New Chapter

Process Pathways for Cryopreservation Research, Application and Commercialization

Terrence R. Tiersch

Introduction

The bulk of all cryopreservation work in aquatic species over the past half century has addressed research-scale efforts at protocol development. This has yielded countless (often idiosyncratic) protocols that have been assembled in a number of outlets (e.g., Lahnsteiner 2000, Tiersch and Mazik 2000, Cabrita et al. 2009). To proceed into the next half century it will be useful to view the activities involved in cryopreservation as belonging to a series of process pathways that can be developed, refined and optimized, and also extended, broadened and envisioned in reverse. It is far less useful to collapse things down to empirical tinkering with the minimal cryobiological parameters (e.g., cryoprotectant and cooling rate) and to try to shoehorn application down this narrow research construct. By this I mean that if true commercial-scale cryopreservation is a goal, sample collection, refrigerated storage, and shipping methods will become just as important as cryoprotectant, and control of sperm-to-egg ratios and calculation of a fertilization unit will become just as important as cooling rate. The related activities that come before and after the actual freezing will be as important as the freezing in dictating overall cryopreservation success. Another important concept that emerges from developing a perspective based on multi-step pathways, is the recognition that cumulative damage (small, sequential, individually insignificant losses in gamete quality) can be just as debilitating to overall success as catastrophic damage (major quality losses that occur in only one or two failed steps).

Thus, cryopreservation becomes a *system of thinking and decision making* based on identification and integration of the activities necessary to propagate robust pathways. And, these process pathways can be configured in general or specific forms. This chapter will address three generalized, exemplary forms to serve as templates for future use and adaptation (Table 1, next page). The first pathway represents an ordered series of experiments and activities used to develop practical methods for species including those that are previously unstudied. It essentially is based on accepting samples in their arrival condition and trying to gain as much useful information as possible to identify problems and opportunities for that particular species. This is the basic approach followed in my laboratory for the past two decades in initial work with more than 100 species of fish and shellfish.

The second pathway builds directly upon the first by linking research results into standardized protocols that can be routinely applied. This pathway also includes additional research necessary to improve gamete quality before collection and after thawing, and thus inherently represents an optimization process focused on improvement of the entire process. *Although this includes optimization of individual steps, it should not do so at the expense of the overall results.* For example, use of a highly specialized container might provide an advantage in post-thaw motility, but if it compromised overall throughput (e.g., handling time), labeling quality, or storage efficiency this container would not be considered to offer overall improvement, and would not be incorporated in the application pathway (perhaps being reserved for research only).

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Table 1. Summary overview of three generalized inter-related pathways for the activities involved in initial development of cryopreservation protocols, small-scale application of the protocols, and commercial-scale application for eventual industry development for genetic resources of aquatic species. These pathways are addressed in more detail in other chapters (e.g., Hu and Tiersch, *Development of High-throughput Cryopreservation for Aquatic Species*). Research steps are indicated in bold.

A Research Pathway (Protocol development)	***An Application Pathway*** (Protocol utilization)	***A Commercialization Pathway*** (Programmatic development)
Gamete collection	**Improve male quality**	**Scaling up for high throughput**
Preliminary assessment	**Shipping**	**Commercial-scale facilities**
Initial motility estimation	Gamete collection	Gamete quality assessment
Dilution & choice of extender	Preliminary assessment	**Quality control & assurance programs**
Estimation of sperm concentration	Initial motility estimation	**Standardization & harmonization**
Motility duration	Estimation of sperm concentration	Establishment of markets
Osmotic & ionic activation curves	Dilution with extender	**Valuation & pricing schedules**
Refrigerated storage	Refrigerated storage & shipping	Providing product for customers
Acute toxicity	Cryoprotectant & equilibration	Providing services and training
Equilibration time	Equilibration motility	**Providing genetic improvement**
Equilibration motility	Labeling, packaging & cooling	**Providing equipment, supplies & reagents**
Packaging & cooling rate	**Storage, inventory & database**	Providing storage & shipping services
Thawing rate	Thawing & post-thaw amendments	Labeling, storage, inventory & databases
Post-thaw amendments	Post-thaw assessment	Biosecurity programs
Post-thaw assessment	Fertilization	Material transfer agreements
Fertilization assays	**Growth & survival**	Treaties & regulatory frameworks
Dilutions & concentrations	**Quality control points**	Access & benefit sharing agreements
Fertilization "unit" calculations	**Biosecurity protocols**	

The third pathway, unlike the first two, is mostly theoretical as we do not yet have large-scale commercialization in aquatic species cryopreservation, nor do we have industrial-scale markets for germplasm and genetic resources. Lastly, I will apologize here in advance for presenting the material below from a personal perspective based on our experience. These views are not universally shared (as is true for any specific research approach), but do represent a tested and reasonable starting point for others to build upon.

A Research Pathway (Protocol Development)

The basic methods and safety information presented in the previous original chapter (Wayman and Tiersch) did not require significant updating because not much has changed in these areas over the past decade. However, in light of the needs for application of technology, it will be useful as we go forward to focus on assembling these activities into standardized, practical platforms. The pathway described below introduces the steps that can be used to establish a basic protocol for a previously unstudied species, or to develop your own protocols after becoming familiar with the literature already available for that species or closely related taxa. However, beware: cryopreservation results are often difficult to transfer from one laboratory to another through published reports due to a burdensome lack of standardization in protocols and reporting (Tiersch et al. 2007, Yang et al. 2010). The collection and study of samples on-site or shipped from a remote location are addressed. We usually plan on addressing all of these activities and studies within a 2-d period with a two-person team, and can streamline the pathway if necessary to fit into a single, long day.

Gamete Collection

Usually sperm from ripe males can be obtained during the spawning season by either stripping or by crushing of dissected testis. Stripping of sperm involves collection directly from the male into a sterile tube. Care must be taken to avoid contamination of the sperm with dirt, feces, water, or urine. Dissection usually involves killing of the male and careful removal of the testis with scissors and forceps. Care must also be taken to avoid contamination with bacteria due to cutting of the intestine. Once removed, the testis is rinsed with extender (or the best guess as to something harmless such as isotonic saline for previously unstudied species) to remove blood, and extra tissues are dissected away. The cleaned testis is weighed and placed in a container with an appropriate amount of extender solution (typically a buffer) before it is crushed. The sperm are released and the solution is filtered to remove pieces of tissue.

Application of pressure under the pectoral fins is often sufficient to induce sperm flow in ripe males and avoids the problems associated with urine and fecal contamination caused by application of pressure along the abdomen in the traditional stripping motion (Tiersch et al. 1997). If working with an unstudied species for which there is no known extender, it may be necessary to aliquot each sample into two or three different extenders and to keep some undiluted to hedge your bets – this is easier at the start of a long spawning season, working with a plentiful species, rather than having a 3-d window to perform research on an endangered species in a remote location. As such, movement through this pathway needs to be and can be adjusted to suit the current situation of the research environment (i.e., there is no one way to do things, but the overall goal remains the same).

Decisions on whether to pool samples from individual males should be made with the overall goal of the project in mind. If the time and economic resources are available, it is most

informative to maintain the samples as separate individuals, especially for research. Alternatively, if the goal is for bulk production of fish, or if there are space limitations in a hatchery that would disallow maintenance of separate, multiple groups of fish it would make more sense to pool the samples. Pooling may also be necessary to provide sufficient volume for automated processing or to save time in processing. It should be noted, as described elsewhere in this volume, that pooling of sperm samples from several males is not a reliable method to produce genetic variation in offspring.

Preliminary Assessment of Samples

Samples should be evaluated when they are collected, before they are shipped, and again immediately upon receipt. With sperm, percent motility and general characteristics (e.g., color, presence of gelling) can be easily evaluated. The presence or absence of odors should be noted, and the temperature of the samples should be measured upon arrival. With experience, a quick assessment of sample quality and sperm concentration can be made by eye. It is important to discern if the samples could have been damaged or contaminated during shipment, for example by water from ice melt making its way into loosely sealed containers, or by inadvertent freezing due to use of -80 ^{o}C packaging (which is usually not necessary). It is often useful (probably essential) to include a sample of the ambient water the organism was collected from. This can be invaluable for the detective work associated with solving problems posed by new species or unanticipated results.

Initial Motility Estimation

Before proceeding with further work it is useful to "triage" samples immediately upon collection or receipt. In this way informed decisions can be made with respect to experimental design by identifying the number and quality of the available samples. A small aliquot of sample can be activated and assessed qualitatively for motility. It is important to ensure that sufficient dilution and mixing of sperm is used to elicit maximal activation for each sample. For estimation of percent motility, only sperm that are actively swimming in a forward motion should be included (some consider this a conservative estimate). Sperm that remain in place with only a vibratory movement should typically not be included (this can depend on the species). The procedure can be practiced to ensure that sperm movements are not due to swirling of the activating solution or random movement. Some microscopic organisms (e.g. bacteria) are motile and can be mistaken for sperm by inexperienced observers (Jenkins and Tiersch 1997).

It is important to identify if the samples are of poor quality early in the research process, and to try to elucidate where and when quality reductions occurred. This is especially important for shipped samples and the detective work will benefit from good communication with the sender. It is wasteful to pre-label 200 containers for an experiment planned for use of samples from ten males only to find that 8 of the samples were ruined by inadvertent freezing during shipping caused by poor packaging. It would be even more wasteful to unknowingly use the ruined samples for experiments that produce spurious results. It should be noted that sometimes samples arrive with poor motility, but can "rebound" somewhat over the next 24 hr. This is often the case for tubes that are completely filled without an airspace. Splitting the sample into two half-filled tubes can sometimes provide necessary aeration.

If working with an unstudied species, this is a good opportunity to make some preliminary observations related to choice of extender. These rough observations can be used to narrow down the appropriate test conditions for the next studies. For example, if aliquots of the

samples were placed into different experimental extenders upon collection, now is a good time to identify those that merit further study (and those that do not). Also it is important to be aware that in some cases, ambient environmental conditions can influence the motility characteristics of sperm. Fish collected at different sites or held under different conditions (such as salinity) can display variation in activation and duration of motility (Tiersch et al. unpublished). New species may require some detective work simply to identify conditions that produce sperm activation. A tip here is that if the sperm appear to be in good morphological condition, different activation solutions can be surveyed (including ambient water) and the slides topped with a coverslip. Activation can be delayed in some species such as a 30-min delay observed for coral sperm (Hagedorn et al. unpublished).

Dilution and Choice of Extender

The term "extender" refers to a solution of salts, sometimes including organic compounds such as sugars that help to maintain sperm viability prior to and during the freezing process (e.g., Hanks' balanced salt solution, HBSS). The nature of the effect of extenders is largely based on the control of osmotic pressure, pH, and ionic concentration as well as a supply of energy, and can extend the functional life and fertilizing capability of the sperm. Usually, the extender is a balanced salt buffer of specific pH and osmotic strength. Sometimes other components such as egg yolk and milk are added, but they usually offer little benefit and can interfere with viewing of the samples with a microscope.

Extenders have been developed for many species. A single extender, HBSS, has been used successfully in our laboratory with sperm of more than 100 species, but simple solutions such as 1% unbuffered salt (sodium chloride) have been used with good results at least for short-term (i.e., < 24 hr) storage. With appropriate testing, extenders can be prepared in large batches and be stored frozen until use. Use of extenders provides increased storage time and dilutes the sperm to a greater volume, making the sperm easier to work with. Extenders can be sterilized by passage through a filter or by autoclaving (if this does not affect the ingredients) and should be refrigerated. This is especially important for extenders that contain sugars. Antibiotics can be added to extenders to reduce the growth of bacteria that reduce sperm viability. Antibiotics can be toxic to sperm cells, and therefore should be optimized for each species. In addition, they can provide a false sense of security and lead to careless sample collection and handling. Dilution studies are placed ahead of sperm concentration estimation in the research pathway because undiluted sperm can rapidly lose quality (Jenkins et al. 2011). This must be established early for previously unstudied species.

Dilution after sperm collection is necessary to optimize the volume for efficient use, to counteract the effects of urine contamination, and for research of various factors. Dilution is often in the range of one part sperm to one to four parts of extender solution. However, excessive dilution of sperm samples (e.g., 1:50) has been found to reduce sperm motility in mammals, fish and oysters (e.g., Paniagua-Chavez et al. 1998). Samples collected by stripping can be contaminated by urine which can activate the sperm and reduce the storage lifetime. For example, urine of freshwater fishes is hypotonic to the body tissues and would activate sperm by reducing the osmotic pressure of the sample. Dilution of these samples in appropriate extender can counteract the activation by urine (usually by moving the osmotic pressure back to the isotonic range).

Samples collected by dissection and crushing of the testis may not benefit as much from dilution in extender due to the lack of urine contamination, and these samples may respond best

to a lower dilution ratio in extender, or no dilution (i.e., if a satisfactory extender has not been identified). Such samples can be diluted somewhere in the cryopreservation process, for example, just prior to addition of cryoprotectant to minimize time in extender. Overall, the decision to dilute or not, and at what ratio is driven in practice by the need to hold samples for at least 24 hr. This is to enable shipping or to avoid rushing during processing, especially when work can be done most efficiently in batches rather than with single individuals.

Estimation of Sperm Concentration

This is an extremely important factor in standardizing work and ensuring quality, but it is usually overlooked or not reported. There are actually three components relevant to this factor. The first is to *estimate the concentration* by some reliable method, such as by use of a counting chamber (e.g., hemocytometer), or spectrophotometry (see chapter by Cuevas-Uribe and Tiersch in this volume, Tan et al. 2010, Dong et al. 2005a). Second is to then *adjust the concentration* to some established value. Third is to accurately *report these concentrations*. Most studies do not estimate concentration, some make estimates but do not make adjustments, and as such, these values are not reported.

From our work over the past 15 yr, there is no doubt that uncontrolled variation in sperm concentration will affect results either by directly reducing post-thaw quality, or by introducing considerable levels of uncontrolled male-to-male variation based on sperm density, not on the response to cryopreservation *per se*. It is possible for high sperm concentrations (e.g., 10^9 and above) to overwhelm the amount of cryoprotectant available. This effect is variable with the relationship between the concentration of sperm and concentration of cryoprotectant, but with a consistent result (i.e., too little cryoprotectant for the number of sperm).

Therefore, sperm concentrations should be set at an optimized value in relation to the cryoprotectant concentration. This value should take into consideration the cryoprotectant toxicity (which will limit cryoprotectant concentration), equilibration time (which should be sufficient for cryoprotectant permeation, while allowing sufficient handling time for samples, yet not be so long as to produce toxic effects), container type (chosen for ease of handling, labeling, and storage), cooling rate (which interacts with cryoprotectant and container), and practical constraints in costs, storage space, shipping capabilities (samples that are excessively dilute require extra containers and space for a given number of sperm), and use for fertilization (avoiding shortages or waste of sperm on a per-female or per-egg basis). This may seem like a lot of factors to balance, but these factors do not disappear if ignored, and as such should be directly addressed. If reliable research results and post-thaw quality are to be goals, the first step is to estimate the sperm concentration.

Motility Duration Studies

As part of the process of working with a new species, or simply assessing samples after collection or shipment, it is useful to identify the duration of motility. As stated previously, motility (especially peak motility) is often short-lived (e.g., < 30 sec). However, it is not unusual for continuous motility to last for longer periods, for example in oysters (Paniagua-Chavez et al. 1998) or live-bearing fishes such as *Xiphophorus* (Yang et al. 2006). The longer duration offers benefits in handling and makes motility assessments easier and more accurate. A straightforward experimental method to characterize motility duration involves two people. An experienced technician can activate the sperm on an uncovered slide and estimate motility at specific time

intervals, while another technician records the values and prompts the estimates at the appropriate times. This experiment can be repeated and the results graphed (Figure 1).

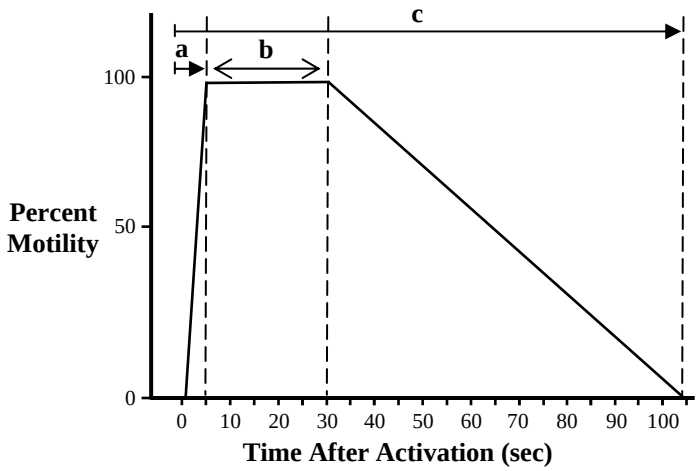

Figure 1. Diagrammatic characterization of sperm motility for a representative freshwater fish. Motility can be divided into three periods: a) time until complete motility (activation); b) duration of complete motility, and c) time until cessation of motility.

This knowledge will benefit planning of experiments by clearly indicating the timing involved for motility estimation or for mixing of gametes to ensure optimum fertilization. Of course these estimates can be made by computer-assisted sperm analysis (CASA) if the instrument can record accurate data within the necessary window of observation. The duration of motility can be expected to be different for sperm at the time of collection, after refrigerated storage, after addition of cryoprotectant, and after thawing. Thus it is valuable to repeat this experiment at those times. In addition, motility duration (and swimming speed) is often affected by osmotic pressure (see below).

Osmotic and Ionic Activation Curves

Most fish spawn by external fertilization, releasing sperm and eggs into the water. Unless certain ions are involved (such as potassium in salmonids), in freshwater species, sperm motility can usually be activated by reducing the salt concentration of the sperm solution in comparison to that of blood. In marine species, motility can be activated by increasing the salt concentration. These are general observations and can serve as a starting point for characterizing sperm motility activation (and perhaps oocyte activation as well) for a new species. It should be noted that aquatic species present a broad variety of biological mechanisms that require individual attention. For example, live-bearing fishes that utilize internal fertilization exhibit a sperm activation zone that is isotonic to body fluids – this may be initially surprising in light of what is observed for freshwater and marine species, but makes sense in that the environment which the sperm needs to be motile (i.e., the female reproductive tract) is isotonic (Figure 2, next page).

As stated above, once activated, aquatic species sperm typically have a short life span. Thus sperm need be maintained in an extender with proper salt concentration (usually nearly isotonic to the blood plasma) to inhibit undesired sperm activation during refrigerated storage or cryopreservation.

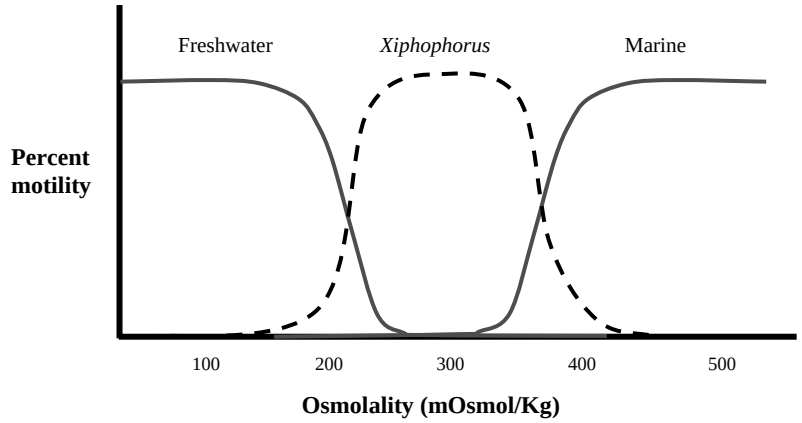

Figure 2. Some generalizations can be made about the osmotic response of sperm motility for species based on the external spawning environment or the mode of reproduction (such as for live-bearing fishes of the genus *Xiphophorus*). Generalizations however, do not substitute for specific knowledge, and motility activation should be characterized for any species being studied.

By evaluating motility across a range of osmotic pressures the basic pattern of activation can be characterized and used to make informed decisions about development of extenders necessary for maintenance of a quiescent state for storage, and the conditions necessary for complete activation of sperm to facilitate fertilization. Our approach is to expose sperm to a graded series of dilutions (spaced approximately at intervals of 30 mOsmol/Kg) of the candidate extender to identify the point of *complete activation*, defined by us as the highest osmolality that elicits the highest motility observed for the sample (this is often not 100%), and the point of *threshold activation*, defined by us in practical terms as 10% motility. A dilution of 1:20 (v:v, sperm:test solution) is typically employed to ensure that the osmotic pressure tested is correct and it is verified with an osmometer by testing a 10-uL aliquot taken directly from the microscope slide immediately after motility assessment (Figure 3).

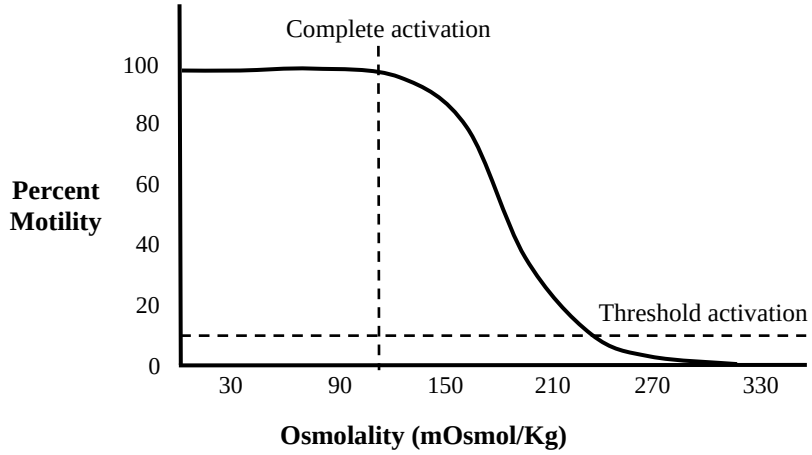

Figure 3. A representative osmotic activation curve produced at 30 mOsmol/Kg intervals for a freshwater species. In this example complete activation occurs at ~110 mOsmol/Kg and below. As such, osmolality of fertilization medium should be reduced below this value to ensure complete activation of gametes. Threshold activation occurs around 240 mOsmol/Kg. As such, extenders should be well above this value (at $\geq$ 300 mOsmol/Kg) to ensure non-activated sperm storage.

Duration and intensity of motility can be affected by osmolality as well as the pattern of activation. For example, motility will be briefest at lower osmotic pressures for a freshwater fish (unless damage occurs from exposure to hypotonic solutions), and more prolonged at higher pressures (until reaching isotonic levels) (Figure 4).

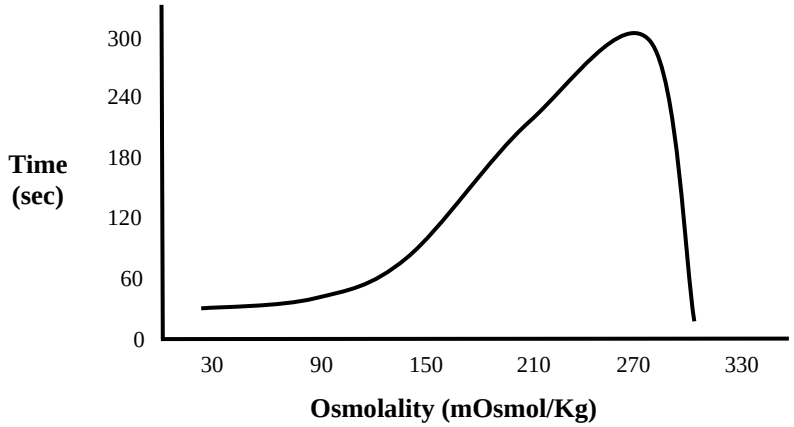

Figure 4. A representation of motility duration across a range of osmotic pressures. This relationship is sometimes used to prolong swimming time with the assumption that it will improve fertilization, but it should be tested for effectiveness in any species (and fertilization medium).

It should not be assumed that a longer swimming time will increase fertilization rates, especially in comparison to other manipulations such as minimizing the fertilization volume (see below in Fertilization Unit section), without evaluating the activation of oocytes (and fertilization) in the same medium. Moreover, a fertilization solution that is used with fresh sperm to prolong motility duration may not be effective with thawed sperm – the osmotic pressure due to the cryoprotectant (even after dilution) can be additive with the osmotic pressure of the fertilization solution and raise the overall value above that which will allow gamete activation (this will lead to the mistaken conclusion that the "cryopreservation did not work"). The activation curve thus offers valuable information to help avoid and troubleshoot problems.

Sometimes an activation curve will indicate that osmotic pressure *per se* is not the dominant factor in controlling motility activation. In these cases, it is possible that the increase or decrease of a particular ion or the presence of another molecule (e.g., egg protein) can be activating motility. This will require more research to elucidate the mechanism of motility activation, and perhaps to identify a suitable extender that allows at least 24 hr of refrigerated storage (see below). A good practical starting point to address this is to test the effect of non-ionic solutions (e.g., sugar solutions) on motility: if motility activation responds to osmotic pressure somewhat or markedly (i.e. similar to Figure 3 for a freshwater fish) it can be hypothesized that the reduction of a particular factor (e.g., a certain ion) is the activating trigger, or that it at least interacts with osmotic pressure; a lack of activation could lead to the hypothesis that the presence of a particular factor is necessary to activate motility. The effect of osmotic pressure on swimming intensity is difficult to assess by eye (beyond simple categories such as "fast" or "slow"), but is ideally suited for CASA. If possible it is best to use darkfield microscopy to evaluate motility with good accuracy and repeatability.

Refrigerated Storage Studies

As indicated above, refrigerated storage is necessary for practical work. Most importantly, it allows shipping of samples from a site such as a working hatchery to a facility that has the specialized equipment and knowledge necessary for research and performance of high quality cryopreservation. Once frozen, the samples can be stored or shipped for use at a later time. Also important is the ability to flexibly schedule cryopreservation for efficient processing in batches for steps that require the same preparation and standardization for one or multiple samples (e.g., estimation of sperm concentration as a single step for multiple samples, rather than a repeated step for individual samples). Refrigerated samples can be calmly assessed for pre-freeze quality (e.g., by CASA) and be adjusted to a optimal working concentration, with careful attention to labeling and coding to avoid the mistakes that come from rushing with samples of rapidly diminishing quality. Indeed, the capability of at least 24 hr of refrigerated storage provides the basis for a routine system of quality control and assurance for commercial-scale cryopreservation. This and the capability for overnight shipping are significant topics that have not received sufficient recognition or research attention in the published literature.

We therefore routinely try to develop refrigerated storage capability for every species that we work with. If dealing with a previously unstudied species, some "clues" should already be available to you at this point from the results of the previous motility and dilution studies described above. If the sperm behaves "typically" (e.g., as in Figure 3) it is possible to test several candidate extenders of the balanced salt buffer type. Research directed at finding an "optimal" extender (and osmotic pressure) can sometimes takes on a life of its own, and can consume considerable time. While this is not necessarily bad for a well-funded, long-term project, it would not be advisable for pilot projects, or for working with species that have short annual spawning seasons. Once again, the prevailing conditions and research goals should be kept in mind when making the decision to test additional candidate extenders or to move forward with the cryopreservation research. Again, good motility (e.g., 50-70%) at 24 hr is probably sufficient to move forward at this point in developing basic protocols (Figure 5).

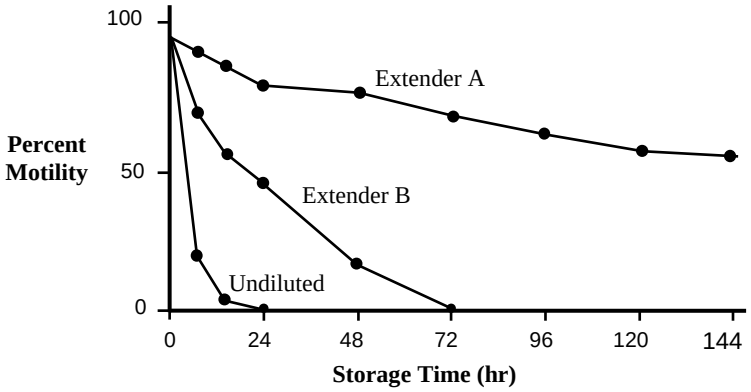

Figure 5. Representation of a refrigerated storage study comparing two extender solutions (at 300 mOsmol/Kg) and undiluted sample collected by stripping. Undiluted, stripped sperm often loses motility capability quickly compared to sperm in extender due to urine contamination. In this case, Extender B is satisfactory for routine storage and shipping (e.g., ~50% motility at 24 hr), while Extender A offers much more utility. If time or resources were in short supply, and Extender B was the best you had, you might consider using it for the cryopreservation studies that follow. The search for Extender A could wait for optimization studies.

Shipping Studies

Shipping is often a violent process. Boxes are sometimes thrown or dropped, left sitting outside in the sun or on a frozen loading dock, or opened for inspection and repackaged improperly. The point here is that storage of samples in the refrigerator for 24 hr does not constitute a realistic shipping evaluation. Practical evaluation would involve using the actual packaging that will eventually be used for samples, and sending the package overnight (even if only to yourself). Often samples are collected elsewhere and shipping is required from the beginning. In short, carefully controlled protocols that do not take the sperm outside of the laboratory may not transfer well to practical application. This could be a weak link in the protocol development pathway so it should receive consideration.

Acute Toxicity of Cryoprotectants and Equilibration Time Studies

Conventional cryopreservation involves the use of cryoprotectants and freezing rates sufficiently slow to produce cellular dehydration and shrinkage (termed “equilibrium cooling”) to avoid intracellular ice formation. Cryoprotectants are chemicals used to protect cells from damage during freezing and thawing, and are classified by whether they penetrate the cell (referred to as “permeating”) or remain outside of the cell (“non-permeating”). Although the mechanisms of action are as yet not completely understood, permeating cryoprotectants such as dimethyl sulfoxide are believed to help reduce the damage caused by formation of ice crystals within sperm cells. They also help reduce the dehydration damage that occurs when water leaves the cell to become ice in the surrounding solution. Non-permeating cryoprotectants such as sugars and polymers are believed to help stabilize the membrane during cryopreservation. Too little cryoprotectant entering the cell before cooling can reduce effectiveness, whereas too much can cause swelling and rupture during thawing and dilution. This is governed by the ability of the cryoprotectant to diffuse into and out of the cells.

In addition, cryoprotectants are often toxic to cells (including osmotic damage), and thus the choice of the types of cryoprotectant and their optimal concentration (usually a balance between cryoprotection and toxicity) has been the focus of numerous studies. After the addition to sperm samples, time is needed for the cryoprotectant to permeate the cells. This is referred to as the *equilibration time*. For most circumstances it can be set at 15 to 30 min (often on ice or at 4 ^{o}C), but it can be varied depending on the type and concentration of cryoprotectant being used, and the number of samples being processed. If the necessary concentration of cryoprotectant is toxic to the cells, the equilibration time of rapidly permeating cryoprotectants such as dimethyl sulfoxide can be shortened to the minimum time required for filling of containers (e.g., 10 min).

For practical purposes we use 15 min as our minimum equilibration time which we define as the time between addition of cryoprotectant and the samples reaching 0 ^{o}C in the freezing process. Some cryoprotectants such as methanol are relatively non-toxic, penetrate rapidly, and do not contribute to the overall osmotic pressure. Others such as dimethyl sulfoxide have some toxicity, penetrate rapidly, and contribute markedly to osmotic pressure. Other cryoprotectants such as dimethyl acetamide, are sometimes so toxic that they are not useful for some species. How can this be determined in a practical manner? We utilize acute toxicity studies (Figure 6) to narrow down the list of cryoprotectants and select the concentrations to be used in subsequent cryopreservation experiments.

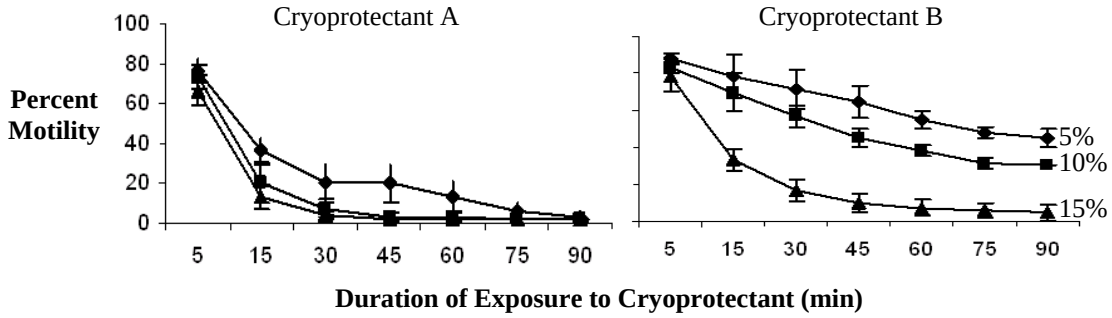

Figure 6. Representation of an acute toxicity study for two cryoprotectants at three concentrations (5, 10 and 15%). Cryoprotectant A reduces motility quickly and does not allow much time for permeation or handling. If other candidates are not available, 5% offers the least toxicity, but may not offer sufficient cryoprotection. Cryoprotectant B however has less effect on motility, and 10% would be a suitable choice for a 15-minute equilibration period. This may be a useful cryoprotectant concentration, especially if the sperm concentration is controlled.

By following this process we can reduce the number of cryoprotectants and concentrations to be tested, greatly speeding the research process. The reasoning behind this is that if the sperm cells are damaged (immotile) by cryoprotectant toxicity before they are cooled, they will not be resurrected by the ensuing freezing and thawing ("do not kill the cells before you freeze them"). We use sufficient dilution to ensure that the osmolality of the aliquots tested is appropriate for motility activation. This is verified by testing the actual sample from the microscope slide with an osmometer (i.e., the loss of motility is not due to an osmotic constraint on activation).

The pursuit of "optimal" cryoprotectants and concentrations can take on a life of its own. Indeed, it is not uncommon for problems in other aspects of the cryopreservation process to be incorrectly ascribed to cryoprotectant selection, and an empirical survey undertaken to test whatever chemicals that are at hand or have been recently been published with exciting results. I view this as a trap and would suggest that practical protocols for the vast majority of aquatic species can be developed using a small number of cryoprotectants such as methanol or dimethyl sulfoxide, and secondarily glycerol or dimethyl acetamide, perhaps in combination with specific sugars. In any event, these should be the starting points in a strong research pathway controlling the major variables. Pursuit of exotic cryoprotectants has a place as an optimization step, or for those recalcitrant species that pose special challenges.

Equilibration Motility

Given, as explained above, that cryoprotectant toxicity can produce non-viable cells prior to freezing, it is essential to be able to identify this effect and not confuse it with the effects of freezing and thawing. For this reason *it is essential* to retain a small sample of the material being frozen and to *always* estimate the motility at the end of the equilibration period as the samples are being cooled. We refer to this as the *equilibration motility* and it is an extremely important piece of information that is not typically collected or reported. Experiments are often incorrectly interpreted as "the cryopreservation did not work" because sperm were immotile after thawing. Without a pre-freeze assessment of equilibration motility it is not possible to know how much of the motility loss came before cryopreservation. It is important to note in this regard that samples can become more sensitive to the toxic effects of cryoprotectants during refrigerated storage or

shipping, and thus samples that initially showed little effect from 10% cryoprotectant could suffer dramatic drops in equilibration motility after 24 or 48 hr. Therefore, as stated above, *always* estimate motility at the end of the equilibration period as the samples are being cooled.

Packaging and Cooling Rate Studies

Proper packaging of cryopreserved samples is important for numerous reasons including standardization of rates for freezing and thawing, efficient use of space for storage and shipment, assurance of permanent sample identification, proper sealing for biosecurity and safety (leaky containers can explode on thawing), and enablement of automated sample processing. Various containers have been used for fish sperm, including drinking straws, glass capillary tubes, and plastic cryovials. Plastic French straws have been in use for livestock sperm for decades. These straws come in 0.5- and 0.25-mL volumes, and are produced in over 20 colors. The use of these containers offers the advantages of efficient and reliable sample identification by permanent printing on colored straws, sample safety by complete sealing, standardization of the cooling and thawing processes because of their thermal properties and large surface area in relation to their volume, and their being designed for use with automated processing equipment. Adoption of the French straw for aquatic species has been limited by a number of factors including the notion that their volume is too small for routine hatchery use (and the idea that "free" straws are available from fast-food restaurants). However when sperm numbers are controlled, especially in relation to egg numbers, this need not be a realistic problem, and the benefits of this packaging greatly outweigh the inconvenience of handling multiple straws.

The choice of cooling rate has been another major focus of numerous studies of sperm cryopreservation. To be considered as optimal, a rate should be slow enough to minimize the amount of ice crystals that form within the sperm cells (below a damaging level) and yet be rapid enough to minimize the length of time cells are exposed to what is referred to as the "solution effect", which is the concentration and precipitation of materials that occurs when solubility limits are exceeded during the dehydration caused by ice formation. There are a number of methods that can be used for freezing. These range from the use of expensive computer-controlled freezers which offer precise and reproducible rates, to simpler and cheaper freezing by suspending samples above liquid nitrogen in a styrofoam cooler, although this is a less reproducible approach. Samples can also be suspended in the neck of a storage dewar. With these latter two methods, the height of the samples above the liquid nitrogen offers control of the temperature and cooling rate. There is no current accepted definition of cooling rate for aquatic species and it is possible that the rates reported are obtained by different means for every study. We typically use the time required *for the sample* to traverse from 0 ^{o}C to -80 ^{o}C to calculate cooling rates. Care should be taken to carefully report the method used to calculate the rate.

As indicated above for other parameters, the pursuit of "optimal" cooling rates can take on a life of its own. Because this is an important cryobiological parameter, specialists (or novices) will sometimes focus on this component at the expense of the other activities outlined in this chapter. The concept of "optimal" is extremely relative for cooling rates because they are so dependent on the choice of container and cryoprotectant. This is one of the reasons why studies seem to disagree on the relative effectiveness of specific protocols even within the same species. Sperm cells are not tremendously different in basic biophysical properties when surveyed across aquatic species, and there is no theoretical reason why each species should *a priori* be considered to be distinct from every other. A lack of confidence in the research area (especially for novices) may lead to hasty conclusions to accept a particular cooling rate or cryoprotectant as being

ineffective because of the mistaken idea that there are unique, optimal conditions awaiting discovery (the "eureka" moment). This may be responsible for adoption of the empirical survey approach often seen in aquatic species research (i.e., trying everything on the shelf) instead of addressing the more useful question of *why* a perfectly reasonable cooling rate or cryoprotectant such as methanol was not effective.

The cooling rate should be measured directly within the container being frozen. This is because large volumes or thick containers will cool at a different rate than small, thin containers when exposed to the exact same freezing conditions. How can cooling rate be tested in a practical manner? Because the factors interact with one another it is important to establish some of them from the beginning. We use plastic straws (0.25 or 0.5 mL) because of their numerous advantages and their capability for automated processing with enables high-throughput approaches. With the container chosen, we proceed to evaluate selected cryoprotectants and concentrations (described above) at specific cooling rates. If time or resources are in short supply we will initially test two rates: 4 ^{o}C ("slow") and 40 ^{o}C ("fast"). Based on these results we will then focus in on the range of cooling rates to be tested or change other parameters (Figure 7).

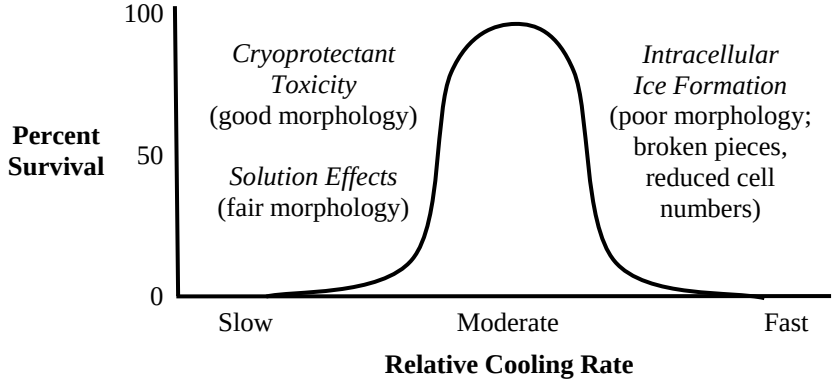

Figure 7. Diagrammatic representation of how to use post-thaw assessment to identify problems in a cryopreservation protocol. Basically, if the cells are intact ("beautiful corpses") but immotile (dead), they were likely victims of cryoprotectant toxicity (check the equilibration motility). If they have fair morphology but little survival they might have been frozen at too slow of a rate and succumbed to the effects of high salt concentration or low pH (e.g., protein denaturation). If there are few cells visible and the background is littered with sperm fragments, they likely were frozen too fast (or had too little cryoprotectant, or the cell concentration was too high) and were destroyed by ice formation. This information can be used to make adjustments in subsequent experiments.

After this initial assessment, we can narrow experiments down to a few cryoprotectants (at a single concentration) and if necessary a reduced range of cooling rates. The interval between rates tested and the reproducibility of the profiles is dictated by the freezing method. We routinely use a computer-controlled freezer to obtain accurate and reproducible rates. This is not essential however, and reasonably reliable cooling can be obtained by a variety of methods including styrofoam boxes, or even dry ice, if proper safeguards and protocols are used. Regardless of the method used, the reasoning behind research decisions will remain the same. For example, cryoprotectants can interact with cooling rate. Certain cryoprotectants may function better or worse than others at specific rates under the conditions being used, and it is possible to inadvertently test outside of the effective rates for specific cryoprotectants (Figure 8).

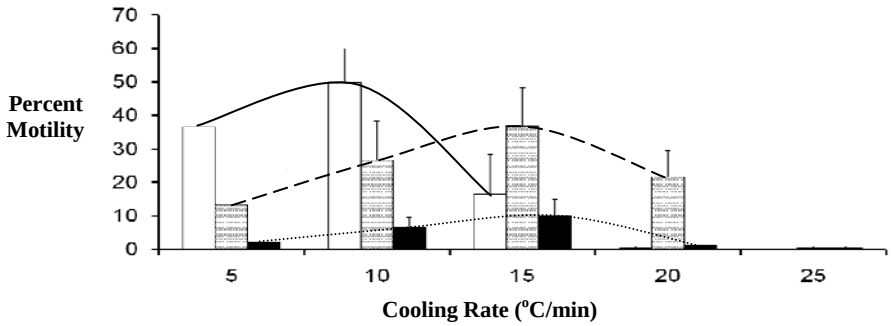

Figure 8. A representative comparison of three cryoprotectants at five cooling rates. Cryoprotectant A (white bars) performed well at the slower rates (best at 10 °C/min). Cryoprotectants B (gray) and C (black) performed better at faster rates (best at 15 °C/min), although cryoprotectant C never achieved the same performance as the other cryoprotectants. From this we can conclude that there is no single "optimal" cooling rate, and that cryoprotectants do not have equal protective abilities. In addition, note the abrupt drop at the 25 °C/min rate for all cryoprotectants. Without careful control of cooling rate, or examination of lower rates, it could be incorrectly concluded that none of these cryoprotectants were effective, and that empirical testing of new, exciting cryoprotectants was necessary.

As stated above, cooling rates should be monitored within a container (preferably within a sample of the actual liquid being frozen) by use of a thermocouple and recorder. Cooling rate should be reported along with the container type, and if possible, the rate or temperature within the chamber (freezer environment) along with the programmed rate (if using a controlled-rate freezer). Also be aware that numerous factors will affect cooling rates including: the ambient room temperature, the number of containers being frozen at one time (thermal mass), whether freezing cycles are being repeated (successive runs can be different), whether frost has accumulated within the freezer (a problem in humid environments like Louisiana), low levels of liquid nitrogen in the pressure tank that drives the controlled-rate freezer, and so on. This list alone provides ample additional reasons why the actual rate within a container should be measured during every cooling cycle.

Thawing Rate Studies

In simple terms, freezing and thawing each present a "danger zone" to cells as they traverse between the temperatures of 0 °C and -40 °C. As such, thawing can be as destructive to cells (albeit for somewhat different reasons) as cooling. In general, rapid thawing is preferred to minimize the damage associated with recrystalization (the coalescence of small ice crystals into large crystals during thawing). Samples should be removed from the storage dewar and transferred immediately to a container such as a styrofoam box that can safely hold liquid nitrogen. This ensures that samples will not thaw prematurely due to handling. For our work, straws are held in a 20 to 40 °C water bath (a thermos or small ice chest will also work) for 7-30 sec. Specific times and temperatures should to be optimized for the particular sample volume, container type (e.g., volume, materials, and geometry all affect the rate of heating), and species (e.g., cold water or warmwater fishes). We sometimes test a range of thawing temperatures (e.g., from 0 to 60 °C) to optimize protocols for a variety of species. As a rule of thumb, samples can be considered to be thawed when air bubbles within the container can move freely within the liquid. The use of transparent or translucent containers will aid in viewing the sample. The

samples should be cool to the touch when thawed (not warm), or even cold when working with coldwater species such as salmonids. For research, motility should be estimated *immediately* after thawing. This is because sperm can sometimes be motile in the container after thawing (without addition of activating solution) and this motility can be lost quickly (i.e., < 15 sec, unpublished observations). As such it also follows that fertilization should be tested immediately after thawing unless experiments have been done to determine the useful lifetime of post-thaw samples (this could be tested as part of the application pathway). On the other hand, when using relatively non-toxic cryoprotectants such as methanol, post-thaw sperm can sometimes be refrigerated for days with satisfactory motility (e.g., Tiersch et al. 1994).

Post-thaw Amendment Studies

Sometimes depending on the species or application it is necessary to evaluate treatment of the sperm after thawing to improve performance. Usually it is sufficient to thaw the samples, place them on eggs, and add water to activate the gametes. For live-bearing fishes of the genus *Xiphophorus*, however, thawed sperm requires washing to remove cryoprotectant, and concentration of the cells (to achieve an appropriate sperm density) and to reduce the sample volume for artificial insemination into the female reproductive tract. To accomplish this, studies were necessary to identify the conditions for proper centrifugation and resuspension of the pellet (e.g., Dong et al. 2006).

Post-thaw Quality Assessment

As stated above, qualitative observations of thawed sperm can be quite helpful in evaluation and troubleshooting of protocols. For example, to review, if the sperm cells are visibly damaged, the cryoprotectant concentration may have been too low, the cell concentration too high, or the cooling rate may have been too rapid. Conversely, if the cell morphology is intact although the sperm are immotile, the concentration of the cryoprotectant may have been too high; this would be confirmed by a reduction in the equilibration motility observed before freezing. Assessment therefore must extend beyond the simple, often incorrect, observation that cryopreservation did or did not “work” (an observation heard countless times).

By considering all of the steps involved the entire process pathway, verifying quality and controlling variables at each step, using screening to narrow down the number of variables in successive experiments, and developing a basic understanding of cryobiological theory, it is possible to evaluate thawed samples and make informed decisions about the problems in the overall process. Perhaps there is a single step that accounts for substantial motility loss, or small quality losses that are accumulating across a number of steps. It can be as simple as looking into the microscope and recognizing that the background is gray, not black as it should be in darkfield microscopy. If the scope is set up properly, this usually indicates that the cells have been destroyed by intracellular ice formation and the cooling rate was too fast (assuming the cryoprotectant was sufficient). In other words, within 1 sec of viewing and without seeing a single sperm cell it is possible to begin to diagnose problems (yes, it can be that simple).

If possible, it would be best to evaluate sperm with a battery of tests such as those characterizing membrane and nuclear integrity, mitochondrial potential (e.g., flow cytometry), motility, and fertilization capacity. This would present a more complete picture of how and where damage has occurred. Even apparently simple cells such as sperm have a variety of compartments that can respond differentially to a set of cryopreservation conditions and would be best characterized by a combination of quality assessment assays (Figure 9).

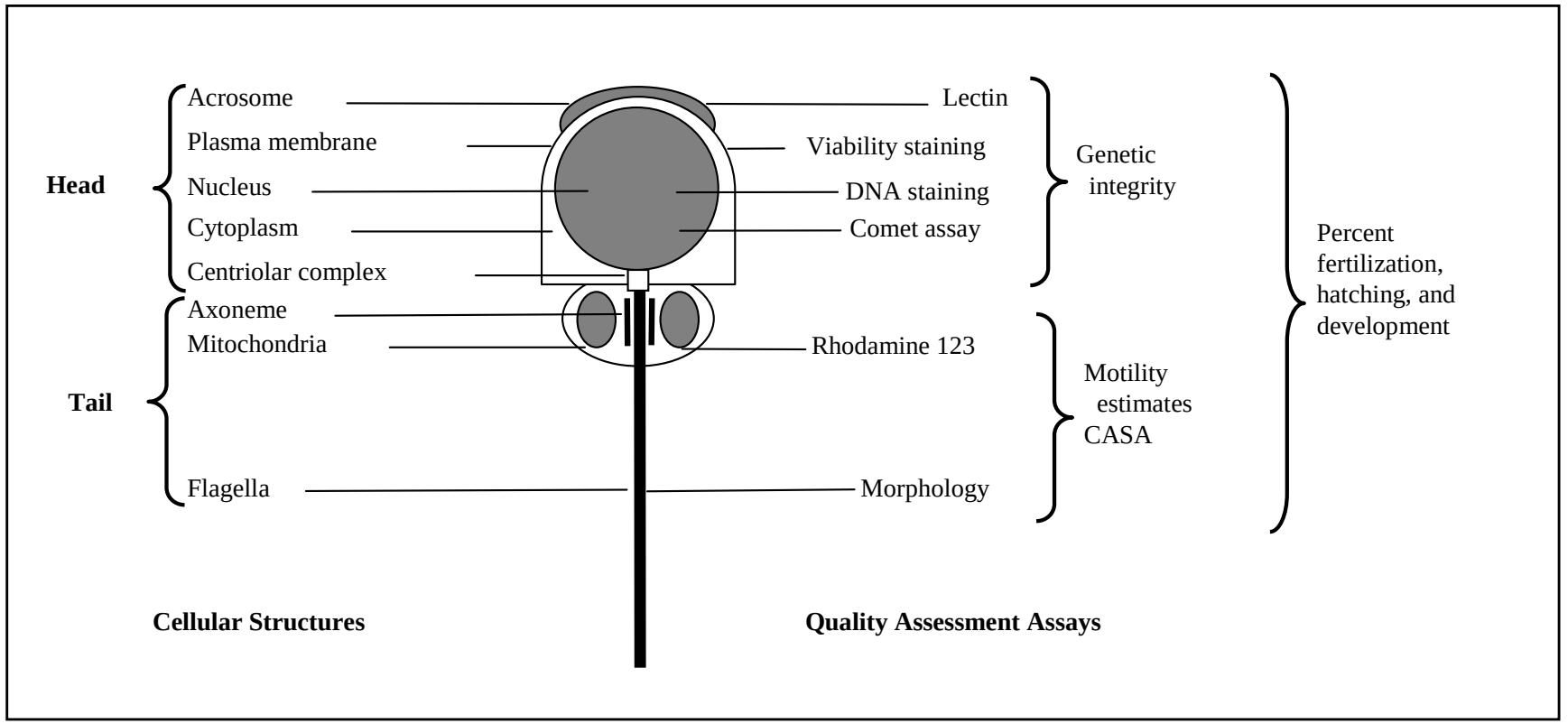

Figure 9. Sperm quality is a generic term that encompasses proper functioning of a combination of cellular structures (indicated on the left of the sperm cell) that can be evaluated individually by specific assays (indicated on the right) or in aggregate by examining factors such as the capacity of sperm to fertilize eggs that hatch and develop normally. Sperm quality can be affected by a number of factors in cryopreservation process pathways. Indeed, damage can occur to specific structures (for example the acrosome or nucleus) that would not be detectable by study using a single assay (such as motility). Assays such as these have been applied in aquatic species, but an integrated battery of assays that would encompass a comprehensive analysis of sperm quality does not yet exist.

An example of the compartmentalization of damage possible for sperm cells during cryopreservation is the gelling of post-thaw samples seen in Pacific oysters *Crassostrea gigas* (e.g., Dong et al. 2007a). Sub-optimal conditions (e.g., cooling rate, cryoprotectant concentration, or cell concentration) can cause damage or activation of the acrosome. This causes the cells to form aggregates, reducing fertilization capacity despite otherwise acceptable cell morphology. For practical purposes in the research pathway, we generally use a 50% recovery of viable (motile) sperm (based on the original starting point) as an acceptable goal.

Fertilization Assay Studies

Artificial spawning involves the collection from females of unfertilized eggs to be combined with sperm. Unless testing has shown otherwise, it is generally best to minimize the time between thawing of sperm and fertilization. After thawing, sperm samples are added to eggs and thoroughly mixed, and the gametes are activated with an appropriate solution (this is called the "dry method"). Other fertilization methods are available and can be evaluated for use with any particular species. Fresh sperm samples can be used to fertilize replicate batches of eggs to serve as a test for egg quality (unless factors such as sperm numbers are controlled it is generally not statistically appropriate to make direct comparison of the fertilization produced by fresh and thawed samples). It is important to quantify the fertilizing ability of each batch of eggs because variation in egg quality across females can be considerable. It is likewise advisable to replicate all treatments (e.g., fresh and thawed from each male) that will be statistically compared with eggs from each female (e.g., the female represents a replicate) and to establish *a priori* the minimum egg quality to be included in statistical analysis (e.g., at least 50% fertilization with fresh sperm). If the females are small and only produce a few eggs (such as medaka *Oryzias latipes* or zebrafish *Danio rerio*) pooled batches from several females can be used to obtain the requisite number of eggs (e.g., Yang et al. 2010). After ~5 min, additional water is added to water-harden the eggs. Percent fertilization can be determined to evaluate sperm quality.

Artificial spawning in this way allows for a variety of crosses such as the use of one male to fertilize eggs from several females, or for the eggs of one female to be fertilized by sperm from several males. This can lead to a breeding matrix where a group of select males can be mated with a group of select females to develop populations with distinctive traits. By having assayed the parents for genetic markers, breeders can develop broodstocks with enhanced characteristics, such as growth rate or disease resistance. Subsequent gene mapping can be used to further enhance and balance desirable phenotypes. Such a process can also be used to cross two different species to develop hybrids with improved traits, although precautions should be taken to prevent the accidental escape of the hybrid fish into the wild.

There is no current accepted definition of percent fertilization. Approaches vary considerably among studies ranging from first mitotic division to hatching and beyond, using absolute values or values relative to controls. Thus it is problematic or impossible to directly compare fertilization values among studies.Care should be given to report clearly the criteria used. In addition, it is advisable to choose a life stage (e.g., neurula) sufficiently developed that it represents post-zygotic activity rather than oocytic activity, yet not so late in development that culture conditions or other non-tested factors could reduce survival in a way that could be confounded with treatment effects.

Establishment of Optimal Dilutions and Concentrations

Estimates of sperm concentration can be made by a number of methods (see chapter by Cuevas-Uribe and Tiersch in this volume), and are essential to collect reliable data necessary to develop protocols (Dong et al. 2007a). In addition, estimation and adjustment of sperm concentration provide the foundation upon which quality assurance and optimization are built. As such, cell concentration should be assessed early in the pathway (described above). This method is vastly superior to simple standardized dilutions of samples. Nonetheless, dilutions are necessary in the pathway (e.g., at sample collection, when adding cryoprotectants, or for fertilization after thawing), and when dilutions are based on an initial sperm concentration they simply become sperm density adjustments.

It is at this stage in the process that some optimization is advisable for sperm concentrations and the dilutions necessary to produce appropriate volumes. Some variables of importance are the concentration and total number of sperm per straw (e.g., can be used to maximize storage efficiency), the sperm numbers (total or percent motile) in relation to the egg numbers (e.g., can be used to match the cryopreservation unit – such as a straw – to the number of eggs available per spawn), the total volume used for individual fertilizations (e.g., it is often useful to minimize this volume to enhance sperm-egg interaction), and the sperm-to egg-ratio (e.g., it can be used to avoid waste of excess gametes, and to standardize assay conditions for direct comparison of treatments). Practical protocols and pathways will pay close attention to optimizing these volumes, dilutions, and concentrations *with respect to overall efficiency*, not just enhancement of any single step.

Calculation of the "Fertilization Unit"

As part of this process it is useful to perform the calculations necessary to match your protocol to how it will be eventually be used. Large-boded fishes that produce hundreds of mL of diluted sperm will need different calculations than will small-bodied aquarium species that produce microliter volumes (Tiersch 2001), and fertilizations used to mass produce offspring in a commercial foodfish hatchery will need different calculations than a conservation program aimed at producing numerous separate families to achieve proscribed levels of genetic diversity. These calculations often will need to strike a balance between the expected levels of gamete quality and the desired numbers of offspring.

This is where research protocols leave the sheltered environment of the laboratory and have to earn a living in the cold, hard world. The earlier in the process that consideration is given to specific forms and levels of application, the more useful the protocol will likely be. For example, it is not efficient to do research with a container that is not suitable for hatchery application, because large amounts of the work will have to be repeated to re-establish the necessary working conditions (e.g., cooling rate, possibly cryoprotectant and concentration, and batching methods). One approach we use is to calculate backwards from the numbers of sperm and eggs needed, and to base the cryopreservation container on the expected number of eggs. For example, calculations could be performed to establish one straw per female as the basic "Fertilization Unit" (Table 2).

Table 2. Sample calculations used to establish the "Fertilization Unit" for zebrafish sperm collected by two methods based on the use of a single 250-ul straw and the eggs (~100) from a single female.

Step in Process	Sample Type	Sample Volume	Concentration (sperm/ml)	Sperm Number	Sperm Number Definition
1) Sperm collection	Testis	4 µL (4 mg)	1.0E+10	4.0E+07	Total # sperm available
	Stripped	2 µL	1.0E+10	2.0E+07	Total # sperm available
2) Initial dilution (1 in 200)	Testis	800 µL	5.0E+07	4.0E+07	Total # sperm available
	Stripped	400 µL	5.0E+07	2.0E+07	Total # sperm available
3) Add cryoprotectant (1 in 2 dilution)	Testis	1600 µL	2.5E+07	4.0E+07	Total # sperm available
	Stripped	800 µL	2.5E+07	2.0E+07	Total # sperm available
4) Load in 250-µL straws	Testis	200 µL (x 8)	2.5E+07	5.0E+06	Sperm in single straw
	Stripped	200 µL (x 4)	2.5E+07	5.0E+06	Sperm in single straw
5) Fertilize 100 eggs (+300 mL activating solution)	Testis	500 µL	1.0E+07	5.0E+06	Sperm in single fertilization
	Stripped	500 µL	1.0E+07	5.0E+06	Sperm in single fertilization

An Application Pathway (Protocol Utilization)

By this time, a fairly robust process pathway would be in place to provide a base for application. Many of the research pathway steps could be retained to ensure quality control, other factors could benefit from optimizations relevant to the specific application, and new research topics could become relevant. Emphasis here should be placed on improvement of overall integration and efficiency. Relevant questions include: who will apply the technology and use the samples, and how will they use them? What will be the scale of the application, and will it function as a self-contained entity, or be part of a larger enterprise? How much will it cost in terms of facilities, equipment, personnel, and supplies (e.g., Caffey and Tiersch 2000)? Does ownership (e.g., of the genetic resources, or the samples themselves) change as samples move through the pathway, and who will derive benefits from the process and products? As such, it is useful to look at an overview of the activities that would be involved (Figure 10).

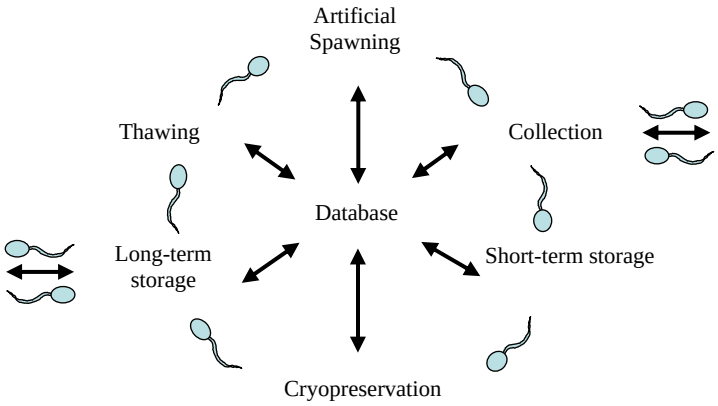

Figure 10. Representative minimal activities necessary for application of sperm cryopreservation at a single location (linked in a circle). Consecutive components are shown as a clockwise flow of sperm, and two-way arrows are used to indicate maintenance of a centralized database for information on biological characteristics, motility assessments, quality control points (shown as sperm cells), fertilization, and inventory control. Refrigerated (right side) and frozen sperm (left) can enter and leave the facility necessitating establishment of a biosecurity program (Caffey and Tiersch 2000).

For the sake of brevity only new topics in the application pathway (identified in Table 1) will be addressed below. Topics previously addressed above, such as assessment of motility at time of collection, after equilibration, and immediately after thawing, and control of sperm concentration at various points although not listed below *remain essential for the application pathway* and should not be disregarded.

Improvement of Male Quality

This is included as an example of a new research topic, and as an example of how the application pathway involves extension beyond the narrow confines of the research pathway. This might include study of control of the broodstock conditioning environment (e.g., photoperiod and temperature), hormonal induction of gonadal or gametic development, dietary manipulations to improve gamete quality, quantity, or freezing resistance, or improved husbandry to maintain family or age groupings. It could also involve multigenerational record-keeping to identify specific individuals or lines that bear advantageous alleles or gene combinations.

Shipping Studies

As indicated above, it is often necessary to ship or transport refrigerated and frozen samples. This becomes even more important when developing an application pathway. First there is the need to protect against or be able to assess damaging or wasteful accidents. Even dewars expressly designed for shipping can be dropped or stored upside down (this greatly reduces the cryogenic storage time). Thus, for valuable samples, a temperature data logger or a thermometer that records maximum and minimum temperatures should be included within the shipping container. We have had several very strong plastic cases shattered during overnight shipping (although the samples remained intact), and have even had shipping dewars arrive with the factory vacuum seal ("bung") smashed (because of the loss of the insulative properties of the vacuum, these samples were thawed and ruined). Next would come the need to achieve efficiency in transport which can come from revisiting the fertilization unit calulations and perhaps manipulating the numbers of sperm per straw. Remember, a change from 10^9 sperm per straw to 10^8 would require ten times the number of straws to transport the same number of sperm. This can quickly add up beyond current capabilities (e.g., the number of shipping dewars that are available) or drive up shipping costs beyond acceptable levels (Hu et al. 2011)

Labeling, Packaging, Storage, Inventory, and Database

Proper labeling of cryopreserved samples is essential for application. Samples can be in storage for weeks, months, or years before they are thawed. Improperly labeled samples can cause delays in processing, and even worse, could cause genetic contamination of pure stocks. The necessity for proper labeling cannot be overemphasized. *The value of samples is directly proportional to the quality of labeling information and record keeping*. Unlabeled or poorly labeled samples are essentially worthless and can even be detrimental. At the minimum, containers should be permanently labeled to indicate species, male identification number, date and facility. With the use of straws and automated processing more sophisticated labeling, such as individualized printing of alphanumeric character strings and bar coding are possible. Containers intended for archiving and breeding uses should receive the best labeling possible in conjunction with establishment of robust databases. A powerful interactive database is being established by the USDA National Animal Germplasm Program for its archival collections (it

can be viewed at: *www.ars.usda.gov/Main/docs.htm?docid=16979*). Maintenance of a current and accurate inventory can become extremely demanding and costly as a consequence of successful application and scaling up of cryopreservation. It is advisable to plan ahead for this. It can also require development of or linkage to uniform coding systems for sample identification (e.g., classification by genetic markers).

Growth and Survival Studies

Studies of this sort will become valuable to evaluate any possible effects of cryoprotectants or cryopreservation on offspring, and to keep track of the phenotypes of the animals produced by the specific matings that cryopreservation can make possible. This could include monitoring of genetic progress in agricultural settings or verification of specific gene mutations or transgenes in reconstituted research lines.

Quality Control Points

As introduced above, it is important to note with respect to cryobiology that sperm have a high degree of internal complexity. The various structures within a sperm cell represent different functional compartments that can each require different optimal conditions, and thus can each exhibit differential responses to cryopreservation. This can cause a variety of damages and outcomes. For example, damage to the tail could interfere with motility whereas damage to the head could interfere with embryonic development. Sperm quality is a generic term that encompasses proper function of a combination of cellular structures (such as the head, membrane, and tail) that can be superficially evaluated individually by specific assays, or in aggregate by examining factors such as the capacity of sperm to fertilize eggs that hatch and develop normally. For this reason it is important to evaluate the quality of thawed sperm by fertilizing eggs and monitoring the development of the offspring, which can be expensive and difficult. This can sometimes be accomplished by use of a surrogate species to provide eggs when the sperm comes from a species that has valuable or limited broodstock, or has a restricted spawning period. Hopefully at some point it would be possible to establish reliable predictive assays of fertilization capacity, such as by CASA or flow cytometry, to standardize and streamline the process.

With development of an application pathway, it is possible to incorporate regular quality control points into the process (e.g., Figure 10). A balance is necessary for the research desire to collect as much information as possible and the practical constraints of time and expense that would dictate collection of data at a few key points along the pathway. These points could be determined on a species or application basis.

Biosecurity Protocols

The collection and transfer of gametes from aquatic species poses concerns for unintended transfer of microbial organisms (Tiersch and Jenkins 2003). Sampling could come from infected broodstock (especially if they have immunosuppression arising from the stress associated with capture, transport, or handling for spawning). Contamination of samples can occur during collection, processing, storage, and transport. Generally, proper sanitation during collection is essential for limiting the spread and growth of microorganisms such as bacteria, viruses, fungi, mycoplasmas, and parasites. Materials and equipment used to freeze samples should be sterile. Establishing and following good practice guidelines for handling and processing of samples is especially important for wild-caught animals where disease-free status

cannot be guaranteed. The research and application communities and stakeholders should initiate a process to develop guiding principles or best practices for this very important topic.

A Commercialization Pathway (Programmatic Development)

This pathway brings us to *terra incognita* for aquatic species cryopreservation and exploitation of genetic resources and consequently the following statements will be brief (which potentially understates their importance). As stated above, programmatic development and commercialization are not yet available for fish and shellfish. Programmatic development can proceed along a large number of paths, but would probably proceed with private sector involvement first utilizing cryopreservation technology to improve current practices and ultimately developing into an industry based on such things as genetic resources and the provision of products (e.g., frozen sperm for increases in hatchery efficiency), genetic value (e.g., germplasm from elite broodstock) technology services (e.g., cryopreservation for hire), training, and information services (e.g., tracking genetic improvement across a given industry such as catfish or oysters). Activities of this sort have developed over the past century for genetic resources of livestock species (Caffey and Tiersch 2000) (Figure 11).

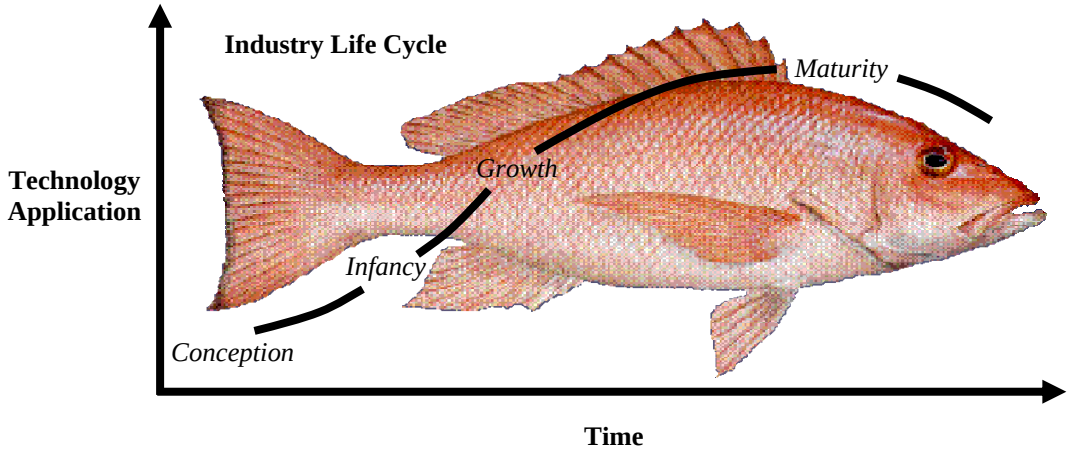

Figure 11. Diagrammatic representation of a prototypical industry life cycle. Industrial development in genetic resources of aquatic species could parallel that experienced by livestock commodities during the 20th century.

Scaling-up for High Throughput, and Establishment of Commercial-scale Facilities

The initial barrier for industrial or programmatic development will be in establishment of the facilities, equipment, and protocols necessary to produce the volume of materials and activities necessary to enable widespread adoption of cryopreservation technology, services and products. The research necessary for scaling up from the laboratory is only beginning in aquatic species, but has been recognized by groups such as the US National Institutes of Health in a recent workshop entitled *Achieving High-Throughput Repositories for Biomedical Germplasm Preservation* (NIH 2007).

Gamete Quality Assessment and Quality Control and Assurance Programs

Quality assessment will assume a much greater role with programmatic development. Rapid and reliable methods will be needed to establish industry standards. Livestock industries

have established minimum requirements promulgated by groups such as Certified Semen Services (CSS 2011), a subsidiary of the National Association of Animal Breeders (*www.naab-css.org/*). Linkage with existing organizations such as this, or development of new organizations created specifically for aquatic species should be considered.

Standardization and Harmonization

These are necessary processes that are only beginning for research–level activities (e.g., for particular equipment or processes) in aquatic species. With forethought these research-oriented approaches could be formulated to be compatible with the needs for subsequent commercial and programmatic development, and for promulgation as industry standards.

Establishment of Markets, Valuation, and Pricing Schedules

Economic research is very much in need for the products and services associated with cryopreservation, as well as for valuation of genetic resources themselves. Extremely basic questions to be addressed include: how much is a 10% improvement in growth worth when it can be purchased in the form of frozen sperm in a straw (see chapter by Boever et al. in this volume)? How much is frozen sperm from a threatened or endangered species worth? For that matter, what is the value of frozen sperm from an extinct species? More mundane questions would address the pricing schedules for batched sperm frozen from commercially relevant males in comparison to that from elite individual high-performance males. What kind of market structures will emerge from this technology? Will they be contained within single commodities (e.g., catfish or salmon), or be grouped by region, or some other consideration such as habitat (e.g., marine or freshwater), sector (e.g., private, governmental, non-governmental organization), or application community (e.g., biomedical research, conservation programs, aquaculture)?

Development of Equipment, Supplies, and Reagents Specialized for Aquatic Species

Currently work has addressed the feasibility of working with existing livestock cryopreservation facilities for use with aquatic species (e.g., Caffey and Tiersch 2000, Lang et al. 2003, Dong et al. 2005b, Dong et al. 2007b). Newer work is addressing adoption of equipment developed for use with mammals for dedicated use with aquatic species (e.g., Hu et al. 2011). With programmatic development in aquatic species, greater need and opportunities will exist to encourage continued innovation in creation and marketing of equipment, supplies and reagents specialized for use with aquatic species.

Treaties, Regulatory Frameworks, Material Transfers, and Access and Benefit Sharing

Large-scale transfer of samples, and more importantly the genetic resources contained within, will open expansive areas of legal and ethical research to address proper approaches and protections for the new capabilities created by the availability of commercial-scale cryopreservation. This will undoubtedly involve international agreements and frameworks to promote access, enable benefit sharing, avoid inequities, and to protect investments and encourage innovation (some of these topics and addressed in this volume in an annotated original chapter by Jenkins, and a new chapter by Long and Blackburn). Existing and pending regulatory frameworks for industries such as livestock or concerns for preservation of biodiversity could supercede those that would be developed by choice within the aquatic community. Potential stakeholders should initiate development of appropriate structures and look to development of

harmonization among the network of regulatory frameworks that could come into play for global commercialization of aquatic genetic resources.

Summary

This chapter is intended to serve as an introduction to the concepts necessary to view cryopreservation research and application in a comprehensive and integrated manner. Fifty yr of previous study of aquatic species cryopreservation has not yet led to commercial application and programmatic development (despite need for them). This is due to a number of reasons including aquatic market structures, breeding and husbandry practices, and a weak base for application due to a narrow and disjointed research focus that emphasizes certain cryobiological parameters over development of balanced and functional pathways. The important conceptual message from this chapter is to promote a larger comprehensive approach for aquatic species, to treat this process as a series of pathways (rather than as independent steps) and to seek integration and balance across the steps necessary for applied goals. Significant contributions to this approach could be realized using technology transfer from established livestock industries.

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