The final publication is available at

https://doi.org/10.1071/RD17460

Additional Information

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

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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.

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

110   kinetics can be used from this perspective.

111   To sum up, in addition to offering an objective and accurate estimation of classical kinetic

112   parameters such as total motility, CASA systems provide a high number of novel sperm

113   motion variables (impossible to detect by subjective evaluation) that can be successfully

114   used in many research areas from fundamental to applied research.

115

116   Technical applications from CASA systems in fish

117   Although the first scientific reports assessing fish sperm quality using a subjective method

118   date from about a century ago, computer-assisted systems for fish did not start to be used

119   until the mid 80's. Since then, more than 700 publications on different topics using sperm

120   motility as a research tool can be found in the literature on fish (Figure 2). In fact, in the

121   last 30 years fish sperm parameters from 170 different species belonging to different

122   families have been studied using these systems, and the results have been applied to many

123   different areas, from ecology to molecular research (Figure 3). However, 20 of these fish

124   species represent more than 50% of published papers, of which salmonids, cyprinids and

125   sturgeons have been the most studied. Moreover, scientists have devoted much more time

126   to studying the former (Figures 4 and 5). Here we present an overview of the most state

127   of the art research areas in which CASA systems have been applied successfully.

128

129   *Sperm physiology*

130   Sperm physiology has been the most investigated factor in sperm studies carried out by

131   CASA systems (Figure 3). In fact, the first study on fish sperm using a uncertain semi-

132   assisted computer system was carried out in rainbow trout (*Oncorhynchus mykiss*) in 1985

133   (Cosson *et al.* 1985), where the authors reported an objective technique for the rapid

134   quantitative assessment of sperm motility using stroboscopic illumination. Since then,

135   this research field has grown continually over the years, and more than 100 physiology-

136   related articles on different species have been published over the last 10 years.

137   The fish sperm activation process has been the key subject within this area, and to learn

138   about the process by which spermatozoa begin to move has been the main goal of fish

139   physiologists (Zuccarelli and Ingermann 2007; Vílchez *et al.* 2016; Pérez *et al.* 2016).

140   Although sperm activation models for different species had previously been discovered

141   and reported thanks to subjective motility evaluation, CASA systems have helped to

142   describe these activation pathways in more depth through sperm kinetic features. For

143 example, some studies have reported that sperm activation in marine fish can be triggered
144 both by electrolyte (e.g., seawater) and non-electrolyte (e.g., glucose-containing) media,
145 but the absence of ions in the extracellular medium caused a general decline in sperm
146 velocities in several species (Detweiler and Thomas 1998: Gallego *et al.* 2013c; Vílchez
147 *et al.* 2017). On the other hand, some studies have shown that *in vitro* temperature can
148 have an important effect on sperm motility parameter. In common carp (*Cyprinus carpio*),
149 spermatozoa activated at 4 ºC showed higher motility rate than sperm activated at 14 and
150 24 ºC, whereas highest swimming velocity was observed at 14 °C (Dadras et al., 2016).
151 Other studies showed similar results, and swimming velocity at high temperatures is often
152 higher in species such as Senegalese sole (*Solea senegalensis*, Diogo et al., 2010) and
153 European perch (*Perca fluviatilis*; Lahnsteiner*, 2011*). Moreover, the propulsion
154 machinery of spermatozoa has been another research focus within sperm physiology
155 studies, and although sperm ATP levels have been correlated with motility, velocity
156 and∕or fertilizing ability in several species like rainbow trout (Lahnsteiner *et al.* 1998),
157 chinook salmon, *Oncorhynchus tshawytscha* (Bencic *et al.* 1999), or sea bass,
158 *Dicentrarchus labrax* (Zilli *et al.* 2004); no correlations between ATP and sperm motility
159 were found in other species such as common bleak, *Alburnus alburnus* (Lahnsteiner *et al.*
160 1996); bluegill, *Lepomis macrochirus* (Burness *et al.* 2005); or Atlantic cod, *Gadus*
161 *morhua* (Butts *et al.* 2010).
162 Summing up, CASA systems have become useful for carrying out studies on fish sperm
163 physiology, providing the user with an in-depth understanding of the activation
164 mechanisms involved in different genus and families, and approaching several factors
165 such as osmolality, temperature, ion plasma composition, etc…
166
167 ***Sperm storage***
168 Sperm storage, both short and long-term, has been the second most investigated field
169 using CASA systems as a research tool (Figure 3). Almost 200 scientific publications
170 reporting kinetic sperm parameters have contributed to the discovery and improvement
171 of sperm storage protocols in more than 80 fish species. Now, these techniques can be
172 seen in a great number of applications, ranging from ecology to aquaculture.
173 With regards to cryopreservation, significant results have been reported in species
174 belonging to the most important families used in aquaculture, and Table 3 summarizes
175 the best results in terms of the pre- and post-thaw motilities (MOT) and velocities (VCL)
176 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

211 freezing-thawing process on genetic material, so fish sperm cryopreservation studies
212 should combine both sperm kinetic assessments and DNA damage studies (Cabrita *et al.*
213 2014; Martínez-Páramo *et al.* 2017).

214

215 ***Broodstock management***
216 Broodstock management involves a large number of factors that contribute to the ultimate
217 aim of enabling a captive group of fish to successfully complete reproductive maturation
218 and fertilization. In this context, sperm motion parameters play an essential role in
219 achieving this objective, and the effect of different rearing factors (temperature, diet,
220 handling, etc.) can be tested through the proper use of CASA systems. Around 100
221 scientific publications focusing on broodstock handling and using these systems have
222 been published, and this section offers an overview of the most studied topics within this
223 area (Figure 6).
224 Temperature and photoperiod are the main environmental factors controlling the
225 development of gametes and gamete quality in most fish species (Migaud *et al.* 2013).
226 With regard to temperature, several studies have shown how under- or over-optimal
227 conditions have negative effects on gamete quality (Alavi and Cosson, 2005). Lahnsteiner
228 and Leitner (2013) reported that in brown trout (*Salmo trutta*), a thermal regime of more
229 than 5 ºC above the natural temperature affects the spermiation process, and causes a
230 reduction in the percentage of spermiating male fish that produce spermatozoa of high
231 quality (in terms of motility and swimming velocity). In European grayling (*Thymalus*
232 *thymallus*), the maturation rate of male fish and their gamete quality depended greatly on
233 the temperature regime, and the highest sperm motilities and velocities were obtained
234 under a creek water temperature regime with natural seasonal fluctuations (Lahnsteiner
235 and Kletzl 2012).
236 On the other hand, when sperm production using environmental treatments is not
237 possible, hormonal induction techniques can be used to enhance spermiation and sperm
238 quality. A wide variety of hormonal treatments (e.g., carp pituitary extract or
239 gonadotropin preparations) have been tested on a great many aquacultural species
240 (Mylonas *et al.* 2017), but CASA systems have mainly been used to test gonadotropin-
241 releasing hormone agonist (GnRHa) treatments. Indeed, GnRHa implants have provided
242 great results in marine species such as Atlantic bluefin tuna (*Thunnus thynnus thynnus*),
243 where GnRHa-implantation therapy increased the percentage of spermiating males and
244 the availability of motile spermatozoa (Mylonas *et al.* 2007). They have also shown

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

253 Broodstock nutrition is another key factor that affects gonadal development and gamete
254 quality in fish (Izquierdo *et al.* 2001). However, although there are many publications
255 linking diet and reproductive success (e.g., fertilization and hatching rates), few reports
256 have been able to make a direct link between broodstock diet and the kinetic
257 characteristics of spermatozoa assessed by a CASA system. In terms of freshwater
258 species, although the dietary regime did not affect the percentage of motile spermatozoa,
259 it significantly affected sperm velocity in common barb (*Barbus barbus*) (Alavi *et al.*
260 2008). In goldfish (*Carassius auratus gibelio*), the addition of vitamins and highly
261 unsaturated fatty acids (HUFA) had a significant effect on sperm parameters such as the
262 duration and percentage of spermatozoa with motility (Kashani and Imanpoor 2012); and
263 in African catfish (*Clarias gariepinus*), a diet formulated with agricultural products
264 provided higher milt volumes and improved sperm velocity in breeding males (Nyina-
265 wamwiza *et al.* 2012). In marine species, such as Senegalese sole (*Solea senegalensis*),
266 Beirão *et al.* (2015) reported that males who had been fed on an enriched diet
267 (polyunsaturated fatty acid, PUFA) showed improvements in sperm motility parameters
268 such as pMOT and VCL. Likewise, in European eel (*Anguilla anguilla*), diets with high
269 levels of arachidonic acid and eicosapentaenoic acid induced better sperm kinetic
270 parameters than did commercial diets (Butts *et al.* 2015; Baeza *et al.* 2015).

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

279 and that of triploid males from 37% to 77%. However, the ploidy level did not result in
280 any significant differences in terms of the velocity of spermatozoa. Conversely, in
281 Atlantic cod (*Gadus morhua*), VCL was higher in the spermatozoa of diploid males
282 compared with that of triploid males, but no differences between ploidies were observed
283 for the remaining sperm motility descriptors (Peruzzi *et al.* 2009). On the other hand, in
284 fish in which atypical combinations of sexual phenotype and genotype has become a
285 useful tool for aquaculture production, the assessment of gamete quality is essential in
286 order to carry out future crosses. In this context, a study performed in Nile tilapia
287 (*Oreochromis niloticus*) showed that sperm kinetic parameters (measured using a CASA
288 system) did not differ between the three different genotypes: XX, XY, and YY (Gennotte
289 *et al.* 2012). Similar results were obtained in a comparative study of sperm quality over
290 all possible sex genotypes in rainbow trout (*Oncorhynchus mykiss*), where sperm motility
291 parameters showed no differences between neo-males (XX genotype) and super-males
292 (YY genotype) (Kowalski *et al.* 2011).
293 To sum up, sperm kinetic parameters have become useful in the evaluation of many
294 aspects relating to broodstock handling, and several factors such as *i)* the environmental
295 rearing conditions, *ii)* the hormonal treatments used, *iii)* the diet requirements of each
296 species, and *iv)* biotechnology and genetic engineering, have been improved through
297 gamete evaluation using CASA systems.
298
Sperm competition
300 Sperm competition is defined as the process in which spermatozoa from two or more
301 males race to fertilize the egg, is a widespread phenomenon that occurs in a wide range
302 of animal taxa, including fish (Stoltz and Neff 2006). This phenomenon is closely related
303 to dominance hierarchies, where male fish can adopt different mating strategies according
304 to their social position (Serrano *et al.* 2006). Although sperm competition has become a
305 recent topic of interest, more than 90 scientific papers on fish species have been published
306 during the last two decades (Figure 3).
307 The trade-off between social investment and sperm performance has been widely studied
308 in fish, and some studies have shown differences in sperm kinetic parameters between
309 males with different social statuses. For example, in Chinook salmon (*Oncorhynchus*
310 *tshawytscha)*, parr males (jacks) invested significantly more of their somatic tissue into
311 gonads compared with anadromous males (hooknoses), and parr males showed higher

312 motility and velocity values (90% and 70 µm/s, respectively) than dominant males (85%
313 and 55 µm/s, respectively). In another study, after examining the sperm characteristics of
314 29 cichlid species, Fitzpatrick *el al.* (2009) showed that species experiencing greater
315 levels of sperm competition have faster-swimming sperm. Nevertheless, even when
316 theory predicts that dominant males might have lower quality spermatozoa, some studies
317 have shown no effects, or even the opposite situation in other species such as rainbow
318 trout (*O. mykiss*), bluegill (*Lepomis macrochirus*) and three-spined sticklebacks
319 (*Gasterosteus aculeatus*) (Cardwell *et al.* 1996; Stoltz and Neff 2006; Mehlis *et al.* 2013).
320 In this sense, sperm motility assessment can serve as a useful tool for studying the
321 evolution of alternative reproductive strategies and mating systems in different fish taxa,
322 and several kinetic parameters such as total motility, swimming velocity and/or motility
323 over time will provide further data for sperm competition studies.
324
325 *Ecotoxicology*
326 Aquatic environments can carry substantial quantities of natural and man-made
327 environmental contaminants (ECs), and evaluating the kinetics of fish sperm via CASA
328 systems has become a key in assessing EC toxicity (Hatef *et al.* 2013). At present, around
329 70 scientific publications reporting the impact of ECs on sperm motion performance have
330 contributed to the understanding of the toxicity mechanisms and action sites of ECs, and
331 this knowledge can now be applied to a wide range of topics. However, is important to
332 note that EC effects are extremely variable among fish taxa and even within species, and
333 several factors such as EC concentrations or the duration of exposure can greatly affect
334 sperm motion performance. In this regard, Table 4 summarizes the main ECs affecting
335 sperm motility as assessed by a CASA system in some fish species, indicating the
336 minimum EC dose at which sperm kinetic parameters were affected significantly.
337 Xenoestrogens are types of xenohormones that imitate oestrogen activity, and they can
338 be produced by both synthetic or natural pathways. Among the most important ECs with
339 oestrogenic effects are bisphenol-A, estradiol, and ethynyloestradiol, and several studies
340 have reported their negative effect on the sperm motion performance in several freshwater
341 species belonging to the Salmonidae and Cyprinidae (see Table 4). On the other hand,
342 heavy metals represent the other EC group with high toxicity levels, and now they are
343 considered the most dangerous pollutants in the world (Hatef *et al.* 2013). In this regard,
344 Lahnsteiner *et al.* (2004) studied the impact of different heavy metals (zinc, mercury, and
345 cadmium) on the sperm motility parameters of four teleosts belonging to the most

346 representative freshwater families (Salmonidae, Cyprinidae, Gadidae, and Clariidae). The
347 authors concluded that toxic concentrations of all the pollutants differed markedly for
348 each species (highlighting species-specific effects of these EC groups).
349 To sum up, sperm motility assessment has become a valuable tool to check and
350 understand toxicity mechanisms and sites of action of different ECs, and changes in sperm
351 motion performance can serve as a potential biomarker for biomonitoring these agents
352 and their potential effects on reproductive function.
353

354 ***Ecology***
355 CASA systems can also be applied to many areas of fish ecology. Although subjective
356 evaluation of sperm motility has been the main method used in this field, more than 50
357 recent publications have used CASA systems and have contributed to the exploration of
358 numerous ecology issues of different fish species from different taxa. In this context, a
359 wide range of topics such as breeder age, seasonal changes, and characterization of
360 populations are going to be approached through a sperm quality perspective.
361 In fish species with an annual reproductive cycle, sperm quality usually oscillates
362 throughout the spawning season both in the wild and in captivity, and sperm motility
363 assessment can give us the optimal period in which they should be collected.. For
364 example, thanks to sperm motility assessment, scientists know (in wild conditions) that
365 there are species in which sperm quality is higher at the beginning of the spawning season,
366 such as halibut (*H. hippoglossus,* (Babiak *et al.* 2006)) or Senegalese sole (*Solea*
367 *senegalensis,* (Beirão *et al.* 2011b)); species in which sperm quality is higher in the
368 middle of the spawning season, such as Atlantic cod (*Gadus morhua,* (Rouxel *et al.*
369 2008)) or European seabass (*Dicentrarchus labrax,* (Dreanno *et al.* 1999b); and species
370 such as common carp (*C. carpio,* (Christ *et al.* 1996)) or European perch (*P. fluviatilis,*
371 (Alavi *et al.* 2010)) in which sperm quality is higher at the end of the spawning season.
372 Furthermore, kinetic parameters provided by CASA systems can also be applied to
373 investigate inter-population differences, either by comparing wild populations to link
374 sperm quality to environmental conditions (Salte *et al.* 2004; Dietrich *et al.* 2014;
375 Biernaczyk *et al.* 2012), or by comparing farmed and wild populations to ascertain the
376 possible impact of escaped farmed fish on wild ecosystems (Lehnert *et al.* 2012; Rideout
377 *et al.* 2004; Butts *et al.* 2010).
378 Concerning inter-species studies, interesting ecology approaches can be made using
379 sperm motility data. Gallego *et al.* (2014) after having analysed the sperm motion

380   parameters of several swimmer and sessile species, reported that the patterns were totally

381   different. In that study, the authors linked the sperm motion patterns to species-specific

382   lifestyles, postulating that sessile organisms (which show limited or no movement) need

383   spermatozoa with a capacity to swim long distances to find the oocytes, while swimming

384   male organisms can move toward the female and release gametes nearby, and as such the

385   spermatozoa do not need to swim for such a long time.

386

Aspects to be improved in CASA systems

388   Although CASA systems are widely accepted by the animal reproductive science

389   community as a valuable research tool for basic sperm biology, an evident lack of

390   standardization in assessing fish sperm motion has often resulted in low reproducibility,

391   making it difficult to interpret and compare intra- and inter-laboratory results (Rosenthal

392   *et al.* 2010). Indeed, a series of biological, technical and CASA settings must be taken

393   into account to harmonize common procedures and establish standardized protocols to be

394   used in many research groups (see Table 5).

395   Biological or handling settings such as how to collect gametes (Aramli *et al.* 2016a),

396   which ejaculate portion to use for analysing (Gallego *et al.* 2013a), the storage

397   temperature before analysis (Sanches *et al.* 2015), and the sperm-to-activation medium

398   ratio (Toth *et al.* 1995) can have a marked influence on evaluating sperm kinetics. In this

399   context, it is important to note that the kinetic characteristics of fish spermatozoa are often

400   species-specific, so biological settings must be linked to the species being evaluated.

401   Technical settings for assessing sperm motility can also involve a wide range of factors

402   (Table 5), but few reports can be found in the literature on fish. For example, microscope

403   settings such as the magnification had a significant effect on the pMOT levels and sperm

404   velocities in European eel (*Anguilla anguilla*) (Gallego *et al.* 2013a); however, the use of

405   different chambers did not affect these same sperm motion parameters when assessed by

406   a CASA system. In common carp (*Cyprinus carpio*), Kowalski *et al.* (2014) reported that

407   adhesion of sperm to a glass surface can be a crucial factor when assessing sperm motion

408   performance by CASA systems; and recommended the use of protein supplements (e.g.,

409   bovine serum albumin) to obtain accurate CASA results for sperm quality prediction.

410   Finally, CASA settings also play a key role in estimating sperm kinetic parameters, and

411   factors such as the recording frame rate (Castellini *et al.* 2011; Gallego *et al.* 2013a;

412   Boryshpolets *et al.* 2013) or even the type of CASA used (Boryshpolets *et al.* 2013) have

413 a notable effect on sperm kinetic results both in freshwater and seawater species.

414 However, there are other CASA settings that have not yet been tested, such as the number

415 of cells sampled per field/capture, the location of the field inside the chamber, or even the

416 focal position of swimming sperm cells inside an open drop. All of these factors could

417 also affect sperm motion results, so further studies are necessary to evaluate the effect of

418 reported and novel factors on a greater number of fish species.

419

420 **5. New challenges for CASA systems in fish research**

421 CASA systems are able to analyse a huge number of spermatozoa per capture, which

422 means thousands of motion tracks reported per sample. However, despite the advantages

423 of working with these extensive databases, most research groups are can only show the

424 mean of the sperm quality parameters (or even some of them), and spermatozoa are

425 considered to represent homogeneous populations. Nevertheless, it has been pointed out

426 that the spermatozoa of some species do not constitute a homogeneous mixture, and

427 several studies in fish have clearly shown the coexistence of different sperm motility-

428 based subpopulations (Martínez-Pastor *et al.* 2008; Beirão *et al.* 2011a; Gallego *et al.*

429 2017). In this context, the study of the variations and distributions of these populations

430 has been applied successfully in several research areas such as sperm physiology, sperm

431 cryopreservation and broodstock management (Beirão *et al.* 2009; Kanuga *et al.* 2012;

432 Gallego *et al.* 2015); moreover, certain sperm subpopulations have been positively and

433 significantly correlated with fertilization and hatching rates in key aquaculture species,

434 such as gilthead seabream (*Sparus aurata*, (Beirão *et al.* 2011a)) or tambaqui (*Colossoma*

435 *macropomum*, (Gallego *et al.* 2017). Just as data modelling techniques (such as

436 clustering) allow for the extraction of information between many variables and patterns

437 relating to the kinetics of spermatozoa, subpopulation studies are becoming a novel tool

438 to be applied in scientific fish and aquaculture matters.

439 Asides from providing us with a large number of sperm motion characteristics (described

440 in Table 1), CASA systems are able to demonstrate other important parameters such as

441 sperm concentrations, morphology, survival (viability) rates, and even the rate of DNA

442 fragmentation. The set of parameters provided by the given CASA program depends on

443 the brand of the product and, overall, by the number of modules purchased by the

444 researcher. We can presently identify more than 20 companies that market CASA

445 systems, and because they focus on a range of areas, from biology and medicine to

446 engineering, computer technology, and mathematics, the future development of these
447 systems will be directed at a combination of related subjects (motility, morphology and/or
448 viability) (Lu *et al.* 2014).
449 To sum up, CASA results in sperm motion analysis boast precision, reliability and
450 reproducibility, providing the scientific community with a useful tool which can be
451 applied both in aquaculture and for ecological purposes. Although sperm motion traits
452 from a large number of species have already been reported in hundreds of articles, future
453 developments in CASA systems (e.g., three-dimensional motion analysis, species-
454 specific software, comfortable and portable systems) will be necessary to expand and
455 deepen our knowledge of the biological functions of fish spermatozoa.
456

457 **Acknowledgements**

461

462 **References**

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

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

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

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

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

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

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

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

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

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

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

495   Beirão, J., Cabrita, E., Pérez-Cerezales, S., Martínez-Páramo, S. and Herráez, M. P.
496   (2011a). Effect of cryopreservation on fish sperm subpopulations. *Cryobiology* **62**,
497   22–31.

498   Beirão, J., Soares, F., Herráez, M. P., Dinis, M. T. and Cabrita, E. (2011b). Changes in
499   *Solea senegalensis* sperm quality throughout the year. *Anim. Reprod. Sci.* **126**, 122–
500   129.

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

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

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

511   Bernáth, G., Żarski, D., Kása, E., Staszny, Á., Várkonyi, L., Kollár, T., Hegyi, Á., Bokor,
512   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., Slowińska, 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

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

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

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

620 Gallego, V., Carneiro, P. C. F., Mazzeo, I., Vílchez, M. C., Peñaranda, D. S., Soler, C.,
621   Pérez, L. and Asturiano, J. F. (2013a). Standardization of European eel (*Anguilla*
622   *anguilla*) sperm motility evaluation by CASA software. *Theriogenology* **79**, 1034–
623   1040.

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

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

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

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

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

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

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

648 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–

717   1297.

718   Lahnsteiner, F., Berger, B., Kletzl, M. and Weismann, T. (2005). Effect of bisphenol A

719   on maturation and quality of semen and eggs in the brown trout, *Salmo trutta* f. fario.

720   *Aquat. Toxicol.* **75**, 213–224.

721   Lahnsteiner, F. (2011). Spermatozoa of the teleost fish *Perca fluviatilis* (perch) have the

722   ability to swim for more than two hours in saline solutions. Aquaculture **314**, 221-

723   224.

724   Lahnsteiner, F., and Kletzl, M. (2012). The effect of water temperature on gamete

725   maturation and gamete quality in the European grayling (*Thymalus thymallus*) based

726   on experimental data and on data from wild populations. *Fish Physiol. Biochem.* **38**,

727   455–467.

728   Lahnsteiner, F., and Leitner, S. (2013). Effect of Temperature on Gametogenesis and

729   Gamete Quality in Brown Trout, *Salmo trutta. J. Exp. Zool. Part A Ecol. Genet.*

730   *Physiol.* **319**, 138–148.

731   Lehnert, S. J., Heath, D. D. and Pitcher, T. E. (2012). Sperm trait differences between

732   wild and farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* **344–**

733   **349**, 242–247.

734   Li, P., Hulak, M., Li, Z. H., Sulc, M., Psenicka, M., Rodina, M., Gela, D. and Linhart, O.

735   (2013). Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces

736   protein phosphorylation in tyrosine and threonine residues. *Theriogenology* **80**, 84–

737   89.

738   Liley, N. R. R., Tamkee, P., Tsai, R. and Hoysak, D. J. J. (2002). Fertilization dynamics

739   in rainbow trout (*Oncorhynchus mykiss*): effect of male age, social experience, and

740   sperm concentration and motility on in vitro fertilization. *Can. J. Fish. Aquat. Sci.*

741   **59**, 144–152.

742   Linhart, O., Rodina, M. and Cosson, J. (2000). Cryopreservation of sperm in common

743   carp *Cyprinus carpio*: sperm motility and hatching success of embryos. *Cryobiology*

744   **41**, 241–250.

745   Linhart, O., Rodina, M., Flajshans, M., Mavrodiev, N., Nebesarova, J., Gela, D. and

746   Kocour, M. (2006). Studies on sperm of diploid and triploid tench, *Tinca tinca* (L.).

747   *Aquac. Int.* **14**, 9–25.

748   Liu, Q. H., Li, J., Xiao, Z. Z., Ding, F. H., Yu, D. De and Xu, X. Z. (2007). Use of

749   computer-assisted sperm analysis (CASA) to evaluate the quality of cryopreserved

750   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

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

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

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

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

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

836 Rurangwa, E., Kime, D. E., Ollevier, F. and Nash, J. P. (2004). The measurement of
837   sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture* **234**,
838   1–28.

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

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

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

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

853   *Can. J. Zool.* **84**, 1019–1024.

854 Stoltz, J. A., and Neff, B. D. (2006). Sperm competition in a fish with external
855   fertilization: The contribution of sperm number, speed and length. *J. Evol. Biol.* **19**,
856   1873–1881.

857 Toth, G. P., Christ, S. A., McCarthy, H. W., Torsella, J. A. and Smith, M. K. (1995).
858   Computer-assisted motion analysis of sperm from the common carp. *J. Fish Biol.*
859   **47**, 986–1003.

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

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

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

868 Verstegen, J., Iguer-Ouada, M. and Onclin, K. (2002). Computer assisted semen
869   analyzers in andrology research and veterinary practice. *Theriogenology* **57**, 149–
870   179.

871 Vílchez, M. C., Morini, M., Peñaranda, D. S., Gallego, V., Asturiano, J. F. and Pérez, L.
872   (2016). Sodium affects the sperm motility in the European eel. *Comp. Biochem.*
873   *Physiol. -Part A Mol. Integr. Physiol.* **198**, 51–58.

874 Vílchez, M. C., Morini, M., Peñaranda, D. S., Gallego, V., Asturiano, J. F. and Pérez, L.
875   (2017). Role of potassium and pH on the initiation of sperm motility in the European
876   eel. *Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol.* **203**, 210–219.

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

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

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

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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

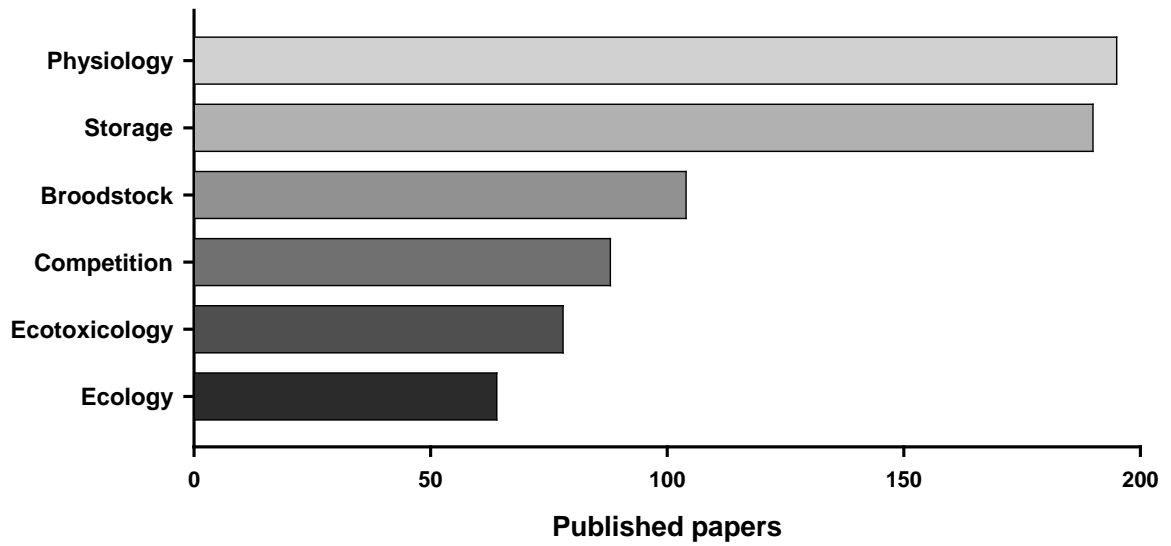892   displacement; BCF, beat/cross frequency.

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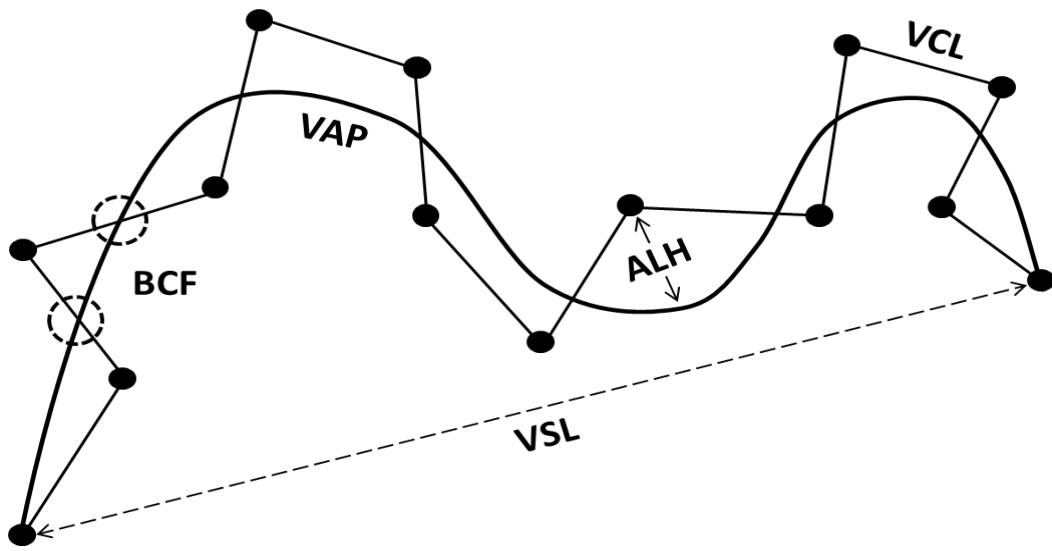
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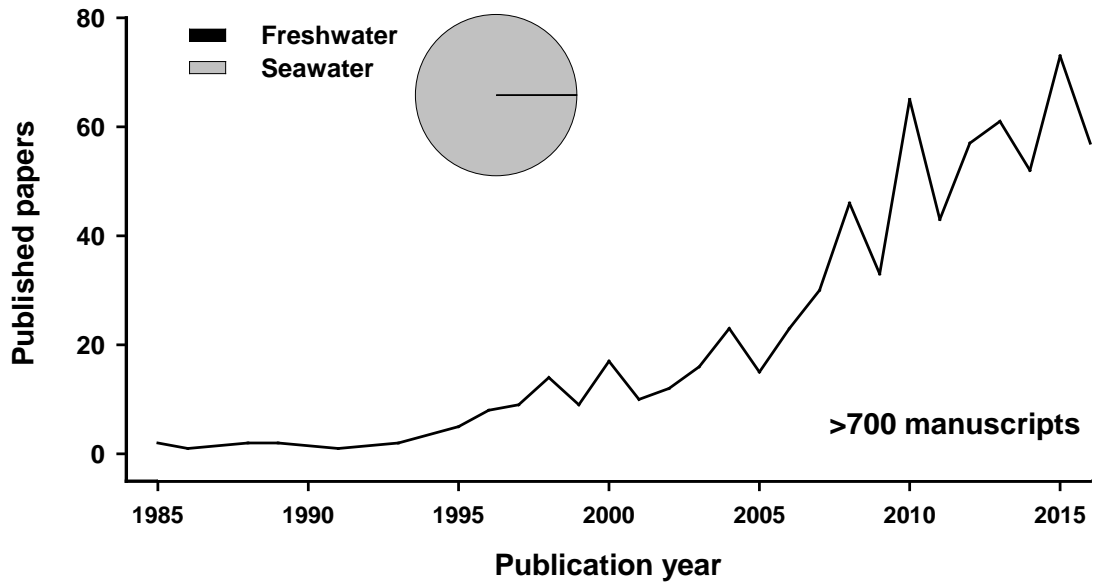
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901 **Figure 3.** Number of manuscripts published by research area (sperm physiology, sperm
902 storage, broodstock management, sperm competition, ecotoxicology, and breeding cycle)
903 in SCI journals using fish sperm motility assessed by CASA systems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

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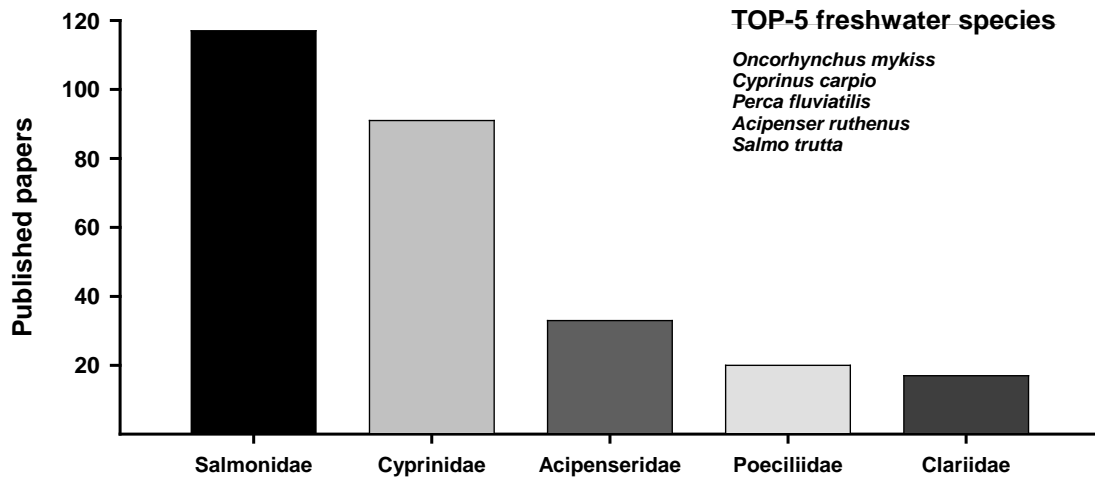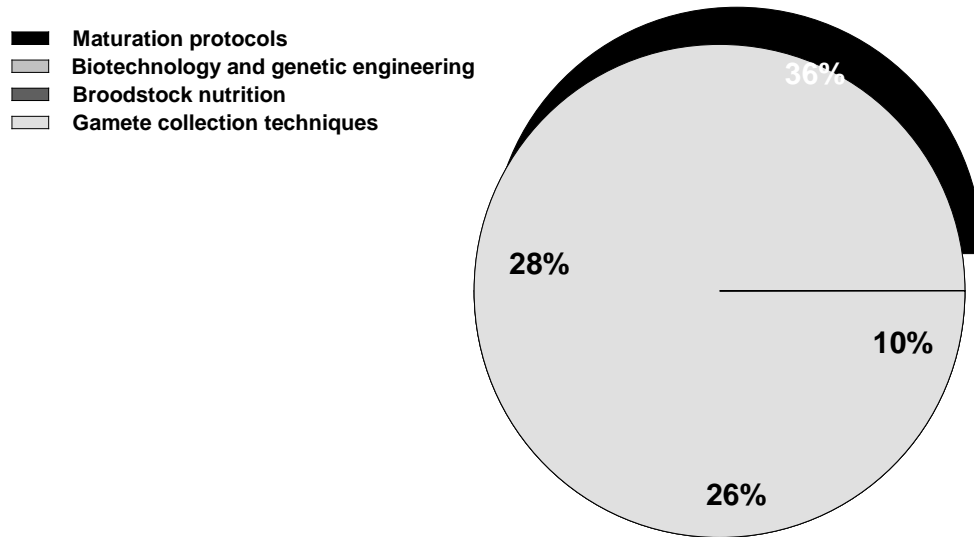

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910 **Figure 5.** Number of manuscripts published on the main seawater fish families used in
911 aquaculture using fish sperm motility assessed by CASA systems as research tool. The
912 insert also shows the five most commonly studied seawater species.
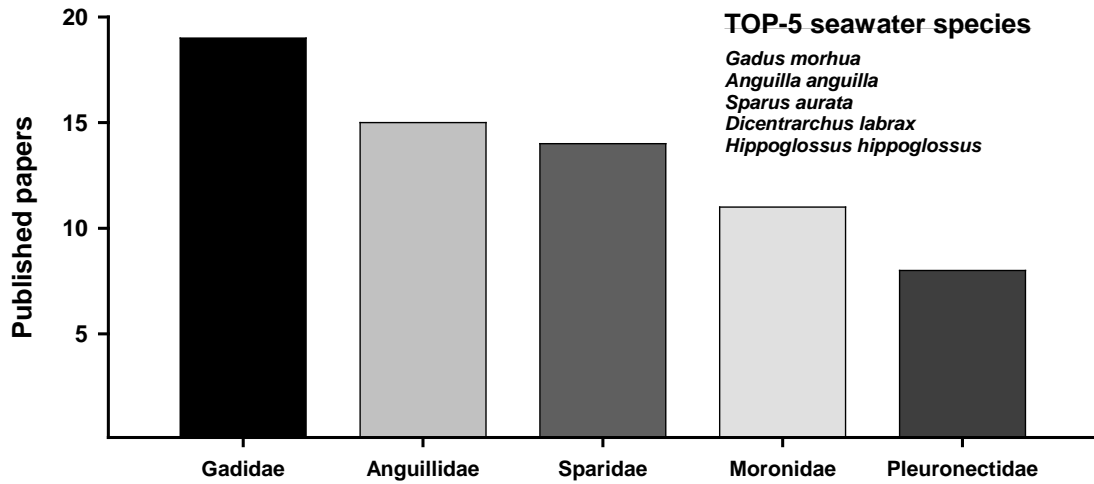
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915   **Figure 6.** Percentage of manuscripts published in the main topics (maturation protocols;
916   broodstock nutrition; biotechnology and genetic engineering; and gamete collection
917   techniques) of broodstock management using fish sperm motility assessed by CASA
918   systems as a research tool.

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