belgium

* This device does not require image capture.

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To produce accurate and reliable results by use of CASA, a series of parameters and thresholds in the system need to be properly established to ensure that sperm cells can be recorded and sorted into appropriate categories such as cell size, contrast, and identification of movement. These settings are essential for the application of CASA, and are based on characteristics such as size, shape, and swimming trajectory of sperm from each species. For most CASA systems, these settings can be validated by playing back of videos in sequence and inspecting the frames in real time to confirm if the cells were categorized correctly.

Some Characteristics of Sperm from Aquatic Species

Compared to mammalian sperm, fish sperm possess some specific characteristics and show great diversity among species. Accordingly, the CASA settings for analysis of fish sperm are different from that for mammalian sperm. With respect to size and morphology, fish sperm heads are usually around 2-5 μm, much smaller than those of mammalian sperm (8-10 um). The morphology of fish sperm, especially ultrastructure, varies enough from species to species to be used as phylogenetic criteria (Jamieson 2009).

For motility activation and swimming duration, fish sperm possess characteristics different from mammal sperm. Generally, fish sperm are quiescent in the testes, and their activation relies on the difference in osmotic pressure or ion levels between the testicular fluid and the outside environment (Morisawa and Suzuki 1980, Coward et al. 2002, Alavi and Cosson 2006) and also can be influenced by factors such as pH and temperature (Alavi and Cosson 2005). Upon activation, fish sperm show only a short swimming duration time (from 30 sec to 5 min, except for sperm from live-bearing fishes and some euryhaline fishes), while mammal sperm usually can swim for d. In addition, fish sperm move faster than the mammalian sperm, and the movement trajectory can be different.

As general approaches, collection of fish sperm samples can be performed by stripping or by crushing of testis. These latter samples can include immature sperm cells or somatic cells which require specific thresholding of parameters to distinguish them from sperm cells. Usually the parameters for recording of movement need to be set manually, and in terms of the specific values chosen, can be fairly subjective. This problem is exacerbated in aquatic species because of the great variability in sperm morphology and physiology (Jamieson 2009), and due to the short time of peak motility duration in most species ($\leq$ 30 sec). Therefore, to achieve accurate results in aquatic species, it is necessary to establish suitable parameters concerning image capture, cell size, speed values, light intensity and contrast, and photometer settings for each species based on sperm characteristics and condition (e.g., fresh, refrigerated, or post-thaw samples).

Current Application of CASA in Aquatic Species

Genetic improvement has driven great production gains in livestock industries such as poultry and dairy, and advances have been made for aquatic species (Burnell and Allen 2009). Preservation of valuable germplasm can improve genetic resources and reproduction, and also can be applied to conservation of imperiled species. So far, sperm cryopreservation has been studied in more than 200 species since its beginning in the 1950's (Blaxter 1953), and has been applied to large-bodied aquaculture fish species and small aquarium fishes (e.g., (Yang and Tiersch 2009). However, evaluation of gamete quality is still an extremely important but highly problematic component in sperm cryopreservation. To evaluate male gamete quality, work began

in the late 1970's in mammals to develop objective, automated technologies to rapidly evaluate sperm movement. This led to development of CASA systems that became widely commercially available in the 1990's, and have been adopted in biomedical applications and for use with high-value livestock.

In the late-1980's CASA was first applied to use in fish. Since then (at time of this writing) there have been 62 publications addressing this topic. Of these, 56 are peer-reviewed primary research articles, and 6 are reviews. The bulk of this research addresses demonstration of the feasibility of CASA application in fish (only 2 publications address invertebrates). The types of research address the following topics: sperm characteristics, motility changes after exposure to toxic chemicals and hormone treatments, sperm enzymology, motility characteristics in relation to storage solutions (e. g. pH, buffer, and osmolality), and sperm motility after cryopreservation. Most of the research utilized fresh sperm collected by stripping or crushing of testis, and only 7 of these reports addressed thawed sperm.

Generally, no standardization of methodology exists for CASA application in fish and shellfish. Indeed, 23 of these publications did not include any statements concerning instrument settings (Table 2), and several publications mentioned only certain parameters such as definitions for progressively motile or static cells. Proper parameter settings are essential to ensure that the images collected and analyzed are the targeted sperm cells. Also, 30 of these publications did not report sperm concentration, while 11 provided a dilution ratio only. The type and depth of viewing slides for loading of samples can affect the concentrations determined by CASA, and potentially influence sperm movement. Most publications described the types of slides used, but with large variation in detail. Temperature can be a factor controlling motility, especially swimming velocity. Of the 56 reviewed publications, 26 did not report sample temperature at the time of images capture. In addition, the time interval prior to the start of image capture after motility initiation and the timing of data collection periods are critical factors for analysis of velocity and motility because fish sperm are often motile for only sec to min, and the duration of burst speed can be short (10-20 sec). Rapid sample handing followed by high speed video recording is required to monitor this window. Of the 56 publications, 46 described this in some way, but most lacked information to clarify even if the starting time and period of video capture used for analysis were within the window of maximal sperm motility.

With respect to the output parameters used for sperm quality in these publications, most reported motility, progressive motility, velocity (μm/s) including average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL), and other parameters such as beat cross frequency (BCF), lateral head displacement (ALH), and swimming duration time.

Overall, the previous studies summarized in this review have demonstrated the feasibility of CASA for aquatic species, and showed that several output parameters are useful for evaluating gamete quality. However, routine application of CASA in aquatic species is limited by: 1) lack of clearly established instrument settings, especially for material other than fresh, stripped sperm of fish; 2) lack of standardized protocols, and 3) consequently because of these deficiencies, not taking advantage of the full range of analysis capabilities of these powerful instruments. These shortcomings need to be addressed by systematic evaluation of representative panels of aquatic species from freshwater, marine, euryhaline, anadromous, and catadromous habits with external and internal fertilization.

Table 2. Summary of previous publications addressing the use of CASA for aquatic species (arranged in chronological order).

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
						Image capture	Data analysis		
Boitano and Omoto 1992	Rainbow trout *Oncorhynchus mykiss*	Fresh	10	--	Regular glass slide	At 10 sec	2 sec	30	Definition of linear, arched, & circular
Toth et al. 1995	Common carp *Cyprinus carpio*	Fresh	23-25	Dilution ratio only	20-um µ-Cell semen chamber (Fertility Technologies)	At 12-14 sec for 1-2 min	15-20 sec; 25-30 sec; 55-60 sec	200	Detailed listing
Christ et al. 1996	Common carp	Fresh	23-25	Hemocy-tometer	20-um µ-Cell semen chamber	--	15-20 sec; 55-60 sec	200	Same as above
Ciereszko et al. 1996	Lake sturgeon *Acipenser fulcescens*	Fresh & thawed	15	Neubauer counting chamber	20- um Microcell (Conception Technologies)	At 5 sec & 5 min for 20-30 sec	25 frames	200	--
Kime et al. 1996	African catfish *Clarias gariepinus*	Fresh	--	Dilution only	--	At 20 sec	15 sec x 4	25	Detailed listing
Ravinder et al. 1997	Common carp	Fresh	2, 3, 25	Dilution only	10-µm Marler chamber (Fertility Technologies)	At 10 sec	--	25 or 60	Detailed listing
Toth et al. 1997	Lake sturgeon	Fresh	12	Dilution only	20-µm µ-cell semen analysis chamber	At 5 sec for 25 sec	--	200	Detailed listing
Creech et al. 1998	Fathead minnow *Pimephelas promelas*	Fresh	RT	60-100 sperm/view	--	At 10 sec x 4	2 sec	30	Cited another reference
Ciereszko et al. 1999	Muskellunge *Esox masquinongy*	Thawed	22	--	20-µm Microcell	At 15-20 sec	25 frames	200	--
Linhart et al. 2000	Common carp	Thawed	--	Dilution only	Regular glass slide	At 10 sec	15 sec	--	Threshold velocity only

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Rurangwa et al. 2001	African catfish	Fresh & thawed	RT	Dilution only	10-well multitest slide with cover slip (ICN Biomedicals)	At 0 sec to 2 min	15 sec at 5 sec after activation	--	Modified settings of Kime et al. 1996
Kime and Tveiten 2002	Spotted wolffish *Anarhichas minor*	Fresh	--	--	12-well slide* with cover slip	At 30 sec	15 sec	--	Detailed listing
Rurangwa et al. 2002	African catfish & common carp	Fresh	--	--	10-well multitest slide with cover slip	At 5 sec to 20 sec	--	--	Kime et al. 1996
Schoenfuss et al. 2002	Goldfish *Carassius auratus*	Fresh	--	--	--	--	--	--	--
Van Look and Kime 2003	Goldfish	Fresh	--	--	12-well slide* with cover slip	At 0 sec	5–20 sec	--	Detailed listing
Elofsson et al. 2003	Fifteen-spined stickleback *Spinachia spinachia*	Fresh	15	--	12-well slide*	At 20 sec	--	--	Detailed listing
Warnecke and Pluta 2003	Common carp	Fresh & thawed	20 ± 1	--	10-µm chamber (Stroemberg/Mika-CMA)	At 15 sec for 5 sec	32 frames	50	Definitions of motility
Aravindaksh an et al. 2004	Spottail shiner *Notropis hudsonius*	Fresh	--	By CASA	--	5 sec	--	--	--
Asturiano et al. 2004	European eel *Anguilla anguilla*	Fresh	--	Hemocy-tometer	Teflon-coated microwells coated with 10% BSA	--	--	--	--

Citation	Species	Sample type	Temp. (°C)	Sperm density	Slide type and depth	Time intervals		Frames per sec	Settings reported
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Burness et al. 2004	Bluegill *Lepomis macrochirus*	Fresh	20	Dilution ratio only	Improved Neubaur hemocytometer	0.5-sec readings x 6 in 90 sec	60 sec after activation	--	Definition of motility
Kleinkauf et al. 2004	Flounder	Fresh	--	--	12-well slide*	At 15 sec	30-45, 45-60, & 60-75 sec	--	--
Le Comber et al. 2004	Three-spined stickleback *Gasterosteus aculeatus*	Fresh	--	Dilution ratio only	12-well slide*	0 to 105 sec at 15-sec intervals	--	--	Detailed listing
Vermeirssen et al. 2004	Atlantic halibut *Hippoglossus hippoglossus*	Fresh	--	--	PTFE-coated slide (ICN Biochemicals)	--	--	--	--
Asturiano et al. 2005	European eel	Fresh	--	Hemocy-tometer	--	--	--	--	--
Burness et al. 2005	Bluegill	Fresh	20 ± 1	Hemocy-tometer	--	10, 20, 30, 45, 60, & 120 sec for 0.5-sec	10 sec after activation	--	Definition for immotile
Dietrich et al. 2005	Rainbow trout	Fresh	20	--	12-well slide* with cover slip	At 5 to 20 sec	--	50	Detailed listing
Urbach et al. 2005	Arctic charr *Salvelinus alpinus*	Fresh	--	--	Micro slide with cover-slip	At 0 sec to 1.5 min	--	50	Contrast,cell size,VAP threshold & VSL
Babiak et al. 2006a	Atlantic halibut	Fresh	6-8	Hemocy-tometer	Counting chamber (Leja products)	At 0 to 105 sec	--	50	Contrast, cell size

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Babiak et al. 2006b	Atlantic halibut	Fresh	20	Dilution ratio only	Burker's chamber	At 30 sec	0.5 sec	--	Defined static cells
Felip et al. 2006	European sea bass	Fresh	--	Dilution ratio only	Regular glass slide	At 5 sec	--	25	Immotile, slow, moderate, & fast
Hu et al. 2006	Amphioxus *Branchiostoma belcheri*	Fresh	RT	Hemocy-tometer	--	At 0.5, 4 & 10 min for 3 sec	--	--	Immotile, swaying, circular & progressive
Kowalski et al. 2006	European smelt *Osmerus eperlanus*	Fresh	4	Dilution ratio only	12-well slide*	At 4 sec for 12 sec	--	--	--
Locatello et al. 2006	Guppy *Poecilia reticulate*	Fresh	26	--	12-µm microcell chamber	--	--	--	Static cells: VAP, VCL & VSL
Holt et al. 2007	Bluegill	Fresh	20	Dilution ratio only	Neubaur hemocytometer	At 0 to 60 sec	5-10 sec	30	--
Liu et al. 2007	Red seabream *Pagrus major*	Fresh & thawed	18-20	--	10-µm chamber (20-ul)	At 10 sec	--	24	Defined motility
Wilson-Leedy and Ingermann 2007	Zebrafish *Danio rerio*	Fresh	20 ± 1	--	12-well (12-µm) slide coated with 1% polyvinyl alcohol with cover slip	At 15 sec	--	97	--
Wojtczak et al. 2007	Common carp	Fresh	--	Spectro-photometer	12-well slide*	At 15 to 30 sec	--	--	--
Cabrita et al. 2008	Senegalese sole *Solea senegalensis*	Fresh	--	--	--	At 15, 30, 45 & 60 sec	--	--	--
Ciereszko et al. 2008	European whitefish *Coregonus lavaretus*	Fresh & thawed	--	--	12-well slide*	At 5 sec	15 sec	--	--

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Dietrich et al. 2008	Rainbow trout	Fresh	RT	Spectro-photometer	12-well slide*with cover slip	At 5 to 20 sec	--	--	Detailed listing
Fitzpatrick et al. 2008	Blue mussel *Mytilus trossulus*	Fresh	--	Yes	1-mm welled slide with cover slip	--	0.33 sec	60	--
Jha et al. 2008	Blue mussel	Fresh	20	--	20-μm chambered slide	--	0.5-sec x 10	60	--
Martinez-Pastor et al. 2008	Senegalese sole	Fresh	--	--	--	At 15, 30, 45 & 60 sec	--	--	--
Singh and Singh 2008	Stinging catfish *Heteropneustes fossilis*	Fresh	--	By CASA	Slide coated with 1% polyvinyl alcohol	At 15 s	--	--	Kime et al. 1996, 2001; Chowdhury and Joy 2001 with modifications
Zilli et al. 2008	Gilthead sea bream *Sparus aurata* & Striped sea bream *Lithognathus mormyrus*	Fresh	--	--	12-well slide* with a cover slip	At 15 sec	45 sec	--	Detailed listing for each species
Gasparini et al. 2009	Guppy	Fresh	--	--	Glass slide coated with silicone with cover slip	--	--	--	Defined static cells
Krol et al. 2009	European smelt *Osmerus eperlanus*	Fresh	--	Yes	Method of Kawalski et al. 2006	--	--	--	--
Ottesen et al. 2009	Atlantic halibut	Fresh	7	--	Standard counting chamber (Leja)	--	--	--	--
Rosengrave et al. 2009a	Chinook salmon *Oncorhynchus tshawytscha*	Fresh	12	--	Regular glass slide with cover slip	At 10 & 20 sec for 0.5 sec	--	--	--

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						Image capture	Data analysis		
Rosengrave et al. 2009b	Chinook salmon	Fresh	--	--	--	At 10 & 20 sec for 0.5 sec	--	--	Defined motility
Schoenfuss et al. 2009	Goldfish	Fresh	22	--	--	--	--	--	--
Wilson-Leedy et al. 2009	Zebrafish	Fresh	--	--	Slides coated with 1% polyvinyl alcohol, 0.5-mm perfusion chamber (Invitrogen)	At 0 sec for 150 sec	--	97	Refers to Wilson-Leedy et al. 2007
Zilli et al. 2009	Gilthead sea bream	Fresh	--	--	12-well slide* with cover slip	--	--	--	--
Dietrich et al. 2010	Vendace *Coregonus albula*	Fresh	6	--	12-well slide* with cover slip	--	--	--	--
Groison et al. 2010	European hake *Merluccius merluccius*	Fresh	22	--	--	At 15 sec for 30 or 120 sec	15 sec	25	Detailed listing
Marchand et al. 2010	Mosambique tilapia *Oreochromis mossambicus* & African catfish	Fresh	RT	--	2-ul Leja chamber	At 0 sec for 50 sec	first 10 sec	30	--

--: Not reported.
RT: Room temperature.
*****12-well slides: 12-well multi-test slide from ICN Biomedicals.

Outlook for Future Application of CASA in Aquatic Species

Sperm quality analysis and control are necessary components for a wide range of programs including aquaculture, cryopreservation, and environmental monitoring. Currently, germplasm cryopreservation, distribution, and development represent a multi-billion dollar global industry for improvement in livestock industries. These activities provide a working blueprint for establishing parallel industries in aquatic species, and allow adoption of the equipment originally developed for mammals for use in fish and shellfish such as CASA systems. The publications summarized in Table 2 demonstrated the potential for application of CASA in fish and shellfish. However, to fully integrate CASA into aquaculture or germplasm programs as a reliable tool for evaluation of gamete quality, more investigation is needed. An approach for integration could include the following:

1) As stated above, *standardized settings* are essential for collection of data used for analysis. Data collection by CASA can be entirely dependent on control of settings (such as brightness and contrast) and protocols (such as timing of data capture). Due to the specific characteristics and diversity of fish sperm compared to mammal sperm, a panel of aquatic species to represent freshwater, marine, and euryhaline habitats (including species with distinct motility characteristics such as live-bearers) needs to be evaluated at controlled conditions (e.g., concentration and temperature). Standardized procedures for CASA parameter settings need to be established, and thus can serve as templates for use with new species in the future. In addition, sperm collected by stripping or dissection of the testis (necessary in some species) in fresh, stored, and thawed conditions needs to be compared for parameter settings.

2) *Standardized procedures* for data collection and analysis are needed to ensure reliable results. This is a large problem for several reasons. For example, most fish sperm are motile for 30 sec or less. However, CASA systems are generally designed for use with mammalian sperm which can be continuously motile for d. Thus, rapid data collection is necessary for fish sperm. The interval timing and duration chosen for analysis is critical to ensure observations are made during the time of peak motility. In addition, the problems associated with proper mixing of samples with activating solutions and development of volumetric chambers suitable for use with sperm of aquatic species need to be addressed.

3) Identification of *output parameters* in CASA analysis is most useful for estimation of gamete quality and prediction of sperm viability during refrigerated storage and shipping, after thawing, and in use for fertilization. After locking in the settings and protocols, it will be necessary to link the output parameters available from CASA analysis to sperm fertility for use with aquatic species.

4) Eventual *integration of instrument settings, protocols, and output parameters* into practical methodology would be the goal for application across a broad range of aquatic species. Such an approach would allow specific, systematic and repeatable analysis profiles for sperm before freezing and after thawing, and would allow work to be directly compared across species and laboratories.

The problems encountered in navigating this pathway to standardized CASA application have been addressed previously in livestock species such as cattle, swine, and horses. As such, they could provide useful templates for planning and implementation of informed approaches relevant to aquatic species (e.g., see the Technical Guide for IVOS, TOX IVOS, and CEROS, version 12.3, August 27, 2007, Hamilton Thorne Biosciences, Beverly, MA, USA).

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