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Chapter 4

Semen Cryopreservation in Brazilian Freshwater Fish: Advances and Main Challenges

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Additional information is available at the end of the chapter

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Abstract

Studies on semen cryopreservation in Brazilian freshwater fish have been growing in number of publications and investigated species. Despite this apparent increase in research, standardization of cryoprotocols is still missing, making it clear that the grounds on the quality of cryopreserved semen has not yet reached a level that guarantee satisfactory results for its replication. This chapter aims to make a critical and reflective analysis on the ways cryopreservation of freshwater fish semen has been conducted in Brazil. The difficulties in standardizing protocols, broodstock, and selection of genetically superior animals; the barriers in transferring technology from laboratory benches to the field and make feasible the use of cryopreserved semen on a commercial scale; the formation of germplasm banks and the responsible use of cryopreserved material are also discussed. We have no intention to point out the successes and mistakes that may have been committed in pursuing development of cryopreservation protocols, but a reflection on the future directions considering what should be pondered on this subject with objectivity and scientific consolidation.

Keywords: Brazilian freshwater species, sperm cryopreservation, postthaw quality evaluation, cryobanking, aquaculture, fish conservation

1. Introduction

The studies on semen cryopreservation from Brazilian freshwater fish had its beginning in the 1980s with *Prochilodus scrofa* = *P. lineatus* e *Salminus maxillosus* = *S. brasiliensis* species [1]. Basically, the freezing solution used was composed by dimethylsulfoxide (DMSO) as permeating and

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glucose as nonpermeating cryoprotectant. Other fish species have been studied at that period, such as *Rhamdia hilarii* = *R. quelen* [2] and *Leporinus silvestrii* [3].

The 1990s began with the studies focused on the species target as potential for production in the fish farming scenario at that time, the *Piaractus mesopotamicus* [4, 5]. However, during this decade, there were virtually no further researches in the area and only in 1999 a study with *Colossoma macropomum* [6] was published. However, the study considered the major milestone in fish semen cryopreservation in Brazil was carried out by Carolsfeld et al. [7], who published a series of compiled results describing the freezing protocols for native species, most of them still being used nowadays. Currently, around 20 Brazilian freshwater species have their semen cryopreserved, some even have different protocols [8], or small variations from the protocols described by Carolsfeld et al. [7].

The studies that guided the composition of a future protocol for fish semen freezing produced a lack of originality, resulting in little progress to date. Some factors have contributed to this scenario, such as: the small number of researchers working in the area; restriction of modern equipment; reduced scientific exchange with international groups and even the lack of criteria for distribution of public resources that result in numerous similar publications without significant progress.

This chapter is not intended to point out the successes and mistakes that may have been committed in the pursuit of development of cryopreservation protocols for fish semen in Brazil, but a reflection on the future directions considering what should be pondered on this subject with objectivity and scientific consolidation.

2. Extender solutions

The extenders are used to meet several requirements necessary for sperm survival during cryostorage. Its function is to provide a favorable microenvironment to maintain the viability of sperm cells, allowing the addition of energy sources (lipids, carbohydrates, and metabolites) to support the cellular metabolism, control of pH and osmolality, and prevention of bacterial growth [9].

Since many factors can influence on semen quality parameters, the extender solution must be carefully formulated [10]. The first extender mediums used for South American fish semen were composed only by 0.8% NaCl [1]. Thereafter, an improvement in the results was noticed when glucose was added to the mediums, jointly with egg yolk or powder milk [7].

Egg yolk is still frequently used as a component of extender solutions. According to Watson [11], due to the low-density lipoprotein (LDL) fraction in its composition, the egg yolk may provide a better stabilization of the sperm membrane by passing the cryopreservation stress, reducing injuries, and the thermal shock. The stabilizing mechanism of the sperm membrane by LDL possibly occurs because these compounds attract the molecules of cholesterol present in seminal plasma, thus preventing cholesterol binds to the phospholipids of the sperm

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membrane, avoiding their destabilization [12]. In *C. macropomum*, the addition of egg yolk in the extender improved sperm motility rate [13].

The composition of the extenders used for preservation of semen from South American fish is quite varied and may be based on salts, glucose, or a bit more complex composition, which includes the addition of energy substrates and antibiotics [14, 15].

The extenders used for the South American fish species are still based on solutions formulated for mammal's semen. Currently, the BTS™ (Beltsville Thawing Solution—Minitub), which is formulated for swine semen is the basic solution that has been used in the composition of extenders [16–19]. Similarly, ACP-104™ (ACP Biotechnology—UECE), which was originally formulated for goats semen was also tested in some fish species [15, 17, 20, 21]. However, seminal viability results obtained in the literature are very variable, indicating that there is a need to seek efforts to formulate specific extenders, based on biochemical composition of seminal plasma from each fish species. Knowledge on the biochemical characteristics of the seminal plasma is essential to understand the spermatozoa requirements, assisting in the preparation of appropriate extender solutions for both short and long-term cryostorage.

3. Cryoprotectants

For performing cryopreservation, either by slow freezing or ultrarapid techniques, the use of permeating and nonpermeating cryoprotectant agents (CPAs) is required [22]. However, CPAs can be very toxic depending on the concentration used and cell exposure to them before freezing should be controlled [22].

The nonpermeable CPA most used by Brazilian researchers for semen from scale fish species is dimethylsulfoxide (DMSO) [8], whereas for nonscale fish species methanol is the most commonly used CPA [7, 23]. In the last years other CPAs have also been tested, such as methyl glycol, glycerol, ethylene glycol, and dimethylformamide (DMF) [8]. We understand that testing new CPAs should be encouraged in research. However, it is important to consider some aspects and thus avoid wasting of time and money. DMSO is no doubt the CPA that has shown the best results to date. We must understand very well the chemical and physical behaviors of a given reagent at low temperatures as well as its permeability and toxicity to sperm cells to be worth DMSO's replacement.

Glucose, as a low molecular weight nonpermeable CPA has been widely used in most protocols in Brazil. However, it is still quite common to observe its use for the formulation of cryoprotectant solutions with egg yolk [8]. Among the cryoprotective effects of glucose on sperm cells are dehydration before cooling, leading to less intracellular ice crystal formation, the increase of effective viscosity of the media, and serving as a protector to membrane integrity. Importantly, glucose is a component commonly added to the cryoprotectant solution and its function as an energy supplier to the spermatozoon and/or cryoprotective action will depend on the concentration used.

We consider as a decisive factor for the composition of the cryomedium not only the choice of cryoprotectants, but also their chemical quality. Although it appears to be negligible, this fact is crucial for a proper and successful protocol. There has been a significant improvement in the supply of chemical reagents, but the delivery time and costs remain a limiting in some parts of the country.

The large number of farmed fish species nowadays in Brazil may be one of the causes of using a high range of different CPAs. However, a standardization of cryoprotocols within a given species should be searched.

4. Freezing and thawing rates

Freezing and thawing rates are determining factors in a gamete cryopreservation protocol. A standard set for the protocols adopted for the South American native fish species is something lacking. In the published studies, we can observe different freezing and thawing rates for different species and even for the same species. Surely, this implies difficulty of adopting a technology that could be replicated and used efficiently by a farmer or even a trained technician. The use of a dry shipper has been established as a common method in research with semen cryopreservation in Brazil [8], since is considered a safe and practical method [24]. Moreover, the dry-shipper container used in research produces similar freezing rates and storage temperatures [13, 25, 26]. However, it is in the thawing rates that the largest variations are noticed, since different temperatures and thawing times are used [8]. For example, Velasco-Santamaría et al. [27] tested 0.5, 1.8, 2.5, and 4.0 ml straws for freezing of *Brycon amazonicus* semen and the results indicated that an increased in thawing temperature from 35 to 80°C for 10 or 90 s could influence sperm motility and fertilization rate. However, for *P. lineatus* the thawing temperature may vary from 30 to 60°C for 8 or 16 s, respectively, without major changes in motility rate when using 0.5 ml straws [18]. For *C. macropomum* semen, a thawing temperature of 45°C for 5–8 s [20, 24] when using 0.25 ml straw and for 0.5 ml straw 37°C/30 s or 60°C/8 s was used [13, 21]. However, at larger scale using 1.6 or 4.5 ml straws these rates can be of 60°C for 90 s [25].

Note that the size of the straw can affect the quality of cryopreserved semen [28] as well as the temperature used for thawing, since high temperatures can denature enzymes and proteins of sperm cells [29]. Therefore, it is necessary to search for a balance between thawing rate and especially size of the container, to be able to maximize the use of cryopreserved semen and have a product in quality and quantity [25].

5. Activating solutions

By having a limited supply of ATP accumulated in the mitochondria [30], the motility duration in freshwater fish spermatozoa is greatly reduced. Motility is a variable that significantly alter the sperm's ability to fertilize the oocyte [31]. Therefore, the use of balanced osmotic media

with substrates that can provide energy to the sperm is desirable [23, 32, 33]. The activating solution may also assist in the reduction of osmotic shock, which is a causative effect of damage in sperm cells [34, 35].

Examples of the use of activating solutions in Brazilian research can be found for *R. quelen* semen [23] using a solution composed of fructose, and for *Pseudoplatystoma corruscans* semen [7] using 1% $NaCHO_3$ solution. For scale fish, the use of solutions based on NaCl (0.29%) and $NaHCO_3$ (1%) are shown to have better results [8].

It is noticed that the use of activating solutions is still not as common in Brazil and the previous studies are focused on the use of solutions based on salts or sugars. Fundamentally, future Brazilian researches should explore the biochemical composition of seminal plasma and ovarian fluid. This knowledge will be instrumental in building a successful activating solution.

6. Artificial fertilization

Most papers on seminal cryopreservation from South American fish do not have the data of fertilization rates using cryopreserved semen [8]. The final validation of applying this technique necessarily culminates with the results of fertilization and hatching rates. Nevertheless, to reach this goal some steps must comply in order to create a favorable environment allowing the encounter of the gametes to occur in an efficient and safe manner. In Brazil, in general the most basic aspects involving fish gametes manipulation are still neglected on fish farms. There are preeminent need to establish guidelines for best practices in fish handling during gametes collection and all subsequent semen handling until freezing.

Generally, fertilization rates are lower when using cryopreserved semen, which is a function of reduced quality of sperm cells. Regarding the lack of studies assessing the fertilization and hatching rates, we can relate: the difficulty of having oocytes available for carrying out the tests considering the maintenance costs of a broodstock; experiments poorly designed and lack of methodology standardization in the studies. It is noteworthy that even a motile sperm is no guarantee of fertilization. From more elaborated analysis, discussed in the next topic, it is possible to check for sperm membrane or DNA damage and how such damage can affect their fertilizing capacity.

Indeed, for development of a commercial protocol using cryopreserved semen in Brazil, even on a small scale, an evolution in the control of the above-mentioned processes will be required. If the ultimate goal is to get a good hatching rate (live larvae), that is directly related to the quality of biological material to be cryopreserved, asepsis of facilities and equipment and an efficient cryopreservation protocol. We must consider that the quality of oocytes should be excellent, ensuring that this is not a factor that negatively interfere to the success of the procedure. Are there any criteria for assessing the spawning quality of fish species studied in Brazil? The answer is no, there are not! Nowadays, there is no effective technique to evaluate the quality of oocytes in fish farming in a practical and affordable way.

The inseminating dose (spermatozoa/oocyte ratio) is an important feature that starts to be focused in some studies [36–38]. The entire control of the artificial fertilization environment and optimizing the use of inseminating dose should be the goal for the near future.

7. Main methods used to evaluate cryopreserved semen

7.1. Sperm quality evaluation in fish

Cryopreservation, although very useful from a production and conservation point of view, produces several types of damage to germ cells, especially to spermatozoa. Therefore, during the last decades huge efforts were done trying to reduce cryodamage, identify its causes and consequences to the sperm cell.

There are several constituents in a germ cell that can be analyzed. These analyses depend on the objective, possible applications, and on the methodology and available equipment. They can be from a simple motility analysis using a subjective scoring (normally used in fish farms) to more sophisticated analysis of motility, involving specific software (e.g., CASA system) or image analysis systems (Image J) [39]. Sperm quality can also be assessed by its constituents: seminal plasma and spermatozoa, indicating damage in specific cellular structures or in the entire cell, by releasing its constituents in the seminal plasma [39]. Determination of seminal plasma constituents and its variations such as lytic, oxidative, metabolic and apoptotic enzymes, metabolites, sugars, vitamins, amino acids, fatty acids, and other inorganic compounds can provide very useful information on sperm status [39]. Other characteristics of these cells should also be evaluated in order to assess sperm fertilization ability, especially when this assay is difficult to perform due to egg availability or long-term embryonic development. Most tests describing cell viability or mitochondrial status are currently performed with the use of fluorescent probes combined with microscopy or flow cytometry [40]. There is a huge list, depending on the main objective and available supplies and equipment, and can be from the determination of reactive oxygen species (ROS) levels, MDA concentration, detecting oxidative events at protein or lipid level, or simple cell structure impairments or ruptures. The assessment of sperm DNA is another test that should be performed since will provide information on sperm fitness being important for offspring quality. Methods to evaluate chromatin integrity include the comet assay (single cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase-nick-end-labeling), SCSA (sperm chromatin structure assay), and the analysis of specific DNA sequences using qPCR [41–43]. In recent years, and as an attempt to define bases for explaining other sperm quality markers at different levels, the characterization and presence of certain transcripts has been used [43]. These new tests can reveal the true meaning of spermatozoa quality.

All these techniques, although very useful in the evaluation of sperm quality and offspring viability, are still on a laboratory scale and need to be adapted for industry. Efforts are still required to transfer current technologies and make sperm banking accessible for fish producers [44]. In the last years, some national and international networks have been trying to get close contact between researchers and fish farm industries [45]. Although, there are still a lot

of work to do, this topic should be considering as a priority step. It is also clear that although fundamental research is moving forward in the design of new methods and techniques to characterize sperm quality, it is important to focus on a process of scaling-up technologies to encourage companies and fish farmers in their use. Aquaculture needs to benefit from reproductive biotechnology.

7.2. Quality evaluation in sperm from Brazilian species

According to Viveiros, Orfão [8] there are 18 Brazilian native species with high potential for cryopreservation, and indeed, several protocols have been developed and the postthaw quality assessed in most of those species (**Table 1**). Most of the work has been conducted in three main species, *P. lineatus, Brycon orbignyanus*, and *C. macropomum*. The first two more dedicated to conservation and slightly less to production and the *C. macropomum* more dedicated to production. In terms of postthaw quality analysis, research conducted in the last years, especially in these species gained high relevance and allowed to detect specific damage associated with cryopreservation. A subjective analysis of motility is still the main parameter analyzed, which in some cases can be responsible for the high variability of results obtained by different groups working in the same species [44]. The complementary analysis of other parameters such as cell viability using flow cytometer of fluorescent microscopy can help in this matter. Recent work in the previous mentioned species allowed to distinguish methyl glycol as the best cryoprotectant for *P. lineatus* and *B. orbignyanus* in terms of motility (63 and 72% motile cells, respectively) and cell viability (57 and 68% viable cells, respectively) [46]. A similar analysis concluded that DMSO and DMF (5 and 8%, respectively) were the best choices for freezing *C. macropomum* sperm, yielding motility and viability rates higher than 50%. Although, some damage was detected in postthaw samples, fertility, and hatching rates were not compromised (91.6 and 87.6%) [24, 47]. Therefore, it seems clear that although it is very important to perform a complete analysis of sperm using several indicators of quality such as motility, cell viability, and functionality and DNA status, fertility, and especially hatching rates are the ultimate tests to characterize the success of any cryopreservation protocol. These assays should be performed in order to demonstrate the viability of this technology to fish farmers, showing its applicability in aquaculture industry.

Species	Assessed parameter	Level of knowledge[1]	References
Brycon amazonicus	Motility (Subjective)	Low	Casallas, Robles [56]
	Motility (Subjective)		Cruz-Casallas, Medina-Robles [57]
	Motility (Subjective), fertilization rate		Velasco-Santamaría, Medina-Robles [27]
Brycon insignis	Motility (Subjective-CASA)	Low	Viveiros, Orfão [58]
	Motility (Subjective), viability		Viveiros, Amaral [59]

Species	Assessed parameter	Level of knowledge[1]	References
Brycon nattereri	Motility (Subjective), ultrastructure	Low	Viveiros, Maria [19]
	motility (Subjective)		Oliveira, Viveiros [60]
Brycon opalinus	Motility (Subjective), viability	Low	Viveiros, Orfão [58]
Brycon orbignyanus	Motility (CASA)	Medium	López, Leal [61]
	Motility (CASA)		Viveiros, Gonçalves [62]
	Motility (CASA), viability, mitochondrial functionality		Viveiros, Nascimento [46]
	Motility, morphology (Subjective)		Andrade, de Jesus [63]
	Motility, morphology (Subjective)		Galo, Streit Jr [64]
	motility (Subjective)		Viveiros, Maria [17]
	Motility (Subjective), fertilization rate		Maria, Viveiros [16]
	Motility (Subjective)		Carolsfeld, et al. [7]
Brycon orthoenia	Motility (Subjective)	Low	Melo and Godinho [65]
Colossoma macropomum	Motility (Subjective), fertilization rate, DNA methylation	High	de Mello, Garcia [66]
	motility (CASA)		Melo-Maciel, Leite-Castro [67]
	Motility (Subjective), fertilization rate, viability, mitochondrial functionality, DNA integrity		Garcia, Vasconcelos [20]
	Motility (Subjective), fertilization rate, viability, mitochondrial functionality, DNA integrity		Varela Junior, Goularte [24]
	Motility (Subjective), fertilization rate, viability, mitochondrial functionality, DNA integrity		Varela Junior, Corcini [47]
	motility (CASA)		Leite, Oliveira [21]
	Motility (Subjective)		Menezes, Queiroz [68]
Leporinus macrocephalus	Motility (Subjective)	Low	Ribeiro and Godinho [69]
Leporinus obtusidens	Motility (Subjective)	Low	Viveiros, Maria [17]
	Motility (Subjective), fertilization rate		Taitson, Chami [26]
Piaractus brachypomus	Motility (Subjective-CASA)	Low	Nascimento, Maria [70]

Species	Assessed parameter	Level of knowledge[1]	References
Piaractus mesopotamicus	Motility, morphology (Subjective)	Low	Andrade, de Jesus [63]
	Morphology (Subjective)		Paulino, Murgas [71]
	Motility (Subjective), morphology (Subjective)		Streit Jr, Benites [72]
	Motility (Subjective)		Carolsfeld, et al. [7]
Prochilodus lineatus	Motility (CASA)	Medium	Viveiros, Gonçalves [62]
	Motility (CASA), viability, mitochondrial functionality		Viveiros, Nascimento [46]
	Motility (Subjective), morphology (Subjective)		Vasconcelos, Felizardo [73]
	Motility, morphology (Subjective)		Andrade, de Jesus [63]
	Motility (Subjective)		Navarro, Navarro [74]
	Motility (Subjective)		Paula, Andrade [75]
	Morphology (Subjective), fertilization rate		Miliorini, Murgas [76]
	Motility (Subjective), morphology (Subjective)		Felizardo, Mello [77]
	Motility (CASA), fertilization rate		Viveiros, Nascimento [15]
	Motility (Subjective), fertilization rate		Viveiros, Orfão [78]
	Motility (Subjective)		Viveiros, Maria [17]
	Motility (Subjective)		Murgas, Miliorini [79]
	Motility (Subjective)		Carolsfeld, et al. [7]
Pseudoplatystoma corruscans	Motility (Subjective), fertilization rate	Low	Cruz-Casallas, Medina-Robles [57]
Rhamdia quelen	Motility (CASA), fertilization rate	Low	Adames, de Toledo [23]
Salminus brasiliensis	Motility (Subjective), fertilization rate	Low	Viveiros, Oliveira [18]
	Fertilization rate		Zanandrea, Weingartner [80]
	Motility (Subjective)		Zanandrea, Weingartner [81]

[1] Level of knowledge according to the used methodology on the postthaw sperm analysis of each species. Data have been collected from Web of Science platform since papers published from 2000 onwards by using as key words "species name + cryopreservation."

Table 1. Methods used for postthaw evaluation of sperm quality in freshwater Brazilian species.

8. Difficulties in broodstock standardization and acquisition of wild or genetic improved fish

Standardizing a broodstock requires much more than just buying animals from different locations. To address this issue there is a need for a holistic knowledge of the whole process. The technical efficiency of a germplasm bank goes far beyond the knowledge of physicochemical characteristics of semen or a well-performed cryopreservation protocol. The first thing to be defined is the goal of germplasm bank: for restocking or fingerlings production to fish farming. When the goal is to restock environments, the most important aspect is the bank composition by animals that have the greatest possible genetic diversity. We should include here wild animals, even though they do not have a good seminal quality, but that make up the nearest setting of natural environment. On the other hand, when we set up a broodstock for commercial production of fingerlings we should seek animals with a greater genetic standardization, once the results will also have less variation. Thus, to establishment of a commercial broodstock we should analyze the genetic similarity degree of the animals before selecting them.

Insert wild fish in a broodstock whose objective is to produce fingerlings for fish farming can be a terrible mistake. Wild animals can carry parasites and therefore introduce them in the farm broodstock. Another important factor that should be considered is the broodstock domestication. According to Ruzzante [48], domestication is a process of adaptation of organisms in the man-made environment. We had an interesting experience a few years ago, when we use a semen bank from wild *P. mesopotamicus* to produce fingerlings from eighth-generation captive females. The generated fingerlings were darker and presented a different behavior compared to those animals produced from domesticated parents. It is important to note that this was just an observation and not an experiment.

The formation of a germplasm bank should be conducted with the support of molecular techniques, which are still timidly used in Brazil [49]. Although being observed a high degree of genetic variability in broodstock of native species that have been formed in the country, their future efficiency may be compromised. We can cite as example the *C. macropomum* broodstock from two large farms in northern Brazil, where there was no problem of genetic variability, even with both having the same founder effect [50]. In another example, Jacometo et al. [51] reported that in addition to the high genetic variability in *C. macropomum* broodstock it was also observed a moderate differentiation and low genetic distance within themselves. The broodstock formation in this species occurred in 1972 [52].

The broodstock of *B. orbignyanus* [53] and *P. mesopotamicus* [54] used for restocking showed no genetic variability problems. We emphasize that even not having a compromising genetic variability degree it is important to consider the degree of genetic distance of the animals to be used as semen donors; once by accident the collection of semen from sibling animals can occur, which could be compromising depending on the ultimate goal of the germplasm bank.

This context serves to show how much we should be careful in setting up a germplasm bank from a random broodstock. It must be considered from the selected animals: the purpose of

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the genebank, age, genetic origin, and location; consanguinity degree, etc. A good application example of these concepts can be found in the article published by Streit et al. [55], in which two germplasm banks were created for use in breeding programs of two Brazilian fish species, *C. macropomum* and *Pseudoplatystoma reticulatum*.

9. The challenge of making viable the use of cryopreserved semen in Brazil

The first question to be asked is: Are there any germplasm banks in Brazil? Yes, there are! They began to be formed in the 1980s by Professor Hugo Godinho and the visit of two Canadian researchers, Brian Harvey and Joachim Carolsfeld, who started extensive work with Brazilian researchers. Among their actions, they have developed cryoprotocols for some species, already recorded in this chapter [7]. For over 30 years some banks of native species semen were formed and has been used mainly as deposit of genetic material for research at universities laboratories and other ones as genetic reserve for restocking (personal communication from: Bombardelli, R.; Maria, A.N.; Murgas, L.D.; Ninhaus-Silveira, A.; Resende, E.K.; Ribeiro, R.P.; Streit Jr., D.P., and Viveiros, A.).

A relevant question concerns the active use of germplasm banks in the country, either for commercial purposes (fish farming) or conservation. Popularize the use of cryopreserved fish semen in the country as far as the use of bovine or equine semen, for instance, is still far away. Some conditions are crucial to this fact: the culture of fingerlings producers not to use a germplasm bank; have actually consistent cryopreservation protocols that can be used effectively; unfamiliarity of the market to absorb the product (frozen semen) and especially the real need for the use of cryopreserved semen in fingerling production farms.

Almost all Brazilian farms are unaware of the genetic characteristics of their broodstocks. Thus, there would be no sense to use semen from another location without any objective criteria to explore genetic gain. Moreover, it is significant that most of the Brazilian migratory species present reproductive asynchrony in captivity. It is quite common to note that at the end of reproductive season there is availability of mature females but not males. Thereby, the germplasm bank could meet this need. However, what is the cost to maintain a germplasm bank? There would be any market demand? Are there concluding protocols ready, tested, and efficient to be used for the main migratory species that are farmed? The last question is not that simple to answer. The quality parameters from cryopreserved semen still vary widely. From this reflection, we must establish priority in research in order to meet the demands, even if they are as wide as the number of species that make up our ichthyofauna. Focus research efforts to establish a secure protocol for commercial application purposes should be a goal.

The germplasm bank significantly facilitates the establishment of a breeding program for fish [55]. Surely, the semen distribution from known strain animals will contribute to spread of genebanks and there may be a specific market to the product cryopreserved semen. We believe that in the future the industry will be aged enough to realize it, especially those fingerlings buyers that will seek fish with known pedigree, even if it is necessary to pay a little more for them. Soon, the company that offers high-quality genetic material can explore this market.

Regarding germplasm banks with restocking purposes, most are circumstantial. They have been created through research and development (R&D) projects, largely in partnership with electric power companies. These gene banks are maintained while the project is running and later are reduced to a small number of samples (as a collection), especially due to maintenance costs. The genebanks that are intended for restocking, nowadays mostly consist of semen from species that are also farmed. On the other hand, it would need to extend to other species, such as the large Amazon catfishes: *Phractocephalus hemioliopterus; Brachyplathystoma filamentosum* and *B. Flavicans*, and others as *Zungaro jahu, Surubim lima* and *Hemisorubim platyrhynchos*; in addition to the species endangered by numerous factors such as the construction of hydroelectric dams, overfishing, and pollution of the aquatic environment.

10. Conclusions, perspectives and future challenges

Although, cryopreservation of Brazilian species has been improving in the last years with more publications coming to this field of research and especially with a far advanced analysis on cryodamage, there is still a long way to follow. This is truly mainly in those species where commercial interests and aquaculture benefits can arise or in species where conservation is a main challenge. One of the main objectives to pursue is for sure a more exhaustive analysis of sperm quality, adapting techniques that are already used in other species. This will give guarantees of quality for producers that will set out the best protocols defined. Another point to take in consideration is standardization of procedures and reporting, not only related with cryopreservation protocol, but especially with the analysis performed. This lack in standardization has been already identified in some of the most reported species around the world and over the last years it has been a struggle to joint researchers in following certain rules. Building a database of standardized information for semen analysis would be crucial. What has been discussed throughout this chapter has significant relevance in the compression of events that concern fish semen cryopreservation in Brazil. The advances made in recent years by efforts of research groups are commendable. However, there still a lot to do, and joint efforts through networking activities can be a good opportunity, not only for training young researchers and technicians, but also to find solutions to the main problems.

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