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Sperm handling in aquatic animals for artificial reproduction

Beirão José [1, *], Boulais Myrina [2], Gallego Victor [3], O'brien Justine K. [4], Peixoto Silvio [5], Robeck Todd R. [6], Cabrita Elsa [7]

[1] Faculty of Biosciences and Aquaculture, Nord University, NO - 8049, Bodø, Norway
[2] University of Brest, CNRS, IRD, Ifremer, LEMAR, rue Dumont d'Urville, F-29280, Plouzané, France
[3] Grupo de Acuicultura y Biodiversidad, Instituto de Ciencia y Tecnología Animal, Universitat Politècnica de València, Valencia, Spain
[4] Taronga Institute of Science and Learning, Taronga Conservation Society, Bradleys Head Rd, Mosman NSW, 2088, Australia
[5] Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco (UFRPE), Recife, Brazil
[6] SeaWorld Species Preservation Lab, SeaWorld Parks and Entertainment, 2595 Ingraham Road, San Diego, CA, 92019, USA
[7] CCMAR, University of Algarve, Campus of Gambelas, 8005-139 Faro, Portugal

* Corresponding author :José Beirão, email address : jose.beirao-dos-santos@nord.no

Abstract :

Artificial reproduction involves collection and handling of gametes in a way that secures their quality and maximizes the fertilization outcome. In addition to initial sperm quality, numerous steps can affect the final result of fertilization, from the sperm collection process until gamete mixing (or co-incubation) when spermatozoon enters or fuses with the oocyte. In this review, we summarize the whole process of sperm handling, from collection until fertilization for fish, penaeid shrimp, bivalve mollusks and marine mammals. To obtain sperm from captive animals, techniques vary widely across taxa, and include stripping by abdominal massage or testis surgical removal in fish, spermatophore collection in penaeid shrimps, gonadal scarification or temperature shock in bivalve mollusks, and voluntary collection via positive reinforcement in mammals. In most cases, special care is needed to avoid contamination by mucus, seawater, urine, or feces that can either activate spermatozoa motility and/or decrease its quality. We also review techniques and extender solutions used for refrigerated storage of sperm across the aforementioned taxa. Finally, we give an overview of the different protocols for in vivo and in vitro fertilization including activation of spermatozoa motility and methods for gamete co-incubation. The present study provides valuable information regarding breeder management either for animal production or species conservation.

Highlights

► *In vitro* and *in vivo* fertilization involve sperm collection and handling until gamete co-incubation ► Practical guidelines for sperm handling in fish, penaeid shrimp, bivalve mollusks and marine mammals are presented ► Sperm refrigeration techniques are revised for the different taxa ► Protocols for gamete co-incubation are discussed across the different taxa

Keywords : Sperm extraction, Spermatozoa:oocyte ratio, In vitro fertilization, In vitro reproduction, Assisted reproduction

1. Introduction

Ideally under captive conditions, males and females would be able to sexually mature and present mating behavior that leads to synchronized gamete release, in the case of external fertilizers, like most fish and mollusks [1], to transference of spermatophores in case of penaeids [2] or to normal copula behavior in case of marine mammals [3]. Artificial fertilization procedures involving the manual collection of sperm may be utilized to address reproductive dysfunction or to maintain population genetic diversity and health. This includes *in vitro* fertilization, as is the case of several fish, mollusks and some penaeid species [1,4,5]; *in vivo* fertilization in most penaeid shrimps [6]; and non-surgical intrauterine insemination in some marine mammals [7]. In addition, production of hybrids, monosex populations, or establishment of selective breeding programs in the aquaculture industry [1,4,8], or genetic management of zoo populations [9], make necessary to control artificial collection, handling, and storage of gametes.

When conducting artificial reproduction, male and female gametes are not always available at the same time. After ovulation or spawning, oocyte quality declines at a faster rate than that of sperm quality, and need to be fertilized in a relatively narrow time interval to ensure fertilization success [1]. Sperm on the other hand, in most cases, can be artificially collected throughout the entire reproductive season and stored for several hours or days depending on the species [1,7,10–13]. Thus, artificial reproduction in aquaculture species and assisted reproductive technologies in mammals involve sperm collection and storage until the time of ovulation or stripping of mature oocytes [1,7,14], requiring the best possible practices in sperm handling to maintain its quality and the general steps are illustrated in Figure 1.

The literature and the knowledge regarding sperm handling in aquatic animals is very extensive. Our intention is to provide practical guidelines about sperm handling procedures across

64 the range of aquatic animals that exist in managed environments, either for animal production or
65 species conservation.

66 **2. Sperm handling in fish**

67 Most of the knowledge about sperm handling in fish has been developed for aquaculture
68 produced species, but different protocols also exist for model species, specially zebrafish *Danio*
69 *rerio*, or for species conservation. All the cited studies in this section are either for teleost or
70 acipenseriforme species from the Osteichthyes class. Only a few studies exist for the
71 Chondrichthyes class and none of them were included in this revision.

72 *2.1. Sperm collection*

73 *2.1.1. Methods for sperm collection*

74 Different methods for sperm collection from fish could be used according to testis morphology
75 and the intend use. The most frequently applied technique is through abdominal massage, or
76 stripping, that helps the sperm to be released from the gonad into the spermatic duct. Using this
77 method and a gentle pressure, only mature cells are released together with the seminal plasma. This
78 technique is commonly used in species with filiform testis where the semen (sperm with the
79 seminal plasma) can be collected with a syringe placed directly outside the urogenital pore, such
80 as gilthead seabream *Sparus aurata* [15]. Nonetheless, some authors reported better sperm quality
81 using a catheter or cannula, because it reduces the risk of sperm contamination by urine, feces and
82 water [16,17]. The cannula is introduced through the urogenital pore and connected to a tube or
83 syringe where the sperm is collected directly (Figure 2c). In these cases, sperm is collected from
84 the spermatic duct or directly from the testis. When the structure and aperture of the urogenital
85 pore does not allow for this type of collection or there is a risk of fish injury by clogging of the
86 spermatic ducts due to internal damage, other modifications to the method can be applied. In the
87 spotted wolffish *Anarhichas minor*, a Pasteur pipette connected to the male papilla was used to

collect sperm after abdominal massage [18] (Figure 2f). In species with few microliters of sperm, glass microcapillary tubes, syringes or micropipette tips can be used to collect sperm such as the case of zebrafish, or Senegalese sole *Solea senegalensis* [19,20] (Figure 2a). However, there is always a risk of urine contamination in these cases and special attention is necessary to discard contaminated samples to avoid negatively impacting quality of the final pooled sample. In species where the contamination with urine is difficult to avoid, such as the European eel *Anguilla anguilla* [21], devices using aspiration have been developed to collect sperm directly into an immobilizing solution (Figure 2e). In European seabass *Dicentrarchus labrax* immobilizing solutions (NAM-non activating media) can be used just after collection to reduce the risk of contamination and to prolong cell viability [14]. Immobilizing solutions are important to maintain the sperm in a quiescent state prior to its use (see sections 2.2. and 2.3.1).

Sperm can also be collected directly from surgically removed testes (Figure 2d). In catfish species, sperm cannot be collected by abdominal massage due to testis structure (wels catfish *Silurus glanis*), which is folded several times within the abdominal cavity [22], or due to the presence of a seminal vesicle preventing sperm stripping (African catfish *Clarias gariepinus*) [23]. In these cases, testes are removed, crushed using a mesh fabric and cells collected into a tube. Testes should be previously cleaned to avoid blood cell contamination. This method is also used in zebrafish or other small sized fish when higher amounts of spermatozoa are needed, since stripping can only produce a few microliters and requires some technical training to avoid urine contamination [24,25]. In both examples, fish are sacrificed to collect the testes, but in recent studies performed in African catfish it was observed that the gonad can recover from a 75% ablation without sacrificing the fish [26]. The collection of testicular sperm has also been used in rainbow trout *Oncorhynchus mykiss* neomales (genetically female fish that are subject to masculinization hormonal treatment to produce semen) that do not possess functional spermatic ducts [27], and in

post-mortem individuals (e.g. Atlantic bluefin tuna *Thunnus thynnus*) [28]. In several instances the use of testicular sperm implies the artificial maturation for the spermatozoa to acquire motility. Some solutions for artificial maturation are available including commercial solutions (for example Storfish®, IMV) used in salmonids [29]. Immediately after sperm collection it is important to access its quality. In case of fishes we will not discuss this aspect as it is subject of another work in this same issue [30].

For the methods described before, spermatozoa needs to be maintained in a quiescent state and contact with water, feces or urine should be avoided. Fish spermatozoa motility for most externally fertilizing species lasts for only a few minutes [31] and any contamination will decrease ATP levels and motility parameters, such as spermatozoa velocity or progressivity [17,32]. Contamination of sperm by bacteria present in fish skin and by anesthesia used to sedate fish during manipulation can be reduced by rinsing the urogenital pore with water and cleaning with paper towels before sperm collection. Some authors also recommend to disinfect the urogenital area before sperm collection to reduce the bacterial counts [33].

2.1.2. Stripping frequency and seasonality

Only a few studies have investigated how many times a male fish can be stripped during the reproductive season or how often this procedure can be done without compromising sperm quality. This will be affected by several factors, including the quality of breeders and husbandry conditions during the reproductive season, species reproductive strategy and intrinsic factors related with each species. One important aspect to take into consideration is that the period between two stripping should allow for adequate recovery of sperm production and an individual's homeostasis after the stress caused by handling. In some flatfish species, sperm could be extracted monthly (turbot *Scophthalmus maximus*) or fortnightly (Senegalese sole) without significant differences in sperm quality [34,35]. In zebrafish AB line (wild type), sperm could be collected from 6 to 8 month old

136 males every 14 days [25], although this interval could change according to fish age and fish line.
137 In other species with restricted periods of spermiation, such as common carp *Cyprinus carpio* some
138 detrimental effects on sperm quality were seen in consecutive samplings in terms of spermatozoa
139 motility and seminal plasma pH [36].

140 Another topic investigated is sperm production seasonality, which in a practical aspect may
141 help to identify the best period for sperm collection. Temperate species regulate sperm production
142 by environmental cues (temperature and photoperiod), and therefore in most fish species sperm is
143 collected in a certain period coinciding usually with female egg release. During this period that
144 may vary depending on the species, sperm quantity and quality changes usually with some
145 improvement throughout the breeding season. This fact was reported for species such as turbot,
146 Atlantic halibut *Hippoglossus hippoglossus*, red porgy *Pagrus pagrus*, rainbow trout, Atlantic cod
147 *Gadus morhua*, with picks of poor quality and production at the beginning and end of reproductive
148 season [37–41].

149 In summary, apart from choosing the correct method for sperm collection it is also important
150 to define the best strategy and timing for extraction, especially if samples will be stored or used for
151 cryopreservation.

152 *2.2. Spermatozoa motility activation*

153 Spermatozoa are maintained in most fish species in a quiescent stage in the testis, and a
154 maturation process occurs when passing through the spermatic ducts in order to acquire motility
155 when in contact with an activation media. Until this process is triggered, it is important to avoid
156 motility activation due to the low duration of motility in most fish species. There are several
157 solutions that maintain sperm quiescence, which consist in the dilution of sperm in solutions that
158 mimic seminal plasma composition, with similar osmolality and pH (see section 2.3.1.).

On the other hand, several other solutions can induce motility activation of fish spermatozoa. Spermatozoa motility can be induced, in most externally fertilizing species, by hypotonic solutions in freshwater fishes or hypertonic solutions in seawater fishes, whereas in other species the concentrations of specific ions is crucial for the initiation of motility [42]. For euryhaline species, (e.g medaka *Oryzias latipes*) spermatozoa motility can be activated by hypertonic, isotonic or hypotonic solutions, depending on fish acclimatized to seawater or freshwater [43]. In internal fertilizing species the sperm can swim in isotonic media [24]. In addition, pH of the activation media can also influence the triggering of spermatozoa motility [44]. The presence of other compounds such as ovarian fluid has been seen to play an important role during motility activation in some species and is the focus of another review in this same issue [45].

Overall, there is not a common standard solution that can activate or inactivate the spermatozoa in all fish species since the activation mechanism reflect the environment fishes are adapted to, but some highly conserved mechanisms (spermatozoa from most marine species being activated by seawater) can help in the design of specific media to improve gamete management and quality in several species.

2.3. Refrigerated storage

Gamete storage is an essential tool for artificial fertilization and breeding programs in aquatic animals as it allows fertilization to be synchronized according to gametes' availability; gametes' transportation between different locations and; facilitates the usage of total volume of the gametes, which is particularly important when sperm volume is a limitation [46,47]. Artificial reproduction techniques normally involve sperm storage until collection of oocytes [14]. In this context, it is relatively easier to preserve sperm of most fish species, either through refrigerated storage, also called short-term storage, [47] or long term storage (cryopreservation) in liquid nitrogen, at -196^{o}C [46]. In this work we will focus exclusively on refrigerated storage, as sperm cryopreservation is

183 the focus of other reviews in this same issue [48,49]. Sperm refrigeration protocols have been
184 established for several marine and freshwater fish species (Table 1).

185 Depending on the species and the initial quality, gametes may be stored unaltered from hours
186 to days at room temperature [50,51]. However, after collection, spermatozoa start to undergo a
187 degenerative process that leads to reduced fertilizing ability. Sperm quality decreases with storage
188 time, also termed as aging, can result from apoptosis and necrosis-related events and is influenced
189 by the storage conditions. Increased incidence of apoptotic spermatozoa in fish has only been
190 studied after cryopreserved storage [52], whereas in mammals this has already been described after
191 refrigerated storage [53]. Thus, the storage conditions need to be manipulated to delay the
192 degradation process. Below we revise the most important conditions affecting fish sperm quality
193 during refrigerated storage.

194 *2.3.1. Extender solution*

195 The first step for the implementation of a refrigeration protocol is usually the selection of an
196 extender solution. To improve sperm storability the extenders will reduce the spermatozoa
197 concentration and thus facilitate the oxygen supply, should not activate spermatozoa motility,
198 should be isotonic, have a good buffering capacity, should include nutrients, antioxidants to control
199 the activity of reactive oxygen species, should include antibacterial substances and stabilizing
200 colloids [47,54]. Although extenders are not essential, several studies have showed that undiluted
201 sperm storability is much lower [14,50,55–59]. As an example, in European seabass sperm
202 storability increase by 2 days for diluted sperm [14] and in meagre *Argyrosomus regius* up to 10
203 days [55]. Whereas, in some case simple solutions of NaCl are used as an extender [55,60,61],
204 other cases more or less complex saline solutions such as Hanks balanced salt solution are preferred
205 [14,16,62], or extenders are develop to resemble the species seminal plasma [59,63]. This approach
206 will guarantee the correct ionic composition to maintain the spermatozoa in a quiescent state [47].

In most cases extenders are isosmotic to the seminal plasma and with similar pH [55,56], but lower osmolalities (200 $mOsm.kg^{-1}$) in the marine red drum *Sciaenops ocellatus* [64] and slightly different pH (7.6 vs 8.1 - seminal plasma pH) in the freshwater piracanjuba *Brycon orbignyanus* [65] improved sperm storability. To keep the pH levels stable during storage, the extenders could be buffered with Tris-based and Hepes components [56,66]. The availability of oxygen for respiration can limit storage time [57,67], but in excess can promote oxidative stress caused by reactive oxygen species (ROS) if the sperm antioxidant system is insufficient to create a balance between ROS elimination and production [51,68,69]. Ultimately these changes lead to a reduction in spermatozoa motility [68,69] and hence fertilization capacity. Oxidative stress is also considered responsible for the reported reductions in the integrity of the plasma membrane and DNA fragmentation during storage [27,68]. Hence, the addition of different types of antioxidants, such as glutathione, to hinder oxidative stress during storage has proved beneficial to some species, with species-specific effectiveness [69,70]. Decrease levels of adenosine triphosphate (ATP), directly affect the activation and maintenance of the flagellar movement and the percentage of motile spermatozoa and spermatozoa velocity [29,71]. Therefore, the addition of energy substrate to the extender, such as glucose or lactate, that can be used by the cells has proven beneficial [56,63,66,71], but this should be carefully considered since it can also stimulate bacterial growth [72]. Indeed, the bacterial growth is proportional to the storage time [55,73], and bacteria compete with the spermatozoa for nutrients and oxygen and degrade the extender media [66,73]. To reduce the fungal and bacterial growth during storage, besides the use of sterile media [55,73], different authors have tested extenders supplemented with antimycotics and antibiotics [60,65,66,72,74]. Usually there is an improvement in the sperm storability with the use of antibiotics, but the dosage needs to be adjusted for each species. Concentrations above 0.5 mg/mL of gentamicin in the Nile tilapia *Oreochromis niloticus* reduced the spermatozoa viability [74] and above 0.1 mg/mL reduced

fertilization rate in piracanjuba [65]. Finally, there are commercially available extenders that may be used (some examples in Table 1). These media are recommended for use in fish farms where preparation of media is more complicated, and standardization of procedures is required.

The sperm dilution in the extender will also affect the storage time [50,55,75]. Extenders are essential for prolonging the spermatozoa viability and low dilution rates, because of the high spermatozoa concentration, can lead to hypoxic conditions [16]. Nonetheless, they also dilute essential protective compounds of the seminal plasma and too higher dilutions decrease the sperm storability [16,47,50,54,55]. For example, in meagre the best sperm storability was achieved with a sperm:extender dilution of 1:4 compared with higher dilutions [55].Thus a species-specific balance should be found for each case.

2.3.2. Storage temperature, atmosphere and handling

Another important step in the creation of a refrigeration protocol is the decision of the storage temperature, essential to reduce spermatozoa metabolism. Lower temperatures, close to but not 0ºC, are usually recommended as they decrease the cells metabolic rate and reduce energy consumption to a minimum without affect cellular structure [16,54]. Nonetheless, most authors use 4ºC, for practical reasons, as this is the normal temperature of household refrigerators (Table 1).

Additionally, the atmosphere in which the sperm is stored can be manipulated. Spermatozoa viability is dependent on aerobic metabolism and oxidative phosphorylation is essential for the ATP levels maintenance [76], thus during storage spermatozoa consume oxygen, and aerobic conditions should be provided [57]. Generally, normal aerobic conditions are used (normoxia), as observed in Table 1, but oxygen is frequently added with positive results [54,57,72,77]. Nevertheless, an oxygen atmosphere could increase ROS production and lipid peroxidation, resulting in cell damage with deterioration of quality. Thus normoxic conditions could result in the best option [78,79]. Alternatively, to avoid oxidative stress, air-limited conditions can be created

by the use of vacuum [63]. The samples handling during storage should also be taken into consideration to secure a renewal of atmosphere and to avoid cells sedimentation [16]. The most commons approach are constant stirring or rocking of the samples [59], daily shake and open the containers for air exchange [55,57] or keeping a maximum depth of 5-6 mm to improve gas exchange [67]. In most cases it has not been empirically shown the efficiency of these techniques, and the decision of the handling procedure is affected by the sperm dilution and ratio of semen volume to atmosphere. Containers that allow for a higher ratio of surface area to volume are frequently preferred to facilitate gas exchange with the selected atmosphere.

To our knowledge, in all the species studied so far, sperm can be stored refrigerated, usually from a few days to weeks, if the correct storage conditions are created. The best protocols are quite variable from species to species and should at least consider extender composition and dilution, storage temperature, atmosphere and handling.

2.4. Measuring and adjusting spermatozoa concentration

To make an optimal use of the available sperm, spermatozoa concentration reported as the number of cells per mL (also known as spermatozoa density) is often adjusted by diluting the sperm samples at a known concentration in an extender solution. Adjustment of spermatozoa concentration can be either done immediately before fertilization to have an optimal spermatozoa:oocyte ratio (see section 2.5.1.) or before sperm cryopreservation to ensure the best ratio of cryoprotectant per cell [80,81]. Spermatozoa concentration adjustment prior to cryopreservation removes technical variability and allows for meaningful comparisons and procedure standardization [80,82].

Several methods to determine spermatozoa concentration have been developed and adapted to different species. Concentration measures with haemocytometer are considered the standard method and involve counting the number of spermatozoa with a microscope and a cell counting

chamber, such as a Neubauer or a Bürker chamber [18,83,84] or specialized spermatozoa counting chambers such as Makler®. This method, despite cheaper, is time consuming and impractical when large sets of samples need to be analysed [84,85]. Spermatocrit, that is the ratio of packed spermatozoa after centrifugation regarding the total volume of semen, is determined with haematocrit capillary tubes, is faster and simpler, and the results are frequently equivalent to spermatozoa concentration [18,85,86]. However, this technique is not as effective in marine fish as in freshwater species because spermatozoa do not sediment efficiently, which may be due to their density being similar to that of seminal plasma [84]. Alternatively, the value of absorbance caused by the turbidity of the sperm suspension, can be measured by spectrophotometry, but a calibration of the appropriate wavelength is required and correlation with concentration needs to be established in advance for each species [18,87,88]. This is an indirect measurement, and the presence of other cells, such as blood cells or immature sperm cells, can cause an overestimation of the spermatozoa concentration [88,89], especially when using testicular sperm [87]. Furthermore, some authors also suggest the use of automatically cell counters (Coulter counter) or flow cytometry [86,90,91], or specialized equipment, such as NucleoCounter SP-100, that make use of fluorescent microscopy [89]. These are highly precise but expensive methods that in most cases allow for simultaneous evaluation of other sperm quality parameters [86,89]. Lastly, CASA (computer assisted sperm analysis) systems, can also be used to determine concentration fast and efficiently and have the advantage that spermatozoa concentration is evaluated simultaneously with sperm motility parameters [19,84,86]. The decision regarding the used method to measure spermatozoa concentration will most of the times depend on the species, objective of the work and available resources.

2.5. In vitro fertilization

In vitro fertilization is part of the process of induced spawning for fish artificial reproduction, and basically consists on the collection of sperm and ova and their mixing together. This process tries to facilitate the gamete encounter, increasing the fertilization success, and is often evaluated by counting the percentage of eggs which reached the 2 or 4-cell stage in relation to the total number used at the beginning of the process.

In vitro fertilization can be carried out by three different fertilization methods in fish species. In the "dry method" -which is the method employed in most hatcheries around the world- sperm is directly poured onto the oocytes and the activating solution is added only at a later stage [92]. Secondly, the "semi-dry method" consists of mixing the sperm with the activation media in a first step (diluted 50 to 200 times), for pouring immediately the activated sperm onto the oocytes [22]. Finally, artificial fertilization can be carried out in reverse, using the "wet method", where oocytes are mixed with the activation solution in a first step and later, the sperm is added. The last method has been reported controversial results in some cyprinid species, thus it is seldom use in fish farms [22].

The success of *in vitro* fertilization is going to depend on a large number of factors, some of them purely biological and related to the quality of gametes (color or buoyancy of oocytes, kinetic features of spermatozoa, etc.), and others factors directly linked to the management of gametes along the *in vitro* fertilization operations already reviewed in the above sections (type of extender, storage time before *in vitro* fertilization, properties of activating media, etc.). Within the last factors, both *i*) the proportion of gametes used on the *in vitro* fertilization environment (also known as spermatozoa:oocyte ratio), and *ii*) the contact time between sperm and oocytes are going to have an essential role for achieving high fertilization rates throughout *in vitro* fertilization trials.

2.5.1. Spermatozoa:oocyte ratio

Spermatozoa to oocyte (or egg) ratio is an essential factor which needs to be taken into account to standardize and optimize the *in vitro* fertilization trials on the aquaculture sector. Generally, an excess of sperm is used in *in vitro* fertilization trials both in freshwater and seawater species, but an appropriate combination of the number of spermatozoa per oocyte must be used in order to enhance the reproductive efficiency in fish farms and avoid wasting sperm when limiting amounts of gametes are available. It is important to note that spermatozoa:oocyte ratio is relatively high and very variable among fish species, and fish sperm quality (usually evaluated as sperm motility) will determine the appropriate ratio (Table 2). In the best scenario, spermatozoa:oocyte ratios not too high (10^2-10^3 spermatozoa per oocyte) are required for achieving successful fertilization rates (>80 %) in several fish species such as wels or African catfish, Atlantic croaker *Micropogonias undulatus*, pufferfish *Takifugu niphobles* or turbot [92–97]. In intermediate cases, much higher ratios (at least of 10^4 spermatozoa per oocyte) are imperative for obtaining notably fertilization rates in species such as sea lamprey *Petromyzon marinus*, Northern pike *Esox lucius* or herring *Clupea harengus* [98–100]; and finally, some marine species such as European eel, Atlantic halibut or Atlantic cod require spermatozoa:oocyte ratios extremely high that exceed 10^5 spermatozoa per ova [101–105]. In addition to the interspecific variability, several studies have reported different optimal spermatozoa:oocyte ratios for the same species. This seems to occur in walleye or rainbow trout (see Table 2), and these disparate results could be attributed to different reasons such as *i*) the gamete management throughout hatchery protocols (i.e. amount of water for activation the gametes, *in vitro* fertilization environment features, etc.) or *ii*) even the gamete quality at the time of stripping (i.e. oocyte buoyancy, sperm kinetic features, etc.). In this respect, spermatozoa motility - considered the best sperm quality biomarker in fish - became the most important factor in order to choose the proper spermatozoa:oocyte ratios throughout the *in vitro* fertilization trials. Even within the same species, if low quality (<50%) sperm is used, spermatozoa:oocyte ratio must

be increased with the aim to keep high fertilization and hatching rates [93]. The most obvious example occurs when fresh and cryopreserved sperm from the same species is used for carrying out *in vitro* fertilization trials. In African catfish, the effective spermatozoa:oocyte ratio reported for fresh sperm was 1.5×10^4 spermatozoa per oocyte [94], while for cryopreserved sperm the optimum ratios ranged from 4.9×10^4 to 1.7×10^6 [106]. In Cyprinidae, the spermatozoa concentration of post-thaw sperm used to achieve optimal fertilization success was approximately 100 times higher than for fresh sperm [107].

Spermatozoa:oocyte ratios published by different authors must be considered as guideline values, and not as absolute or unchangeable data for carrying out *in vitro* fertilization trials. In this respect, it is important to highlight that even keeping the same spermatozoa:oocyte ratio through IVF trials, the final volume of activating solution can be a crucial factor for achieving the expected fertilization rates. An excess of activation media could hinder the meeting of oocyte and spermatozoa (too much space to look for the oocyte), whereas the lack of activation media will affect the spermatozoa activation process or cause an erratic displacement to reach the micropyle. Thus, some protocols include a step for spermatozoa concentration adjustment in the extender solution (see section 2.4 and references within). Summing up, the spermatozoa:oocyte ratio approaches will improve the aquaculture sector by *i*) promoting a rational use of gametes, *ii*) limiting the number of breeding fish and therefore, *iii*) reducing the production costs and complying with a more sustainable production.

2.5.2. Gamete contact time

In addition to the amount of gametes (oocyte and spermatozoa) added to the fertilization environment, the contact time between them became a key factor for reaching high fertilization rates throughout *in vitro* fertilization trials. Common sense suggests that longer contact times, increase probabilities of successful encounters between gametes. However, it is important to keep

in mind two basic premises: *i*) how long spermatozoa are able to move through the activation media (sperm longevity), and *ii*) how long oocyte is receptive to be fertilized by a spermatozoon (oocyte receptivity). In this sense, if gamete contact time surpass one of those periods, fertilization rates will not be improved due either the (exhausted) immotile spermatozoa that will not be able to reach the oocyte or motile spermatozoa will reach the blocked oocyte.

Contrary to what happens with spermatozoa:oocyte ratio, there are scarce studies concerning the effects of gamete contact time on fertilization success in fish species. In Atlantic cod when the number of spermatozoa per oocyte is limited, gamete contact time had a significant effect on fertilization success [101]. Specifically, using spermatozoa:oocyte ratios of $1:10^5$ the authors recommended gamete contact times of around 5 min; while for lower spermatozoa:oocyte ratios ($1:10^3$ or $1:10^4$), the contact times suggested for achieving high fertilization rates were about 30 min. In turbot, for 6×10^3 spermatozoa per oocyte, maximum fertilization success was usually recorded after 1 min of contact between gametes [95]. However, for lower spermatozoa:oocyte ratios ($1.5:10^3$), maximum fertilization success was scattered but mainly observed after 2 or 3 min. For spotted wolffish, gametes contact time of 2 h using at least 5×10^5 spermatozoa per oocyte were recommended for achieving fertilization rates over 80%, but an increased contact time (6 h) could be used to compensate for lower spermatozoa:oocyte ratios [18]. On the contrary, in herring, the fertilization success varied only slightly within the contact times tested (15, 30, 60 and 120 s), and the results also show that a contact time between oocytes and sperm of only 15 s was sufficient to achieve a high fertilization rates at proper spermatozoa concentration [98].

Therefore, it seems evident that a number of factors (spermatozoa:oocyte ratio, gamete quality, contact time, aqueous environment, etc.) should be taken into account throughout *in vitro* fertilization trials in order to maximize the fertilization success. In this sense, a successful

396 combination of the different factors will make possible to obtain notably fertilization rates, using
397 the gametes in an optimized way and enhancing the reproductive efficiency in fish farms.

398 **3. Sperm handling in penaeid shrimp**

399 *3.1. Spermatophore extrusion*

400 The spermatozoa of penaeid shrimp are transported along the vas deferens as a compact mass,
401 packed into several tissue layers and stored in the terminal ampoule, forming the spermatophore,
402 which will be expelled through the genital pores at the base of the fifth pair of pereopods [2,4,108].
403 The petasma and the masculine appendices are external reproductive structures located on the first
404 and second pair of pleopods, respectively, near the genital pores and are responsible for transferring
405 the spermatophore formed in the terminal ampoules to the female thelycum during mating [2].

406 The spermatozoa can vary in concentration and distribution in the spermatophore depending
407 on the reproductive characteristics of each penaeid species. The structure is similar among the five
408 open thelycum species (sub genus *Litopenaeus*), with the spermatozoa found in the center of the
409 spermatophore. Some closed thelycum species, such as the brown shrimps *Farfantepenaeus*
410 *subtilis* and *Farfantepenaeus aztecus*, also have a longitudinal spermatozoa, but it is distributed
411 along the periphery of the spermatophore near the outer cuticle [108,109].

412 The term "spermatophore extrusion" is specifically used in penaeid shrimps for sperm
413 collection. Regardless the spermatophore structure, two extrusion methods have been used to assess
414 sperm quality for reproductive performance trials, the development of refrigeration and
415 cryopreservation protocols and artificial insemination procedures. Manual extrusion of the
416 spermatophore is performed by gently pressing around the coxas of the fifth pair of pereiopods
417 [6,110] (Figure 3a and b). Extrusion can also be performed by electrical stimulation (4.5 to 9 Volts)
418 applied to the same area [110]. Both extrusion methods can cause damage to the reproductive tract
419 if not performed correctly, which could compromise the quality of regenerated spermatophores

after multiple extrusions in the same male. Although manual extrusion is a very simple procedure, its success depends on the skill and experience of the handler. The electrical method has the advantage of being quick and efficient regardless of the operator's experience, but electricity may have harmful effects in terms of animal stress and the sperm quality of regenerated spermatophores [110,111].

In a study comparing the two methods for the extrusion of spermatophores from the pink shrimp *Farfantepenaeus paulensis*, manual extrusion was recommended for maintaining a stable number of spermatozoa, spermatophore weight, body weight and the spermatosomatic index after the regeneration of the spermatophores [110]. Lending support to this recommendation, similar quality spermatophores were formed in 16 days without molting and in 24 h with molting after manual extrusion from the pink shrimp [112]. Artificial ejaculation may also avoid the process of spermatophore deterioration as substitutes the molt-dependent spermatophore renovation mechanism in the pacific white shrimp *Litopenaeus vannamei* [4]. However, the replacement process of the ejaculated spermatophore may be affected by several factors, such as temperature and molting stage [4].

Spermatophores have also been retrieved from cadaveric shrimp within up to 1 h after death for the banana shrimp *Fenneropenaeus indicus* [113], 24 h for pacific white shrimp [10] and 48 h for the white shrimp *Litopenaeus schmitti* [114] after which a significant deterioration in the quality was observed. Nonetheless, the retrieval of a viable cadaveric spermatophore requires knowledge regarding the postmortem time interval, cause of death and storage conditions of the shrimp [10,113]. Therefore, the different authors recommend this procedure only to recover gametes from healthy males that die unexpectedly in captivity or from recently caught wild penaeids.

3.2. Refrigerated storage

443 Also in penaeid shrimp, the development of refrigeration protocols for spermatophores can be
444 very useful for artificial insemination in breeding programs [10,11,115,116] (see also section 2.2.)
445 and has been successful applied to a few penaeid species [10,116].

446 Freezing temperatures (-18°C) are not recommended for the maintenance of spermatophores
447 from pacific white shrimp, but cadaveric spermatozoa viability remained high (~90%) for 48 h
448 after death, when the shrimp were kept at a cooling temperature of 4°C [10]. However, membrane
449 cell damage has been reported when pacific white shrimp spermatophores were maintained
450 between 2 and 5°C [117]. A low percentage (~8%) of abnormal and non-viable spermatozoa of
451 pacific white shrimp was obtained when maintaining the spermatophores cooled at 14°C for 27 h
452 [116].

453 The use of extender solutions has been proposed during refrigerated storage of spermatophores
454 and increase the storability for artificial insemination procedures [11,115,116,118]. Although these
455 extenders are usually based on a saline solution [11,115], other substances have been tested to
456 control bacterial proliferation [116,118]. When mineral oil with 0.1% penicillin-streptomycin was
457 added to prevent bacterial proliferation in chilled (2 to 4°C) pacific white shrimp spermatophores,
458 spermatozoa kept the apparent viability for 35 days [11]. However, the mineral oil causes the loss
459 of the adhesive properties of the spermatozoa, which are critical for artificial insemination [116].
460 Alternatively, pacific white shrimp spermatophores were transported in microtubes containing a
461 complex saline solution with an antibiotic and antimycotic solution, placed in a polystyrene box to
462 keep the material cooled (14°C) and viable for up to 27 h [116].

463 *3.3. Sperm quality*

464 Decapod crustacean spermatozoa are unique and different from the spermatozoa of other
465 animals. In most penaeid species, these non-motile aflagellate spermatozoa show a spherical main

466 body containing an uncondensed nucleus and acrosomal vesicles that included the acrosomal cap
467 and spike [119].

468 Spermatozoa counting and the characterization of morphological features (e.g., twisted or
469 missing spikes) are the simplest methods for determining spermatophore quality and reproductive
470 potential in penaeids [120]. For such, the spermatophores are homogenized in a calcium-free saline
471 solution and spermatozoa are counted using a hemocytometer under a light microscope [121]. This
472 procedure can be also combined with a colorimetric technique by adding trypan blue [120,121] or
473 eosin-nigrosin [122] to evaluate the cell membrane integrity and thus estimate the percentage of
474 dead and live spermatozoa.

475 Fluorescence microscopy with the fluorescent probes propidium iodide (PI) and
476 carboxyfluorescein diacetate (CFDA) has been used to assess membrane integrity of penaeid
477 spermatozoa. In this case, intact cells membranes are stained green due to the binding of CFDA to
478 esterases, whereas damaged spermatozoa are stained red due to the binding of PI to nuclear DNA
479 [122]. DNA integrity has also been evaluated under a fluorescence microscope by adding acridine
480 orange to the sperm solution of banana shrimp [113].

481 Flow cytometry has been used to determine spermatozoa viability of pacific white shrimp
482 based on cytoplasmic membrane integrity [116,123]. Although cytometry is reported to be more
483 sensitive and less prone to observer error, a positive correlation between morphotype analysis
484 performed by light microscopy and sperm viability using flow cytometry has been found [123].
485 However, cells undergoing a change from an intact to disrupted cytoplasmic membrane
486 (transitional spermatozoa populations) of pacific white shrimp, may not show evident
487 morphological changes for detection by microscopy, and this type of damage would only be
488 detected by flow cytometry [116].

Sperm quality diagnostic techniques in penaeids have changed little in recent decades (see Table 3), and have often overestimated the fertilizing capability of spermatozoa [124]. Recently, a sperm chromatin dispersion test for the assessment of sperm DNA fragmentation was applied in the giant tiger shrimp *Penaeus monodon* [125]. Therefore, additional efforts for the application of new techniques and more sophisticated approaches are envisaged (e.g., the analysis of sperm DNA fragmentation and ROS) to determine sperm quality in penaeids [125].

3.4. Artificial insemination

Artificial insemination has several advantages for the reproductive management and breeding programs for penaeids, such as overcome a lack of mating and improve nauplii production in some species such as pink shrimp [6]. Artificial insemination has been used, for example, in hybridization trials between two closed [126] or two open thelycum species [127,128]. However, most crossbreeding has produced few or no hybrid embryos, suggesting that our understanding of the spermatozoa-oocyte compatibility mechanism needs to be improved [128].

During the mating of closed thelycum penaeids, the male inserts the spermatophores into the thelycum of the female soon after molting (ecdysis), whereas the spermatophores are attached externally in open thelycum females in the intermolt stage a few hours before spawning [2]. Therefore, artificial insemination procedures must respect the natural reproductive cycle of the genus *Penaeus*. In closed thelycum sub-genera, a recently molted female is identified (e.g., exuviae with coded cuts on uropods or tags) and a spermatophore is implanted in the soft thelycum with a spatula [6,129] (Figure 3c and d). Artificial insemination in the open thelycum sub-genus (*Litopenaeus*) has been performed by placing the spermatophore with forceps into the thelycum of a mature (ready-to-spawn) female, which is located at the base of the fifth pair of pereopods [124].

Successful oocytes fertilization and hatching after artificial insemination have been observed using freshly collected spermatophores from pink shrimp [6] or following refrigerated storage from

pacific white shrimp [115] as well as cryopreserved spermatophores from giant tiger shrimp [129]. However, a high spermatozoa survival rate based on membrane integrity may not ensure adequate fertilizing capacity for artificial insemination procedures in penaeids. Low hatching rates (~12%) and impaired embryo development were found in pacific white shrimp when using for artificial insemination spermatophores with high rates (~90%) of cell membrane integrity after the vitrification [122,124].

4. Sperm handling in bivalve mollusks

4.1. Sperm collection

Most bivalve species are broadcast spawners, releasing synchronously oocytes and free-swimming spermatozoa in the water column where external fertilization occurs. However, some bivalves including species of the genus *Ostrea* release sperm clusters called spermatozeugmata which are acellular structures in which spermatozoa heads are embedded. Both spermatophores and spermatozeugmata transfer sperm but spermatophores differ from spermatozeugmata in that the spermatozoa are enclosed by a sheath or capsule of varying complexity in the former [130]. Spermatozeugmata dissociation is triggered in seawater, releasing free-swimming spermatozoa (for details see [131]). In bivalve hatcheries, sperm collection is a current practice for the purpose of *in vitro* fertilization. Spermatogenesis is seasonal in bivalves, and can be accelerated or delayed by environmental factors including temperature and photoperiod [132,133]. In the Pacific oyster *Crassostrea gigas*, hatchery methods allow the sperm production throughout the year [133]. Sperm can be collected in bivalves during the reproduction period by scarification of the gonad or following induction of sperm release using chemicals or thermal shock. Scarification of the gonad is the easiest method to quickly collect a high concentration of spermatozoa. After opening the shells with a knife, the animal is moved to a Petri dish and sperm are collected by scarification of the gonad (Figure 4). Spermatozoa are immotile in the gonad and motility is triggered by dilution

of sperm in seawater [134]. In the Pacific oyster and the black-lip pearl oyster *Pinctada Margaritifera*, 5 to 15 × 10^9 spermatozoa mL^{-1} can be collected by scarification of a gonad when oysters are sexually ripe [134]. The disadvantage of using this method is that male germ cells (e.g., spermatogonia) can be collected among mature spermatozoa because their proliferation is continuous throughout the reproductive period [135]. On the other hand, controlling the time of sperm collection and motility activation using the scarification method is of significant benefit for hatchery practices.

For some species, including simultaneous hermaphrodite bivalves, such as the great scallop *Pecten maximus*, scarification approach does not allow collection of motile spermatozoa after their dilution in seawater [136]. To acquire motility capacity, a maturation process of spermatozoa is required during their transit along the genital tract [5,136]. In this case, sperm release can be triggered by thermal shock or by direct injection of the neurotransmitter serotonin (i.e., 5-hydroxytryptamine) in the gonad. A sharp increase in seawater temperature, from 19 °C to 30 °C over a half-hour period [137], initiates gamete release in sexually ripe bivalves, but spawning response to thermal shock is highly variable and can takes hours. Otherwise, injection of serotonin (100 µL at 2 to 10 mM) into the gonad is an efficient and convenient method to induce sperm release in bivalves. After injection, the animal is placed in seawater and ejaculation occurs in 10 to 30 min post injection [5,13]. This approach is effective for scallop species since their gonad is easy to reach with a syringe and without killing the animal. Induction of sperm release by thermal shock or serotonin injection allows sampling free-swimming sperm near the gonopore. These methods do not impact sperm fertilizing ability as demonstrated in the Pacific oyster, great scallop [127] and the Caribbean scallops *Argopecten nucleus* and *Nodipecten nodosus* [138].

Following sperm collection, concentration of spermatozoa can be determined after dilution to 1/500 or 1/1000 in seawater by counting spermatozoa using a Coulter counter [5,139], a flow

561 cytometer [12,140], or by directly counting spermatozoa in a known small volume using a
562 Neubauer or Malassez hemocytometer [131,141]. For ripe oysters, concentration of spermatozoa
563 is estimated to be 6×10^7 and 5 to 15×10^9 spermatozoa mL^{-1} for ejaculated and gonadal sperm,
564 respectively [134].

565 *4.2. Refrigerated storage*

566 Compared with fish, where spermatozoa usually only swim for a few minutes, bivalve sperm
567 motility lasts for hours, up to 24 h in the Pacific oyster [5,12]. Collected sperm can be stored for
568 some hours or days according to spermatozoa concentration, and temperature and chemical
569 composition of the storage medium. Undiluted sperm collected by scarification can be kept longer
570 than diluted sperm, such as ejaculated sperm, which is suggested to be related to the dilution of
571 protective components of the seminal plasma [142,143]. Undiluted Pacific oyster sperm was stored
572 for 7 days when refrigerated but the mean percentage of motile spermatozoa decreased from 62 ±
573 23 % to 37 ± 24 % between the beginning and day seven of storage [143]. Similarly, motility of
574 black-lip pearl oyster spermatozoa was reported 13 days after collection when kept at 4 °C [144].
575 Spermatozoa of the Portuguese oyster *Crassostrea angulate* can be stored for 3 days at 4 °C without
576 losing motility [145]. Temperature lower or higher than seawater temperature during natural
577 spawning period may reduce sperm quality [146]. Since spermatozoa of bivalves are characterized
578 by a long motility phase, refrigerated storage is recommended only if sperm is to be kept longer
579 than a few hours. The fertilizing ability of Pacific oyster sperm stored at 4 °C for a 12 day period
580 was similar to that assessed for fresh spermatozoa [147]. Chemical composition of the storage
581 medium plays an important role in improving sperm storability by controlling motility. In marine
582 bivalves, spermatozoa motility is triggered by changes in external pH and ionic composition
583 between the gonad and seawater [148,149]. Indeed, acidic pH (pH < 6.0) and high concentration
584 of K^+ (ie, > of seawater K^+ concentration) inhibit spermatozoa motility initiation in the testis [150].

585 Changing pH and ionic composition of artificial seawater can enhance sperm storability and help
586 to manage artificial reproduction in bivalve aquaculture.

587 *4.3. Artificial fertilization*

588 High fertilization success (> 70 %) is usually obtained from *in vitro* fertilization since the high
589 fecundity of bivalve species compensates for the inter-breeder variability of sperm quality.
590 Variability of fertilization success is partly explained by spermatozoa ATP content and viability
591 [151,152]. Intracellular ATP is required for flagellar beating via dynein-ATPase activity and
592 positively correlated to spermatozoa fertilizing ability (R^2 =0.40) in the Pacific oyster [151].
593 Intracellular ATP level can be determined by bioluminescence on sperm samples stored in liquid
594 nitrogen. Sperm quality is also related to its viability, which is assessed by flow cytometry using a
595 dual staining with SYBR-14 and propidium iodide [140]. Populations of live (labelled with SYBR-
596 14 only, cells with intact plasma membranes), dying (labelled with SYBR-14 and PI, cells with
597 damaged plasma membranes) and dead (labelled with PI only) spermatozoa are determined for
598 each male by drawing three regions on the cytogram of SYBR-14 and PI fluorescences. In the
599 Pacific oyster, fertilizing ability of spermatozoa is positively correlated to percentage of live
600 spermatozoa (R^2= 0.55), and negatively correlated with percentage of dying spermatozoa (R^2=
601 0.57) [151]. A negative relationship was reported between viability and DNA integrity (Sperm
602 Chromatin Structure Assay) in spermatozoa of the green-lipped mussel *Perna canaliculus* [153]
603 and DNA damage and fertilization rate are negatively correlated in the Pacific oyster [154]. Sperm
604 viability and DNA damage assays are valuable tool for assessing sperm quality in bivalve
605 aquaculture production and cryopreservation. Compared with penaeids, morphological parameters
606 of spermatozoa are not involved in sperm quality [151]. Finally, ROS production assessed by flow
607 cytometry using 2′7′-dichlorofluorescein diacetate (DCFH-DA) is not involved in spermatozoa
608 quality in the Pacific oyster [151].

609 The *in vitro* fertilization success varies depending on the spermatozoa:oocyte ratio. High
610 fertilization success is reported using sperm collected by scarification at 100 spermatozoa per
611 oocyte in the scallop *Mizuhopecten yessoensis* (fertilization rate > 70%) [155] and the black-lip
612 pearl oyster (80% fertilization rate) [156]. It is recommended to increase the spermatozoa:oocyte
613 ratio when using cryopreserved compared to fresh sperm. In the black-lip pearl oyster, a
614 spermatozoa:oocyte ratio of 100000:1 is required to reach 80 % fertilization rates when using
615 cryopreserved sperm compared to 100:1 for fresh sperm [156]. In oysters, optimal
616 spermatozoa:oocyte ratio is between 400:1 and 5000:1 for high fertilization rate (> 70%) in a
617 volume between 10 to 200 mL of seawater and a contact time between 10 to 30 min [130,138], and
618 below 200 spermatozoa per oocyte for the blood clam *Tegillarca granosa* [158]. Using higher
619 spermatozoa:oocyte ratio may decrease the larval yield probably due to polyspermy [158–160].

620 **5. Sperm handling in Marine Mammals**

621 Among marine mammals, key reproductive technologies for maintaining zoo-based
622 population genetic diversity, including sperm collection, preservation and artificial insemination,
623 have been developed in a small number of species, primarily cetaceans, where 52 calves across
624 five species have been born from such procedures to date [7]. Crucial understanding of sperm
625 biology and male reproductive health has evolved from such studies. This knowledge has been
626 obtained as sperm samples have been analyzed for their fertility *in vivo* (via artificial insemination)
627 whilst simultaneously undergoing a suite of *in vitro* assessments, often in concert with serum
628 hormone measurements. Repeated over time, these assessments of males with known reproductive
629 and health histories have been used to build species-specific reproductive health databases.

630 In this section, sperm handling and assessment research will be presented for the taxon where
631 the most comprehensive information is available, namely cetaceans, including Delphinidae,
632 (dolphins) and Monodontidae (beluga). Relevant knowledge from other marine mammals where

limited studies have been conducted, including the walrus *Odobenus rosmarus* (Odobenidae) and the polar bear *Ursus maritimus* (Ursidae), will also be included.

5.1. Sperm collection

5.1.1. Timing of collection

The degree of a species' reproductive seasonality must be considered when determining the timing of sperm collection attempts in pubertal and sexually mature males [7]. Many cetaceans display a diffuse pattern of reproductive seasonality, meaning that collections yielding high quality sperm samples can be performed throughout the year (e.g. bottlenose dolphin *Tursiops truncatus*, killer whale *Orcinus orca*). In contrast, marine mammal species whose reproductive activity appears to be heavily influenced by the annual light cycle (with postulated impact of geographic origin of founder stock), such as most pinnipeds and some delphinids, such as the Pacific white-sided dolphin *Lagenorhynchus obliquidens*, have more discrete mating periods during which sperm can be collected [161]. Beluga (Monodontidae, *Delphinapterus leucas*) and polar bear (Ursidae) also display seasonal mating periods but spermatogenesis occurs year-round in captive beluga [162] and likely year-round in polar bears, with sperm having been collected during the non-breeding season on two occasions [163].

5.1.2. Collection method

The method used for collecting sperm is dependent on the tractability of the species. For tractable species like delphinids and beluga, males are trained for voluntary sperm collection using a positive-reinforcement schedule combined with operant conditioning [9,162,164]. Males receive various tactile stimulations to elicit voluntary extrusion of the penis from the genital groove whilst positioned in dorsal recumbency adjacent to the pool wall, and after an erection is obtained on a consistent basis, are conditioned to ejaculate into a sterile Whirl-Pak® bag (NASCO, Fort Atkinson, WI, USA) (Figure 5). To minimise saltwater contamination, the penis

tip is wiped dry with sterile cotton gauze, with or without a pre-rinse in HEPES-TALP medium for 2-3 s. Custom-made rings with latex lining can also be used to prevent saltwater contamination [162], and sperm creatinine and osmolality measurements can distinguish urine contamination from that of saltwater contamination, information of which is necessary to refine the collection technique to minimize the deleterious impact of both contaminants on sperm quality (Table 4).

Choice of lubricant placed on the gloved hand used during the collection process (and during the insemination process as described) is another important consideration since some brands claimed to be non-spermicidal go on to negatively impact sperm quality over time. The lubricant Pre-Seed® (INGFertility, Valleyford, WA, USA), originally developed for use with equine sperm, has been used without detrimental impact on mammal sperm (aquatic and terrestrial).

It should be noted that the time taken to train a male for voluntary sperm collection is highly influenced by male- and trainer-associated variation, and can take a month to more than a year to achieve collection of non-contaminated sperm (with no or negligible contamination of saltwater and urine) on a consistent basis. Despite considerable training efforts being dedicated to the voluntary collection of sperm from the Pacific walrus *O. r. divergens*, sperm samples have not yet been collected on a consistent basis (Robeck et al. unpublished). Fluid smears taken from the glans penis of one Pacific walrus at 8 weeks from the initiation of a 14 week gonadotropin treatment contained considerable numbers of morphologically normal spermatozoa [165].

Urethral catheterization of alpha-2 adrenergic agonist anaesthetized males is the collection method of choice in non-tractable species like the polar bear, as electroejaculation has been predominantly unsuccessful [163].

5.2. Use of sperm function tests for estimating individual and population reproductive health

When estimating the *in vivo* fertility potential of a cetacean male, important sperm traits to assess include *in vitro* spermatozoa motility characteristics (including longevity), spermatozoa morphology and DNA integrity. CASA has revealed species- and individual variation in spermatozoa motility parameters and as in other taxa, this motility analysis approach has been useful for refining sperm preservation methods [162]. Cetacean ejaculates typically exhibit spermatozoa with high rates of progressive motility and normal morphology (> 80 %). Cetacean spermatozoa DNA quality was also high based on measurements using the Sperm Chromatin Structure Assay (SCSA; 15 ± 3% DNA fragmentation index) [166]. The DNA fragmentation index (DFI) as determined by the SCSA is designated an estimated threshold, above which a detrimental impact on fertility is observed. This threshold varies across species, being as little as 6 % DFI for pigs to ~28 % for the horse [167,168]. An almost 30% decline in pregnancy rate was observed in the pig when the DFI exceeded 12%, compared to pigs with a DFI < 6% [167].

For the bottlenose dolphin, the aquatic species where the most information exists, fertile males displayed a DFI of 5.3 ± 1.3 %, whereas, males considered to have poor fertility potential, including clinically diagnosed orchitis and reduced spermatozoa motility and morphology (< 50 %), presented a DFI of 36.0 ± 20.8 % (O'Brien, Robeck, Montano, unpublished data). Clearly, if non-voluntary sperm collection methods become available, sperm function tests hold promise for integrating into longitudinal population health assessments of free-ranging cetaceans [169], to allow detection of changes in fertility. Long-term databases are critical for understanding the impact of intrinsic factors (e.g. animal age) as well as extrinsic, anthropogenic-related factors such environmental pollutant loads, which accumulate in the blubber of many marine species and may impact spermatozoa DNA integrity and ensuing normalcy of pregnancy after conception.

5.3. Refrigerated storage

5.3.1. Extender type and preparation

In vitro spermatozoa parameters (motility, membrane and acrosome integrity) of cetaceans are well-maintained during refrigerated storage at 4 to 6 °C when held in species-specific egg yolk based extenders (Table 5). As with other marine species (see sections 2.3.1., 3.2. and 4.2.), extender composition (and ensuing pH and osmolality) significantly affect the success of sperm refrigerated storage in marine mammals. Extenders are typically prepared by ultracentrifugation (10 000 *g* for 1 h at 10 °C) and filtering of the supernatant (0.22 μm) to facilitate CASA analysis and/or sex-sorting [166].

5.3.2. Storage temperature and duration

Though controlled studies concerning the impact of prolonged refrigerated storage on fertility are lacking for cetaceans, *in vivo* fertility has been maintained for 1 to 4 days post-storage (Table 5). Bottlenose dolphin ejaculates stored at 5-12 °C over 32 to 36 h prior to cryopreservation, to allow for overnight sperm transport and sex-sorting, resulted in a 57 % conception rate after artificial insemination [170].

5.4. Artificial insemination

Intrauterine inseminations have been conducted using a non-surgical, endoscopic method comprising specialized flexible endoscope preparation (to allow complete removal of spermicidal agents) and custom-made catheters that facilitate uterine sperm deposition in cetaceans [7] and the polar bear (O'Brien and Robeck, unpublished). The minimum effective intrauterine dose has been examined most extensively using sex-sorted then frozen-thawed sperm in the bottlenose dolphin [170]. Currently, an insemination dose of 200 million progressively motile spermatozoa is recommended to achieve conception rates exceeding 50 % for this species. Frozen-thawed samples are normally inseminated into the uterus within 30 min of thawing (and maintained at 15 to 18 °C during that time). Inseminations are timed to occur within 12 h prior to ovulation.

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Figure 1 – Schematic representation of the most common sperm handling procedures for artificial reproduction in fish, penaeid shrimp, bivalve mollusks and marine mammals.

Figure 2 – Different techniques for sperm collection in fish by: (a) small volume stripping with the help of a syringe in Senegalese sole *Solea senegalensis*, (b) large volume stripping directly into a falcon tube in tambaqui *Colossoma macropomum*, (c) with a cannula in rainbow trout *Oncorhynchus mykiss*, (d) by surgical removal of the testes in wels catfish *Silurus glanis* (photo by Ákos Horváth), (e) collection with aspiration directly into an immobilizing solution in European eel *Anguilla anguilla* and, (f) with a Pasteur pipette connected to the male papilla in spotted wolffish *Anarhichas minor*.

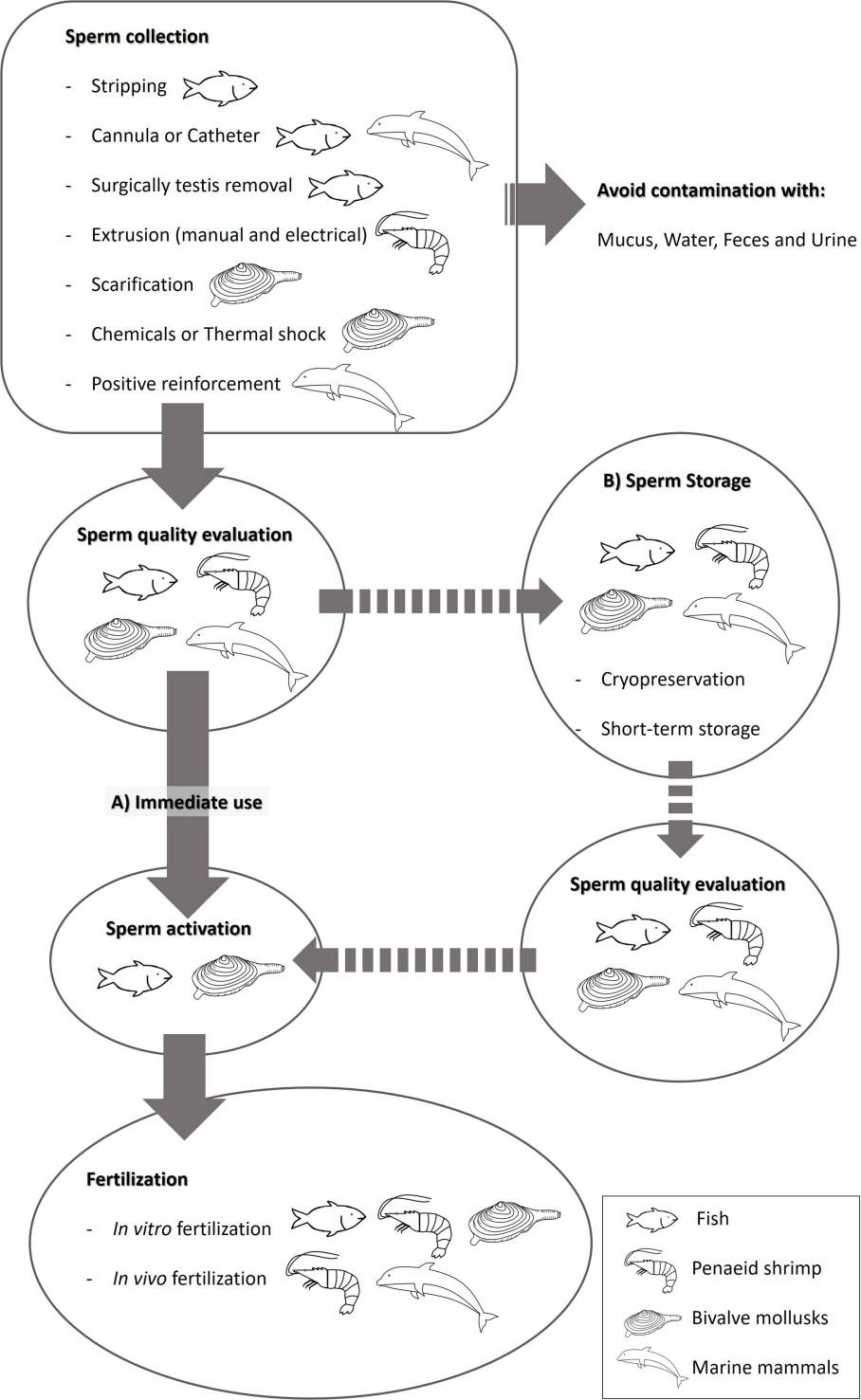
Figure 3 - Artificial reproduction in closed thelycum penaeid. (a) Manual extrusion of the spermatophores by gently pressing around the coxas of the fifth pair of pereiopods. (b) A pair of fresh spermatophores, extruded immediately before artificial insemination. (c) Artificial insemination is performed by placing the spermatophore in the thelycum of recently molted females, located between the base of the fifth pair of pereopods. (d) The spermatophore is then artificially implanted into the soft thelycum with the aid of a spatula.

Figure 4 – (a) Morphology of the Pacific oyster, *Crassostrea gigas*, after upper shell removal. The gonad is the white part of the animal (see arrow). (b) Sperm is collected by scarification of the gonad.

Figure 5 – Sperm collection from a bottlenose dolphin. Note the stimulation of the perineal area (a) and the collection of semen by manipulation and direction of the penis into the collection bag (b) [9].

Highlights

- *In vitro* and *in vivo* fertilization involve sperm collection and handling until gamete co-incubation
- Practical guidelines for sperm handling in fish, penaeid shrimp, bivalve mollusks and marine mammals are presented
- Sperm refrigeration techniques are revised for the different taxa
- Protocols for gamete co-incubation are discussed across the different taxa


Sperm collection
- Stripping
- Cannula or Catheter
- Surgically testis removal
- Extrusion (manual and electrical)
- Scarification
- Chemicals or Thermal shock
- Positive reinforcement
Avoid contamination with:
Mucus, Water, Feces and Urine
Sperm quality evaluation
B) Sperm Storage
- Cryopreservation
- Short-term storage
A) Immediate use
Sperm activation
Sperm quality evaluation
Fertilization
- In vitro fertilization
- In vivo fertilization
Fish
Penaeid shrimp
Bivalve mollusks
Marine mammals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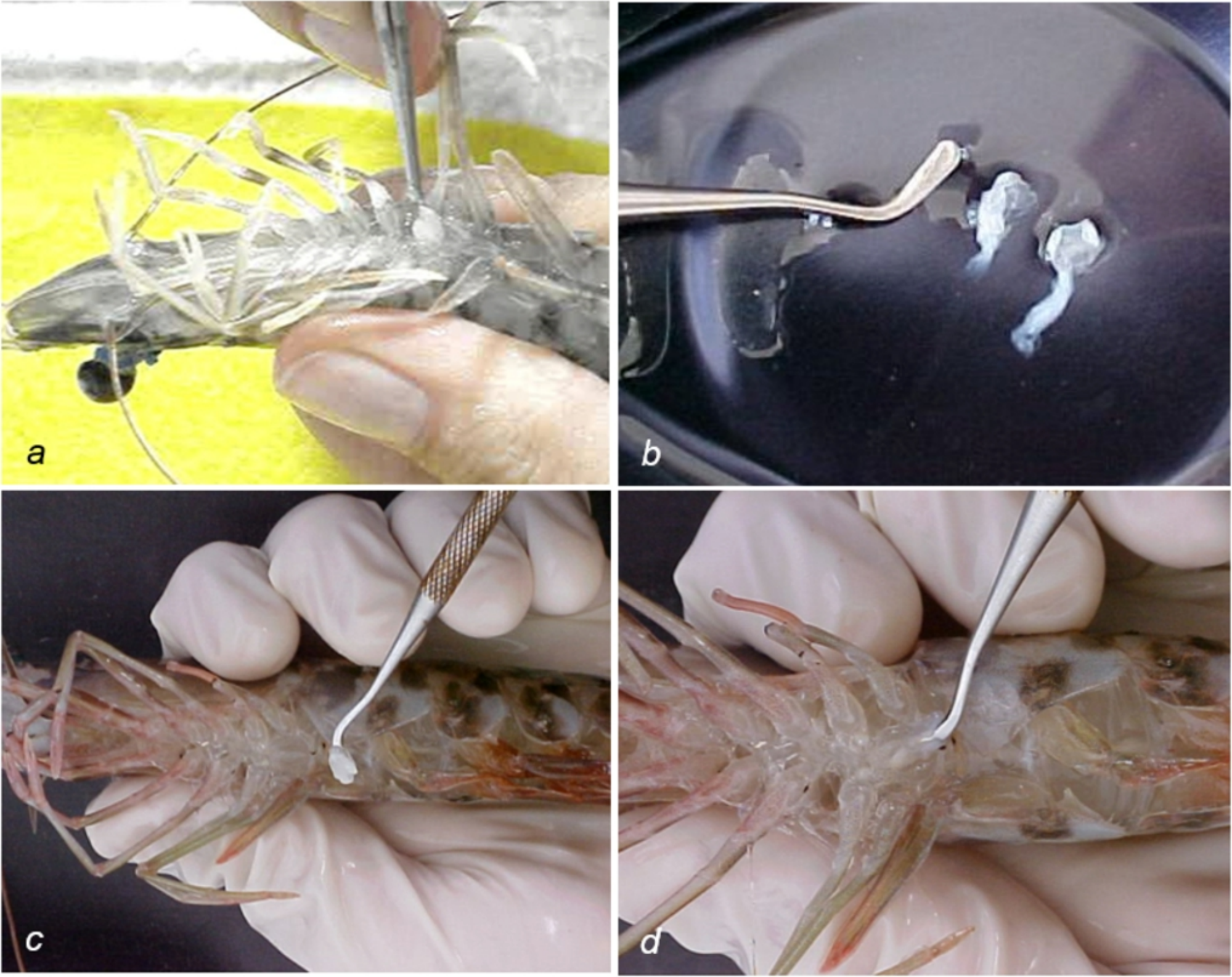
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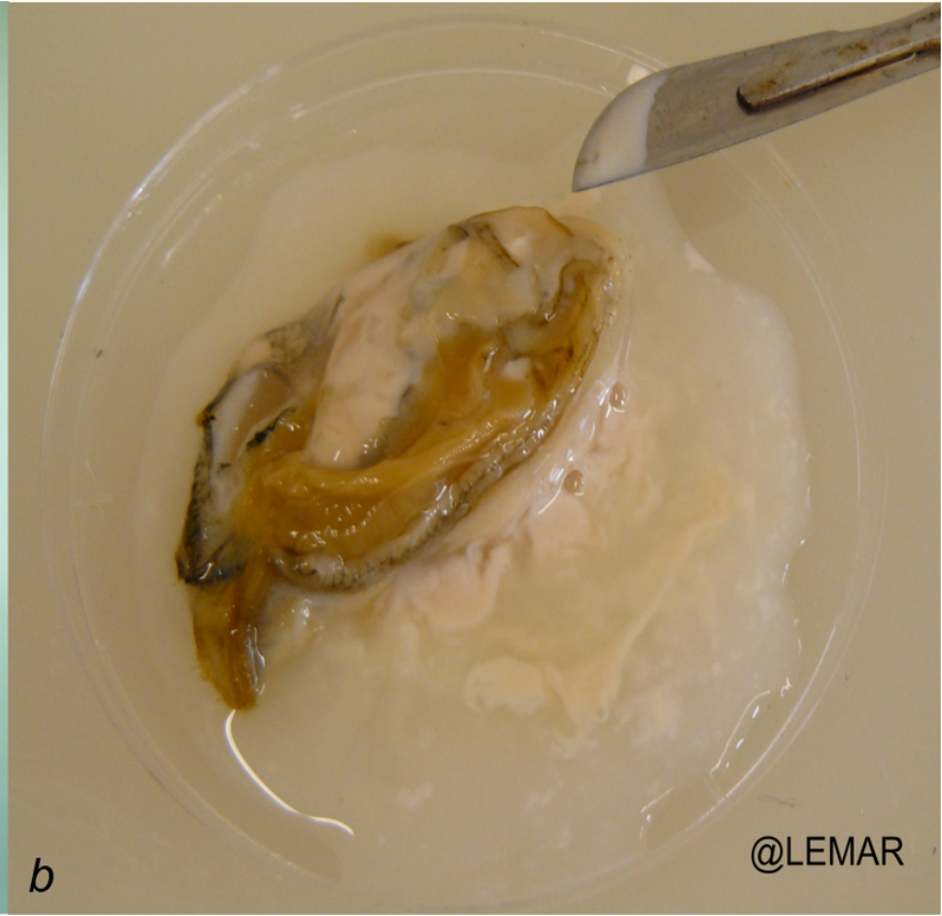

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a
b

Table 1. Some examples of sperm refrigeration protocols for different fish species.

Species	Extender	pH	Osmolality mOsm/kg	Dilution semen:medium	Temp °C	Atmosphere	Days of storage[1]	Motility	Fertilization	Reference
Acipenseriformes										
Acipenser oxyrinchus	Based on sturgeon seminal plasma[2]	7.5	100	1:3	4	oxygen	21	above 50%		[57]
Acipenser brevirostrum	Based on sturgeon seminal plasma[2]	7.3-7.5	100	1:3	4	air	28	40-65%	30-65% at 21 days	[59]
Polyodon spathula	NaCl	7.6	310	1:1	1	air	25	50 %	61% at 25 days	[61]
Anguiliformes										
Anguilla anguilla	Specific extender[3]	8.5	330	1:50	4	air-limited conditions	7	77,90 %		[63]
Anguilla japonica	Isotonic solution with 25mM K	8.2	338	1:50	3		28	35 %		[171]
Characiformes										
Prochilodus lineatus	Androstar®	7.6	311	1:10	4 to 6	air	4	53 %	26 to 61%	[66]
Cichliformes										
Oreochromis niloticus	Ringer + antibiotics	8.0	318		4		7	close to 20%		[74]
Oreochromis mossambicus	Egg yolk-citrate diluent + glucose + glycine + sucrose + antibiotics	7.4	416	1:1	4 to 5		17	10 %	59 %	[172]
Cypriniformes										
Carassius auratus	No extender			_	4	air	2			[83]

Danio rerio	HBSS[4]		300		4	air	1	41.4% at 24h		[62]
Labeo chrysophekadion	Specific extender[5]	8.11	252	1:3	4	air	3	61.2 at 3 days	40 % at 3 days	[56]
Cyprinus carpio	Saline solution[6] + antibiotics	8		1:10	4	oxygen	16		close to 20%	[173]
Cyprinodontifromes										
Poecilia reticulata	HBSS[4] without Ca	5.6-7.8	300	1:50 to 1:500	4			close to 20% at 48h		[24]
Gadiformes										
Gadus morhua	Modified Mounib extender[7]		324	1:3	3		10 to 40	42% at 10 days, 3% at 40 days		[174]
Melanogrammus aeglefinus	Modified Mounib extender[7]		324	1:3	3		10 to 38	52% at 10 days, 3% at 38 days		[174]
Perciformes										
Dicentrarchus labrax	Leibovitz culture medium + gentamycine	7.3-8.1	213	1:3	4	air	2	close to 50%	75-90%	[14]
Argyrosomus regius	0.9% NaCl	7.7	300	1:4	4	air	4 or 10	40% at 4 days, 10% at 10 days		[55]
Micropogonias undulatus	HBSS[4]		200	1:3	4					[88]

Sciaenops ocellatus	HBSS[4]		200		1	air	3	close to 30%		[64]
Perca fluviatilis	Kobayashi buffer[8] + 5mM glutathione + antibiotics	9.5		1:9	4	air	17	57 %		[70]
Morone saxatilis	Specific extender[9]	7.6	350	1:3	4	oxygen	2	38 %		[175]
Pleuronectiformes										
Hippoglossus hippoglossus	HBSS[4] + antibiotics	7.2	281	1:5	0 to 1	air	70	close to 20%	54 %	[16]
Scophthalmus maximus	Ringer	8.1	204	1:9	0	air	45h			[79]
Salmoniformes										
Oncorhychus mykiss	Storfish®			1:2	4	air	5 (optimal), 14 (drop in quality)	over 70%, close to 20% at day 14		[29]
Salmo salar	Stopmilt®			1:2	4	air	5	motility over 50%		[176]
Siluriformes										
Clarias macrocephalus	Calcium free HBSS[4] + antibiotics	7.6	301	1:4	4	air	4 or 6	56% at 4 days, 44% at 6 days	52% at 4 days, 19% at 6 days	[50]
Clarias gariepinus	Specific extender[10]	7.5		1:10	4		6			[71]
Tetraodontiformes										

Takifugu niphobles	Saline solution[11]	7.5		1:50	4	air	7	close to 80%	[58]

[1] Maximum recommended days of storage in the described protocol
[2] 1g/L NaCl, 0.2g/L KCl, 0.5g/L $NaHCO_3$, 0.05/L $CaCl_2$, 0.05g/L $MgSO_4$, 0.15g/L NaH_2PO_4, 0.15g/L Na_2HPO_4, 17.2g/L Sucrose or 9.0g/L Glucose
[3] 80mM $NaHCO_3$, 2.5mM $MgCl_2$, 1mM $CaCl_2$, 30mM KCl, 300mM Glucose, 2%BSA
[4] HBSS – Hanks' Balanced Salt Solution
[5] 111.43mM NaCl, 2.79mM KCl, 4.86mM $NaHCO_3$, 1.39mM $CaCl_2.2H_2O$, 0.65mM $MgSO_4.7H_2O$, 17.78mM Glucose, 3.22mM Tris-HCl
[6] 125mM NaCl, 0.1mM $CaCl_2$, 20mM Tris
[7] 0.1g/L $KHCO_3$, 0.02g/L Glutathione, 0.427g/L Sucrose
[8] 7.6g/L NaCl, 2.98g/L KCl, 0.37g/L $CaCl_2.2H_2O$, 0.31g/L $MgCl_2.6H_2O$, 0.21g/L $NaHCO_3$
[9] 240mM NaCl, 5.4mM KCl, 23.8mM $NaHCO_3$, 5.5mM Glucose, 75mM Glycine
[10] 94mM NaCl, 27mM KCl, 50mM Lactate, 5mM Pyruvate, 15mM Tris–HCl
[11] 130mM NaCl, 5mM KCl, 10mM HEPES, 1mM $CaCl_2$

Note: For several species more than one protocol is available in the literature and we decided to cite the protocol that reports longer storage time, if enough details from this protocol were available.

Table 2. The optimal number of spermatozoa needed to fertilize an ovum in fish in several teleost species. Spermatozoa:oocyte ratio reflects the number of spermatozoa after which adding more spermatozoa to the oocytes will not significantly increase the fertilization rates. FR: Fertilization rate.

Species	Spermatozoa:oocyte ratio	Motility (%)	FR (%)	Reference
Cypriniformes				
Barbus barbus	1300000*	>77	90	[177]
Chalcalburnus chalcalburnus	700000*	>77	80	[177]
Cyprinus carpio	8500-25000	-	>70	[178]
Chondrostoma nasus	600000*	>77	65	[177]
Rutilus meidingerii	1300000*	>77	□75	[177]
Salmoniformes				
Salmo trutta	43000	-	>80	[99]
Oncorhynchus mykiss	75000	-	>80	[91]
Oncorhynchus mykiss	300000	-	>80	[179]
Siluriformes				
Clarias gariepinus	15000	-	80	[94]
Clarias macrocephalus	4000-8000	-	>60	[180]
Silurus glanis	800	-	80-90	[96]
Marine fish				
Gadus morhua	100000	□80	>60	[93]
Gadus morhua	300000	□90	>70	[102]
Micropogonias undulatus	1000	-	□60	[97]
Hippoglossus hippoglossus	10000	-	□60	[105]
Hippoglossus hippoglossus	940000	-	90-100	[103]
Anarhichas lupus	200000	-	>90	[181]
Anarhichas minor	500000	>60	□90	[18]
Anguilla anguilla	240000	>75	□60	[104]
Dicentrarchus labrax	66000	>95	□30	[182]
Clupea harengus	64000	-	>75	[98]
Takifugu niphobles	1000	80	85	[93]
Scophthalmus maximus	3000-4000	20-100	□85	[84]
Scophthalmus maximus	6000	-	□90	[95]
Pseudopleuronectes americanus	34000	80-100	□80	[183]
Others				
Esox Lucius	26000	-	>80	[99]

Piaractus mesopotamicus	7000	50-55	□70	[184]
Petromyzon marinus	50000	70-90	>80	[100]
Acipenser ruthenus	43000	60-65	□70	[185]
Sander vitreus	2500	75-85	□80	[168]
Sander vitreus	25000	>90	>70	[187]

*Spermatozoa:oocyte ratios lower than indicated were not checked in that study, thus probably a smaller amount of spermatozoa per oocyte could be enough for achieving notably fertilization rates in these species

Table 3. Summary of sperm quality evaluation techniques in penaeids.

Species	Parameter	Technique	References
Farfantepenaeus brasiliensis	Abnormal spermatozoa / Membrane integrity	Light microscopy	[112]
Farfantepenaeus paulensis	Spermatozoa count	Light microscopy	[110]
Fenneropenaeus indicus	Membrane integrity	Fluorescence microscopy	[113]
Litopenaeus schmitti	Membrane integrity	Light microscopy	[114]
Litopenaeus setiferus	Membrane integrity	Light microscopy	[111]
Litopenaeus vannamei	Abnormal spermatozoa / Membrane integrity	Light microscopy / Flow cytometry	[121,123]
	Membrane integrity	Fluorescence microscopy / Flow cytometry	[10,11,116,122]
	Membrane integrity / Fertilization and hatching	Fluorescence microscopy / Artificial insemination	[115,124]
Penaeus monodon	Abnormal sperm / Membrane integrity / Fertilization and hatching	Light microscopy / Artificial insemination	[129,188]
	Spermatozoa count / Abnormal spermatozoa / Acrosome reaction	Light microscopy/ Artificial insemination	[120]
	Spermatozoa DNA quality	Spermatozoa chromatin dispersion	[125]

Table 4. Typical sperm characteristics of osmolality and creatinine concentration for manually collected non-contaminated and contaminated cetacean semen.

Species	Parameter Osmolality (mOsm/kg) Creatinine (mg Cr/ml)	Non-contaminated (negligible contamination of urine or saltwater[a])	Urine-contaminated	Saltwater-contaminated
ODONTOCETES				
Bottlenose dolphin	Osmolality	320 - 345	> 345	> 345
Tursiops truncatus	Creatinine	≤ 0.015	≥ 0.015	≤ 0.015
Pacific white-sided				
Lagenorhynchus obliquidens				
Killer whale	Osmolality	335 - 375	> 375	> 375
Orcinus orca	Creatinine	≤ 0.015	≥ 0.015	≤ 0.015
MONODONTOCETES				
Beluga	Osmolality	335 – 365	> 365	> 365
Delphinapterus leucas	Creatinine	≤ 0.015	≥ 0.015	≤ 0.015

[a] Negligible contamination: sperm samples displaying these osmolality and creatinine parameters are suitable for chilled storage and cryopreservation. Note that osmolality of cetacean urine and saltwater from zoo-based habitats typically exceeds 1000 mOsm/kg.

Table 5. Male marine mammal genome (sperm) storage methods (modified from [7]).

Species	Optimum base extender(s) (components)	Duration of chilled storage (4-6°C) prior to conceptive AI (days)
ODONTOCETES	Test-yolk buffer (TYB; TES, Tris, fructose, egg yolk)	2
Bottlenose dolphin [9] *Tursiops truncatus*		
Pacific white-sided dolphin [161] *Lagenorhynchus obliquidens*	Platz Diluent Variant (PDV; lactose, egg yolk) or TYB	Estimated 1-2 days
Killer whale [189] *Orcinus orca*	Beltsville extender (BF5F; TES, Tris, glucose, fructose, egg yolk)	4
MONODONTOCETES	BF5F+ hyaluronic acid (HA)	1
Beluga [162] *Delphinapterus leucas*		