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# Sperm handling in aquatic animals for artificial reproduction

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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 the 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 sperm 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 sperm motility and methods for gamete co-incubation. The present study provides valuable information regarding breeder management either for animal production or species conservation.



## 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

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steps are illustrated in Fig. 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 the range of aquatic animals that exist in managed environments, either for animal production or species conservation.

## 2. Sperm handling in fish

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

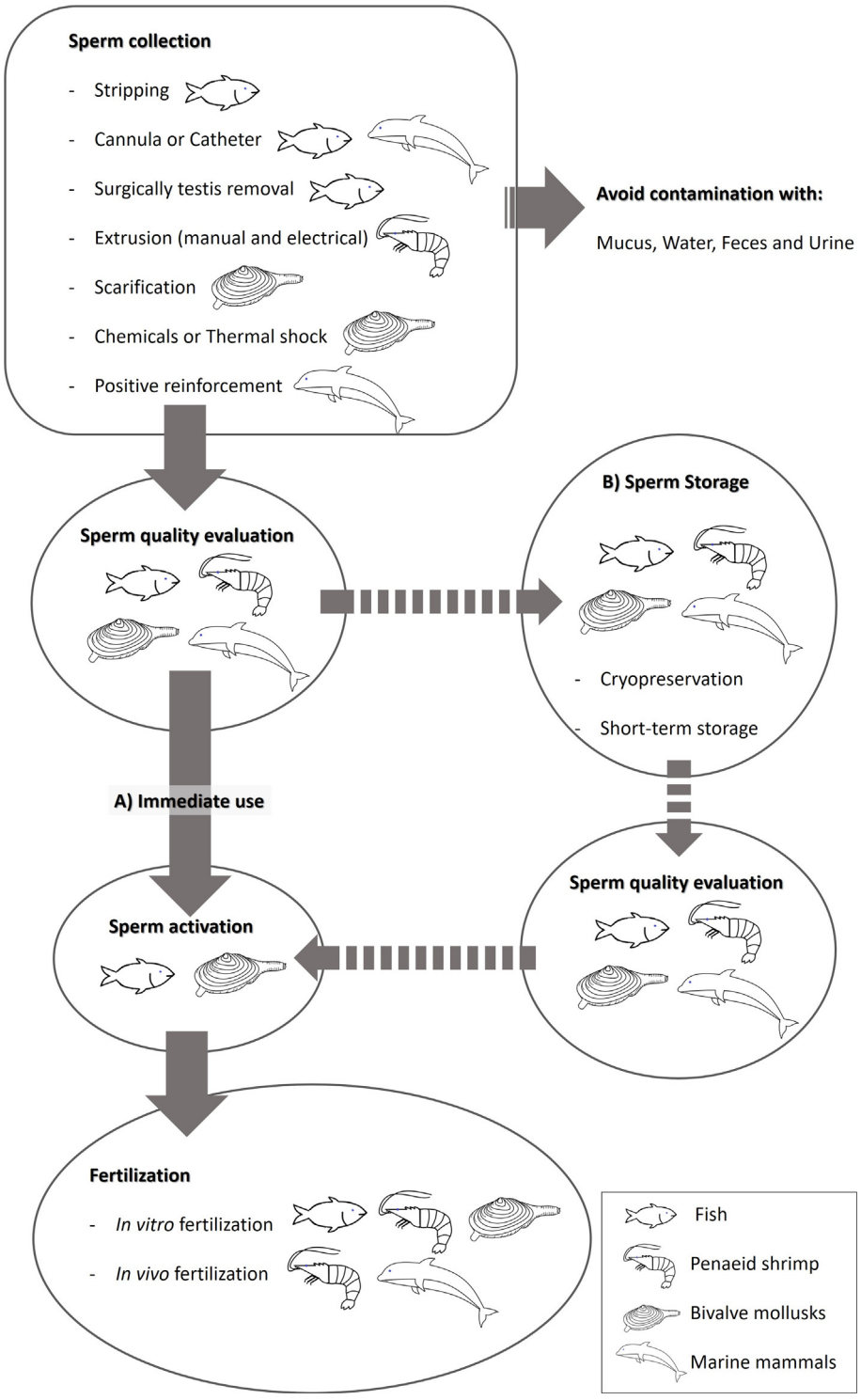


**Fig. 1.** Schematic representation of the most common sperm handling procedures for artificial reproduction in fish, penaeid shrimp, bivalve mollusks and marine mammals.

them were included in this revision.

### 2.1. Sperm collection

#### 2.1.1. Methods for sperm collection

Different methods for fish sperm collection could be used according to testis morphology and the intend use. The most frequently applied technique is through abdominal massage, or stripping, that helps the sperm to be released from the gonad into the spermatic duct. Using this method and a gentle pressure, only mature cells are released together with the seminal plasma. This technique is commonly used in species with filiform testis where the semen (spermatozoa with the seminal plasma) can be collected with a syringe placed directly outside the urogenital pore, such as in gilthead seabream *Sparus aurata*, or directly into a collection tube as in tambaqui *Colossoma macropomum* [15] (Fig. 2b). Nonetheless, some authors reported better sperm quality using a catheter or cannula, because it reduces the risk of sperm contamination by urine, feces and water [16,17]. The cannula is introduced through the urogenital pore and connected to a tube or syringe where the sperm is collected directly (Fig. 2c). In these cases, sperm is collected from the spermatic duct or directly from the testis. When the structure and aperture of the urogenital pore does not allow for this type of collection or there is a risk of fish injury by clogging of the spermatic duct due to internal damage, other modifications to the method can be applied. In the spotted wolffish *Anarhichas minor*, a Pasteur pipette connected to the male papilla was used to collect sperm after abdominal massage [18] (Fig. 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] (Fig. 2a).


**Fig. 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*.

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

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

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

### 2.2. Sperm motility activation

Spermatozoa are maintained in most fish species in a quiescent stage in the testis, and a maturation process occurs when passing through the spermatic ducts in order to acquire motility when in contact with an activation media. Until this process is triggered, it is important to avoid motility activation due to the low duration of motility in most fish species. There are several solutions that maintain sperm quiescence, which consist in the dilution of sperm in solutions that 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. Sperm 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*) sperm 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 sperm 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 °C [46]. In this work we will focus exclusively on refrigerated storage, as sperm cryopreservation is the focus of other reviews in this same issue [48,49]. Sperm refrigeration protocols have been established for several marine and freshwater fish species (Table 1).

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

#### 2.3.1. Extender solution

The first step for the implementation of a refrigeration protocol is usually the selection of an extender solution. To improve sperm storability the extenders will reduce the sperm concentration and thus facilitate the oxygen supply, should not activate sperm motility, should be isotonic, have a good buffering capacity, should include nutrients, antioxidants to control the activity of reactive oxygen species, should include antibacterial substances and stabilizing colloids [47,54]. Although extenders are not essential, several studies have showed that undiluted sperm storability is much lower [14,50,55–59]. As an example, in European seabass sperm storability increase by 2 days for diluted sperm [14] and in meagre *Argyrosomus regius* up to 10 days [55]. Whereas, in some cases simple solutions of NaCl are used as an extender [55,60,61], in other cases more or less complex saline solutions such as Hanks balanced salt solution are preferred [14,16,62], or extenders are develop to resemble the species seminal plasma [59,63]. This approach 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 sperm 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 sperm velocity [29,71]. Therefore, the addition of energy substrate to the extender, such as glucose or

**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[a] | Motility | Fertilization | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| **Acipenseriformes** | | | | | | | | | | |
| *Acipenser oxyrinchus* | Based on sturgeon seminal plasma[b] | 7.5 | 100 | 1:3 | 4 | oxygen | 21 | above 50% | | [57] |
| *Acipenser brevirostrum* | Based on sturgeon seminal plasma[b] | 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* | Specific extender[c] | 8.5 | 330 | 1:50 | 4 | air-limited conditions | 7 | 77,90% | | [63] |
| *Anguilla japonica* | Isotonic solution with 25 mM K | 8.2 | 338 | 1:50 | 3 | | 28 | 35% | | [170] |
| **Characiformes** | | | | | | | | | | |
| *Prochilodus lineatus* | Androstar® | 7.6 | 311 | 1:10 | 4 to 6 | air | 4 | 53% | 26–61% | [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% | [171] |
| **Cypriniformes** | | | | | | | | | | |
| *Carassius auratus* | No extender | | | – | 4 | air | 2 | | | [83] |
| *Danio rerio* | HBSS[d] | | 300 | | 4 | air | 1 | 41.4% at 24 h | | [62] |
| *Labeo chrysophekadion* | Specific extender[e] | 8.11 | 252 | 1:3 | 4 | air | 3 | 61.2 at 3 days | 40% at 3 days | [56] |
| *Cyprinus carpio* | Saline solution[f] + antibiotics | 8 | | 1:10 | 4 | oxygen | 16 | | close to 20% | [172] |
| **Cyprinodontifromes** | | | | | | | | | | |
| *Poecilia reticulata* | HBSS[d] without Ca | 5.6–7.8 | 300 | 1:50 to 1:500 | 4 | | | close to 20% at 48 h | | [24] |
| **Gadiformes** | | | | | | | | | | |
| *Gadus morhua* | Modified Mounib extender[g] | | 324 | 1:3 | 3 | | 10 to 40 | 42% at 10 days, 3% at 40 days | | [173] |
| *Melanogrammus aeglefinus* | Modified Mounib extender[7] | | 324 | 1:3 | 3 | | 10 to 38 | 52% at 10 days, 3% at 38 days | | [173] |
| **Perciformes** | | | | | | | | | | |
| *Dicentrarchus labrax* | Leibovitz culture medium + gentamicine | 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[d] | | 200 | 1:3 | 4 | | | | | [88] |
| *Sciaenops ocellatus* | HBSS[d] | | 200 | | 1 | air | 3 | close to 30% | | [64] |
| *Perca fluviatilis* | Kobayashi buffer[h] + 5 mM glutathione + antibiotics | 9.5 | | 1:9 | 4 | air | 17 | 57% | | [70] |
| *Morone saxatilis* | Specific extender[i] | 7.6 | 350 | 1:3 | 4 | oxygen | 2 | 38% | | [174] |
| **Pleuronectiformes** | | | | | | | | | | |
| *Hippoglossus hippoglossus* | HBSS[d] + 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 | 45 h | | | [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% | | [175] |
| **Siluriformes** | | | | | | | | | | |
| *Clarias macrocephalus* | Calcium free HBSS[d] + 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[j] | 7.5 | | 1:10 | 4 | | 6 | | | [71] |
| **Tetraodontiformes** | | | | | | | | | | |
| *Takifugu niphobles* | Saline solution[k] | 7.5 | | 1:50 | 4 | air | 7 | close to 80% | | [58] |

**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.

[a] Maximum recommended days of storage in the described protocol.

[b] 1 g/L NaCl, 0.2 g/L KCl, 0.5 g/L $NaHCO_3$, 0.05/L $CaCl_2$, 0.05 g/L $MgSO_4$, 0.15 g/L $NaH_2PO_4$, 0.15 g/L $Na_2HPO_4$, 17.2 g/L Sucrose or 9.0 g/L Glucose.

[c] 80 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 30 mM KCl, 300 mM Glucose, 2%BSA.

[d] HBSS – Hanks' Balanced Salt Solution.

[e] 111.43 mM NaCl, 2.79 mM KCl, 4.86 mM $NaHCO_3$, 1.39 mM $CaCl_2.2H_2O$, 0.65 mM $MgSO_4.7H_2O$, 17.78 mM Glucose, 3.22 mM Tris-HCl.

[f] 125 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris.

[g] 0.1 g/L $KHCO_3$, 0.02 g/L Glutathione, 0.427 g/L Sucrose.

[h] 7.6 g/L NaCl, 2.98 g/L KCl, 0.37 g/L $CaCl_2.2H_2O$, 0.31 g/L $MgCl_2.6H_2O$, 0.21 g/L $NaHCO_3$.

[i] 240 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, 5.5 mM Glucose, 75 mM Glycine.

[j] 94 mM NaCl, 27 mM KCl, 50 mM Lactate, 5 mM Pyruvate, 15 mM Tris–HCl.

[k] 130 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$.

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 sperm 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 sperm viability and low dilution rates, because of the high sperm 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 sperm metabolism. Lower temperatures, close to but not 0 °C, are usually recommended as they decrease the cells metabolic rate and reduce energy consumption to a minimum without affecting cellular structure [16,54]. Nonetheless, most authors use 4 °C, 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. Sperm 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 common 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 sperm concentration

To make an optimal use of the available sperm, sperm concentration reported as the number of cells per mL (also known as sperm density) is often adjusted by diluting the sperm samples at a known concentration in an extender solution. Adjustment of sperm 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]. Sperm concentration adjustment prior to cryopreservation removes technical variability and allows for meaningful comparisons and procedure standardization [80,82].

Several methods to determine sperm 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 sperm 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 sperm 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 sperm 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 sperm concentration is evaluated simultaneously with sperm motility parameters [19,84,86]. The decision regarding the used method to measure sperm 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. For the last method, controversial results have been reported 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, sperm 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 $1.5 \times 10^4$ spermatozoa per oocyte [94], while for cryopreserved sperm the optimum ratios ranged from $4.9 \times 10^4$ to $1.7 \times 10^6$ [106]. In common carp, the sperm 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 oocytes and spermatozoa (too much space to look for the oocyte), whereas the lack of activation media will affect the sperm activation process or cause an erratic displacement to reach the micropyle. Thus, some protocols include a step for sperm 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 (oocytes 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 (energy depleted) immotile spermatozoa that will not be able to reach the oocyte or motile spermatozoa will reach the blocked (non-receptive) 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 $10^5$ the authors recommended gamete contact times of around 5 min; while for lower spermatozoa:oocyte ratios ($10^3$ or $10^4$), the contact times suggested for achieving high fertilization rates were about 30 min. In turbot, for $6 \times 10^3$ spermatozoa per oocyte, maximum fertilization success was usually recorded after 1 min of contact between gametes [95]. However, for lower spermatozoa:oocyte ratios ($1.5 \times 10^3$), 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 $5 \times 10^5$ 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 sperm 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 combination of the different factors will make possible to obtain notably fertilization rates, using the

**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[a] | >77 | 90 | [176] |
| *Chalcalburnus chalcalburnus* | 700000[a] | >77 | 80 | [176] |
| *Cyprinus carpio* | 8500–25000 | – | >70 | [177] |
| *Chondrostoma nasus* | 600000[a] | >77 | 65 | [176] |
| *Rutilus meidingerii* | 1300000[a] | >77 | 75 | [176] |
| **Salmoniformes** | | | | |
| *Salmo trutta* | 43000 | – | >80 | [99] |
| *Oncorhynchus mykiss* | 75000 | – | >80 | [91] |
| *Oncorhynchus mykiss* | 300000 | – | >80 | [178] |
| **Siluriformes** | | | | |
| *Clarias gariepinus* | 15000 | – | 80 | [94] |
| *Clarias macrocephalus* | 4000–8000 | – | >60 | [179] |
| *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 | [180] |
| *Anarhichas minor* | 500000 | >60 | 90 | [18] |
| *Anguilla* | 240000 | >75 | 60 | [104] |
| *Dicentrarchus labrax* | 66000 | >95 | 30 | [181] |
| *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 | [182] |
| **Others** | | | | |
| *Esox Lucius* | 26000 | – | >80 | [99] |
| *Piaractus mesopotamicus* | 7000 | 50–55 | 70 | [183] |
| *Petromyzon marinus* | 50000 | 70–90 | >80 | [100] |
| *Acipenser ruthenus* | 43000 | 60–65 | 70 | [184] |
| *Sander vitreus* | 2500 | 75–85 | 80 | [185] |
| *Sander vitreus* | 25000 | >90 | >70 | [186] |

[a] 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.

gametes in an optimized way and enhancing the reproductive efficiency in fish farms.

## 3. Sperm handling in penaeid shrimp

### 3.1. Spermatophore extrusion

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

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

The term “spermatophore extrusion” is specifically used in penaeid shrimps for sperm collection. Regardless the spermatophore structure, two extrusion methods have been used to assess sperm quality for reproductive performance trials, the development of refrigeration and cryopreservation protocols and artificial insemination procedures. Manual extrusion of the spermatophore is performed by gently pressing around the coxas of the fifth pair of pereiopods [6,110] (Fig. 3a and b). Extrusion can also be performed by electrical stimulation (4.5–9 V) applied to the same area [110]. Both extrusion methods can cause damage to the reproductive tract 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

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

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

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

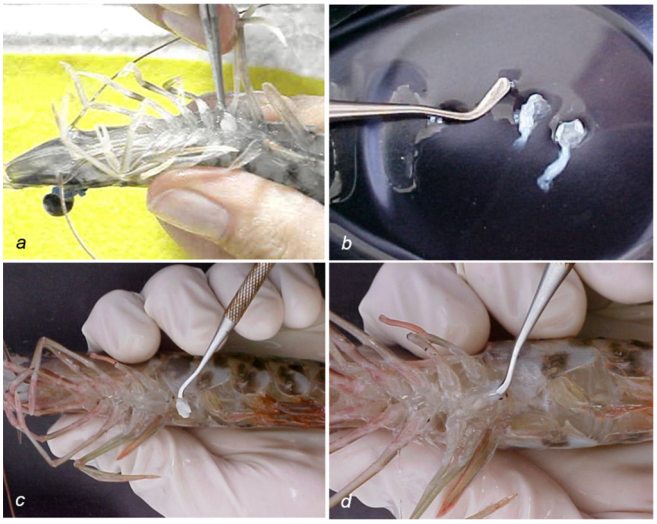


**Fig. 3.** Artificial reproduction in closed thelycum penaeid. (a) Manual extrusion of the spermatophores by gently pressing around the coxas of the fifth pair of pereiopods. (b) A pair of fresh spermatophores, extruded immediately before artificial insemination. (c) Artificial insemination is performed 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.

### 3.3. Sperm quality

Decapod crustacean spermatozoa are unique and different from the spermatozoa of other animals. In most penaeid species, these non-motile aflagellate spermatozoa show a spherical main body containing an uncondensed nucleus and acrosomal vesicles that included the acrosomal cap and spike [119].

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

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

Flow cytometry has been used to determine sperm viability of pacific white shrimp based on cytoplasmic membrane integrity [116,123]. Although cytometry is reported to be more sensitive and less prone to observer error, a positive correlation between morphotype analysis performed by light microscopy and sperm viability using flow cytometry has been found [123]. However, cells undergoing a change from an intact to disrupted cytoplasmic membrane (transitional spermatozoa populations) of pacific white shrimp, may not show evident morphological changes for detection by microscopy, and this type of damage would only be 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 cross-breeding 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] (Fig. 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 sperm 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 Ref. [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 (Fig. 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 $15 \times 10^9$ 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 2–10 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 10–30 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 cytometer [12,140], or by directly counting spermatozoa in a known small volume using a Neubauer or Malassez hemocytometer [131,141]. For ripe oysters, concentration of spermatozoa is estimated to be $6 \times 10^7$ and 5 to $15 \times 10^9$ spermatozoa $mL^{-1}$ for ejaculated and gonadal sperm, respectively [134].

### 4.2. Refrigerated storage

Compared with fish, where spermatozoa usually only swim for a few minutes, bivalve sperm motility lasts for hours, up to 24 h in the Pacific oyster [5,12]. Collected sperm can be stored for some hours

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

| Species | Parameter | Technique | Reference |
|---|---|---|---|
| *Farfantepenaeus brasiliensis* | Sperm morphology/Membrane integrity | Light microscopy | [112] |
| *Farfantepenaeus paulensis* | Sperm 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* | Sperm morphology/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* | Sperm morphology/Membrane integrity/Fertilization and hatching | Light microscopy/Artificial insemination | [129,187] |
| | Sperm count/Sperm morphology/Acrosome reaction | Light microscopy/Artificial insemination | [120] |
| | Sperm DNA quality | Sperm chromatin dispersion | [125] |

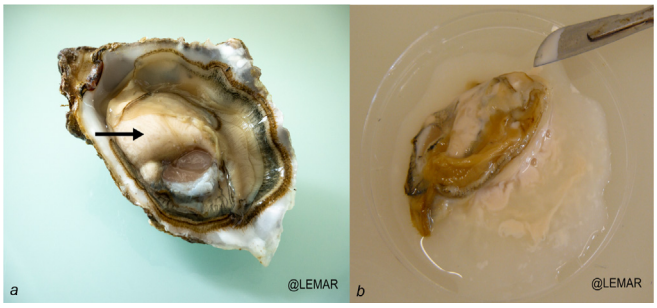


**Fig. 4.** (a) Morphology of the Pacific oyster, *Crassostrea gigas*, after upper shell removal. The gonad is the white part of the animal (see arrow). (b) Sperm is collected by scarification of the gonad.

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

### 4.3. Artificial fertilization

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

The *in vitro* fertilization success varies depending on the spermatozoa:oocyte ratio. High fertilization success is reported using sperm collected by scarification at 100 spermatozoa per oocyte in the scallop *Mizuhopecten yessoensis* (fertilization rate > 70%) [155] and the black-lip pearl oyster (80% fertilization rate) [156]. It is recommended to increase the spermatozoa:oocyte ratio when using cryopreserved compared to fresh sperm. In the black-lip pearl oyster, a spermatozoa:oocyte ratio of 100000:1 is required to reach 80% fertilization rates when using cryopreserved sperm compared to 100:1 for fresh sperm [156]. In oysters, optimal spermatozoa:oocyte ratio is between 400:1 and 5000:1 for high fertilization rate (>70%) in a volume between 10 and 200 mL of seawater and a contact time between 10 and 30 min [130,138], and below 200 spermatozoa per oocyte for the blood clam *Tegillarca granosa* [157]. Using higher spermatozoa:oocyte ratio may decrease the larval yield probably due to polyspermy [157–159].

## 5. Sperm handling in marine mammals

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

In this section, sperm handling and assessment research will be presented for the taxon where the most comprehensive information is available, namely cetaceans, including Delphinidae, (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, including the Pacific white-sided dolphin *Lagenorhynchus obliquidens*, have more discrete mating periods during which sperm can be collected [160]. Beluga (Monodontidae, *Delphinapterus leucas*) and polar bear (Ursidae) also display seasonal mating periods but spermatogenesis occurs year-round in captive beluga [161] and likely year-round in polar bears, with sperm having been collected during the non-breeding season on two occasions [162].

*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 [9,161,163]. 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) (Fig. 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 [161], 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 minimise 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

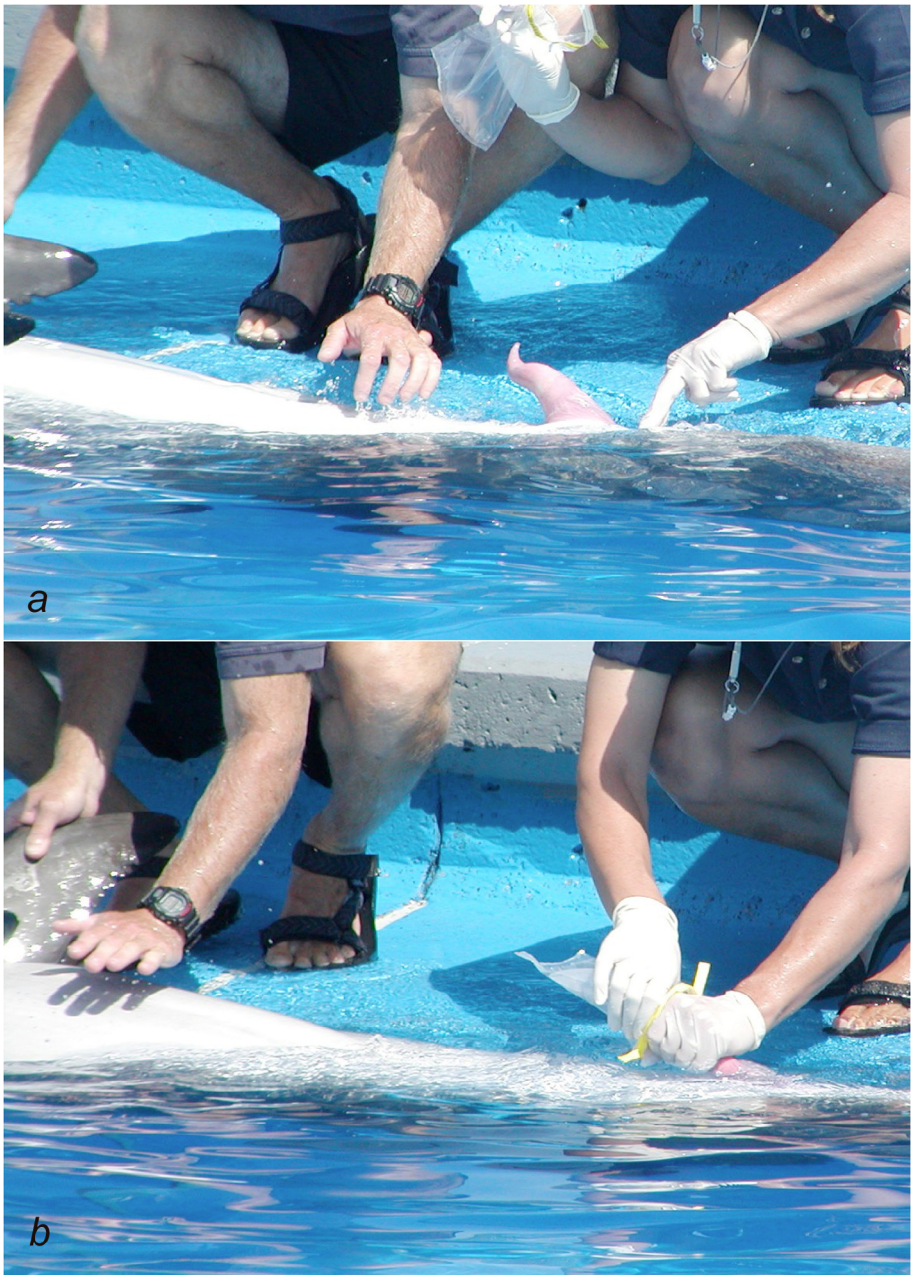


**Fig. 5.** Sperm collection from a bottlenose dolphin. Note the stimulation of the perineal area (a) and the collection of semen by manipulation and direction of the penis into the collection bag (b) [9].

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

| Species | Parameter<br>Osmolality (mOsm/kg)<br>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.

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 [164].

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 [162].

### 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* sperm motility characteristics (including longevity), sperm morphology and DNA integrity. CASA has revealed species- and individual variation in sperm motility parameters and as in other taxa, this motility analysis approach has been useful for refining sperm preservation methods [161]. Cetacean ejaculates typically exhibit spermatozoa with high rates of progressive motility and normal morphology (>80%). Cetacean sperm DNA quality was also high based on measurements using the Sperm Chromatin Structure Assay (SCSA; 15 ± 3% DNA fragmentation index) [165]. 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 [166,167]. An almost 30% decline in pregnancy rate was observed in the pig when the DFI exceeded 12%, compared to pigs with a DFI <6% [166].

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 sperm 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 [168], 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 sperm DNA integrity and ensuing normalcy of pregnancy after conception.

### 5.3. Refrigerated storage

#### 5.3.1. Extender type and preparation

*In vitro* sperm parameters (motility, membrane and acrosome integrity) of cetaceans are well-maintained during refrigerated storage at 4–6 °C when held in species-specific egg yolk based extenders (Table 5). As with other marine species (see sections

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

| Species [Reference] | Optimum base extender(s) (components) | Duration of chilled storage (4-6 °C) prior to conceptive AI (days) |
|---|---|---|
| **ODONTOCETES** | | |
| Bottlenose dolphin [9]<br>*Tursiops truncatus* | Test-yolk buffer (TYB; TES, Tris, fructose, egg yolk) | 2 |
| Pacific white-sided dolphin [160]<br>*Lagenorhynchus obliquidens* | Platz Diluent Variant (PDV; lactose, egg yolk) or TYB | Estimated 1–2 days |
| Killer whale [188]<br>*Orcinus orca* | Beltsville extender (BF5F; TES, Tris, glucose, fructose, egg yolk) | 4 |
| **MONODONTOCETES** | | |
| Beluga [161]<br>*Delphinapterus leuca* | BF5F + hyaluronic acid (HA) | 1 |

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 [165].

### 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 1–4 days post-storage (Table 5). Bottlenose dolphin ejaculates stored at 5–12 °C over 32–36 h prior to cryopreservation, to allow for overnight sperm transport and sex-sorting, resulted in a 57% conception rate after artificial insemination [169].

## 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 [169]. 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 15–18 °C during that time). Inseminations are timed to occur within 12 h prior to ovulation.

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## References

[1] Mylonas CC, Duncan NJ, Asturiano JF. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 2017;472:21–44. https://doi.org/10.1016/j.aquaculture.2016.04.021.
[2] Dall W, Hill BJ, Rothlisberg PC, Sharples DJ. Advances in marine biology. The biology of the penaeidae. London: Academic Press; 1990.
[3] Schaeff C. Courtship and mating behavior. In: Miller DL, editor. Reproductive biology and phylogeny of cetacea: whales, porpoises and dolphins. CRC Press; 2007. p. 349–70.
[4] Alfaro-Montoya J. The reproductive conditions of male shrimps, genus Penaeus, sub-genus Litopenaeus (open thelyca penaeoid shrimps): a review. Aquaculture 2010;300:1–9. https://doi.org/10.1016/j.aquaculture.2009.12.008.
[5] Suquet M, Quere C, Mingant C, Lebrun L, Ratiskol D, Miner P, et al. Effect of sampling location, release technique and time after activation on the movement characteristics of scallop (*Pecten maximus*) sperm. Aquat Living Resour 2013;26:215–20. https://doi.org/10.1051/alr/2013048.
[6] Peixoto S, Cavalli RO, Krummenauer D, Wasielesky W, D'Incao F. Influence of artificial insemination on the reproductive performance of *Farfantepenaeus paulensis* in conventional and unisex maturation systems. Aquaculture 2004;230:197–204. https://doi.org/10.1016/S0044-8486(03)00431-9.
[7] Robeck TR, O'Brien JK, Atkinson S. Reproduction. In: Gulland FMD, Dierauf LA, Whitman KL, editors. Handbook in marine mammal medicine. third ed. CRC Press; 2018. p. 169–208.
[8] Cabrita E, Robles V, Herráez P. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: CRC Press; 2009.
[9] Robeck TR, O'Brien JK. Effect of cryopreservation methods and pre-cryopreservation storage on bottlenose dolphin (*Tursiops truncatus*) spermatozoa. Biol Reprod 2004;70:1340–8. https://doi.org/10.1095/biolreprod.103.025304.
[10] Castelo-Branco T, Quinto BPT, Soares R, Guerra MMP, Peixoto S. Cadaveric sperm viability in the white shrimp *Litopenaeus vannamei*. Aquacult Res 2016;47:3350–1. https://doi.org/10.1111/are.12770.
[11] Nimrat S, Siriboonlamom S, Zhang S, Xu Y, Vuthiphandchai V. Chilled storage of white shrimp (*Litopenaeus vannamei*) spermatophores. Aquaculture 2006;261:944–51. https://doi.org/10.1016/j.aquaculture.2006.08.018.
[12] Boulais M, Soudant P, Le Goic N, Quere C, Boudry P, Suquet M. Involvement of mitochondrial activity and OXPHOS in ATP synthesis during the motility phase of spermatozoa in the pacific oyster, *Crassostrea gigas*. Biol Reprod 2015;93:118. https://doi.org/10.1095/biolreprod.115.128538.
[13] Suquet M, Malo F, Queau I, Ratiskol D, Quere C, Le Grand J, et al. Seasonal variation of sperm quality in Pacific oyster (*Crassostrea gigas*). Aquaculture 2016;464:638–41. https://doi.org/10.1016/j.aquaculture.2016.07.016.
[14] Fauvel C, Boryshpolets S, Cosson J, Leedy JGW, Labbé C, Haffray P, et al. Improvement of chilled seabass sperm conservation using a cell culture medium. J Appl Ichthyol 2012;28:961–6. https://doi.org/10.1111/jai.12071.
[15] Zilli L, Beirão J, Schiavone R, Herráez MP, Cabrita E, Storelli C, et al. Aquaporin inhibition changes protein phosphorylation pattern following sperm motility activation in fish. Theriogenology 2011;76:737–44. https://doi.org/10.1016/j.theriogenology.2011.04.006.
[16] Babiak I, Ottesen O, Rudolfsen G, Johnsen S. Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. - I: optimizing the protocol. Theriogenology 2006;66:2025–35. https://doi.org/10.1016/j.theriogenology.2006.06.003.
[17] Sarosiek B, Dryl K, Krejszeff S, Zarski D. Characterization of pikeperch (*Sander lucioperca*) milt collected with a syringe and a catheter. Aquaculture 2016;450:14–6. https://doi.org/10.1016/j.aquaculture.2015.06.040.
[18] Beirão J, Ottesen OH. Optimization of a fertilization protocol for spotted wolffish (*Anarhichas minor*). Aquaculture 2018;484:133–8. https://doi.org/10.1016/j.aquaculture.2017.11.004.
[19] Beirão J, Soares F, Herráez MP, Dinis MT, Cabrita E. Changes in *Solea senegalensis* sperm quality throughout the year. Anim Reprod Sci 2011;126:122–9. https://doi.org/10.1016/j.anireprosci.2011.04.009.
[20] Diogo P, Martins G, Quinzico I, Nogueira R, Gavaia PJ, Cabrita E. Electric ultrafreezer (− 150 °C) as an alternative for zebrafish sperm cryopreservation and storage. Fish Physiol Biochem 2018;44:1443–55. https://doi.org/10.1007/s10695-018-0500-6.
[21] Asturiano JF. Different protocols for cryopreservation of European eel (*Anguilla anguilla*) sperm. In: Cabrita E, Robles V, Herráez P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: CRC Press; 2009. p. 415–21.
[22] Urbányi B, Horváth Á, Bokor Z. Artificial fertilization in aquaculture species: from normal practice to chromosome manipulation. In: Cabrita E, Robles V, Herráez P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: CRC Press; 2009. p. 183–216.
[23] Viveiros ATM, Komen J. Semen cryopreservation of the African catfish, Clarias gariepinus. In: Cabrita E, Robles V, Herráez P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: CRC Press; 2009. p. 403–7.
[24] Sun C, Huang C, Su X, Zhao X, Dong Q. Optimization of handling and refrigerated storage of guppy *Poecilia reticulata* sperm. J Fish Biol 2010;77:54–66. https://doi.org/10.1111/j.1095-8649.2010.02658.x.
[25] Diogo P, Martins G, Eufrásio A, Silva TS, Cabrita E, Gavaia P. Selection criteria of zebrafish male donors for sperm cryopreservation. Zebrafish 2019;16:189–96. https://doi.org/10.1089/zeb.2018.1660.
[26] Adebayo OT, Fasakin EA, Adewumi JA. Reproductive performance of partial gonadectomized male African catfish, *Clarias gariepinus* broodstocks. Theriogenology 2012;77:1050–5. https://doi.org/10.1016/j.theriogenology.2011.09.027.
[27] Pérez-Cerezales S, Martínez-Páramo S, Cabrita E, Martínez-Pastor F, de Paz P, Herráez M. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 2009;71:605–13. https://doi.org/10.1016/j.theriogenology.2008.09.057.
[28] Suquet M, Cosson J, de la Gandara F, Mylonas CC, Papadaki M, Lallemant S, et al. Sperm features of captive Atlantic bluefin tuna (*Thunnus thynnus*). J Appl Ichthyol 2010;26:775–8. https://doi.org/10.1111/j.1439-0426.2010.01533.x.
[29] Trigo P, Merino O, Figueroa E, Valdebenito I, Sánchez R, Risopatrón J. Effect of short-term semen storage in salmon (*Oncorhynchus mykiss*) on sperm functional parameters evaluated by flow cytometry. Andrologia 2015;47:407–11. https://doi.org/10.1111/and.12276.
[30] Kowalski RK, Cejko BI. Sperm quality in fish: determinants and affecting factors. Theriogenology 2019. in press.
[31] Browne RK, Kaurova SA, Uteshev VK, Shishova NV, McGinnity D, Figiel CR, et al. Sperm motility of externally fertilizing fish and amphibians. Theriogenology 2015;83:1–13. https://doi.org/10.1016/j.theriogenology.2014.09.018.
[32] Król J, Żarski D, Bernáth G, Palińska-Żarska K, Krejszeff S, Długoński A, et al. Effect of urine contamination on semen quality variables in Eurasian perch *Perca fluviatilis L.* Anim Reprod Sci 2018;197:240–6. https://doi.org/10.1016/j.anireprosci.2018.08.034.

[33] Boonthai T, Khaopong W, Sangsong J, Sooksawat T, Nimrat S, Vuthiphandchai V. Semen collection methods affect the bacterial composition of post-thawed semen of silver barb (*Barbodes gonionotus*). Anim Reprod Sci 2016;166:90–8. https://doi.org/10.1016/j.anireprosci.2016.01.007.

[34] Suquet M, Omnes MH, Normant Y, Fauvel C. Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture 1992;101:177–85. https://doi.org/10.1016/0044-8486(92)90241-C.

[35] Beirão J, Soares F, Pousão-Ferreira P, Diogo P, Dias J, Dinis MT, et al. The effect of enriched diets on *Solea senegalensis* sperm quality. Aquaculture 2015;435:187–94. https://doi.org/10.1016/j.aquaculture.2014.09.025.

[36] Cejko BI, Sarosiek B, Krejszeff S, Kowalski RK. Multiple collections of common carp *Cyprinus carpio* L. semen during the reproductive period and its effects on sperm quality. Anim Reprod Sci 2018;188:178–88. https://doi.org/10.1016/j.anireprosci.2017.12.002.

[37] Butts IAE, Litvak MK, Trippel EA. Seasonal variations in seminal plasma and sperm characteristics of wild-caught and cultivated Atlantic cod, *Gadus morhua*. Theriogenology 2010;73:873–85. https://doi.org/10.1016/j.theriogenology.2009.11.011.

[38] Babiak I, Ottesen O, Rudolfsen G, Johnsen S. Quantitative characteristics of Atlantic halibut, *Hippoglossus hippoglossus* L., semen throughout the reproductive season. Theriogenology 2006;65:1587–604. https://doi.org/10.1016/j.theriogenology.2005.09.004.

[39] Suquet M, Dreanno C, Dorange G, Normant Y, Quemener L, Gaignon JL, et al. The ageing phenomenon of turbot spermatozoa: effects on morphology, motility and concentration, intracellular ATP content, fertilization, and storage capacities. J Fish Biol 1998;52:31–41. https://doi.org/10.1111/j.1095-8649.1998.tb01550.x.

[40] İnanan BE, Öğretmen F, İnanan T, Yılmaz F. Total antioxidant capacity, catalase activity, and lipid peroxidation changes in seminal plasma of sex-reversed female and male rainbow trout (*Oncorhynchus mykiss*) during spawning season. Theriogenology 2016;86:1975–82. https://doi.org/10.1016/j.theriogenology.2016.06.014.

[41] Mylonas CC, Papadaki M, Divanach P. Seasonal changes in sperm production and quality in the red porgy *Pagrus pagrus* (L.). Aquacult Res 2003;34:1161–70. https://doi.org/10.1046/j.1365-2109.2003.00922.x.

[42] Alavi S, Cosson J. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 2006;30:1–14. https://doi.org/10.1016/j.cellbr.2005.06.004.

[43] Yang H, Tiersch TR. Sperm motility initiation and duration in a euryhaline fish, medaka (*Oryzias latipes*). Theriogenology 2009;72:386–92. https://doi.org/10.1016/j.theriogenology.2009.03.007.

[44] Alavi S, Cosson J. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol Int 2005;29:101–10. https://doi.org/10.1016/j.cellbi.2004.11.021.

[45] Zadmajid V, Myers JN, Sørensen SR, Butts IAE. Ovarian fluid and its impacts on sperm performance in fish: a review. Theriogenology 2019;132:144–52. https://doi.org/10.1016/j.theriogenology.2019.03.021.

[46] Cabrita E, Sarasquete C, Martínez-Páramo S, Robles V, Beirão J, Pérez-Cerezales S, et al. Cryopreservation of fish sperm: applications and perspectives. J Appl Ichthyol 2010;26:623–35. https://doi.org/10.1111/j.1439-0426.2010.01556.x.

[47] Bobe J, Labbé C. Chilled storage of sperm and eggs. In: Cabrita E, Robles V, Herráez P, editors. Methods in reproductive aquaculture: marine and freshwater species. Boca Raton: CRC Press; 2009. p. 219–36.

[48] Herranz-Jusdado JG, Gallego V, Morini M, Rozenfeld C, Pérez L, Müller T, Horváth Á, Ohta H, Asturiano JF. Eels sperm cryopreservation: an overview. Theriogenology 2019. in press.

[49] Judycka S, Nynca J, Ciereszko A. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. Theriogenology 2019;132. https://doi.org/10.1016/j.theriogenology.2019.03.022.

[50] Vuthiphandchai V, Thadsri I, Nimrat S. Chilled storage of walking catfish (*Clarias macrocephalus*) semen. Aquaculture 2009;296:58–64. https://doi.org/10.1016/j.aquaculture.2009.07.018.

[51] Risopatrón J, Merino O, Cheuquemán C, Figueroa E, Sánchez R, Farías JG, et al. Effect of the age of broodstock males on sperm function during cold storage in the trout (*Oncorhynchus mykiss*). Andrologia 2018;50:e12857. https://doi.org/10.1111/and.12857.

[52] Beirão J, Pérez-Cerezales S, Martínez-Páramo S, Herráez MP. Detection of early damage of sperm cell membrane in Gilthead seabream (*Sparus aurata*) with the nuclear stain YO-PRO 1. J Appl Ichthyol 2010;26:794–6. https://doi.org/10.1111/j.1439-0426.2010.01560.x.

[53] Gallardo Bolanos JM, Miro Moran A, Balao da Silva CM, Morillo Rodriguez A, Plaza Davila M, Aparicio IM, et al. Autophagy and apoptosis have a role in the survival or death of stallion spermatozoa during conservation in refrigeration. PLoS One 2012;7. https://doi.org/10.1371/journal.pone.0030688. e30688.

[54] Mansour N, Lahnsteiner F, Berger B. Characterization of the testicular semen of the African catfish, *Clarias gariepinus* (Burchell, 1822), and its short-term storage. Aquacult Res 2004;35:232–44. https://doi.org/10.1111/j.1365-2109.2004.00993.x.

[55] Santos M, Soares F, Moreira M, Beirão J. Evaluation of different extenders for the cold storage of meagre (*Argyrosomus regius*) semen. Aquacult Res 2018;49:2723–31. https://doi.org/10.1111/are.13733.

[56] Ladoktha P, Ponchunchoovong S, Udomkarn C. Preservation of black sharkminnow, *Labeo chrysophekadion* (Bleeker, 1849) spermatozoa. Aquacult Res 2017;48:3837–47. https://doi.org/10.1111/are.13211.

[57] Dorsey KM, Guthrie HD, Welch GR, Mohler J, Theisen DD, Siewerdt F, et al. Quality Assessment of wild atlantic sturgeon semen under conditions of short-term storage. N Am J Aquacult 2011;73:418–25. https://doi.org/10.1080/15222055.2011.629945.

[58] Gallego V, Pérez L, Asturiano JF, Yoshida M. Study of pufferfish (*Takifugu niphobles*) sperm: development of methods for short-term storage, effects of different activation media and role of intracellular changes in Ca2+ and K+ in the initiation of sperm motility. Aquaculture 2013;414–415:82–91. https://doi.org/10.1016/j.aquaculture.2013.07.046.

[59] Park C, Chapman FA. An extender solution for the short-term storage of sturgeon semen. N Am J Aquacult 2005;67:52–7. https://doi.org/10.1577/FA03-068.1.

[60] Chang YJ, Chang YJ, Lim HK, Lee JK, Park YJ. Cold storage of milt from four species of flatfish. Fish Aquat Sci 2002;5:64–74.

[61] Brown G, Mims S. Storage, transportation, and fertility of undiluted and diluted paddlefish milt. Prog Fish-Cult 1995;57:64–9. https://doi.org/10.1577/1548-8640(1995)057<0064:STAFOU>2.3.CO;2.

[62] Sadeghi S, Nunez J, Soler C, Angel Silvestre M. Effect of the activation media with different osmolality and cool storage on spermatozoa motility parameters over time in zebrafish, *Danio rerio*. Turk J Fish Aquat Sci 2017;17:111–20. https://doi.org/10.4194/1303-2712-v17_1_13.

[63] Peñaranda DS, Marco-Jiménez F, Pérez L, Gallego V, Mazzeo I, Vicente JS, et al. Evaluation of different diluents for short-term storage of European eel sperm under air-limited conditions. J Appl Ichthyol 2010;26:659–64. https://doi.org/10.1111/j.1439-0426.2010.01530.x.

[64] Wayman WR, Tiersch TR, Thomas RG. Refrigerated storage and cryopreservation of sperm of red drum, *Sciaenops ocellatus* L. Aquacult Res 1998;29:267–73. https://doi.org/10.1111/j.1365-2109.1998.tb01131.x.

[65] Viveiros ATM, Isau ZA, Figueiredo HCP, Leite MAS, Maria AN. Gentamycin controls bacterial growth during refrigerated storage of piracanjuba, *Brycon orbignyanus*, semen. J World Aquacult Soc 2010;41:57–65.

[66] Orfão LH, Maria AN, Nascimento AF, Isau ZA, Viveiros ATM. Sperm fertility of the subtropical freshwater streaked prochilod *Prochilodus lineatus* (Characiformes) improved after dilution and cold storage. Aquacult Res 2010;41:e679–87. https://doi.org/10.1111/j.1365-2109.2010.02597.x.

[67] McNiven MA, Gallant RK, Richardson GF. Fresh storage of rainbow trout (*Oncorhynchus mykiss*) semen using a non-aqueous medium. Aquaculture 1993;109:71–82. https://doi.org/10.1016/0044-8486(93)90487-J.

[68] Shaliutina A, Hulak M, Gazo I, Linhartova P, Linhart O. Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Anim Reprod Sci 2013;139:127–35. https://doi.org/10.1016/j.anireprosci.2013.03.006.

[69] Lahnsteiner F, Mansour N. A comparative study on antioxidant systems in semen of species of the Percidae, Salmonidae, Cyprinidae, and Lotidae for improving semen storage techniques. Aquaculture 2010;307:130–40. https://doi.org/10.1016/j.aquaculture.2010.07.011.

[70] Sarosiek B, Dryl K, Kucharczyk D, Żarski D, Kowalski RK. Motility parameters of perch spermatozoa (*Perca fluviatilis* L.) during short-term storage with antioxidants addition. Aquacult Int 2014;22:159–65. https://doi.org/10.1007/s10499-013-9679-9.

[71] Zietara MS, Slominska E, Swierczynski J, Rurangwa E, Ollevier F, Skorkowski EF. ATP content and adenine nucleotide catabolism in african catfish spermatozoa stored in various energetic substrates. Fish Physiol Biochem 2004;30:119–27. https://doi.org/10.1007/s10695-005-2493-1.

[72] Christensen JM, Tiersch TR. Refrigerated storage of channel catfish sperm. J World Aquacult Soc 1996;27:340–6. https://doi.org/10.1111/j.1749-7345.1996.tb00617.x.

[73] Jenkins JA, Tiersch TR. A preliminary bacteriological study of refrigerated channel catfish sperm. J World Aquacult Soc 1997;28:282–8. https://doi.org/10.1111/j.1749-7345.1997.tb00644.x.

[74] Segovia M, Jenkins JA, Paniagua-Chavez C, Tiersch TR. Flow cytometric evaluation of antibiotic effects on viability and mitochondrial function of refrigerated spermatozoa of nile tilapia. Theriogenology 2000;53:1489–99. https://doi.org/10.1016/S0093-691X(00)00291-0.

[75] Ramachandran B, Natesan M. Assessment of sperm quality and short term preservation of sperm in grey mullet *Mugil cephalus* Linnaeus, 1758. Indian J Fish 2016;63:140–4. https://doi.org/10.21077/ijf.2016.63.4.61001-23.

[76] Bencic DC, Krisfalusi M, Cloud JG, Ingermann RL. ATP levels of chinook salmon (*Oncorhynchus tshawytscha*) sperm following in vitro exposure to various oxygen tensions. Fish Physiol Biochem 1999;20:389–97. https://doi.org/10.1023/A:1007749703803.

[77] Bencic DC, Krisfalusi M, Cloud JG, Ingermann RL. Short-term storage of salmonid sperm in air versus oxygen. N Am J Aquacult 2000;62:19–25. https://doi.org/10.1577/1548-8454(2000)062<0019:STSOSS>2.0.CO;2.

[78] Bencic DC, Ingermann RL, Cloud JG. Does CO2 enhance short-term storage success of Chinook salmon (*Oncorhynchus tshawytscha*) milt? Theriogenology 2001;56:157–66.

[79] Chereguini O, Cal RM, Dreanno C, Baulny BO de, Suquet M, Maisse G. Short-term storage and cryopreservation of turbot (*Scophthalmus maximus*) sperm. Aquat Living Resour 1997;10:251–5. https://doi.org/10.1051/alr:1997028.

[80] Judycka S, Żarski D, Dietrich MA, Palińska-Żarska K, Karol H, Ciereszko A. Standardized cryopreservation protocol of European perch (*Perca fluviatilis*)

semen allows to obtain high fertilization rates with the use of frozen/thawed semen. Aquaculture 2019;498:208–16. https://doi.org/10.1016/j.aquaculture.2018.08.059.
[81] Torres L, Hu E, Tiersch TR. Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development. Reprod Fertil Dev 2016;28:1105–15. https://doi.org/10.1071/RD15388.
[82] Torres L, Liu Y, Guitreau A, Yang H, Tiersch TR. Challenges in development of sperm repositories for biomedical fishes: quality control in small-bodied species. Zebrafish 2017;14:552–60. https://doi.org/10.1089/zeb.2017.1426.
[83] Bernáth G, Ittzés I, Szabó Z, Horváth Á, Krejszeff S, Lujić J, et al. Chilled and post-thaw storage of sperm in different goldfish types. Reprod Domest Anim 2017;52:680–6. https://doi.org/10.1111/rda.12951.
[84] Fauvel C, Suquet M, Cosson J. Evaluation of fish sperm quality. J Appl Ichthyol 2010;26:636–43. https://doi.org/10.1111/j.1439-0426.2010.01529.x.
[85] Agarwal NK, Raghuvanshi SK. Spermatocrit and sperm density in snowtrout (*Schizothorax richardsonii*): correlation and variation during the breeding season. Aquaculture 2009;291:61–4. https://doi.org/10.1016/j.aquaculture.2009.03.002.
[86] Sorensen SR, Gallego V, Perez L, Butts IAE, Tomkiewicz J, Asturiano JF. Evaluation of Methods to determine sperm density for the European eel, *Anguilla anguilla*. Reprod Domest Anim 2013;48:936–44. https://doi.org/10.1111/rda.12189.
[87] Tan E, Yang H, Tiersch TR. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. Zebrafish 2010;7:233–40. https://doi.org/10.1089/zeb.2010.0655.
[88] Leclercq E, Antoni L, Bardon-Albaret A, Anderson CR, Somerset CR, Saillant EA. Spectrophotometric determination of sperm concentration and short-term cold-storage of sperm in Atlantic croaker *Micropogonias undulatus* L. broodstock. Aquacult Res 2014;45:1283–94. https://doi.org/10.1111/are.12073.
[89] Nynca J, Ciereszko A. Measurement of concentration and viability of brook trout (*Salvelinus fontinalis*)spermatozoa using computer-aided fluorescent microscopy. Aquaculture 2009;292:256–8. https://doi.org/10.1016/j.aquaculture.2009.04.020.
[90] Nynca J, Dietrich GJ, Liszewska E, Judycka S, Karol H, Dobosz S, et al. Usefulness of a portable flow cytometer for sperm concentration and viability measurements of rainbow trout spermatozoa. Aquaculture 2016;451:353–6. https://doi.org/10.1016/j.aquaculture.2015.09.027.
[91] Rakitin A, Ferguson M, Trippel E. Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. Aquaculture 1999;170:349–58. https://doi.org/10.1016/S0044-8486(98)00417-7.
[92] Chereguini O, De La Banda IG, Rasines I, Fernandez A. Artificial fertilization in turbot, *Scophthalmus maximus* (L.): different methods and determination of the optimal sperm–egg ratio. Aquacult Res 1999;30:319–24. https://doi.org/10.1046/j.1365-2109.1999.00326.x.
[93] Gallego V, Pérez L, Asturiano JF, Yoshida M. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture 2013;416–417:238–43. https://doi.org/10.1016/j.aquaculture.2013.08.035.
[94] Rurangwa E, Roelants I, Huyskens G, Ebrahimi M, Kime DE, Ollevier F. The minimum effective spermatozoa:egg ratio for artificial insemination and the effects of mercury on sperm motility and fertilization ability in *Clarias gariepinus*. J Fish Biol 1998;53:402–13. https://doi.org/10.1111/j.1095-8649.1998.tb00989.x.
[95] Suquet M, Billard R, Cosson J, Normant Y, Fauvel C. Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture 1995;133:83–90. https://doi.org/10.1016/0044-8486(94)00395-5.
[96] Linhart O, Gela D, Rodina M, Kocour M. Optimization of artificial propagation in European catfish, *Silurus glanis* L. Aquaculture 2004;235:619–32. https://doi.org/10.1016/j.aquaculture.2003.11.031.
[97] Gwo J, Strawn K, Longnecker M, Arnold C. Cryopreservation of atlantic croaker spermatozoa. Aquaculture 1991;94:355–75. https://doi.org/10.1016/0044-8486(91)90179-B.
[98] Rosenthal H, Klumpp D, Willführ J. Influence of sperm density and contact time on herring egg fertilization. J Appl Ichthyol 1988;4:79–86. https://doi.org/10.1111/j.1439-0426.1988.tb00470.x.
[99] Erdahl AW, Graham EF. Fertility of teleost semen as affected by dilution and storage in a seminal plasma-mimicking medium. Aquaculture 1987;60:311–21. https://doi.org/10.1016/0044-8486(87)90296-1.
[100] Ciereszko A, Dabrowski K, Toth GP, Christ SA, Glogowski J. Factors affecting motility characteristics and fertilizing ability of sea lamprey Spermatozoa. Trans Am Fish Soc 2002;131:193–202. https://doi.org/10.1577/1548-8659(2002)131<0193:FAMCAF>2.0.CO;2.
[101] Butts IAE, Trippel EA, Litvak MK. The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L. Aquaculture 2009;286:89–94. https://doi.org/10.1016/j.aquaculture.2008.09.005.
[102] Babiak I, Marschhäuser V, Ottesen O, Rudolfsen G, Eggen B, Babiak J. Effects of extender, storage and sperm-to-egg ratio on cryopreservation success of Atlantic cod (*Gadus morhua* L.) sperm. J Appl Ichthyol 2012;28:941–7. https://doi.org/10.1111/jai.12063.
[103] Tvedt HB, Benfey TJ, Martin-Robichaud DJ, Power J. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 2001;194:191–200. https://doi.org/10.1016/S0044-8486(00)00516-0.
[104] Butts IAE, Sørensen SR, Politis SN, Pitcher TE, Tomkiewicz J. Standardization of fertilization protocols for the European eel, *Anguilla anguilla*. Aquaculture 2014;426–427:9–13. https://doi.org/10.1016/j.aquaculture.2014.01.020.
[105] Vermeirssen ELM, Mazorra C, Shields R, Norberg B, Scott AP, Kime DE. The application of GnRHa implants in male Atlantic halibut; effects on steroids, milt hydration, sperm motility and fertility. In: Norberg B, editor. Proceedings of the 6th international symposium on the reproductive physiology of fish, Norway; 1999. p. 399–401.
[106] Viveiros ATM, So N, Komen J. Sperm cryopreservation of African catfish, *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology 2000;54:1395–408.
[107] Lubzens E, Daube N, Pekarsky I, Magnus Y, Cohen A, Yusefovich F, et al. Carp (*Cyprinus carpio* L.) spermatozoa cryobanks - strategies in research and application. Aquaculture 1997;155:13–30. https://doi.org/10.1016/S0044-8486(97)00106-3.
[108] Bauer RT, Cash CE. Spermatophore structure and anatomy of the ejaculatory duct in *Penaeus setiferus, P. duorarum*, and *P. aztecus* (Crustacea: Decapoda): homologies and functional significance. Trans Am Microsc Soc 1991;110:144–62. https://doi.org/10.2307/3226751.
[109] Castelo-Branco T, Silva EF, Calazans N, Soares R, Peixoto S. Scanning electron microscopic investigation of the spermatophore and spermatozoa of the shrimp *Farfantepenaeus subtilis* (Decapoda: penaeidae). Invertebr Reprod Dev 2014;58:190–2. https://doi.org/10.1080/07924259.2014.889766.
[110] Nakayama C, Peixoto S, Lopes D, Vita G, Krummenauer D, Foes G, et al. Manual and electrical spermatophore extrusion methods of the pink shrimp *Farfantepenaeus paulensis* (Decapoda: penaeidae) wild broodstock. Ciência Rural 2008;38:2018–22. https://doi.org/10.1590/S0103-84782008000700034.
[111] Rosas C, Sanchez A, Chimal MAE, Saldaña G, Ramos L, Soto LA. The effect of electrical stimulation on spermatophore regeneration in white shrimp *Penaeus setiferus*. Aquat Living Resour 1993;6:139–44. https://doi.org/10.1051/alr:1993013.
[112] Braga A, Lopes DLA, Poersch LH, Wasielesky W. Spermatophore replacement of pink shrimp *Farfantepenaeus brasiliensis* after manual extrusion: effect of molting. Aquaculture 2014;433:313–7. https://doi.org/10.1016/j.aquaculture.2014.06.032.
[113] Narasimman S, Krishnamoorthy D, Buelah C D, Natesan M. Retrieval and cryopreservation of sperm in spermatophores from cadaveric Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). Anim Reprod Sci 2018;192:185–92. https://doi.org/10.1016/j.anireprosci.2018.03.008.
[114] Bambozzi AF, Mattos LA, Mello M, Oshiro LMY. Post-mortem spermatophore and sperm cryopreservation of the white shrimp *Litopenaeus schmitti*. Bol Inst Pesca Sao Paulo 2014;40:49–60.
[115] Morales-Ueno K, Montaldo HH, Ortega AM, Paniagua-Chávez CG, Castillo-Juárez H. An extender solution for the short-term storage of *Litopenaeus vannamei* sperm to be used in artificial insemination. Aquacult Res 2013;44:1254–8. https://doi.org/10.1111/j.1365-2109.2012.03126.x.
[116] Morales-Ueno K, Paniagua-Chávez CG, Martínez-Ortega A, Castillo-Juárez H, Alfaro-Montoya J. A simple method for short-term storage and transportation of spermatophores of Pacific white shrimp (*Litopenaeus vannamei*). Hidrobiologica 2016;26:9–14.
[117] Salazar M, Lezcano M, Granja C. Protocol for cryopreservation of Penaeus vannamei sperm cells. Methods Reprod. Aquac. Mar. Freshw. Species. Boca Raton: CRC Press; 2009. p. 505.
[118] Nimrat S, Vuthiphandchai V. Role of bacterial in the chilled storage and cryopreservation of sperm in aquatic animals: a review. In: Schwartz SH, editor. Aquaculture research trends. New York: Nova Science Publisher; 2008. p. 149–84.
[119] Camargo TR, Rossi N, Castilho AL, Costa RC, Mantelatto FL, Zara FJ. Sperm ultrastructure of shrimps from the family Penaeidae (Crustacea: dendrobranchiata) in a phylogenetic context. Arthropod Struct Dev 2017;46:588–600. https://doi.org/10.1016/j.asd.2017.01.006.
[120] Leelatanawit R, Uawisetwathana U, Khudet J, Klanchui A, Phomklad S, Wongtripop S, et al. Effects of polychaetes (*Perinereis nuntia*) on sperm performance of the domesticated black tiger shrimp (*Penaeus monodon*). Aquaculture 2014;433:266–75. https://doi.org/10.1016/j.aquaculture.2014.06.034.
[121] Braga A, Lopes D, Magalhães V, Klosterhoff M, Romano L, Poersch L, et al. Infertility of biofloc-reared *Litopenaeus vannamei* males associated with a spermatophore mycobacterial infection: description of the pathological condition and implications for the broodstock management and larval production. Aquaculture 2018;492:357–60. https://doi.org/10.1016/j.aquaculture.2018.04.039.
[122] Castelo-Branco T, Batista AM, Guerra MMP, Soares R, Peixoto S. Sperm vitrification in the white shrimp *Litopenaeus vannamei*. Aquaculture 2015;436:110–3. https://doi.org/10.1016/j.aquaculture.2014.11.005.
[123] Lezcano M, Granja C, Salazar M. The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). Cryobiology 2004;48:349–56. https://doi.org/10.1016/j.cryobiol.2004.03.003.
[124] Castelo-Branco T, Guerra MMP, Soares R, Peixoto S. Sperm vitrification of *Litopenaeus vannamei*: effect of cryoprotectant solutions on sperm viability and spawning quality after artificial insemination. Aquacult Int 2018;26:

913–20. https://doi.org/10.1007/s10499-018-0258-y.
[125] Feng T, Gosálvez J, Lopez-Fernandez C, Arroyo F, Paterson B, Johnston S. Sperm chromatin dispersion test (SCDt) for the assessment of sperm DNA fragmentation in black tiger prawn, *Penaeus monodon*. Aquaculture 2018;491:281–8. https://doi.org/10.1016/j.aquaculture.2018.03.041.
[126] Benzie JAH, Kenway M, Ballment E, Frusher S, Trott L. Interspecific hybridization of the tiger prawns *Penaeus monodon* and *Penaeus esculentus*. Aquaculture 1995;133:103–11. https://doi.org/10.1016/0044-8486(95)00013-R.
[127] Perez-Velazquez M, González-Félix ML, Zúñiga-Panduro M, Barraza-Guardado RH. Evidence of pre-zygotic barriers in attempts to cross-breed *Penaeus* (*Litopenaeus*) *vannamei* (Boone) and *P.* (*Litopenaeus*) *stylirostris* (Stimpson) by means of artificial insemination. Aquaculture 2010;304:100–3. https://doi.org/10.1016/j.aquaculture.2010.03.021.
[128] Ulate K, Alfaro-Montoya J. Sperm–egg incompatibility in inter-specific inseminations of *Penaeus* (*Litopenaeus*) *occidentalis*, *P.* (*Litopenaeus*) *stylirostris* and *P.* (*Litopenaeus*) *vannamei*. Aquaculture 2010;309:290–2. https://doi.org/10.1016/j.aquaculture.2010.08.029.
[129] Bart AN, Choosuk S, Thakur DP. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). Aquacult Res 2006;37:523–8. https://doi.org/10.1111/j.1365-2109.2006.01460.x.
[130] Foighil DO. Role of spermatozeugmata in the spawning ecology of the brooding oyster *Ostrea edulis*. Gamete Res 1989;24:219–28. https://doi.org/10.1002/mrd.1120240209.
[131] Suquet M, Pouvreau S, Queau I, Boulais M, Le Grand J, Ratiskol D, et al. Biological characteristics of sperm in European flat oyster (*Ostrea edulis*). Aquat Living Resour 2018;31:20. https://doi.org/10.1051/alr/2018008.
[132] Dohmen MR. Gametogenesis. In: Mollusca, vol. 3. New York: Academic Press Inc; 1983. p. 1–48. Dev.
[133] Fabioux C, Huvet A, Le Souchu P, Le Pennec M, Pouvreau S. Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. Aquaculture 2005;250:458–70. https://doi.org/10.1016/j.aquaculture.2005.02.038.
[134] Demoy-Schneider M, Schmitt N, Suquet M, Labbé C, Boulais M, Prokopchuk G, et al. Biological characteristics of sperm in two oyster species: the Pacific oyster, *Crassostrea gigas*, and the Black-Lip Pearl oyster, *Pinctada margaritifera*. Spermatozoa. Nova Science Publishers; 2014. p. 259.
[135] Franco A, Jouaux A, Mathieu M, Sourdaine P, Lelong C, Kellner K, et al. Proliferating cell nuclear antigen in gonad and associated storage tissue of the Pacific oyster *Crassostrea gigas*: seasonal immunodetection and expression in laser microdissected tissues. Cell Tissue Res 2010;340:201–10. https://doi.org/10.1007/s00441-009-0923-6.
[136] Faure C, Devauchelle N, Girard J. Ionic factors affecting motility, respiration and fertilization rate of the sperm of the bivalve pecten-maximus (l). J Comp Physiol B Biochem Syst Environ Physiol 1994;164:444–50. https://doi.org/10.1007/BF00714581.
[137] Wilbur PB, Robert EM. Hatchery manual for the pacific oyster. Oregon State University; 1975.
[138] Velasco LA, Barros J, Acosta E. Spawning induction and early development of the Caribbean scallops *Argopecten nucleus* and *Nodipecten nodosus*. Aquaculture 2007;266:153–65. https://doi.org/10.1016/j.aquaculture.2007.02.015.
[139] Song YP, Suquet M, Queau I, Lebrun L. Setting of a procedure for experimental fertilisation of Pacific oyster (*Crassostrea gigas*) oocytes. Aquaculture 2009;287:311–4. https://doi.org/10.1016/j.aquaculture.2008.10.018.
[140] Le Goic N, Hegaret H, Fabioux C, Miner P, Suquet M, Lambert C, et al. Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive output: application of flow cytometry assays on spermatozoa. Aquat Living Resour 2013;26:221–8. https://doi.org/10.1051/alr/2013047.
[141] Smith JF, Adams SL, Gale SL, McGowan LT, Tervit HR, Roberts RD. Cryopreservation of Greenshell (TM) mussel (*Perna canaliculus*) sperm. I. Establishment of freezing protocol. Aquaculture 2012;334:199–204. https://doi.org/10.1016/j.aquaculture.2011.12.027.
[142] Paniagua-Chavez CG, Buchanan JT, Tiersch TR. Effect of extender solutions and dilution on motility and fertilizing ability of Eastern oyster sperm. J Shellfish Res 1998;17:231–7.
[143] Dong QX, Eudeline B, Huang CJ, Allen SK, Tiersch TR. Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. Cryobiology 2005;50:1–16. https://doi.org/10.1016/j.cryobiol.2004.09.003.
[144] Demoy-Schneider M, Leveque A, Schmitt N, Le Pennec M, Cosson J. Motility activation and metabolism characteristics of spermatozoa of the black-lip-pearl oyster *Pinctada margaritifera* var: cumingii (Jameson, 1901). Theriogenology 2012;77:53–64. https://doi.org/10.1016/j.theriogenology.2011.07.014.
[145] Riesco MF, Félix F, Matias D, Joaquim S, Suquet M, Cabrita E. First study in cryopreserved *Crassostrea angulata* sperm. Gen Comp Endocrinol 2017;245:108–15. https://doi.org/10.1016/j.ygcen.2016.05.003.
[146] Greenwood P, Bennett T. Some effects of temperature-salinity combinations on the early development of the sea-urchin *Parechinus-Angulosus* (leske) fertilization. J Exp Mar Biol Ecol 1981;51:119–31. https://doi.org/10.1016/0022-0981(81)90124-6.
[147] Brizard R, Bernardi M, Boudry P, Haffray P, Labbe C, Maisse G, et al. Projet CRYOYSTER: optimisation, standardisation et validation de la congélation de laitance d'huître creuse Crassostrea gigas à des fins de conservation et de diffusion génétique - appel d'offre de l'OFIMER du 8 Novembre 2001 - rapport final 2004.
[148] Alavi SMH, Matsumura N, Shiba K, Itoh N, Takahashi KG, Inaba K, et al. Roles of extracellular ions and pH in 5-HT-induced sperm motility in marine bivalve. Reproduction 2014;147:331–45. https://doi.org/10.1530/REP-13-0418.
[149] Boulais M, Suquet M, Arsenault-Pernet EJ, Malo F, Queau I, Pignet P, et al. pH controls spermatozoa motility in the Pacific oyster (*Crassostrea gigas*). Biol Open 2018;7. https://doi.org/10.1242/bio.031427. UNSP bio031427.
[150] Boulais M, Demoy-Schneider M, Alavi SMH, Cosson J. Spermatozoa motility in bivalves: signaling, flagellar beating behavior, and energetics. Theriogenology 2019.
[151] Boulais M, Soudant P, Le Goïc N, Quéré C, Boudry P, Suquet M. ATP content and viability of spermatozoa drive variability of fertilization success in the Pacific oyster (*Crassostrea gigas*). Aquaculture 2017;479:114–9. https://doi.org/10.1016/j.aquaculture.2017.05.035.
[152] Paniagua-Chávez CG, Jenkins J, Segovia M, Tiersch TR. Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes. Cryobiology 2006;53:128–38. https://doi.org/10.1016/j.cryobiol.2006.05.001.
[153] Smith JF, Adams SL, McDonald RM, Gale SL, McGowan LT, Tervit HR. Cryopreservation of Greenshell™ Mussel (*Perna canaliculus*) sperm. II. Effect of cryopreservation on fertility, motility, viability and chromatin integrity. Aquaculture 2012;364–365:322–8. https://doi.org/10.1016/j.aquaculture.2012.08.039.
[154] Gwo J-C, Wu C-Y, Chang W-SP, Cheng H-Y. Evaluation of damage in Pacific oyster (*Crassostrea gigas*) spermatozoa before and after cryopreservation using comet assay. Cryo Lett. 2003;24:171–80.
[155] Vaschenko MA, Syasina IG, Zhadan PM, Medvedeva LA. Reproductive function state of the scallop *Mizuhopecten yessoensis* jay from polluted areas of Peter the Great Bay, Sea of Japan. Hydrobiologia 1997;352:231–40. https://doi.org/10.1023/A:1003087610120.
[156] Hui B, Vonau V, Moriceau J, Tetumu R, Vanaa V, Demoy-Schneider M, et al. Hatchery-scale trials using cryopreserved spermatozoa of black-lip pearl oyster, *Pinctada margaritifera*. Aquat Living Resour 2011;24:219–23. https://doi.org/10.1051/alr/2011117.
[157] Dong Y, Yao H, Lin Z, Zhu D. The effects of sperm-egg ratios on polyspermy in the blood clam, *Tegillarca granosa*. Aquacult Res 2012;43:44–52. https://doi.org/10.1111/j.1365-2109.2011.02799.x.
[158] Gruffydd LD, Beaumont AR. Determination of the optimum concentration of eggs and spermatozoa for the production of normal larvae in *Pecten maximus* (Mollusca, Lamellibranchia). Helgol Wiss Meeresunters 1970;20:486–97. https://doi.org/10.1007/BF01609924.
[159] Stephano J, Gould M. Avoiding polyspermy in the oyster (*Crassostrea gigas*). Aquaculture 1988;73:295–307.
[160] Robeck TR, Steinman KJ, Greenwell M, Ramirez K, Van Bonn W, Yoshioka M, et al. Seasonality, estrous cycle characterization, estrus synchronization, semen cryopreservation, and artificial insemination in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). Reproduction 2009;138:391–405. https://doi.org/10.1530/REP-08-0528.
[161] O'Brien JK, Steinman KJ, Schmitt T, Robeck TR. Semen collection, characterisation and artificial insemination in the beluga (*Delphinapterus leucas*) using liquid-stored spermatozoa. Reprod Fertil Dev 2008;20:770–83. https://doi.org/10.1071/RD08031.
[162] Curry E, Roth TL. 118 A rapid, minimally invasive method of collecting semen from polar bears. Reprod Fertil Dev 2016;28. https://doi.org/10.1071/RDv28n2Ab118. 189–189.
[163] Keller J. Training atlantic bottlenose dolphins (*Tursiops truncatus*) for artificial insemination. Naval ocean systems center san diego CA; 1988.
[164] Muraco HS, Coombs LD, Procter DG, Turek PJ, Muraco MJ. Use of human chorionic gonadotropin in a male pacific walrus (*Odobenus rosmarus divergens*) to induce rut and achieve a pregnancy in a nulliparous female. J Androl 2012;33:789–97. https://doi.org/10.2164/jandrol.111.015032.
[165] Montano GA, Kraemer DC, Love CC, Robeck TR, O'Brien JK. Evaluation of motility, membrane status and DNA integrity of frozen-thawed bottlenose dolphin (*Tursiops truncatus*) spermatozoa after sex-sorting and recryopreservation. Reproduction 2012;143:799–813. https://doi.org/10.1530/REP-11-0490.
[166] Didion BA, Kasperson KM, Wixon RL, Evenson DP. Boar fertility and sperm chromatin structure status: a retrospective report. J Androl 2009;30:655–60. https://doi.org/10.2164/jandrol.108.006254.
[167] Kenney RM, Evenson DP, Garcia MC, Love CC. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate. Biol Reprod 1995;52:647–53. https://doi.org/10.1093/biolreprod/52.monograph_series1.647.
[168] Wells RS, Smith CR, Sweeney JC, Townsend FI, Fauquier DA, Stone R, et al. Fetal survival of common bottlenose dolphins (*Tursiops truncatus*) in sarasota bay, Florida. Aquat Mamm 2014;40:252–9. https://doi.org/10.1578/AM.40.3.2014.252.
[169] Robeck TR, Montano GA, Steinman KJ, Smolensky P, Sweeney J, Osborn S, et al. Development and evaluation of deep intra-uterine artificial insemination using cryopreserved sexed spermatozoa in bottlenose dolphins (*Tursiops truncatus*). Anim Reprod Sci 2013;139:168–81. https://doi.org/10.1016/j.anireprosci.2013.04.004.
[170] Ohta H, Izawa T. Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. Aquaculture 1996;142:107–18. https://doi.org/

10.1016/0044-8486(95)01246-X.
[171] Harvey B, Kelley RN. Chilled storage of *Sarotherodon mossambicus* milt. Aquaculture 1984;36:85–95. https://doi.org/10.1016/0044-8486(84)90056-5.
[172] Saad A, Billard R, Theron MC, Hollebecq MG. Short-term preservation of carp (*Cyprinus carpio*) semen. Aquaculture 1988;71:133–50. https://doi.org/10.1016/0044-8486(88)90280-3.
[173] DeGraaf J, Berlinsky D. Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa. Aquaculture 2004;234:527–40. https://doi.org/10.1016/j.aquaculture.2003.11.037.
[174] Guthrie HD, Welch GR, Woods LC. Effects of frozen and liquid hypothermic storage and extender type on calcium homeostasis in relation to viability and ATP content in striped bass (*Morone saxatilis*) sperm. Theriogenology 2014;81:1085–91. https://doi.org/10.1016/j.theriogenology.2014.01.035.
[175] Parodi J, Guerra G, Cuevas M, Ramírez-Reveco A, Romero F. Effects of storage time on the motility, mortality and calcium levels of Atlantic salmon *Salmo salar* spermatozoa. J Fish Biol 2017;90:1506–16. https://doi.org/10.1111/jfb.13245.
[176] Lahnsteiner F, Berger B, Weismann T. Effects of media, fertilization technique, extender, straw volume, and sperm to egg ratio on hatchability of cyprinid embryos, using cryopreserved semen. Theriogenology 2003;60:829–41. https://doi.org/10.1016/S0093-691X(02)01300-6.
[177] Linhart O, Rodina M, Gela D, Kocour M, Rodriguez M. Improvement of common carp artificial reproduction using enzyme for elimination of egg stickiness. Aquat Living Resour 2003;16:450–6. https://doi.org/10.1016/S0990-7440(03)00083-4.
[178] Billard R. L'insémination artificielle de la truite *Salmo gairdneri* Richardson. V. - effets de la dilution et définition du rapport optimum gamètes/dilueur. - V. - effets de la dilution et définition du rapport optimum gamètes/dilueur. Bull Fr Piscic 1975:121–35. https://doi.org/10.1051/kmae:1975008.
[179] Tambasen-Cheong MVP, Tan-Fermin JD, Garcia LMB, Baldevarona RB. Milt-egg ratio in artificial fertilization of the Asian freshwater catfish, *Clarias macrocephalus* , injected salmon gonadotropin-releasing hormone analogue and domperidone. Aquat Living Resour 1995;8:303–7. https://doi.org/10.1051/alr:1995031.
[180] Moksness E, Pavlov DA. Management by life cycle of wolffish, *Anarhichas lupus* L., a new species for cold-water aquaculture: a technical paper. Aquacult Res 1996;27:865–83. https://doi.org/10.1046/j.1365-2109.1996.00810.x.
[181] Fauvel C, Savoye O, Dreanno C, Cosson J, Suquet M. Characteristics of sperm of captive seabass in relation to its fertilization potential. J Fish Biol 1999;54:356–69. https://doi.org/10.1006/jfbi.1998.0873.
[182] Butts IAE, Roustaian P, Litvak MK. Fertilization strategies for winter flounder: effects of spermatozoa density and the duration of gamete receptivity. Aquat Biol 2012;16:115–24. https://doi.org/10.3354/ab00439.
[183] Sanches EA, Baggio DM, Piana PA, de Souza BE, Bombardelli RA. Artificial fertilization of oocytes and sperm activation in pacu: effects of the spermatozoa:oocyte ratio, water volume, and in natura semen preservation. Rev Bras Zootec 2011;40:1–6. https://doi.org/10.1590/S1516-35982011000100001.
[184] Siddique MAM, Butts IAE, Psenicka M, Linhart O. Effects of pre-incubation of eggs in fresh water and varying sperm concentration on fertilization rate in sterlet sturgeon, *Acipenser ruthenus*. Anim Reprod Sci 2015;159:141–7. https://doi.org/10.1016/J.ANIREPROSCI.2015.06.008.
[185] Casselman SJ, Schulte-Hostedde AI, Montgomerie R. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). Can J Fish Aquat Sci 2006;63:2119–25. https://doi.org/10.1139/F06-108.
[186] Rinchard J, Dabrowski K, Van Tassell JJ, Stein RA. Optimization of fertilization success in *Sander vitreus* is influenced by the sperm : egg ratio and ova storage. J Fish Biol 2005;67:1157–61. https://doi.org/10.1111/j.0022-1112.2005.00800.x.
[187] Nimrat S, Sangnawakij T, Vuthiphandchai V. Preservation of black tiger shrimp *Penaeus monodon* spermatophores by chilled storage. J World Aquacult Soc 2005;36:76–86. https://doi.org/10.1111/j.1749-7345.2005.tb00133.x.
[188] Robeck TR, Gearhart SA, Steinman KJ, Katsumata E, Loureiro JD, O'Brien JK. In vitro sperm characterization and development of a sperm cryopreservation method using directional solidification in the killer whale (*Orcinus orca*). Theriogenology 2011;76:267–79. https://doi.org/10.1016/j.theriogenology.2011.02.003.