Sperm motility in fish: technical applications and perspectives through CASA systems

V. Gallego and J.F. Asturiano*

Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal. Universitat Politècnica de València. Camino de Vera s/n, 46022 Valencia, Spain.

* Corresponding author:
Juan F. Asturiano Nemesio
Universitat Politècnica de València
Instituto de Ciencia y Tecnología Animal (Edificio 7G)
Camino de Vera s/n 46022 Valencia (Spain)
email: jfastu@dca.upv.es
Phone: +34 96 387 9385

Abstract

Although a relatively high number of sperm quality biomarkers have been reported over the years in several fish species, sperm motility is nowadays considered the best biomarker for fish spermatozoa. The first scientific reports focusing on fish sperm motility date from a century ago, but the objective assessment allowed by computer-assisted sperm analysis (CASA) systems was not applied to fish species until the mid 1980's. Since this date, a high number of sperm kinetic parameters from more than 170 fish species have already been reported in more than 700 scientific articles, covering a wide range of topics such as *i)* sperm physiology, *ii)* sperm storage, *iii)* broodstock management, *iv)* the phenomenon of sperm competition, *v)* ecotoxicology studies, and *vi)* understanding the life cycle of the species. To sum up, the sperm kinetic parameters provided by CASA systems can serve as a powerful and useful tool for aquaculture and ecological purposes, and this review gives an overview of the major research areas in which fish sperm motility assessed by a CASA system have been applied successfully.

Keywords:

Spermatozoa; velocity; sperm quality; kinetic, CASA

Sperm motility as a sperm qualitative biomarker in fish

Over the years, a relatively high number of sperm parameters have been used to assess sperm quality in fish (reviewed by Fauvel *et al.* 2010). These sperm biomarkers have so far been documented in scientific articles, and several traits, such as osmolality, plasma composition; enzymatic activity; ATP concentration; sperm density or sperm morphology have been linked to the ability of sperm to fertilize ova (Cabrita *et al.* 2014). However, sperm motility is currently considered the most useful parameter for assessing sperm quality in fish (Rurangwa *et al.* 2004), and more than 1500 scientific articles focusing on a large number of topics have been published over the last century. The most commonly used technique for assessing sperm motion in these articles has been subjective evaluation, but some problems have emerged from this method (Verstegen *et al.* 2002). Subjective assessment depends on an experienced observer, and several aspects such as sperm density, sperm velocity, and drift can be over- or underestimated (Rosenthal *et al.* 2010). Therefore, the low reproducibility of motility analyses that use subjective evaluation (which can result in variations of 30–60% in the same sample) often makes it difficult to interpret and compare the results between labs (Verstegen *et al.* 2002).

In this sense, the gradual appearance of computer assisted sperm analysis (CASA) systems has made it possible to estimate a higher number of sperm kinetic parameters using objective, sensitive and accurate techniques (Table 1). These systems are the evolution of multiple photomicrograph exposures and videomicrography techniques for sperm tracking, and with the benefits of a computer equipped with imaging software, detailed information on sperm kinetics can be extracted (Cabrita *et al.* 2009). Although CASA systems were first introduced in the 1970's for mammalian spermatozoa (Katz and Dott 1975; Dubois *et al.* 1974), they have only been successfully adapted for fish spermatozoa in the last two decades. The differences in the biology of fish and mammalian spermatozoa might explain this delay in the release of adequate tools for the measurement of fish sperm motility. Nevertheless, CASA systems are now being applied and validated successfully for a wide range of animal groups such as marine invertebrates (Gallego *et al.* 2014), birds (Lüpold *et al.* 2009), marine mammals (Robeck *et al.* 2011), reptiles (Tourmente *et al.* 2011) and even insects (Al-Lawati *et al.* 2009).

CASA parameters: an approach from fish spermatozoa

Many years ago, an experimented observer was able to estimate, in a subjective way, only

two sperm motion traits: *i)* the percentage of motile sperm cells and *ii)* the total duration of sperm movement. Then, faced with the difficult task of estimating correct and accurate sperm motility values, researchers used to make an arbitrary scale of criteria usually comprising of four to five categories at most. Now, CASA systems are able to quantify the percentage of motile spermatozoa in a concrete sample accurately and instantaneously and, in addition, computerized software is also able to estimate many other additional sperm kinetic parameters from the same sample, including some that cannot be detected by visual inspection (Figure 1 and Table 1). Although there are several companies marketing CASA products, the parameters provided by the systems are almost identical, and high correlations between most of them and fertilization or hatching rates have been reported in both freshwater and seawater fish species (Table 2).

The most commonly used parameters for fish sperm analysis were revised by Kime *et al.* (2001). The percentages of motile (TM or MOT) and progressive motile spermatozoa (PM or pMOT) can provide a general overview about the quality of a sperm sample (Rurangwa *et al.* 2004). MOT means any spermatozoa showing any movement while pMOT is determined as spermatozoa swimming in a progressive way. Although MOT and pMOT have been the most used motion parameters in sperm motility analyses, other authors consider sperm velocities better biomarkers of sperm quality (Rurangwa et al. 2001; Viveiros et al., 2010; Gallego et al., 2017a). In this respect, curvilinear velocity (VCL) is defined as the actual velocity along the real sperm trajectory, and straight-line velocity (VSL) means the straight-line distance between the start and end of the track divided by the time taken from start to finish. In essence, if the trajectory is a straight line, VCL and VSL are identical (Rurangwa *et al.* 2004). Finally, VAP (angular path velocity) is the velocity along a derived smoothed path. VAP is actually of little use in most fish because the sperm tracks are generally smooth curves, so VAP and VCL are very similar. However, depending on the fertilization microenvironment, spermatozoa can follow a much more erratic path so in some fish species both VCL and VAP become useful measurements (Kime and Tveiten 2002).

In addition to sperm velocities, CASA systems are able to provide us with several kinetic ratios such us linearity (LIN), straightness (STR) and wobble (WOB), all of which have been widely used to define fish sperm subpopulations. Although this topic (sperm subpopulations) has mostly been studied in mammals, the few reports in fish have clearly shown the coexistence of distinct motility-based sperm subpopulations (Beirão *et al.* 2009; Kanuga *et al.* 2012; Gallego *et al.* 2015), and new approaches based on sperm

kinetics can be used from this perspective.

To sum up, in addition to offering an objective and accurate estimation of classical kinetic parameters such as total motility, CASA systems provide a high number of novel sperm motion variables (impossible to detect by subjective evaluation) that can be successfully used in many research areas from fundamental to applied research.

Technical applications from CASA systems in fish

Although the first scientific reports assessing fish sperm quality using a subjective method date from about a century ago, computer-assisted systems for fish did not start to be used until the mid 80's. Since then, more than 700 publications on different topics using sperm motility as a research tool can be found in the literature on fish (Figure 2). In fact, in the last 30 years fish sperm parameters from 170 different species belonging to different families have been studied using these systems, and the results have been applied to many different areas, from ecology to molecular research (Figure 3). However, 20 of these fish species represent more than 50% of published papers, of which salmonids, cyprinids and sturgeons have been the most studied. Moreover, scientists have devoted much more time to studying the former (Figures 4 and 5). Here we present an overview of the most state of the art research areas in which CASA systems have been applied successfully.

Sperm physiology

Sperm physiology has been the most investigated factor in sperm studies carried out by CASA systems (Figure 3). In fact, the first study on fish sperm using a uncertain semi-assisted computer system was carried out in rainbow trout (*Oncorhynchus mykiss*) in 1985 (Cosson *et al.* 1985), where the authors reported an objective technique for the rapid quantitative assessment of sperm motility using stroboscopic illumination. Since then, this research field has grown continually over the years, and more than 100 physiology-related articles on different species have been published over the last 10 years.

The fish sperm activation process has been the key subject within this area, and to learn about the process by which spermatozoa begin to move has been the main goal of fish physiologists (Zuccarelli and Ingermann 2007; Vílchez *et al.* 2016; Pérez *et al.* 2016). Although sperm activation models for different species had previously been discovered and reported thanks to subjective motility evaluation, CASA systems have helped to describe these activation pathways in more depth through sperm kinetic features. For

example, some studies have reported that sperm activation in marine fish can be triggered both by electrolyte (e.g., seawater) and non-electrolyte (e.g., glucose-containing) media, but the absence of ions in the extracellular medium caused a general decline in sperm velocities in several species (Detweiler and Thomas 1998: Gallego *et al.* 2013c; Vílchez *et al.* 2017). On the other hand, some studies have shown that *in vitro* temperature can have an important effect on sperm motility parameter. In common carp (*Cyprinus carpio*), spermatozoa activated at 4 ºC showed higher motility rate than sperm activated at 14 and 24 ºC, whereas highest swimming velocity was observed at 14 °C (Dadras et al., 2016). Other studies showed similar results, and swimming velocity at high temperatures is often higher in species such as Senegalese sole (*Solea senegalensis*, Diogo et al., 2010) and European perch (*Perca fluviatilis*; Lahnsteiner, 2011). Moreover, the propulsion machinery of spermatozoa has been another research focus within sperm physiology studies, and although sperm ATP levels have been correlated with motility, velocity and⁄or fertilizing ability in several species like rainbow trout (Lahnsteiner *et al.* 1998), chinook salmon, *Oncorhynchus tshawytscha* (Bencic *et al.* 1999), or sea bass, *Dicentrarchus labrax* (Zilli *et al.* 2004); no correlations between ATP and sperm motility were found in other species such as common bleak, *Alburnus alburnus* (Lahnsteiner *et al.* 1996); bluegill, *Lepomis macrochirus* (Burness *et al.* 2005); or Atlantic cod, *Gadus morhua* (Butts *et al.* 2010).

Summing up, CASA systems have become useful for carrying out studies on fish sperm physiology, providing the user with an in-depth understanding of the activation mechanisms involved in different genus and families, and approaching several factors such as osmolality, temperature, ion plasma composition, etc…

Sperm storage

Sperm storage, both short and long-term, has been the second most investigated field using CASA systems as a research tool (Figure 3). Almost 200 scientific publications reporting kinetic sperm parameters have contributed to the discovery and improvement of sperm storage protocols in more than 80 fish species. Now, these techniques can be seen in a great number of applications, ranging from ecology to aquaculture.

With regards to cryopreservation, significant results have been reported in species belonging to the most important families used in aquaculture, and Table 3 summarizes the best results in terms of the pre- and post-thaw motilities (MOT) and velocities (VCL) obtained from each species. The most studied family has been that of the Salmonidae and

177 excellent sperm motion results have been reported using CASA systems in key
178 aquaculture species such as the Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*)
179 and brown trout (*Salmo trutta*) (see Table 3). In this context, although the
180 cryopreservation process often generates a significant decrease in MOT values, other
181 sperm kinetic parameters were not affected by the freezing process (Nynca *et al.* 2016).
182 For example, in Atlantic salmon, CASA systems have revealed a decrease in VCL and an
183 increase in LIN after cryopreservation, while no differences were observed in the VAP
184 or VSL values in post-thawed sperm. In brown trout (*S. trutta*) and rainbow trout (*O.*
185 *mykiss*), increases in VAP, VSL, and LIN were detected while a decrease in ALH was
186 also reported. In brook trout (*S. fontinalis*), lower values of VCL were seen in
187 cryopreserved sperm in comparison with fresh semen, whereas VAP, VSL, LIN and ALH
188 were similar in both fresh and cryopreserved sperm. Regarding the Cyprinidae, CASA
189 systems have helped in the creation and development of many of the species-specific
190 cryopreservation protocols that are currently being used in fish farms. In the Eurasian
191 perch (*Perca fluviatilis*), for example, an optimized commercial-scale cryopreservation
192 protocol was developed successfully, and although fresh sperm showed significantly
193 higher pMOT (85±5%) and VCL (139±7 μm/s) than cryopreserved sperm, similar
194 fertilization rates were achieved by both fresh and cryopreserved samples (Bernáth *et al.*
195 2016a). In common carp (*C. carpio*), post-thawed motility and sperm velocity were also
196 significantly lower when compared with fresh sperm, but the use of DMSO generated
197 better results than those provided by ethylene glycol (Li *et al.* 2013).
198 On the other hand, marine species have received much less attention than freshwater
199 species with regards to the development of cryopreservation protocols, and much of this
200 research has been concentrated in the last few years. In gilthead seabream (*Sparus aurata*)
201 kinetic data provided by a CASA system showed that sperm composition in terms of
202 subpopulations was differentially affected by the cryopreservation technique, and an
203 optimal protocol for them was established based on sperm motility-based subpopulations
204 (Beirão *et al.* 2011a). In seabass (*Dicentrarchus labrax*), notable post-thawed motility
205 values (~60%) were obtained using vitamins and amino acids to the cryopreservation
206 media (Cabrita *et al*., 2011).
207 To sum up, methods for fish sperm freezing have progressed in the last couple of decades,
208 and the use of CASA systems to assess sperm kinetic parameters is now recognized as
209 key in evaluating the validity of cryopreservation protocols. However, new techniques
210 are emerging in order to provide in-depth information on the negative effects of the

freezing-thawing process on genetic material, so fish sperm cryopreservation studies should combine both sperm kinetic assessments and DNA damage studies (Cabrita *et al.* 2014; Martínez-Páramo *et al.* 2017).

Broodstock management

Broodstock management involves a large number of factors that contribute to the ultimate aim of enabling a captive group of fish to successfully complete reproductive maturation and fertilization. In this context, sperm motion parameters play an essential role in achieving this objective, and the effect of different rearing factors (temperature, diet, handling, etc.) can be tested through the proper use of CASA systems. Around 100 scientific publications focusing on broodstock handling and using these systems have been published, and this section offers an overview of the most studied topics within this area (Figure 6).

Temperature and photoperiod are the main environmental factors controlling the development of gametes and gamete quality in most fish species (Migaud *et al.* 2013). With regard to temperature, several studies have shown how under- or over-optimal conditions have negative effects on gamete quality (Alavi and Cosson, 2005). Lahnsteiner and Leitner (2013) reported that in brown trout (*Salmo trutta*), a thermal regime of more than 5 ºC above the natural temperature affects the spermiation process, and causes a reduction in the percentage of spermiating male fish that produce spermatozoa of high quality (in terms of motility and swimming velocity). In European grayling (*Thymalus thymallus*), the maturation rate of male fish and their gamete quality depended greatly on the temperature regime, and the highest sperm motilities and velocities were obtained under a creek water temperature regime with natural seasonal fluctuations (Lahnsteiner and Kletzl 2012).

On the other hand, when sperm production using environmental treatments is not possible, hormonal induction techniques can be used to enhance spermiation and sperm quality. A wide variety of hormonal treatments (e.g., carp pituitary extract or gonadotropin preparations) have been tested on a great many aquacultural species (Mylonas *et al.* 2017), but CASA systems have mainly been used to test gonadotropin-releasing hormone agonist (GnRHa) treatments. Indeed, GnRHa implants have provided great results in marine species such as Atlantic bluefin tuna (*Thunnus thynnus thynnus*), where GnRHa-implantation therapy increased the percentage of spermiating males and the availability of motile spermatozoa (Mylonas *et al.* 2007). They have also shown

benefits in Atlantic halibut (*Hippoglossus hippoglossus*), although there were no significant differences in sperm motility between the two experimental groups treated with different GnRHa doses (5 and 25 µg/kg), the curvilinear velocity (VCL) assessed by a CASA system was significantly higher in males treated with a high dose (Vermeirssen *et al.* 2004). In European smelt (*Osmerus eperlanus*), GnRHa treatments resulted in the stimulation of a higher sperm volume and higher percentages of motility. However, the CASA systems did not reveal any statistical differences in CASA parameters between the control and hormonally treated groups (Król *et al.* 2009).

Broodstock nutrition is another key factor that affects gonadal development and gamete quality in fish (Izquierdo *et al.* 2001). However, although there are many publications linking diet and reproductive success (e.g., fertilization and hatching rates), few reports have been able to make a direct link between broodstock diet and the kinetic characteristics of spermatozoa assessed by a CASA system. In terms of freshwater species, although the dietary regime did not affect the percentage of motile spermatozoa, it significantly affected sperm velocity in common barb (*Barbus barbus*) (Alavi *et al.* 2008). In goldfish (*Carassius auratus gibelio*), the addition of vitamins and highly unsaturated fatty acids (HUFA) had a significant effect on sperm parameters such as the duration and percentage of spermatozoa with motility (Kashani and Imanpoor 2012); and in African catfish (*Clarias gariepinus*), a diet formulated with agricultural products provided higher milt volumes and improved sperm velocity in breeding males (Nyina-wamwiza *et al.* 2012). In marine species, such as Senegalese sole (*Solea senegalensis*), Beirão *et al.* (2015) reported that males who had been fed on an enriched diet (polyunsaturated fatty acid, PUFA) showed improvements in sperm motility parameters such as pMOT and VCL. Likewise, in European eel (*Anguilla anguilla*), diets with high levels of arachidonic acid and eicosapentaenoic acid induced better sperm kinetic parameters than did commercial diets (Butts *et al.* 2015; Baeza *et al.* 2015).

In the last few years, biotechnology and genetic engineering have contributed greatly to fish culture, allowing the production of triploid, tetraploid, haploid, gynogenetic or androgenetic fish through the application of novel breeding techniques (Foresti 2000). However, this type of technique involves small to large changes in the genetic material of affected cells, often having a negative impact on gamete quality (Pandian and Koteeswaran 1998). For example, in common tench (*Tinca tinca*), Linhart *et al.* (2006) reported that the ploidy level significantly influenced the percentage of motile spermatozoa: with the motile sperm count of diploid males ranging from 93% to 100%

and that of triploid males from 37% to 77%. However, the ploidy level did not result in any significant differences in terms of the velocity of spermatozoa. Conversely, in Atlantic cod (*Gadus morhua*), VCL was higher in the spermatozoa of diploid males compared with that of triploid males, but no differences between ploidies were observed for the remaining sperm motility descriptors (Peruzzi *et al.* 2009). On the other hand, in fish in which atypical combinations of sexual phenotype and genotype has become a useful tool for aquaculture production, the assessment of gamete quality is essential in order to carry out future crosses. In this context, a study performed in Nile tilapia (*Oreochromis niloticus*) showed that sperm kinetic parameters (measured using a CASA system) did not differ between the three different genotypes: XX, XY, and YY (Gennotte *et al.* 2012). Similar results were obtained in a comparative study of sperm quality over all possible sex genotypes in rainbow trout (*Oncorhynchus mykiss*), where sperm motility parameters showed no differences between neo-males (XX genotype) and super-males (YY genotype) (Kowalski *et al.* 2011).

To sum up, sperm kinetic parameters have become useful in the evaluation of many aspects relating to broodstock handling, and several factors such as *i)* the environmental rearing conditions, *ii)* the hormonal treatments used, *iii)* the diet requirements of each species, and *iv)* biotechnology and genetic engineering, have been improved through gamete evaluation using CASA systems.

Sperm competition

Sperm competition is defined as the process in which spermatozoa from two or more males race to fertilize the egg, is a widespread phenomenon that occurs in a wide range of animal taxa, including fish (Stoltz and Neff 2006). This phenomenon is closely related to dominance hierarchies, where male fish can adopt different mating strategies according to their social position (Serrano *et al.* 2006). Although sperm competition has become a recent topic of interest, more than 90 scientific papers on fish species have been published during the last two decades (Figure 3).

The trade-off between social investment and sperm performance has been widely studied in fish, and some studies have shown differences in sperm kinetic parameters between males with different social statuses. For example, in Chinook salmon (*Oncorhynchus tshawytscha),* parr males (jacks) invested significantly more of their somatic tissue into gonads compared with anadromous males (hooknoses), and parr males showed higher

motility and velocity values (90% and 70 µm/s, respectively) than dominant males (85% and 55 µm/s, respectively). In another study, after examining the sperm characteristics of 29 cichlid species, Fitzpatrick *el al.* (2009) showed that species experiencing greater levels of sperm competition have faster-swimming sperm. Nevertheless, even when theory predicts that dominant males might have lower quality spermatozoa, some studies have shown no effects, or even the opposite situation in other species such as rainbow trout (*O. mykiss*), bluegill (*Lepomis macrochirus*) and three-spined sticklebacks (*Gasterosteus aculeatus*) (Cardwell *et al.* 1996; Stoltz and Neff 2006; Mehlis *et al.* 2013). In this sense, sperm motility assessment can serve as a useful tool for studying the evolution of alternative reproductive strategies and mating systems in different fish taxa, and several kinetic parameters such as total motility, swimming velocity and/or motility over time will provide further data for sperm competition studies.

Ecotoxicology

Aquatic environments can carry substantial quantities of natural and man-made environmental contaminants (ECs), and evaluating the kinetics of fish sperm via CASA systems has become a key in assessing EC toxicity (Hatef *et al.* 2013). At present, around 70 scientific publications reporting the impact of ECs on sperm motion performance have contributed to the understanding of the toxicity mechanisms and action sites of ECs, and this knowledge can now be applied to a wide range of topics. However, is important to note that EC effects are extremely variable among fish taxa and even within species, and several factors such as EC concentrations or the duration of exposure can greatly affect sperm motion performance. In this regard, Table 4 summarizes the main ECs affecting sperm motility as assessed by a CASA system in some fish species, indicating the minimum EC dose at which sperm kinetic parameters were affected significantly.

Xenoestrogens are types of xenohormones that imitate oestrogen activity, and they can be produced by both synthetic or natural pathways. Among the most important ECs with oestrogenic effects are bisphenol-A, estradiol, and ethynyloestradiol, and several studies have reported their negative effect on the sperm motion performance in several freshwater species belonging to the Salmonidae and Cyprinidae (see Table 4). On the other hand, heavy metals represent the other EC group with high toxicity levels, and now they are considered the most dangerous pollutants in the world (Hatef *et al.* 2013). In this regard, Lahnsteiner *et al.* (2004) studied the impact of different heavy metals (zinc, mercury, and cadmium) on the sperm motility parameters of four teleosts belonging to the most

representative freshwater families (Salmonidae, Cyprinidae, Gadidae, and Clariidae). The authors concluded that toxic concentrations of all the pollutants differed markedly for each species (highlighting species-specific effects of these EC groups).

To sum up, sperm motility assessment has become a valuable tool to check and understand toxicity mechanisms and sites of action of different ECs, and changes in sperm motion performance can serve as a potential biomarker for biomonitoring these agents and their potential effects on reproductive function.

Ecology

CASA systems can also be applied to many areas of fish ecology. Although subjective evaluation of sperm motility has been the main method used in this field, more than 50 recent publications have used CASA systems and have contributed to the exploration of numerous ecology issues of different fish species from different taxa. In this context, a wide range of topics such as breeder age, seasonal changes, and characterization of populations are going to be approached through a sperm quality perspective.

In fish species with an annual reproductive cycle, sperm quality usually oscillates throughout the spawning season both in the wild and in captivity, and sperm motility assessment can give us the optimal period in which they should be collected.. For example, thanks to sperm motility assessment, scientists know (in wild conditions) that there are species in which sperm quality is higher at the beginning of the spawning season, such as halibut (*H. hippoglossus,* (Babiak *et al.* 2006)) or Senegalese sole (*Solea senegalensis,* (Beirão *et al.* 2011b)); species in which sperm quality is higher in the middle of the spawning season, such as Atlantic cod (*Gadus morhua,* (Rouxel *et al.* 2008)) or European seabass (*Dicentrarchus labrax,* (Dreanno *et al.* 1999b); and species such as common carp (*C. carpio,* (Christ *et al.* 1996)) or European perch (*P. fluviatilis,* (Alavi *et al.* 2010)) in which sperm quality is higher at the end of the spawning season.

Furthermore, kinetic parameters provided by CASA systems can also be applied to investigate inter-population differences, either by comparing wild populations to link sperm quality to environmental conditions (Salte *et al.* 2004; Dietrich *et al.* 2014; Biernaczyk *et al.* 2012), or by comparing farmed and wild populations to ascertain the possible impact of escaped farmed fish on wild ecosystems (Lehnert *et al.* 2012; Rideout *et al.* 2004; Butts *et al.* 2010).

Concerning inter-species studies, interesting ecology approaches can be made using sperm motility data. Gallego *et al.* (2014) after having analysed the sperm motion

parameters of several swimmer and sessile species, reported that the patterns were totally different. In that study, the authors linked the sperm motion patterns to species-specific lifestyles, postulating that sessile organisms (which show limited or no movement) need spermatozoa with a capacity to swim long distances to find the oocytes, while swimming male organisms can move toward the female and release gametes nearby, and as such the spermatozoa do not need to swim for such a long time.

Aspects to be improved in CASA systems

Although CASA systems are widely accepted by the animal reproductive science community as a valuable research tool for basic sperm biology, an evident lack of standardization in assessing fish sperm motion has often resulted in low reproducibility, making it difficult to interpret and compare intra- and inter-laboratory results (Rosenthal *et al.* 2010). Indeed, a series of biological, technical and CASA settings must be taken into account to harmonize common procedures and establish standardized protocols to be used in many research groups (see Table 5).

Biological or handling settings such as how to collect gametes (Aramli *et al.* 2016a), which ejaculate portion to use for analysing (Gallego *et al.* 2013a), the storage temperature before analysis (Sanches *et al.* 2015), and the sperm-to-activation medium ratio (Toth *et al.* 1995) can have a marked influence on evaluating sperm kinetics. In this context, it is important to note that the kinetic characteristics of fish spermatozoa are often species-specific, so biological settings must be linked to the species being evaluated.

Technical settings for assessing sperm motility can also involve a wide range of factors (Table 5), but few reports can be found in the literature on fish. For example, microscope settings such as the magnification had a significant effect on the pMOT levels and sperm velocities in European eel (*Anguilla anguilla*) (Gallego *et al.* 2013a); however, the use of different chambers did not affect these same sperm motion parameters when assessed by a CASA system. In common carp (*Cyprinus carpio*), Kowalski *et al.* (2014) reported that adhesion of sperm to a glass surface can be a crucial factor when assessing sperm motion performance by CASA systems; and recommended the use of protein supplements (e.g., bovine serum albumin) to obtain accurate CASA results for sperm quality prediction.

Finally, CASA settings also play a key role in estimating sperm kinetic parameters, and factors such as the recording frame rate (Castellini *et al.* 2011; Gallego *et al.* 2013a; Boryshpolets *et al.* 2013) or even the type of CASA used (Boryshpolets *et al.* 2013) have

a notable effect on sperm kinetic results both in freshwater and seawater species. However, there are other CASA settings that have not yet been tested, such as the number of cells sampled per field/capture, the location of the field inside the chamber, or even the focal position of swimming sperm cells inside an open drop. All of these factors could also affect sperm motion results, so further studies are necessary to evaluate the effect of reported and novel factors on a greater number of fish species.

5. New challenges for CASA systems in fish research

CASA systems are able to analyse a huge number of spermatozoa per capture, which means thousands of motion tracks reported per sample. However, despite the advantages of working with these extensive databases, most research groups are can only show the mean of the sperm quality parameters (or even some of them), and spermatozoa are considered to represent homogeneous populations. Nevertheless, it has been pointed out that the spermatozoa of some species do not constitute a homogeneous mixture, and several studies in fish have clearly shown the coexistence of different sperm motility-based subpopulations (Martínez-Pastor *et al.* 2008; Beirão *et al.* 2011a; Gallego *et al.* 2017). In this context, the study of the variations and distributions of these populations has been applied successfully in several research areas such as sperm physiology, sperm cryopreservation and broodstock management (Beirão *et al.* 2009; Kanuga *et al.* 2012; Gallego *et al.* 2015); moreover, certain sperm subpopulations have been positively and significantly correlated with fertilization and hatching rates in key aquaculture species, such as gilthead seabream (*Sparus aurata*, (Beirão *et al.* 2011a)) or tambaqui (*Colossoma macropomum*, (Gallego *et al.* 2017). Just as data modelling techniques (such as clustering) allow for the extraction of information between many variables and patterns relating to the kinetics of spermatozoa, subpopulation studies are becoming a novel tool to be applied in scientific fish and aquaculture matters.

Asides from providing us with a large number of sperm motion characteristics (described in Table 1), CASA systems are able to demonstrate other important parameters such as sperm concentrations, morphology, survival (viability) rates, and even the rate of DNA fragmentation. The set of parameters provided by the given CASA program depends on the brand of the product and, overall, by the number of modules purchased by the researcher. We can presently identify more than 20 companies that market CASA systems, and because they focus on a range of areas, from biology and medicine to

engineering, computer technology, and mathematics, the future development of these systems will be directed at a combination of related subjects (motility, morphology and/or viability) (Lu *et al.* 2014).

To sum up, CASA results in sperm motion analysis boast precision, reliability and reproducibility, providing the scientific community with a useful tool which can be applied both in aquaculture and for ecological purposes. Although sperm motion traits from a large number of species have already been reported in hundreds of articles, future developments in CASA systems (e.g., three-dimensional motion analysis, species-specific software, comfortable and portable systems) will be necessary to expand and deepen our knowledge of the biological functions of fish spermatozoa.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 642893 (ETN IMPRESS). VG has a postdoc grant from the UPV (PAID-10-16).

References

Abascal, F. J., Cosson, J. and Fauvel, C. (2007). Characterization of sperm motility in sea bass: The effect of heavy metals and physicochemical variables on sperm motility. *J. Fish Biol.* **70**, 509–522.

Al-Lawati, H., Kamp, G. and Bienefeld, K. (2009). Characteristics of the spermathecal contents of old and young honeybee queens. *J. Insect Physiol.* **55**, 116–121.

Alavi, S. M. H., and Cosson, J. (2005). Sperm motility in fishes. I. Effects of temperature and pH: a review. *Cell Biol. Int.*, **29 (2)**, 101-110.

Alavi, S. M. H., Psenicka, M., Policar, T., Rodina, M., Kozak, P. and Linhart, O. (2008). Sperm characteristics in *Barbus barbus* as a function of nutrition throughout the reproductive season. *Cybium* **32**, 200–201.

Alavi, S. M. H., Rodina, M., Hatef, A., Stejskal, V., Policar, T., Hamáčková, J. and Linhart, O. (2010). Sperm motility and monthly variations of semen characteristics in *Perca fluviatilis* (Teleostei: Percidae). *Czech J. Anim. Sci.* **55**, 174–182.

Aramli, M. S., Golshahi, K., Banan, A. and Sotoudeh, E. (2016a). Reliable collection of Caspian brown trout (*Salmo trutta caspius*) sperm using a catheter. *Reprod. Domest. Anim.* **51**, 831–834.

Aramli, M. S., Golshahi, K., Nazari, R. M., Golpour, A. and Aramli, S. (2016b). Influence of glutamine supplementation on motility and fertilization success of frozen–thawed Persian sturgeon (*Acipenser persicus*) sperm. *Reprod. Domest. Anim.* **51**, 474–477.

Babiak, I., Ottesen, O., Rudolfsen, G. and Johnsen, S. (2006). Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. *Theriogenology* **65**, 1587–1604.

Babiak, I., Marschhäuser, V., Ottesen, O., Rudolfsen, G., Eggen, B. and Babiak, J. (2012). Effects of extender, storage and sperm-to-egg ratio on cryopreservation success of Atlantic cod (*Gadus morhua* L.) sperm. *J. Appl. Ichthyol.* **28**, 941–947.

Baeza, R., Mazzeo, I., Vílchez, M. C., Gallego, V., Peñaranda, D. S., Pérez, L. and Asturiano, J. F. (2015). Relationship between sperm quality parameters and the fatty acid composition of the muscle, liver and testis of European eel. *Comp. Biochem. Physiol. Part A* **181**, 79–86.

Beirão, J., Soares, F., Herráez, M. P., Dinis, M. T. and Cabrita, E. (2009). Sperm quality evaluation in *Solea senegalensis* during the reproductive season at cellular level. *Theriogenology* **72**, 1251–1261.

Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S. and Herráez, M. P. (2011a). Effect of cryopreservation on fish sperm subpopulations. *Cryobiology* **62**, 22–31.

Beirão, J., Soares, F., Herráez, M. P., Dinis, M. T. and Cabrita, E. (2011b). Changes in *Solea senegalensis* sperm quality throughout the year. *Anim. Reprod. Sci.* **126**, 122–129.

Beirão, J., Soares, F., Pousão-Ferreira, P., Diogo, P., Dias, J., Dinis, M. T., Herráez, M. P. and Cabrita, E. (2015). The effect of enriched diets on *Solea senegalensis* sperm quality. *Aquaculture* **435**, 187–194.

Bencic, D. C., Krisfalusi, M., Cloud, J. G., and Ingermann, R. L. (1999). ATP levels of chinook salmon (*Oncorhynchus tshawytscha*) sperm following in vitro exposure to various oxygen tensions. Fish. Physiol. Biochem. **20**, 389-397.

Bernáth, G., Bokor, Z., Zarski, D., Várkonyi, L., Hegyi, A., Staszny, A., Urbányi, B., Radóczi Ifj., J. and Horváth, A. (2016a). Commercial-scale out-of-season cryopreservation of Eurasian perch (*Perca fluviatilis*) sperm and its application for fertilization. *Anim. Reprod. Sci.* **170**, 170–177.

Bernáth, G., Żarski, D., Kása, E., Staszny, Á., Várkonyi, L., Kollár, T., Hegyi, Á., Bokor, Z., Urbányi, B. and Horváth, Á. (2016b). Improvement of common carp (*Cyprinus*

513 *carpio*) sperm cryopreservation using a programable freezer. *Gen. Comp.*
514 *Endocrinol.* **237**, 78–88.
515 Biernaczyk, M., Formicki, K., Bartel, R. and Mongialo, Z. (2012). Characteristics of
516 gametes of the Atlantic salmon (*Salmo salar* L.) restored in northern Poland. *J. Appl.*
517 *Ichthyol.* **28**, 66–74.
518 Billard, R., Cosson, J. and Crim, L. W. (1993). Motility of fresh and aged halibut sperm.
519 *Aquat. Living Resour.* **6**, 67–75.
520 Boryshpolets, S., Kowalski, R. K., Dietrich, G. J., Dzyuba, B. and Ciereszko, A. (2013).
521 Different computer-assisted sperm analysis (CASA) systems highly influence sperm
522 motility parameters. *Theriogenology* **80**, 758–765.
523 Bozkurt, Y., and Secer, S. (2006). Relationship between spermatozoa motility, egg size,
524 fecundity and fertilization success in brown trout (*Salmo trutta fario*). *Pakistan J.*
525 *Biol. Sci.* **9**, 2141–2144.
526 Burness, G., Moyes, C. D., and Montgomerie, R. (2005). Motility, ATP levels and
527 metabolic enzyme activity of sperm from bluegill (*Lepomis macrochirus*). *Comp.*
528 *Biochem. Physiol. A Mol. Integr. Physiol.* **140**(1), 11-17.
529 Butts, I. A. E., Litvak, M. K. and Trippel, E. A. (2010). Seasonal variations in seminal
530 plasma and sperm characteristics of wild-caught and cultivated Atlantic cod, *Gadus*
531 *morhua*. *Theriogenology* **73**, 873–885.
532 Butts, I. A. E., Baeza, R., Strotrup, J. G., Krüger-Johnsen, M., Jacobsen, C., Pérez, L.,
533 Asturiano, J. F. and Tomkiewicz, J. (2015). Impact of dietary fatty acids on muscle
534 composition, liver lipids, milt composition and sperm performance in European eel.
535 *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **183**, 87–96.
536 Cabrita, E., Robles, V. and Herráez, M. P. (2009). Sperm quality assessment, in *Methods*
537 *Reprod. Aquac. Mar. Freshw. Species*, (CabritaE., RoblesV., HerráezM. P., eds), pp.
538 93–148. CRC Press (Taylor and Francis Group), Boca Raton, Florida, USA.
539 Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., and Dinis, M. T.
540 (2011). The influence of certain aminoacids and vitamins on post-thaw fish sperm
541 motility, viability and DNA fragmentation. *Anim. Reprod. Sci*. **125**, 189-195.
542 Cabrita, E., Martínez-Páramo, S., Gavaia, P. J., Riesco, M. F., Valcarce, D. G.,
543 Sarasquete, C., Herráez, M. P. and Robles, V. (2014). Factors enhancing fish sperm
544 quality and emerging tools for sperm analysis. *Aquaculture* **432**, 389–401.
545 Cardwell, J. R., Sorensen, P. W., Van der Kraak, G. J., Liley, N. R. R., Cabrita, E., Robles,
546 V., Cuñado, S., Wallace, J. C., Sarasquete, C. and Herráez, M. P. (1996). Effect of

547 dominance status on sex hormone levels in laboratory and wild-spawning male trout.
548 *Cryobiology* **50**, 273–284.
549 Casselman, S. J., Schulte-Hostedde, A. I. and Montgomerie, R. (2006). Sperm quality
550 influences male fertilization success in walleye (*Sander vitreus*). *Can. J. Fish. Aquat.*
551 *Sci.* **63**, 2119–2125.
552 Castellini, C., Dal Bosco, A., Ruggeri, S. and Collodel, G. (2011). What is the best frame
553 rate for evaluation of sperm motility in different species by computer-assisted sperm
554 analysis? *Fertil. Steril.* **96**, 24–7.
555 Chen, J., Saili, K. S., Liu, Y., Li, L., Zhao, Y., Jia, Y., Bai, C., Tanguay, R. L., Dong, Q.
556 and Huang, C. (2017). Developmental bisphenol A exposure impairs sperm function
557 and reproduction in zebrafish. *Chemosphere* **169**, 262–270.
558 Christ, S., Toth, G., McCarthy, H., Torsella, J. and Smith, M. (1996). Monthly variation
559 in sperm motility in common carp assessed using computer-assisted sperm analysis
560 (CASA). *J. Fish Biol.* **48**, 1210–1222.
561 Chyb, J., and Kime, D. (2000). The influence of zinc on sperm motility of common carp-
562 a computer assisted studies. *Arch. Rybactwa* **8**, 5–14.
563 Chyb, J., Kime, D. E., Szczerbik, P., Mikoajczyk, T. and Epler, P. (2001). Computer-
564 assisted analysis (CASA) of common carp *Cyprinus carpio* L. spermatozoa motility
565 in the presence of cadmium. *Arch. Polish Fish.* **9**, 173–182.
566 Ciereszko, A., Toth, G. P., Christ, S. A. and Dabrowski, K. (1996). Effect of
567 cryopreservation and theophylline on motility characteristics of lake sturgeon
568 (*Acipenser fulvescens*) spermatozoa. *Theriogenology* **45**, 665–72.
569 Ciereszko, A., Dietrich, G. J., Nynca, J., Dobosz, S., and Zalewski, T. (2014).
570 Cryopreservation of rainbow trout semen using a glucose-methanol extender.
571 *Aquaculture*. **420**, 275-281.
572 Cosson, M. P., Billard, R., Gatti, J. L. and Christen, R. (1985). Rapid and quantitative
573 assessment of trout spermatozoa motility using stroboscopy. *Aquaculture* **46**, 71–75.
574 Cosson, J., Groison, A. L., Suquet, M., Fauvel, C., Dreanno, C. and Billard, R. (2008).
575 Studying sperm motility in marine fish: An overview on the state of the art. *J. Appl.*
576 *Ichthyol.* **24**, 460–486.
577 Dadras, H., Dzyuba, V., Cosson, J., Golpour, A., and Dzyuba, B. (2016). The *in vitro*
578 effect of temperature on motility and antioxidant response of common carp *Cyprinus*
579 *carpio* spermatozoa. *J Therm Biol.* **59**, 64-68.
580 Detweiler, C., and Thomas, P. (1998). Role of ions and ion channels in the regulation of

581 atlantic croaker sperm motility. *J. Exp. Zool.* **281**, 139–148.
582 Dietrich, G. J., Dietrich, M., Kowalski, R. K., Dobosz, S., Karol, H., Demianowicz, W.
583 and Glogowski, J. (2010). Exposure of rainbow trout milt to mercury and cadmium
584 alters sperm motility parameters and reproductive success. *Aquat. Toxicol.* **97**, 277–
585 284.
586 Dietrich, M. A., Dietrich, G. J., Hliwa, P. and Ciereszko, A. (2011). Carp transferrin can
587 protect spermatozoa against toxic effects of cadmium ions. *Comp. Biochem. Physiol.*
588 *- C Toxicol. Pharmacol.* **153**, 422–429.
589 Dietrich, G. J., Pecio, A., Sikorska, J., Wolnicki, J., Słowińska, M., Hliwa, P., Kamiński,
590 R., Andronowska, A. and Ciereszko, A. (2014). Characterization of lake minnow
591 Eupallasella percnurus semen in relation to sperm morphology, regulation of sperm
592 motility and interpopulation diversity. *J. Fish Biol.* **85**, 446–455.
593 Dietrich, G. J., Nynca, J., Szczepkowski, M., Dobosz, S., Szczepkowska, B. and
594 Ciereszko, A. (2016). The effect of cryopreservation of semen from whitefish
595 (*Coregonus lavaretus*) and northern pike (*Esox lucius*) using a glucose-methanol
596 extender on sperm motility parameters and fertilizing ability. *Aquaculture* **464**, 60–
597 64.
598 Diogo, P.A.C. (2010). Sperm motility in *Solea senegalensis*: effect of temperature,
599 salinity, pH and ovarian fluid (Doctoral Thesis).
600 Dreanno, C., Cosson, J., Suquet, M., Seguin, F., Dorange, G. and Billard, R. (1999a).
601 Nucleotide content, oxydative phosphorylation, morphology, and fertilizing
602 capacity of turbot (*Psetta maxima*) spermatozoa during the motility period. *Mol.*
603 *Reprod. Dev.* **53**, 230–243.
604 Dreanno, C., Suquet, M., Fauvel, C., Coz, J. R. Le, Dorange, G., Quemener, L. and
605 Billard, R. (1999b). Effect of the aging process on the quality of sea bass
606 (*Dicentrarchus labrax*) semen. *J. Appl. Ichthyol.* **15**.
607 Dubois, M., Jouannet, P., Berge, P. and David, G. (1974). Spermatozoa motility in human
608 cervical mucus. *Nature* **252**, 711–713.
609 Dzyuba, B., Boryshpolets, S., Cosson, J., Dzyuba, V., Fedorov, P., Saito, T., Psenicka,
610 M., Linhart, O. and Rodina, M. (2014). Motility and fertilization ability of sterlet
611 *Acipenser ruthenus* testicular sperm after cryopreservation. *Cryobiology* **69**, 339–
612 341.
613 Fabbrocini, A., D'Adamo, R., Pelosi, S., Oliveira, L. F. J., Prete, F. Del, Silvestri, F.,
614 Vitiello, V. and Sansone, G. (2015). Sperm motility evaluation following long-term

storage (5 years) of cryopreserved sea bream (*Sparus aurata* L., 1758) semen. *J. Appl. Ichthyol.* **31**, 104–107.

Fauvel, C., Suquet, M. and Cosson, J. (2010). Evaluation of fish sperm quality. *J. Appl. Ichthyol.* **26**, 636–643.

Foresti, F. (2000). Biotechnology and fish culture. *Hydrobiologia* **420**, 45–47.

Gallego, V., Carneiro, P. C. F., Mazzeo, I., Vílchez, M. C., Peñaranda, D. S., Soler, C., Pérez, L. and Asturiano, J. F. (2013a). Standardization of European eel (*Anguilla anguilla*) sperm motility evaluation by CASA software. *Theriogenology* **79**, 1034–1040.

Gallego, V., Pérez, L., Asturiano, J. F. and Yoshida, M. (2013b). Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). *Aquaculture* **416**–**417**, 238–243.

Gallego, V., Pérez, L., Asturiano, J. F. and Yoshida, M. (2013c). Study of pufferfish (*Takifugu niphobles*) sperm: Development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca^{2+} and $^{K+}$ in the initiation of sperm motility. *Aquaculture* **414**–**415**, 82–91.

Gallego, V., Pérez, L., Asturiano, J. F. and Yoshida, M. (2014). Sperm motility parameters and spermatozoa morphometric characterization in marine species: A study of swimmer and sessile species. *Theriogenology* **82**, 668–676.

Gallego, V., Vílchez, M. C., Peñaranda, D. S., Pérez, L., Herráez, M. P., Asturiano, J. F. and Martínez-Pastor, F. (2015). Subpopulation pattern of eel spermatozoa is affected by post-activation time, hormonal treatment and the thermal regimen. *Reprod. Fertil. Dev.* **27**, 529–543.

Gallego, V., Cavalcante, S. S., Fujimoto, R. Y., Carneiro, P. C. F., Azevedo, H. C. and Maria, A. N. (2017). Fish sperm subpopulations: Changes after cryopreservation process and relationship with fertilization success in tambaqui (*Colossoma macropomum*). *Theriogenology* **87**, 16–24.

Gárriz, Á., Menéndez-Helman, R. J. and Miranda, L. A. (2015). Effects of estradiol and ethinylestradiol on sperm quality, fertilization, and embryo-larval survival of pejerrey fish (*Odontesthes bonariensis*). *Aquat. Toxicol.* **167**, 191–199.

Gennotte, V., François, E., Rougeot, C., Ponthier, J., Deleuze, S. and Mélard, C. (2012). Sperm quality analysis in XX, XY and YY males of the Nile tilapia (*Oreochromis niloticus*). *Theriogenology* **78**, 210–217.

Giardina, A., Larson, S. F., Wisner, B., Wheeler, J. and Chao, M. (2009). Long-term and

649 acute effects of zinc contamination of a stream on fish mortality and physiology.
650 *Environ. Toxicol. Chem.* **28**, 287.
651 Hatef, A., Alavi, S. M. H., Linhartova, Z., Rodina, M., Policar, T. and Linhart, O. (2010).
652 In vitro effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis*
653 L. (Percidae; Teleostei). *J. Appl. Ichthyol.* **26**, 696–701.
654 Hatef, A., Alavi, S. M. H., Butts, I. A. E., Policar, T. and Linhart, O. (2011). Mechanism
655 of action of mercury on sperm morphology, adenosine triphosphate content, and
656 motility in *Perca fluviatilis* (Percidae; Teleostei). *Environ. Toxicol. Chem.* **30**, 905–
657 914.
658 Hatef, A., Alavi, S. M. H., Abdulfatah, A., Fontaine, P., Rodina, M. and Linhart, O.
659 (2012). Adverse effects of bisphenol A on reproductive physiology in male goldfish
660 at environmentally relevant concentrations. *Ecotoxicol. Environ. Saf.* **76**, 56–62.
661 Hatef, A., Alavi, S. M. H., Golshan, M. and Linhart, O. (2013). Toxicity of environmental
662 contaminants to fish spermatozoa function in vitro-A review. *Aquat. Toxicol.* **140**–
663 **141**, 134–144.
664 Izquierdo, M. S., Fernández-Palacios, H. and Tacon, A. G. J. (2001). Effect of broodstock
665 nutrition on reproductive performance of fish. *Aquaculture* **197**, 25–42.
666 Judycka, S., Szczepkowski, M., Ciereszko, A. and Dietrich, G. J. (2015). New extender
667 for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. *Cryobiology* **70**,
668 184–189.
669 Kanuga, M. K., Drew, R. E., Wilson-Leedy, J. G. and Ingermann, R. L. (2012).
670 Subpopulation distribution of motile sperm relative to activation medium in
671 steelhead (*Oncorhynchus mykiss*). *Theriogenology* **77**, 916–925.
672 Kashani, Z. H., and Imanpoor, M. R. I. (2012). Long-Term Effects and Interactions of
673 Different Levels of Dietary Vitamin C and E and Highly Unsaturated Fatty Acid on
674 Sperm Parameters in Goldfish (*Carassius auratus gibelio*). *J. Aquac. Res. Dev.* **3**,
675 3–6.
676 Katz, D. F., and Dott, H. M. (1975). Methods of measuring swimming speed of
677 spermatozoa. *J. Reprod. Fertil.* **45**, 263–72.
678 Kime, D. E., Ebrahimi, M., Nysten, K., Roelants, I., Rurangwa, E., Moore, H. D. M. and
679 Ollevier, F. (1996). Use of computer assisted sperm analysis (CASA) for monitoring
680 the effects of pollution on sperm quality of fish; application to the effects of heavy
681 metals. *Aquat. Toxicol.* **36**, 223–237.
682 Kime, D. E., Van Look, K. J. W., McAllister, B. G., Huyskens, G., Rurangwa, E. and

683 Ollevier, F. (2001). Computer-assisted sperm analysis (CASA) as a tool for
684 monitoring sperm quality in fish. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.*
685 **130**, 425–433.
686 Kime, D. E., and Tveiten, H. (2002). Unusual motility characteristics of sperm of the
687 spotted wolffish. *J. Fish Biol.* **61**, 1549–1559.
688 Kowalski, R. K., Sarosiek, B., Demianowicz, W., Judek, J., Goryczko, K., Dobosz, S.,
689 Kuźmiński, H., Demska-Zakeś, K., Babiak, I. and Glogowski, J. (2011).
690 Quantitative characteristics of rainbow trout, *Oncorhynchus mykiss*, neo-males (XX
691 genotype) and super-males (YY genotype) sperm. *World Acad. Sci. Eng. Technol.*
692 **53**, 1162–1169.
693 Kowalski, R. K., Cejko, B. I., Krejszeff, S., Sarosiek, B., Judycka, S., Targońska, K.,
694 Kucharczyk, D. and Glogowski, J. (2014). Effect of albumin and casein
695 supplementation on the common carp *Cyprinus carpio* L. sperm motility parameters
696 measured by CASA. *Aquac. Int.* **22**, 123–129.
697 Krasznai, Z., Márián, T., Balkay, L., Gáspár, R. and Trón, L. (1995). Potassium channels
698 regulate hypo-osmotic shock-induced motility of common carp (*Cyprinus carpio*)
699 sperm. *Aquaculture* **129**, 123–128.
700 Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L. and Morisawa,
701 M. (2000). Membrane hyperpolarization removes inactivation of Ca^{2+} channels,
702 leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common
703 carp. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2052–7.
704 Król, J., Kowalski, R. K., Hliwa, P., Dietrich, G. J., Stabiński, R. and Ciereszko, A.
705 (2009). The effects of commercial preparations containing two different GnRH
706 analogues and dopamine antagonists on spermiation and sperm characteristics in the
707 European smelt *Osmerus eperlanus* (L.). *Aquaculture* **286**, 328–331.
708 Lahnsteiner, F., Berger, B., Weismann, T., and Patzner, R. A. (1996). Motility of
709 spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal
710 plasma composition and sperm metabolism. *Fish Physiol. Biochem.* ***15***, 167-179.
711 Lahnsteiner, F., Berger, B., Weismann, T., and Patzner, R.A. (1998). Determination of
712 semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility,
713 seminal plasma parameters, and spermatozoal metabolism. Aquaculture, **163**, 163-
714 181.
715 Lahnsteiner, F., Mansour, N. and Berger, B. (2004). The effect of inorganic and organic
716 pollutants on sperm motility of some freshwater teleosts. *J. Fish Biol.* **65**, 1283–

1297.

Lahnsteiner, F., Berger, B., Kletzl, M. and Weismann, T. (2005). Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta* f. fario. *Aquat. Toxicol.* **75**, 213–224.

Lahnsteiner, F. (2011). Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the ability to swim for more than two hours in saline solutions. Aquaculture **314**, 221-224.

Lahnsteiner, F., and Kletzl, M. (2012). The effect of water temperature on gamete maturation and gamete quality in the European grayling (*Thymalus thymallus*) based on experimental data and on data from wild populations. *Fish Physiol. Biochem.* **38**, 455–467.

Lahnsteiner, F., and Leitner, S. (2013). Effect of Temperature on Gametogenesis and Gamete Quality in Brown Trout, *Salmo trutta*. *J. Exp. Zool. Part A Ecol. Genet. Physiol.* **319**, 138–148.

Lehnert, S. J., Heath, D. D. and Pitcher, T. E. (2012). Sperm trait differences between wild and farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* **344–349**, 242–247.

Li, P., Hulak, M., Li, Z. H., Sulc, M., Psenicka, M., Rodina, M., Gela, D. and Linhart, O. (2013). Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. *Theriogenology* **80**, 84–89.

Liley, N. R. R., Tamkee, P., Tsai, R. and Hoysak, D. J. J. (2002). Fertilization dynamics in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and sperm concentration and motility on in vitro fertilization. *Can. J. Fish. Aquat. Sci.* **59**, 144–152.

Linhart, O., Rodina, M. and Cosson, J. (2000). Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. *Cryobiology* **41**, 241–250.

Linhart, O., Rodina, M., Flajshans, M., Mavrodiev, N., Nebesarova, J., Gela, D. and Kocour, M. (2006). Studies on sperm of diploid and triploid tench, *Tinca tinca* (L.). *Aquac. Int.* **14**, 9–25.

Liu, Q. H., Li, J., Xiao, Z. Z., Ding, F. H., Yu, D. De and Xu, X. Z. (2007). Use of computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved sperm in red seabream (*Pagrus major*). *Aquaculture* **263**, 20–25.

751 Liu, Q., Wang, X., Wang, W., Zhang, X., Xu, S., Ma, D., Xiao, Z., Xiao, Y. and Li, J.
752 (2015). Effect of the addition of six antioxidants on sperm motility, membrane
753 integrity and mitochondrial function in red seabream (*Pagrus major*) sperm
754 cryopreservation. *Fish Physiol. Biochem.* **41**, 413–422.
755 Lu, J. C., Huang, Y. F. and Lü, N. Q. (2014). Computer-aided sperm analysis: Past,
756 present and future. *Andrologia* **46**, 329–338.
757 Lujić, J., Bernath, G., Marinovic, Z., Radojkovic, N., Simic, V., Ćirković, M., Urbbnyi,
758 B. and Horvarth, A. (2017). Fertilizing capacity and motility of tench Tinca tinca
759 (L., 1758) sperm following cryopreservation. *Aquac. Res.* **48**, 102–110.
760 Lüpold, S., Calhim, S., Immler, S. and Birkhead, T. R. (2009). Sperm morphology and
761 sperm velocity in passerine birds. *Proceedings. Biol. Sci.* **276**, 1175–1181.
762 Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet,
763 M., et al. (2017). Cryobanking of aquatic species. *Aquaculture* **472**, 156–177.
764 Martínez-Pastor, F., Cabrita, E., Soares, F., Anel, L. and Dinis, M. T. (2008). Multivariate
765 cluster analysis to study motility activation of Solea senegalensis spermatozoa: A
766 model for marine teleosts. *Reproduction* **135**, 449–459.
767 Mehlis, M., Hilke, L. K. and Bakker, T. C. M. (2013). Attractive males have faster sperm
768 in three-spined sticklebacks *Gasterosteus aculeatus*. *Curr. Zool.* **59**, 761–768.
769 Migaud, H., Bell, G., Cabrita, E., Mcandrew, B., Davie, A., Bobe, J., Herráez, M. P. and
770 Carrillo, M. (2013). Gamete quality and broodstock management in temperate fish.
771 *Rev. Aquac.* **5**, S194–S223.
772 Montgomery, T. M., Brown, A. C., Gendelman, H. K., Ota, M. and Clotfelter, E. D.
773 (2014). Exposure to 17α-ethinylestradiol decreases motility and ATP in sperm of
774 male fighting fish *Betta splendens*. *Environ. Toxicol.* **29**, 243–252.
775 Mylonas, C. C., Bridges, C., Gordin, H., Rios, A. B., Garcia, A., la Gándara, F. De,
776 Fauvel, C., et al. (2007). Preparation and administration of gonadotropin-releasing
777 hormone agonist (GnRHa) implants for the artificial control of reproductive
778 maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*).
779 *Rewiews Fish. Sci.* **15**, 183–210.
780 Mylonas, C. C., Duncan, N. J. and Asturiano, J. F. (2017). Hormonal manipulations for
781 the enhancement of sperm production in cultured fish and evaluation of sperm
782 quality. *Aquaculture* **472**, 21–44.
783 Nyina-wamwiza, L., Milla, S., Pierrard, M. A., Rurangwa, E., Mandiki, S. N. M., Van
784 Look, K. J. W. and Kestemont, P. (2012). Partial and total fish meal replacement by

785 agricultural products in the diets improve sperm quality in African catfish (*Clarias*
786 *gariepinus*). *Theriogenology* **77**, 184–194.
787 Nynca, J., Dietrich, G. J., Dobosz, S., Grudniewska, J. and Ciereszko, A. (2014). Effect
788 of cryopreservation on sperm motility parameters and fertilizing ability of brown
789 trout semen. *Aquaculture* **433**, 62–65.
790 Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., Grudniewska, J., Arai, K., Fujimoto,
791 T. and Ciereszko, A. (2016). Utility of different sugar extenders for cryopreservation
792 and post-thaw storage of sperm from Salmonidae species. *Aquaculture* **464**, 340–
793 348.
794 Okuno, M., and Morisawa, M. (1989). Effects of calcium on motility of rainbow trout
795 sperm flagella demembranated with Triton X-100. *Cell Motil. Cytoskeleton* **14**, 194–
796 200.
797 Oropesa, A. L., Martín-Hidalgo, D., Fallola, C. and Gil, M. C. (2015). Effects of exposure
798 to 17-alpha-ethynylestradiol on sperm quality of tench (*Tinca tinca*). *Ecotoxicol.*
799 *Environ. Saf.* **120**, 318–325.
800 Ottesen, O. H., Babiak, I. and Dahle, G. (2009). Sperm competition and fertilization
801 success of Atlantic halibut (*Hippoglossus hippoglossu*s L.). *Aquaculture* **286**, 240–
802 245.
803 Pandian, T. J., and Koteeswaran, R. (1998). Ploidy induction and sex control in fish.
804 *Hydrobiologia* **384**, 167–243.
805 Pérez, L., Vílchez, M. C., Gallego, V. V., Morini, M., Peñaranda, D. S. and Asturiano, J.
806 F. (2016). Role of calcium on the initiation of sperm motility in the European eel.
807 *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **191**, 98–106.
808 Peruzzi, S., Rudolfsen, G., Primicerio, R., Frantzen, M. and Kaurić, G. (2009). Milt
809 characteristics of diploid and triploid Atlantic cod (*Gadus morhua* L.). *Aquac. Res.*
810 **40**, 1160–1169.
811 Rideout, R. M., Trippel, E. A. and Litvak, M. K. (2004). Relationship between sperm
812 density, spermatocrit, sperm motility and spawning date in wild and cultured
813 haddock. *J. Fish Biol.* **65**, 319–332.
814 Robeck, T. R., Gearhart, S. A., Steinman, K. J., Katsumata, E., Loureiro, J. D. and
815 O'Brien, J. K. (2011). In vitro sperm characterization and development of a sperm
816 cryopreservation method using directional solidification in the killer whale (*Orcinus*
817 *orca*). *Theriogenology* **76**, 267–279.
818 Rosenthal, H., Asturiano, J. F., Linhart, O. and Horváth, Á. (2010). On the biology of fish

gametes: Summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. *J. Appl. Ichthyol.* **26**, 621.

Routray, P., Dash, S. N., Dash, C., Swain, P., Sarkar, S. K. and Sarangi, N. (2008). Cryopreservation of silver barb Puntius gonionotus (Bleeker) spermatozoa: Effect of extender composition, cryoprotective agents and freezing rate on their postthawing fertilization ability. *Aquac. Res.* **39**, 1597–1605.

Rouxel, C., Suquet, M., Cosson, J., Severe, A., Quemener, L. and Fauvel, C. (2008). Changes in Atlantic cod (*Gadus morhua* L.) sperm quality during the spawning season. *Aquac. Res.* **39**, 434–440.

Rurangwa, E., Volckaert, F. A. M., Huyskens, G., Kime, D. E. and Ollevier, F. (2001). Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*). *Theriogenology* **55**, 751–769.

Rurangwa, E., Biegniewska, A., Slominska, E., Skorkowski, E. F. and Ollevier, F. (2002). Effect of tributyltin on adenylate content and enzyme activities of teleost sperm: A biochemical approach to study the mechanisms of toxicant reduced spermatozoa motility. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **131**, 335–344.

Rurangwa, E., Kime, D. E., Ollevier, F. and Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture* **234**, 1–28.

Salte, R., Galli, A., Falaschi, U., Fjalestad, K. T. and Aleandri, R. (2004). A protocol for the on-site use of frozen milt from rainbow trout (*Oncorhynchus mykiss* Walbaum) applied to the production of progeny groups: Comparing males from different populations. *Aquaculture* **231**, 337–345.

Sanches, E. A., Okawara, R. Y., Caneppele, D., Toledo, C. P. R., Bombardelli, R. A. and Romagosa, E. (2015). Sperm characteristics of *Steindachneridion parahybae* (Steindachner, 1877) throughout 112 h of storage at four temperatures. *J. Appl. Ichthyol.* **31**, 79–88.

Schultz, I. R., Battelle, P. N. N. L., Richland, W. A., Drum, A. S., Battelle, M. S. L., Sequim, W. A., Hayton, W. L. and Orner, G. A. (2000). Environmental estrogens: Dose-response relationships for vitellogenin formation and reproductive toxicity in male rainbow trout. *Mar. Environ. Res.* **50**, 192–193.

Serrano, J. V, Folstad, I., Rudolfsen, G. and Figenschou, L. (2006). Do the fastest sperm within an ejaculate swim faster in subordinate than in dominant males of Arctic char?

Can. J. Zool. **84**, 1019–1024.

Stoltz, J. A., and Neff, B. D. (2006). Sperm competition in a fish with external fertilization: The contribution of sperm number, speed and length. *J. Evol. Biol.* **19**, 1873–1881.

Toth, G. P., Christ, S. A., McCarthy, H. W., Torsella, J. A. and Smith, M. K. (1995). Computer-assisted motion analysis of sperm from the common carp. *J. Fish Biol.* **47**, 986–1003.

Tourmente, M., Giojalas, L. C. and Chiaraviglio, M. (2011). Sperm parameters associated with reproductive ecology in two snake species. *Herpetologica* **67**, 58–70.

Van Look, K. J. W., and Kime, D. E. (2003). Automated sperm morphology analysis in fishes: The effect of mercury on goldfish sperm. *J. Fish Biol.* **63**, 1020–1033.

Vermeirssen, E. L. M., Mazorra De Quero, C., Shields, R. J., Norberg, B., Kime, D. E. and Scott, A. P. (2004). Fertility and motility of sperm from Atlantic halibut (*Hippoglossus hippoglossus*) in relation to dose and timing of gonadotrophin-releasing hormone agonist implant. *Aquaculture* **230**, 547–567.

Verstegen, J., Iguer-Ouada, M. and Onclin, K. (2002). Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology* **57**, 149–179.

Vílchez, M. C., Morini, M., Peñaranda, D. S., Gallego, V., Asturiano, J. F. and Pérez, L. (2016). Sodium affects the sperm motility in the European eel. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **198**, 51–58.

Vílchez, M. C., Morini, M., Peñaranda, D. S., Gallego, V., Asturiano, J. F. and Pérez, L. (2017). Role of potassium and pH on the initiation of sperm motility in the European eel. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **203**, 210–219.

Viveiros, A. T. M., Nascimento, A. F., Orfão, L. H. and Isaú, Z. A. (2010). Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. *Theriogenology* **74**, 551–556.

Zilli, L., Schiavone, R., Zonno, V., Storelli, C., and Vilella, S. (2004). Adenosine triphosphate concentration and β-d-glucuronidase activity as indicators of sea bass semen quality. Biol. Reprod. **70**, 1679-1684.

Zuccarelli, M. D., and Ingermann, R. L. (2007). Calcium-induced quiescence of sperm motility in the bluegill (*Lepomis macrochirus*). *J. Exp. Zool. Part A Ecol. Genet. Physiol.* **307**, 590–599.

888 **Figure 1.** Schematic diagram of some kinetic parameters recorded by CASA system.
889 Black circles represent successive positions of the head of motile spermatozoa through
890 the video recording. Sperm motion parameters: VCL, curvilinear velocity; VAP,
891 averaged path velocity; VSL, straight-line velocity; ALH, amplitude of lateral head
892 displacement; BCF, beat/cross frequency.
893

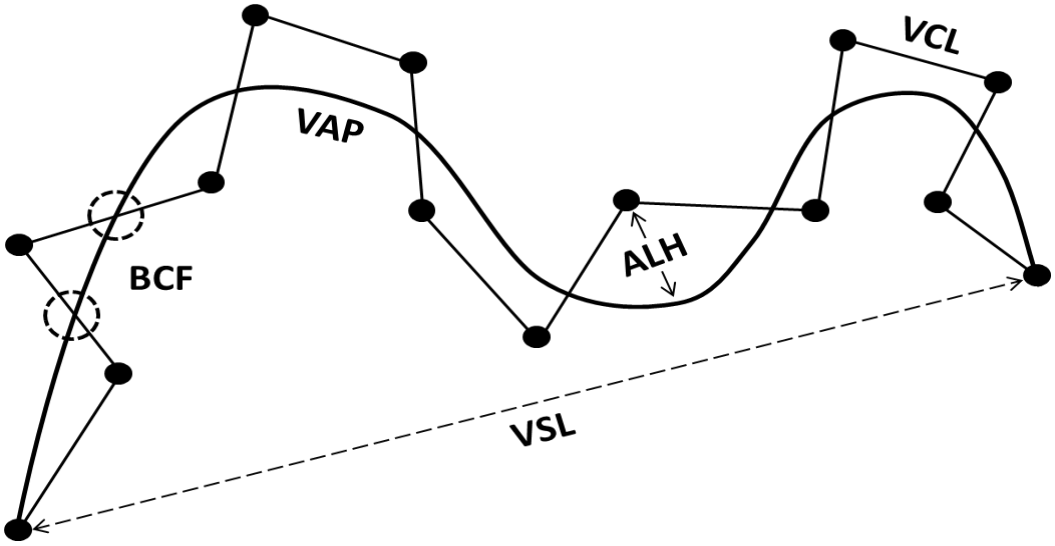

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Figure 2. Evolution of number of scientific manuscripts published from 1985 to 2016 in journals selected in the Science Citation Index (SCI) using fish sperm motility assessed by CASA systems as a research tools. The pie chart indicates the percentages of manuscripts focusing on freshwater or seawater species.

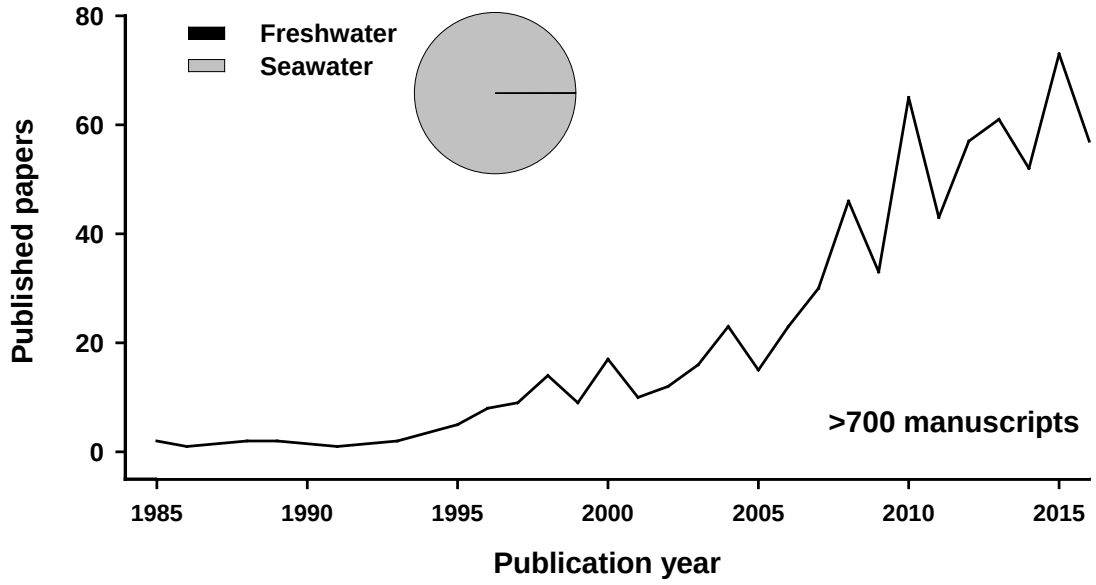


Figure 3. Number of manuscripts published by research area (sperm physiology, sperm storage, broodstock management, sperm competition, ecotoxicology, and breeding cycle) in SCI journals using fish sperm motility assessed by CASA systems as research tools.

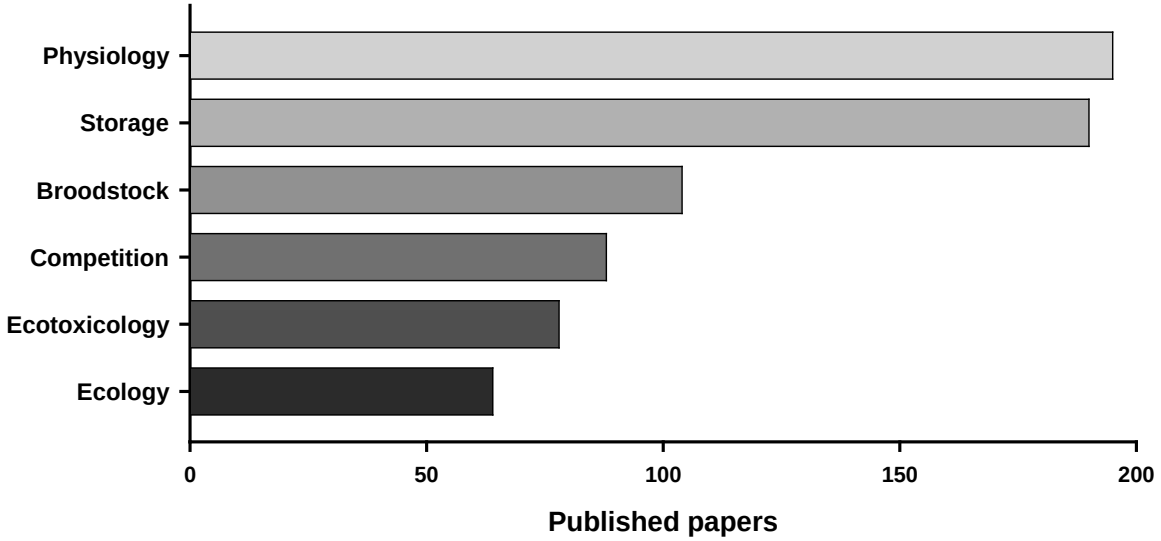


Figure 4. Number of manuscripts published on the main freshwater fish families used in aquaculture using fish sperm motility assessed by CASA systems as research tool. The insert also shows the five most commonly studied seawater species.

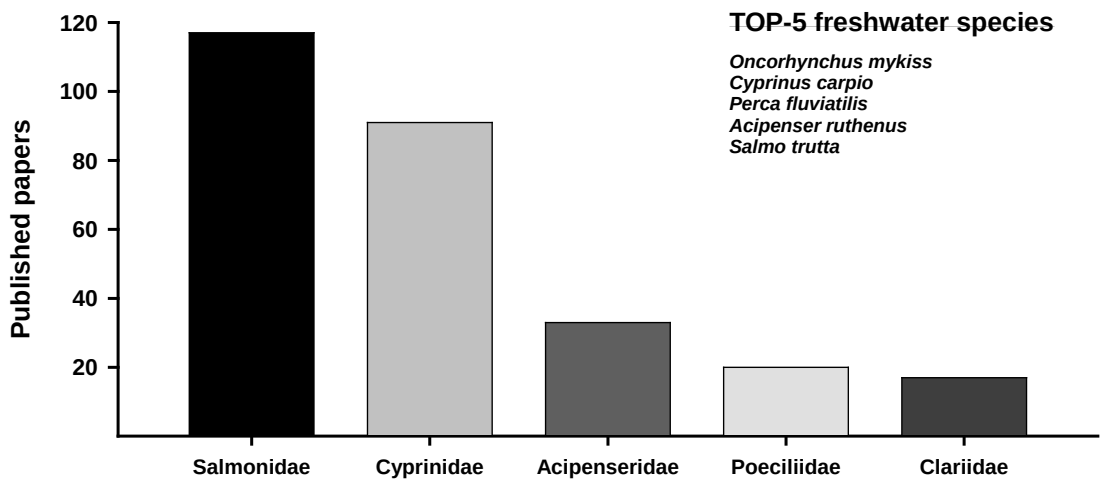
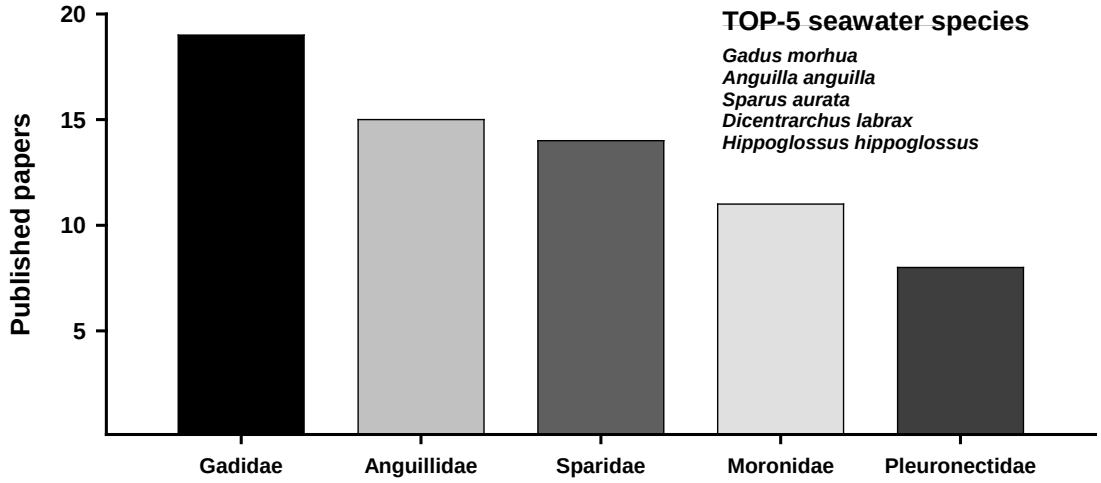
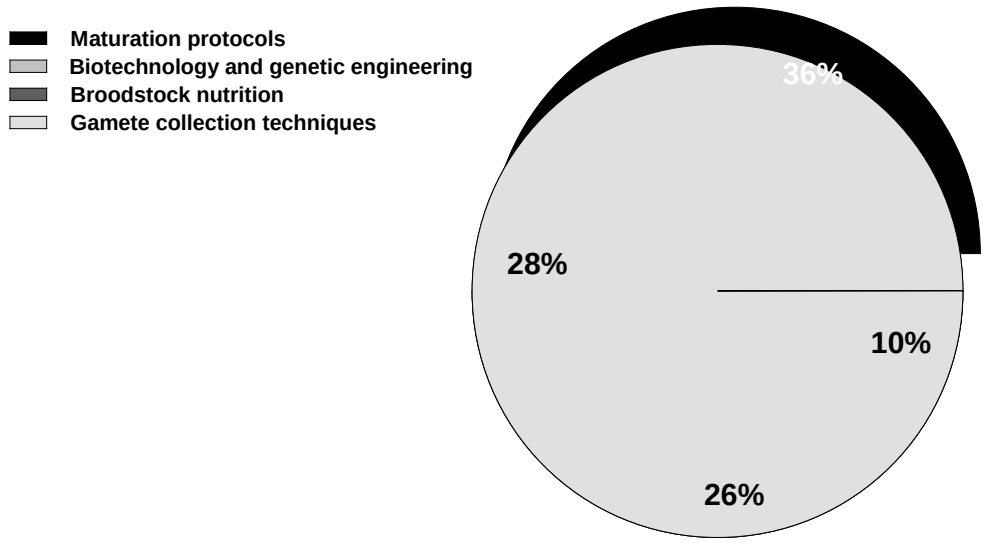


Figure 5. Number of manuscripts published on the main seawater fish families used in aquaculture using fish sperm motility assessed by CASA systems as research tool. The insert also shows the five most commonly studied seawater species.



915 **Figure 6.** Percentage of manuscripts published in the main topics (maturation protocols;
916 broodstock nutrition; biotechnology and genetic engineering; and gamete collection
917 techniques) of broodstock management using fish sperm motility assessed by CASA
918 systems as a research tool.
919



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