BIOSECURITY AND REGULATORY CONSIDERATIONS FOR TRANSFER OF CRYOPRESERVED SPERM AND EARLY LIFE STAGES OF AQUATIC SPECIES

Terrence R. Tiersch
Aquaculture Research Station, Louisiana State University
Agricultural Center, Louisiana Agricultural Experiment Station,
Baton Rouge, Louisiana 70820, USA
E-mail: ttiersch@agctr.lsu.edu

Jill A. Jenkins
National Wetlands Research Center, Biological Resources Division,
United States Geological Survey, Lafayette, Louisiana 70506, USA

ABSTRACT

This paper addresses four types of biosecurity problems that result in varying degrees of uniqueness from cryopreservation of material from aquatic species: (a) disease transmission, (b) introduction of exotic species, (c) genetic consequences for target species, and (d) genetic consequences for ecosystems. Cryopreservation has been studied in aquatic species since the 1950s. Interest in frozen storage of gametes and early life stages of aquatic organisms has expanded recently and advances have made the technology available for commercial application. Integration of cryopreservation into commercial aquaculture has begun and could expand rapidly in response to global demands for improved broodstock and consistent production of seedstock. Frozen bull semen represents a multi-billion dollar global industry that provides a clear example of how a corresponding industry could develop for aquaculture species. This is because it is much simpler and cheaper to ship straws containing millions of frozen gametes or larvae than it is to ship even a few broodstock animals. These, and other benefits of cryopreservation, offer advantages for development, maintenance, and distribution of improved lines, but they also present a biosecurity challenge to guard against transfer of pathogens and other problems through space and time. Diseases of today could be disseminated rapidly throughout the world, and diseases of yesterday could re-emerge in the future through frozen samples. The potential for disease transfer could eventually present a stumbling block to the responsible development of this valuable technology for aquaculture. It is also possible that a lack of forethought will lead to unnecessary transfer of diseases or nonnative species with cryopreserved samples before proper awareness can be developed. The lack of specific guidelines and regulations, and the rapid expansion of cryopreservation research and application intensify this problem. Fortunately, regulatory frameworks, treaties, and agreements addressing the transfer of aquatic animals and aquaculture products exist through organizations such as the United States Department of Agriculture, the United States Fish and

Lee, C.-S., and O'Bryen, P. J. editors. 2003. Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. The World Aquaculture Society, Baton Rouge, Louisiana, United States.

Wildlife Service, and the Office International des Epizooties, the world organization for animal health that works to harmonize transfer regulations in over 150 member countries. Such mechanisms can be updated to include oversight of cryopreserved materials, especially with concern for the risks of temporal transfer of organisms (e.g., years or decades after collection), and the expanded opportunities for transfer provided by cryopreservation (e.g., sperm samples that would otherwise only survive for a few days). Existing procedures are also available to eliminate or minimize contamination of aquaculture broodstock, gametes, and early life stages. Responsible development of biosecurity programs for cryopreservation in aquatic species can occur before significant commercial application takes place. This effort would be assisted greatly by transferring successful practices from the existing cryopreservation industries established for livestock.

INTRODUCTION

The second half of the 20th century produced revolutionary technological advances in computing and communications linking world markets and fueling an unprecedented expansion of the global economy (Friedman 1999). The removal of barriers to communications and trade has enabled dramatic expansion in travel, allowing people, plants, animals, microbes, and materials to move freely across the planet while the human population has more than doubled. These trends, collectively referred to as globalization, have placed great demands on the natural systems and processes of the Earth and represent an escalating threat to wild populations and ecosystems. These general trends of human demands on the natural environment and the effects of expanding trade and travel define the biosecurity concerns relevant to aquaculture. This paper addresses the specific concerns related to the widespread application of cryopreservation to aquatic species. Many of the biosecurity concerns discussed pertain to aquaculture in general, but some, especially those related to travel through time, are unique to cryopreservation.

Transport across broad expanses of time and distance becomes possible when samples are cryopreserved. The effects of cryopreservation on biological materials range from subtle alterations to major disruptions, but survival at cellular and even organismal levels (e.g., embryos) is commonplace. Modern cryopreservation techniques have existed for 50 years, and it is likely that some of the first samples frozen would retain biological activity today. In fact, it is reasonable to assume that samples frozen today would be viable hundreds or thousands of years from now.

Biosecurity, which includes the cumulative measures taken to prevent movement of infectious disease agents into and away from potentially susceptible populations, incorporates physical, chemical, biological, and policy safeguards with regulatory considerations. Cryopreservation technology provides new opportunities by which gametes and early life stages can be transported throughout ecosystems, and it strikes a unique chord in the biosecurity arena. Cryopreserved materials offer a means by which aquatic organisms can be selectively bred, restored, and translocated, but exogenous material, inadvertently frozen, can also be transmitted.

A hallmark of a successful cryopreservation program is the minimization of threats of disease transmission. Microorganisms are routinely present in semen of domestic livestock (Foote 1996) as well as aquatic species (Jenkins 2000a), although samples from aquatic species are not routinely screened (Jenkins and Tiersch 1997). Unlike the artificial insemination industry for domestic livestock, the emerging area of aquatic cryopreservation must consider external fertilization by which male and female gametes make contact in the aquatic environment. Thus biosecurity considerations extend beyond the cryopreserved material to the surrounding aquatic environment. The integrity of aquatic ecosystems worldwide has been challenged by broad-scale alterations due to increased demands for water use and encroaching development (Hughes and Noss 1992), and unanticipated inputs of pathogens or exotic microbes into fragile ecosystems could alter biotic assemblages (Cunningham 1996).

Because inadvertent transmission of pathogens or non-native species can occur with any transfer of aquatic organisms, national and international regulations and guidelines have been instituted (Jenkins 2000b). Modification of these existing mechanisms will offer biosecurity safeguards for programs yet to be developed for aquatic species cryopreservation. The objectives of this paper are to: (a) define and provide examples of biosecurity problems that emerge from the application of cryopreservation to aquaculture, and (b) suggest some precautionary approaches, along with a review of existing and future biosecurity procedures and regulations.

BIOSECURITY PROBLEMS POSED BY CRYOPRESERVATION

It is important to remember that the problems posed by cryopreservation basically come from the removal of barriers to travel across distance (frozen samples are more stable and have longer working lifetimes than do fresh samples, and can thus be more widely and easily transferred) and across time (potentially hundreds or thousands of years). This paper views biosecurity as a tool to anticipate future problems in order to prevent or minimize their effects. Accordingly, problems beyond disease transmission through cryopreservation will be discussed. The rationale is that the broader picture of animal and ecosystem health should be addressed within biosecurity considerations. Thus, four types of biosecurity and regulatory problems that are posed by cryopreservation of material from aquatic species are addressed: (a) disease transmission, (b) introduction of exotic species, (c) genetic consequences for target species, and (d) genetic consequences for ecosystems. Disease transmission is the most likely of these problems, but discussion is also given to the other less-appreciated concerns that could arise through large-scale application of cryopreservation in aquatic species.

Disease Transmission

Only those microbes that multiply in or on a host and cause tissue damage are considered to be pathogenic, and manifestation of infection is host-dependent. Fish pathogens can be disseminated naturally by migration (e.g., anadromy), illegal transfers of live animals, transport of commercially caught fish for processing, transport of animals for establishment of recreational fisheries or aquaculture, as part of the ornamental fish trade, or through unauthorized or accidental introductions (Ganzhorn et al. 1992). Most commonly, diseases of aquatic

organisms are spread horizontally through contact of infected and uninfected individuals (Avault 1996), but some agents are transmitted vertically from parents to progeny, and it is these pathogenic microbes that are of greatest concern for gamete storage and artificial breeding (Jenkins 2000a).

Because microbes can be stored concurrently with sperm or other samples during cryopreservation procedures, pathogens or other organisms could remain viable after freezing and thawing. Since inadvertent transmission of undesired microorganisms (e.g., viral, bacterial, or parasitic agents) is possible through cryopreserved materials, implementation of biosecurity measures for aquatic species is inherent to successful cryopreservation programs.

Viral Agents

Viruses contained in tissues from animals can be stored at ultra-low temperatures without further treatment. In addition, viral diseases are more infectious than are bacterial or protozoan diseases and have a greater ability to surmount global barriers (Gibbs 1981). There are a number of viral diseases affecting fish and crustaceans (Table 1), with those of salmonids receiving the most attention in regulatory guidelines concerning the distribution of animals and gametes (USFWS 1995; OIE Fish Diseases Commission 2001; Code of Federal Regulations 1997). The Office International des Epizooties (OIE) lists notifiable, diseases, and those that are of economic importance or of widespread geographical distribution (OIE Fish Diseases Commission 2001). Some viral diseases that have been shown to be vertically transmitted are infectious hematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN), viral hemorrhagic septicemia (VHS), and *Oncorhynchus masou* virus (OMV).

Bacterial Agents

Cryopreservation is widely used to store microbial samples in the laboratory. During freezing, water is removed from the bacteria and surrounding media, and the dehydrated cells are stored at low temperatures, where they are genetically stable. Approximately 50 species of bacteria have been isolated from diseased fish (Fryer and Bartholomew 1996), and most are Gram negative.

Vertically transmitted disease microorganisms elicit the most concern with regard to gamete storage and artificial breeding. *Cytophaga psychrophila* has been implicated in vertical transmission via eggs and sperm of the Atlantic salmon, *Salmo salar* (Symula et al. 1999). *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD) in salmonids, is transmitted vertically via eggs from infected parents.

Another group of concern is the rickettsia, which are obligate intracellular parasites. Although they have been found in the gonads of infected fish, vertical transmission of the most characterized species, *Piscirickettsia salmonis*, has not been clearly demonstrated (Fryer and Mauel 1997). *Piscirickettsia salmonis* is an OIE-notifiable disease agent, and was the first of previously unrecognized rickettsial pathogens demonstrated to be the etiologic agent of epizootic disease (Fryer and Bartholomew 1996).

Table 1. Examples of viral diseases of concern for fish and crustaceans

Diseases of Fish Notifiable to the Office International des Epizooties[a]
Epizootic hematopoietic necrosis
Infectious hematopoietic necrosis (IHN)
Oncorhynchus masou virus disease (OMVD)
Spring Viremia of carp (SVC)
Viral hemorrhagic septicemia (VHS)
Other Significant Diseases of Fish (as categorized by OIE)
Channel catfish virus disease
Viral encephalopathy and retinopathy
Infectious pancreatic necrosis (IPN)
Infectious salmon anemia (ISA)
Red sea bream iridoviral disease
White sturgeon iridoviral disease
Diseases of Crustaceans Notifiable to the OIE
White spot disease
Taura syndrome
Yellowhead disease
Other Significant Diseases of Crustaceans
Infectious hypodermal and hematopoietic virus
Baculoviral midgut gland necrosis virus

[a] *International Aquatic Animal Health Code* (OIE Fish Diseases Commission, 2001).

Rickettsiae can also affect other aquatic species such as molluscs. *Candidatus xenohaliotis californiensis* causes withering syndrome (WS), which affects wild species including the black abalone, *Haliotis cracherodii*, and cultured species such as the red abalone, *H. rufescens*. *Coxiella burnetti* has been isolated from humans exposed to sheep infected with Q fever, and these rickettsia were later isolated from semen and urine. In mice, *C. burnetti* was found to be adherent to sperm cells (Tylewska-Wierzbanowska and Kruszewska 1990), and it is sexually transmitted in humans, mice, and cattle. Similar routes of transmission could exist for rickettsial diseases of aquatic species.

Fungal Agents

Most fungi produce spores that are the primary unit of transmission, often through the air. Fungi, such as *Saproglegnia* and *Achlya*, are found on dead fish eggs and are common in culture situations. Fungal mycelia, such as those of *Branchiomyces*, are not uncommon in necrotic fish gills. Fungi are resistant to damage from ice crystal formation and could thus survive cryopreservation. Specific management of broodstock and gamete collection may be necessary to prevent contamination of samples.

Parasitic Agents

Parasitic protozoa are intolerant to drying. Thus, more parasitic protozoa affect aquatic species than infect terrestrial animals. Cryopreservation of protozoan parasites has been reported. For example, the flagellate *Trichomonas vaginalis* exhibited survival after cryopreservation with human sperm, and thus could be transmitted by artificial insemination with cryopreserved human semen (Sherman et al. 1991).

Whirling disease caused by *Myxobolus cerebralis* is one of the histozoic parasitic diseases that has been listed as notifiable for fish health inspectors. It was first reported in Europe in the early 1900s, probably arrived in Pennsylvania in the 1950s via frozen, processed fish (Heckmann 1993), and has spread throughout the United States. It has been proposed that the dispersal of *M. cerebralis* occurs through spores contaminating trout eggs, or through frozen trout (Ganzhorn et al. 1992). Waterborne spores of *M. cerebralis* are infective. The parasite is well adapted to natural environments where salmonids are found, and has a complex life cycle involving the fish and oligochete hosts. Although treatment at infected facilities includes ultraviolet irradiation of water supplies, spores of *M. cerebralis* are resistant to disinfection methods, which suggests that they could be resistant to damage caused by cryopreservation.

Other Disease Concerns

Alterations to the aquatic environment can result in increases in waterborne disease outbreaks (Epstein et al. 1994). Microbial agents, such as *Vibrio* species that cause human disease, are sometimes transferred by aquatic species such as oysters and copepods. The use of integrated systems in aquaculture, such as those that link the raising of fowl, swine and fish, has raised questions about the potential for transfer of disease agents (primarily viral) among terrestrial and aquatic organisms (e.g., Velasquez 1980), and reinforces the concept that biosecurity for aquaculture cryopreservation extends beyond the target species to addressing concerns about human disease.

Another biosecurity concern is the potential for non-pathogenic organisms not necessarily screened for in detection programs to cause disease in other geographic regions. For example, it is likely that transfers of crayfish from the United States in the 1860s brought to Europe a fungus, *Aphanomyces astaci*, which was non-pathogenic to crayfish in North America. Over the next hundred years, this fungus caused a disease known as "crayfish plague" which devastated the native European species including the noble crayfish, *Astacus astacus* (Ackefors and Lindqvist 1994). Transfers of cryopreserved materials could thus yield unforeseen disease problems in other taxa, while the inadvertent transfer of organisms can have effects other than disease transmission in animals and ecosystems.

Introduction of Exotic Species

The consequences of the introduction and establishment of non-native "exotic" species are familiar in fisheries, and were addressed in a recent book published by the American Fisheries

Society (Schramm and Piper 1995). Cryopreserved samples can harbor a variety of non-pathogenic organisms distributable across space and time. Spatial distribution of species outside of their natural range can cause significant alteration in the new habitat, including detrimental effects on the resident organisms. Distribution of organisms across time can lead to introduction and establishment of species that may or may not represent extensions of historical ranges (Wachtel and Tiersch 2000).

Spatial Exotics

In a limited sense, the straws or containers used to hold frozen samples can be viewed as being analogous to the ballast tanks of bulk cargo vessels (Carlton and Geller 1993) responsible for transfer of non-indigenous aquatic organisms throughout the world. Transfer of exotic species is thus an unintended consequence of global trade and could be an unintended consequence of cryopreservation. This concern is not limited to the potential transmission of microbial contaminants as described above. Early life stages of multicellular organisms have been successfully cryopreserved. For example, trochophore larvae of the eastern oyster, *Crassostrea virginica* (Paniagua-Chavez et al. 1998; 2000), and juveniles of the marine polychaete *Nereis virens* (Olive and Wang 1997) have been shown to develop normally after cryopreservation. Other organisms such as rotifers (genus *Brachionus*) are common in euryhaline systems and have been shown to survive freezing and thawing (Toledo and Kurokura 1990). While it would seem unlikely that cryopreservation could lead to the unintended establishment of organisms such as these outside of their native ranges, it would be wise to address this possibility through biosecurity planning before widespread application and distribution of cryopreserved samples occurs in aquatic species. The rapid invasion and alteration of ecosystems wrought by organisms such as the zebra mussel, *Dreissena polymorpha,* in the United States, and the ctenophore comb jelly, *Mnemiopsis leidyi,* in the Black Sea, provide examples of the spatial distribution of exotic species (Carlton 1996).

Temporal Exotics

If cryopreservation is considered as a form of time travel, another concern is possible: the temporal exotic, i.e., a species transferred through time to a new area, or re-established in its natural range some time subsequent to its extirpation from that range. The degree of ecological displacement of this species would be related to the degree of temporal displacement (Wachtel and Tiersch 2000). The severity of ecological displacement would likely increase with the duration of storage. It is possible that a species thawed 25 or 50 years after cryopreservation would not be able to survive in its previous environment due to the absence of habitat or food items, for example, the reintroduction of an extirpated large predator into its former range. Species extinct in the wild, but existing within cryogenic storage, could be recovered and distributed with unknown effects on ecosystems if techniques for cryopreservation of embryos could be developed (Hagedorn et al. 1997; Hagedorn and Kleinhans 2000).

Other examples of temporal exotics mentioned above include formerly benign commensal organisms such as fungi or bacteria, that when reintroduced some years after extirpation, could

assume new roles as pathogens. Moreover, the inadvertent transfer of today's pathogens to the future could be dramatic. The reemergence of human diseases, such as smallpox, polio, or tuberculosis would be disastrous to individuals without vaccination or previous exposure to the diseases, and the risks would escalate with time (outbreaks in 50 years would presumably be more intense than those in 5 years). These concerns have recently received considerable attention due to attempts to obtain isolates from the frozen bodies (buried in the permafrost of northern settlements) of victims of the Spanish Flu of 1918, which caused the deaths of between 25 and 40 million people worldwide. Thus, the possibility, if not the likelihood, that that the organisms and germ cells frozen today can have detrimental effects in the future must be considered, even if the organisms and germ cells are innocuous in the present.

Genetic Consequences for Target Species

Loss of Within-population Variability

Cryopreservation enables collection and storage of gametes from particular animals for use in production of far greater numbers of offspring than would otherwise be possible. For example, sperm from a small number of superior males could be collected, frozen, and distributed widely. These animals could have a disproportionate influence on succeeding generations of offspring, leading to an industry-wide, regional or global reduction in genetic variability. Although inbreeding is not currently considered to be a problem with aquaculture broodstocks, it could be in the future. Established cattle, swine, and poultry industries have already had to face this problem, which was exacerbated by widespread application of cryopreservation. The effects of inbreeding can be as debilitating as disease outbreaks to aquaculture or wild species, and may indeed reduce disease resistance, thus warranting biosecurity consideration for cryopreservation.

A special consideration due to transfer of samples through time that is especially relevant for endangered species should be pointed out here. Populations or species threatened with extirpation typically have diminished genetic diversity, and with variability in sperm collection, it is likely that a few males could contribute a disproportionate excess to the total volume of sperm cryopreserved. In this case, the loss of genetic variation would be multiplied across generations. With recurrent thawing and breeding over time, it is possible for sperm from single over-represented males to fertilize eggs produced by their offspring for successive generations, yielding multi-generational inbreeding by overuse of single sires (and a form of "recurrent founder effect"). This is not an unlikely event, nor one without precedent. For example, in the 1970s within the dairy industry, sperm of a single Holstein bull (named "Elevation") was used in over a million matings. While inbreeding in domesticated animals may be desirable to some extent to establish specific traits, it is not desirable for natural populations at risk for loss of genetic diversity. On the other hand, genetic diversity can be increased to inappropriate levels as well.

Loss of Among-population Variability

Cryopreservation can allow widespread distribution of samples beyond that possible by shipment of fresh (unfrozen) sperm, which typically have a useful lifetime of days if kept refrigerated. This could allow crossing of populations, lines, or strains that would be unlikely or impossible if transfers were limited to fresh gametes or live broodstock. This interbreeding of distinct stocks increases genetic variation, but does so at the potential cost of disrupted adaptations to local environments (Busack and Currens 1995) and can produce homogenization of genetic resources. This can be especially harmful to species such as salmonids with highly organized population structures and many distinct subspecies and strains (Lutz 2001b). Cryopreservation would, for example, enable crosses of salmonid populations, such as spring and fall migration runs, that would not otherwise overlap in spawning season. While outbreeding involves crosses within a particular species, cryopreservation can also be used to produce crosses between species.

Genetic Consequences for Ecosystems

Hybridization (Introgression)

Although the interbreeding of individuals from different species is desired in some settings, such as in the generation of hybrid vigor for agricultural purposes, it can have disastrous consequences for species survival. Again salmonids provide a good example, because many species will interbreed naturally if geographic barriers are removed. Past stocking practices placed rainbow trout, *Oncorhynchus mykiss*, in native regions of cutthroat trout, *O. clarki*, resulting in extirpation of pure stocks of cutthroat trout (Leary et al. 1995). Cryopreservation enables wide transfer of samples and allows crossing of species that do not have naturally overlapping spawning seasons or mutually compatible spawning behaviors. A further concern comes from the release of fertile hybrids capable of backcrossing with the natural parental populations. The resulting genetic admixtures further disrupt adaptive genetic combinations, and are capable of introducing foreign genetic material into wild populations, a process referred to as "introgression." Backcrossing and introgression are sometimes used in culture settings for breed formation (Tave 1993; Lutz 2001a). Cryopreservation would facilitate this practice and could increase the potential for escape of these animals.

Genetically Modified Organisms

The ability to produce genetically modified organisms (GMOs) by methods such as the insertion of new genes has been referred to as the "gene revolution," and has raised considerable controversy among scientists (Reichhardt 2000), policy makers, advocacy groups, and the general public (Nash 2000). Products such as insect-resistant corn, pesticide-resistant cotton, and disease-resistant soybeans have entered the marketplace, and transgenic animals are soon to follow. Transgenic animals can provide sperm capable of producing transgenic offspring, thus creating a market for cryopreserved sperm or larvae. The transfer of cryopreserved straws is considerably easier than the transport of broodstock animals. Such

transfers are not adequately addressed by current regulations and could move control of production outside of regulated facilities. Escape of GMOs could cause contamination of wild populations with foreign genetic material (Hallerman and Kapuscinski 1993). This has become a heated issue for advocacy groups such as Greenpeace and has resulted in the continued association in the popular media of aquaculture and GMOs (Tiersch and Hargreaves 2002). Negative media coverage can affect consumer demand and damage the credibility of aquaculture industries regarding concerns about environmental health and human food safety. While the availability of cryopreservation will speed the application of transgenic organisms in aquaculture by aiding development, maintenance, and distribution of transgenic lines, development of methods to minimize the possible environmental effects of escaped GMOs will become necessary.

Tetraploid Broodstocks

Crossing tetraploid organisms with normal diploids to directly produce triploid offspring is likely to be adapted for use with cryopreserved sperm, because it would allow marketing of GMOs with greatly reduced potential for unwanted reproduction of escaped individuals. Theoretically, customers would purchase sperm derived from transgenic tetraploid broodstocks to produce sterile triploids in their own facilities. In addition, triploidy would protect intellectual property and business investments in improved lines by preventing unauthorized spawning of GMOs. A biosecurity concern here would be the escape or release of tetraploid individuals, which could breed with wild diploids and affect small populations by reducing the numbers of fertile offspring. By assisting development, maintenance, and distribution of tetraploid lines and their sperm, cryopreservation will be an important part of the process of establishing biosecure aquaculture production protocols.

PRECAUTIONARY APPROACHES

Applicability of Existing Regulations, Agreements, and Treaties

An exhaustive compendium of agencies and groups responsible for fish disease regulations and agreements is outside of the scope of this paper. We will provide, however, the names of agencies, programs, and member organizations to serve as examples of the groups that can be consulted for cryopreservation biosecurity efforts in aquatic species. Viral, bacterial, and parasitic agents such as rickettsia and mycoplasmas are often isolated from semen of domestic livestock (Jenkins 2000a). For this reason, national and international regulatory requirements and guidelines have been instituted for the translocation of stored gametes (Jenkins 2000b). Biosecurity in aquatic species is an emerging topic resulting from globalization of the world economy, rapid increases in communications and trade, viral pandemics in domestic agriculture (Ferguson et al. 2001), yearly increases in aquaculture production as a worldwide food source, bioterrorism, and increased awareness of declining natural resources (Hughes and Noss 1992). Given the many challenges confronting agriculture and natural resources, successful biosecurity efforts for cryopreservation programs for aquatic species will require integration and cooperation across all sectors and levels.

At the global level, the OIE, the world organization for animal health (www.oie.int), created in 1924 and headquartered in Paris, has more than 150 member countries. It has been a leader in defining international health standards for terrestrial and aquatic animals, as well as harmonizing regulations for trade in animals and animal products, including aquaculture species. The OIE has also developed guidelines to assist in the identification of disease-free mammalian breeding stocks to provide pathogen-free semen that could be adapted for application to aquatic species. The International Animal Health Code Commission, one of the OIE specialist commissions, deals with regulatory issues. Reference laboratories and collaborating centers provide OIE member countries with scientific and technical advice related to disease surveillance and control. In addition to establishing international standards, the OIE has addressed issues such as diseases transmissible by semen and embryo transfer techniques (Hare 1985), and rules for trade in animals and animal products (OIE 1986).

The OIE Fish Diseases Commission (FDC) compiles information on diseases of fish, crustaceans, and molluscs, and on methods to control these diseases. The FDC has published a *Diagnostic Manual for Aquatic Animal Diseases* (OIE Fish Diseases Commission 2000) and *International Aquatic Animal Health Code* (OIE Fish Diseases Commission 2001). The FDC code contains information on international trade in fish, molluscs, and crustaceans, including sections on import risk analysis and import/export procedures. Models of international certificates for trade in gametes, as well as live and dead aquatic animals are also provided in the FDC code. Diseases that qualify for notification to the OIE and other significant diseases are listed. The FDC manual provides recommendations for diagnostic methods for all diseases that appear in the FDC code. Both are updated as new information becomes available.

Since the 1980s, the European Union (EU) has encouraged cooperation between countries and some international health standardization has been achieved (Gibbs 1981). Trade in wildlife and animal parts has become a large global industry with oversight from the Convention on International Trade in Endangered Species (CITES) and the EU Wildlife Trade Regulation (EUWTR). The CITES, in effect since 1975, is the only global convention focusing on the protection of plant and animal species from unregulated international trade. Although CITES and the EUWTR each address animal parts and trade, there are no published guidelines for cryopreserved gametes.

In the United States, the bulk of the responsibility for managing fishery resources lies with the individual states (Jenkins 2000b), which often have different procedures. For instance, there are no consistent guidelines for timely reporting of disease outbreaks of regulatory concern under current federal and state guidelines. The federal responsibility for managing fishery resources in the United States lies with the United States Fish and Wildlife Service (USFWS). The Fish Health Policy (1995) serves as the basis for USFWS efforts at restricting the movement or introduction of animals, and minimizing the impacts of fish pathogens and diseases within the National Fish Hatchery System. Gametes, fertilized eggs, and fish are shipped or accepted only in compliance with the Fish Health Policy, and provisions are established by area disease control programs, regional stock transfer policies, and state regulations.

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) provides leadership to ensure the health and care of animals and plants thereby improving agricultural productivity. The Veterinary Services arm of APHIS assists aquaculture industries by issuing export health certificates for live fish and fish eggs, and ensures that veterinary biological products are safe and effective. Within the United States, unlike the authority it exhibits with livestock and crops, Veterinary Services does not have the authority over aquaculture to prevent the introduction and dissemination of harmful pests and diseases. Permits from the USDA are required for the importation of amphibians, reptiles, shellfish, and aquatic species, including their blood, tissues, serum, feces, extracts, venom, and urine. For importation of livestock, including their semen and embryos, a health certificate must be issued by the exporting country. With regard to cryopreserved gametes and embryos of aquatic species, clear permitting and health certification protocols are not available.

The Code of Federal Regulations (CFR) is a codification of the rules published in the *Federal Register*, the legal daily newspaper of the Office of the Federal Register, National Archives and Records Administration. Specific guidelines are provided in Title 50 (wildlife and fisheries), Chapter I (USFWS, Department of the Interior), Part 16 (injurious wildlife), Subpart B (importation or shipment of injurious wildlife), Section 16.13 (importation of live or dead fish, mollusks, and crustaceans, or their gametes or fertilized eggs) of the CFR (1997). The guidelines are more general for species other than salmonids. If gametes are to be shipped into the United States, the lot must be accompanied by a certification performed by a fish pathologist from the country of origin as per Title 50. The Code states that no progeny or eggs of imported species may be released into the wild, except by the state wildlife conservation agency that has jurisdiction over the area of release, or by persons having prior written permission from such an agency.

Much detailed information is given in the CFR. Guidelines are provided for specific sampling requirements for shipment of gametes and fish, and for virus assay methods. General sample processing requirements list concentrations and types of antibiotics and antifungal agents that may be added at the time of collection to tissue samples for control of microbial contamination. The CFR specifies what is required in the certificate to accompany importations. Information concerning particulars of importation and certification requirements may be obtained by contacting the U. S. Department of the Interior, USFWS, Division of Fish Hatcheries, 4401 North Fairfax Drive, Arlington, Virginia 22203, USA.

The Magnuson-Stevens Fishery Management and Conservation Act mandates that the National Marine Fisheries Service, regional management councils, and other federal agencies delineate "essential fish habitat" for important anadromous fishes. These delineations would provide a cornerstone for site consideration in the use of cryopreserved gametes. Another useful site categorization would be "habitat areas of particular concern," or those that provide unique and important ecological functions, and are sensitive to human-induced environmental degradation.

Between the United States and Canada, The North American Commission (NAC) of the North Atlantic Salmon Conservation Organization (NASCO) sets forth protocols that implement commitments to take measures necessary for maintaining fish health, genetic integrity, and ecological integrity regarding native Atlantic salmon stocks (Windsor and Hutchinson 1994). Included in the guidelines are that Atlantic salmon adults, eggs, and gametes are not to be imported from IHN-enzootic areas without thorough demonstration of the absence of IHN. Also, prior to transfer of eggs or fish (but not sperm), at least three health inspections of the donor facility must be completed within a two-year period preceding the transfer in order to ensure the absence of restricted pathogens. Milt from European stocks is not restricted from entering the state of Maine, thus enabling expansion of the use of crosses between European and North American Atlantic salmon in aquaculture.

At the state level there are several other examples of cooperation. The New England Salmonid Health Committee is a group of fish health specialists charged with responsibility for all regional salmonid health issues. The Maine Conservation Plan addresses threats to wild salmon with regard to genetic and disease concerns and outlines specific actions, such as maintenance and improvement of the current state and federal fish health protocols, development of an emergency disease eradication program, and compilation of industry practices into a Fish Health Code of Practices. Salmonid health regulations in Maine, implemented in 1999, along with the New England Salmon Health Guidelines establish a detailed framework for monitoring the health of salmonid fish held at hatcheries and fish farms. They set forth a permitting system to ensure that salmonids being transferred within or introduced into state waters meet minimum fish health standards. The regulations also establish the requirements for reporting and the procedures for responding to outbreaks of diseases of regulatory concern.

The Great Lake Fishery Commission (GLFC) has managed fish disease control in the Great Lakes Basin. It has a control policy and model program (Hnath 1993) that delineates ways to prevent and control infectious diseases. It also has a protocol that proposes recommendations to minimize the risk of introducing disease agents with the importation of salmonids (Horner and Eshenroder 1993). The GLFC has aided in the development of legislation and regulations to minimize risks associated with introducing disease agents along with imports of salmonids.

Finally, the World Health Organization, Food and Agriculture Organization, and the World Trade Organization are other agencies with particular concerns of aquatic species. The Fish Health Section of the American Fisheries Society, and other international professional organizations involved with aquatic animal health should be among the participants intimately involved with formulating guidelines for biosecurity policies regarding cryopreserved gametes. Other professional organizations include the World Aquaculture Society, European Association of Fish Pathologists, International Association for Aquatic Animal Medicine, Japanese Society of Fish Pathology, Asian Fisheries Society, and the National Shellfisheries Association. At present there are considerable gaps in the coverage afforded by existing regulations, treaties, agreements, and plans regarding the biosecurity concerns posed by widespread application of cryopreservation in aquatic species.

Potential Regulatory Modifications

Widespread application of cryopreservation in aquatic species will necessitate modification of regulations to address several points. First, cryopreserved samples can be distributed more widely than fresh samples because of the extended storage time and smaller package sizes. This will likely expand transfer into new areas that do not have experience in handling the biosecurity of imports and exports and poses an important concern regarding the spread of pathogens and exotic species. Second, cryopreservation creates a new set of concerns about temporal transport of current and potential pathogens and exotic species. Third, regulations should address the genetic concerns posed by application of cryopreservation to aquatic species, such as inbreeding, hybridization and introgression, and widespread distribution of GMOs.

The OIE guidelines provide essential background for creating appropriate methods for preventing the introduction and spread of known diseases to known hosts in the aquatic environment. The International Council for the Exploration of the Sea (ICES) is the official arm of the North Atlantic Salmon Conservation Organization that is charged with the international management of Atlantic salmon stocks on the high seas. The ICES promotes research and provides sound management recommendations for the conservation of North Atlantic salmon stocks. The ICES Code of Practice on Introductions (ICES 1995) provides useful information on handling as yet unidentified disease transfer threats and could be adapted for cryopreserved materials.

Diseases and disease agents of importance to artificial insemination and embryo transfer in livestock have been categorized by their known risks of transmission (Thacker et al. 1984; Philpott 1993). Similar categorizations should be established for use with aquatic species. Documents from the Great Lakes Fishery Commission provide examples of this approach. A pathogen coding system, as described in the National Fish Health Policy (USFWS 1995), designates broodstock facilities and fish stocks according to the presence of the specific disease agents listed in the policy. Biosecure cryopreservation systems should employ such methods to assign categorization to levels of risk.

The artificial insemination industries developed for domestic livestock species have recognized ethical obligations, as well as economic and genetic incentives to practice excellent husbandry practices, and have established strict guidelines to minimize disease agent transfers. The National Association of Animal Breeders (NAAB), formed in 1946, is a well-established organization in Missouri (www.naab-css.org) whose programs could be modified for artificial breeding practices with aquatic species. Certified Semen Services, Inc. is a subsidiary of NAAB, and was established to provide members with an inspection program and other services for such things as disease control of semen produced for artificial insemination. These services provide a minimum industry standard for artificial insemination health management. For instance, bulls are required to be tested for disease prior to entry into the program, during an isolation period, and semi-annually throughout their tenure as sperm donors. Specific guidelines are offered for semen collection, handling, shipment, and storage. Concerns raised by temporal transport of samples have been addressed by the NAAB during the past half-century.

Management plans are available for protection of evolutionarily significant units (ESUs or distinctive population groups), such as the Hatchery and Genetic Management Plans developed for Pacific salmon by the National Marine Fisheries Service (available at the website www.nwr.noaa.gov/1hgmp/index.html). These Plans could be updated to include application of cryopreservation to conservation efforts. In addition, Performance standards for safely conducting research with genetically modified fish and shellfish have been developed through the USDA Agricultural Biotechnology Research Advisory Committee, aiding researchers and institutions in assessing the ecological and evolutionary safety in genetic modification of fish, crustaceans, and molluscs (ABRAC 1995). These Performance Standards consist of three documents: flowcharts to provide a decision pathway; supporting text to provide detailed information necessary for decisions, and a worksheet summarizing the researcher's decision process. Modification of the Standards to include consideration of the potential effects of cryopreservation in the development and distribution of GMOs could be beneficial.

Biosecurity Procedures

Research on cryopreservation began in fish 50 years ago (Blaxter 2000), yet it remains essentially a research activity for which empirical methods have been described (e.g., Wayman and Tiersch 2000). Commercial application is just beginning (Tiersch 2001). It is expected that large-scale cryopreservation programs would perform a number of linked activities (e.g., Caffey and Tiersch 2000a) with prescribed flows of information and samples and established quality control points (Fig. 1). Cryopreservation will likely be integrated into a germplasm repository system combining the activities of several physical locations (Fig. 2). In such a system, specialization is possible and a centralized location can be responsible for addressing important biosecurity concerns that might be neglected at smaller sites. The USDA plant germplasm system (www.ars-grin.gov/npgs) provides an excellent model for the development of a germplasm repository system for aquatic species. At present, four groupings of aquatic species (warm water fishes, cold water fishes, molluscs, and crustaceans) have been identified for inclusion in the USDA National Animal Germplasm Program (www.ars-grin.gov/nag). Some suggestions for biosecurity procedures follow.

Suggestions for Personnel and Facilities

Adequately trained professionals are needed to gather reliable data such as estimates of motility, and to process specimens properly. For example, it is possible that untrained personnel could misidentify bacterial contamination (by motile *Pseudomonas*) as motile sperm (Jenkins and Tiersch 1997). If gametes are to be collected at a facility that is also planning to perform analytical and diagnostic microbiology, additional rules should apply (Jenkins 2000c). Areas should be designated for cell and bacterial cultures, media preparation, glass washing, sterilization, storage, microscopy, and record keeping. Animal facilities should be physically separated from culture facilities, and traffic should be controlled in and between areas. Separate containment areas are needed to handle hazardous and potentially contaminated cultures, and protocols must be established for disposal of contaminated samples. All media coming in contact with gamete samples and unused samples, should be decontaminated before disposal.

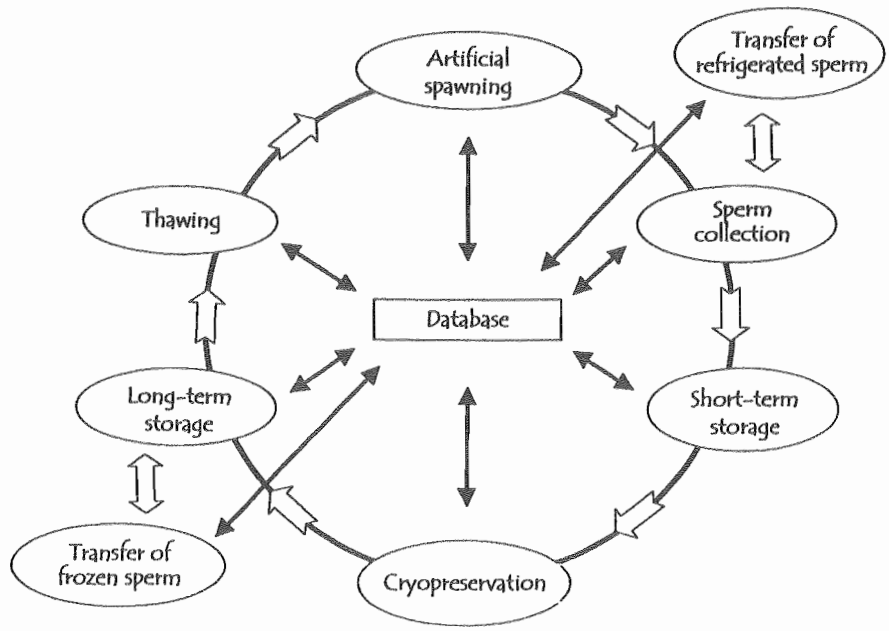

Figure 1. Activities (shown in ovals) involved in cryopreservation of fish sperm at a single location (linked by circle). Transfers of samples between consecutive activities are represented by a clockwise rotation with associated quality control points (white arrows). Information flow in and out of a centralized database (black arrows) enables record keeping for pertinent data such as sperm motility, fertilization and inventory. Refrigerated and frozen sperm can enter or leave the facility.

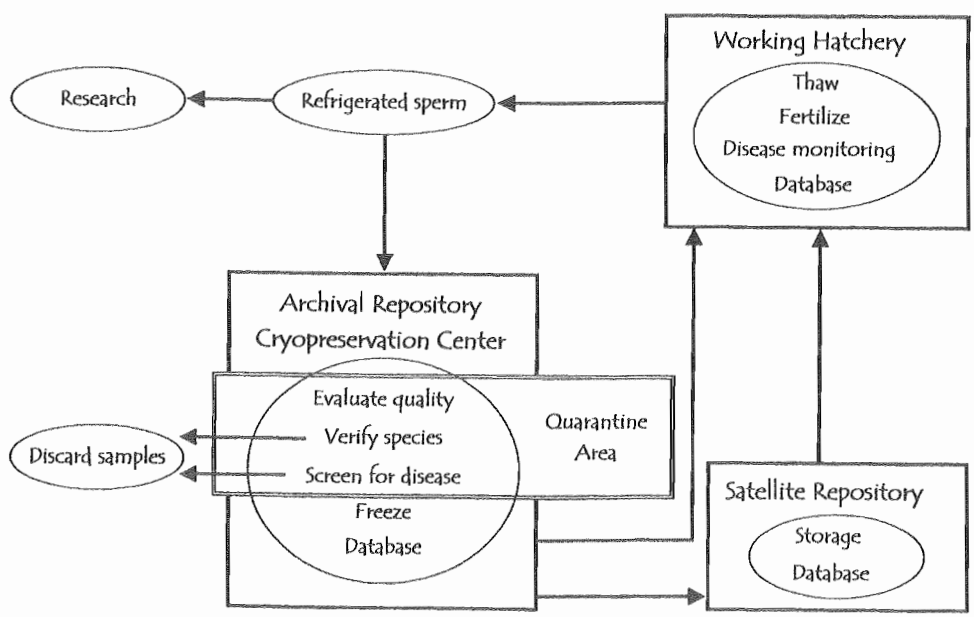

Figure 2. A scheme for a simple germplasm repository system based on three physical locations (shown in rectangles) providing a quarantine area for incoming samples, archival storage, duplicative storage (satellite repository), and production and use of sperm (working hatchery). Activities are shown in ovals, and transfers of samples are shown by arrows.

Semen collection equipment that comes in contact with fish should be thoroughly disinfected after each use. Effective engineering controls for handling sperm samples can include general room ventilation and high-efficiency particulate air (HEPA) filters. Periodic internal and external sterilization (with 70% ethanol) of the storage dewars will reduce contamination.

Suggestions for Broodstock Programs

Many biosecurity concerns can be addressed by maintenance of high quality broodstock. Efforts to exclude or control diseases at broodstock facilities are essential. Pathogen testing can be performed according to procedures outlined in the *Blue Book* (AFS 1997) and animal health status reports (OIE 2001; USFWS 1995).

Good record keeping is essential for proper genetic management. Ecological and life history data are important for wild broodstocks, especially for threatened and endangered species (Gorman 2000). Recently, the USFWS and the United States Geological Survey have produced National Fish Strain Registries to provide information databases on managed wild and cultured fish species (Kincaid et al. 1998). In 1990, the USDA organized the National Genetic Resources Program to acquire, characterize, preserve, document, and distribute germplasm important for food and agricultural production. The USDA Germplasm Resources Information Network (GRIN) web server provides germplasm information about plants, animals, microbes and invertebrates (www.ars-grin.gov). These and other sources can serve as models for gathering and archiving of information in searchable databases (Kincaid 2000).

Efforts should be directed at improving reproductive traits of broodstock, such as sperm production, to ensure the availability of gametes and offspring when needed, and to assist with scheduling and coordination among facilities. Robust safeguards should be in place to prevent escape of genetically modified organisms, hybrids, or tetraploids. Limits could be set (*a priori* if possible) on the volume of sperm frozen from any one male regardless of the population level of a species. Frozen samples could be viewed as a means to reconstitute genetic variation in the form of fish. The frozen lifetime of sperm could be established (also *a priori*) and the sons produced from cryopreserved sperm could be used to provide sperm to be frozen for future reconstitution. Thus frozen sperm from any one male would be carried forward for only a generation or two. Even inferior sperm samples could be used to produce small breeding populations in this scheme, which would then provide gametes for production of fishes and for cryopreservation using newer improved techniques. This concept of using sperm for reconstituting lines for subsequent breeding is of special relevance for genetic management of threatened and endangered species.

Cryopreserved sperm also provides a source of non-invasive DNA samples for utilization in genetic management programs (Pittman-Cooley and Tiersch 2000). Analysis of frozen samples could be integrated into breeding or conservation programs to ensure specific genetic composition within populations. Integration of cryopreservation with newer genetic technologies, such as microchip gene arrays, could enable rapid and comprehensive analyses and use of sperm from specific males even after their death.

Suggestions for Collecting and Handling of Samples

Contamination of semen can occur during collection, processing, storage, and transport. Some preventative measures have been described for limiting the spread and amplification of bacteria, viruses, fungi, mycoplasmas, rickettsia, and parasites (Jenkins 2000c). Generally, only animals with a clean health history should be used for gamete collection, both to maximize the quality of gametes (Jenkins 2000c) and minimize microbe presence. Broodstock management practices have been delineated for suspect diseased animals (USFWS 1995). Sanitation during collection is essential, and thus materials and equipment used to handle semen should be sterile. Good practice guidelines for handling and processing samples are especially important for non-domestic animals when disease-free status cannot be guaranteed and unsophisticated technology is used (Russell et al. 1997). The quality of semen may be affected by the health of the donor (AFS Fish Health Section 1997), collection technique, and sample handling. Collected semen should be free of water, blood, mucus, or fecal matter. Withholding feed prior to milt collection has been suggested (Rana 1995).

Suggestions for Extender Solutions and Cryoprotectants

Sperm are generally collected into extenders, or supportive buffering media. Solutions should be sterilized (by filtration or autoclaving), and either stored at 4 C and used within 1 week (Rana 1995), or frozen at -20 C or below. High-purity water should be used to prepare solutions. Storage of water before use can result in contamination with bacteria such as *Pseudomonas* (Jenkins and Tiersch 1997). It is useful to ensure that sperm are not activated by the extender solution (Bates et al. 1996) and that the osmotic pressure is correct to ensure that sperm remain viable. Chemicals such as glycerol, dimethyl sulfoxide, methanol and ethylene glycol, called "cryoprotectants," are used to minimize damage to the biological material during freezing and thawing. They should be of good chemical grade and stored appropriately (glycerol, for example, is easily contaminated by microorganisms).

Suggestions for Control of Microbial Contamination

To control infectious agents that are transmissible with sperm, the addition of antimicrobial compounds is common in breeding practices with cattle and stallions (Jenkins 2000a). Standard combinations specific for particular species have been configured taking into account seminal quality (e.g., Segovia et al. 2000) and fertility. Cyroprotectants, however, may render antibiotics and antimycotics less effective (Bartlett 1991). Research is required to identify the antibiotic and antimycotic combinations and concentrations to control microbes in the sperm of aquatic species. If antibiotics are added to semen in powdered form, they should be dissolved in sterile distilled water and an appropriate contact time should be allowed before processing for cryopreservation. There should not be an indiscriminate addition of antibiotics, and those added should provide broad-spectrum activity. The selection of antibiotics is best made following cytotoxicity studies that delineate the appropriate type and concentration that favor storage of sperm from the species under study. It is also worth noting that successful

cryopreservation methods for one microbial strain or species are not necessarily applicable to similar cells or species. Preventative measures can capitalize on this fact. If an excludable pathogen species is a potential threat, the specific cryopreservation procedure can be tested with the unwanted microbe. The cryopreservation method may not allow survival of the pathogen after thawing.

Suggestions for Sample Labeling and Storage

Proper labeling of cryopreserved samples is essential. Usually samples are in storage for days, months, or even 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. At the minimum, straws used for research should be labeled to indicate fish identification number, cryoprotectant, and cryoprotectant concentration. If possible, more sophisticated labeling, such as pre-printed straws, should be considered even for research applications (Fig. 3). Straws intended for archiving and breeding uses should receive the best labeling possible. Good practice guidelines for packaging of samples must also be established. Leakage of contents and experimental contaminants has been demonstrated (Russell et al. 1997), and infectious agents can be transferred during storage in liquid nitrogen (Thacker et al. 1984).

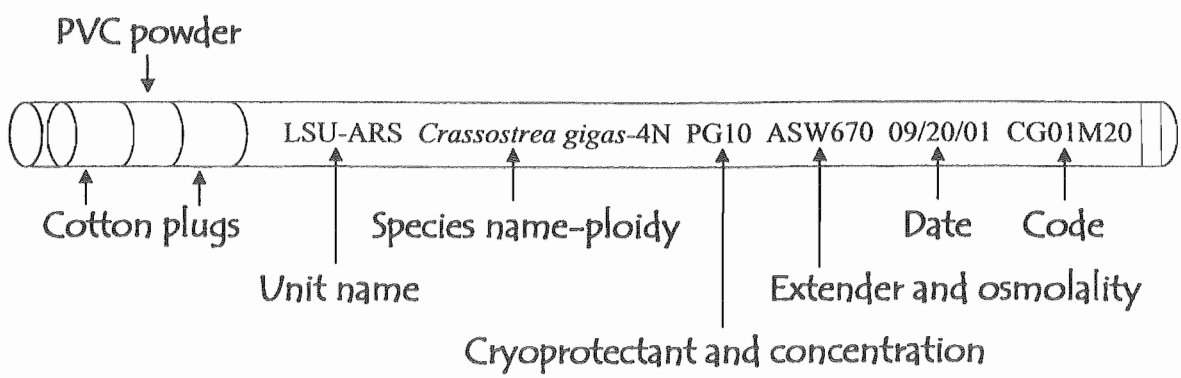

Figure 3. An example of the labeling possible for use with standard 0.5-ml French straws (133 mm long; 3 mm wide). One end comes with a factory plug that is sealed by moistening with sperm suspension. Labeling of this sort is produced by a specialized laser printer. In this example, sperm from a tetraploid (4N) Pacific oyster (*Crassostrea gigas*) were frozen using 10% propylene glycol (PG10) as the cryoprotectant and artificial sea water prepared at 670 mOsmol/kg (ASW670) as the extender. A standardized inventory code has been used for identification of individual animals, where "CG01M20" refers to "*C. gigas* 2001 (year) male 20."

Suggestions for Sample Transfers

Unintentional transfers or releases of gametes, early life stages, juveniles and adults of aquatic species are possible through the transfer of cryopreserved samples. A biosecurity program should evaluate and rank the potential and severity for any such transfers and employ appropriate safeguards. With respect to intentional transfers, the paperwork for a Request for Transfer should categorize the sites for use of cryopreserved materials. Identification of stressed ecosystems or critical regions where potential pathogen transmission would be of concern would also be useful, as would identification of ecosystems that could assimilate the potential pathogen if it is already endemic. Some agencies will require delineation of Distinct Population Segments into which fish resulting from cryopreservation efforts will be transferred. Consideration must be given to the particular situation at hand. All regional, state, federal, and international guidelines for gamete transport (OIE 1997a 1997b, Code of Federal Regulations 1997) should be followed. In the United States, the USFWS National Fish Health Policy (USFWS 1995) can be consulted for information on containing, controlling, and minimizing impacts of fish diseases and pathogens. Systematic disinfection methods can be used at hatcheries, but this sort of control cannot be applied in natural ecosystems. Existing biosecurity programs for development and transfer of broodstocks, such as those in place for shrimp, could be adapted for the inclusion of cryopreserved samples (Fig. 4).

CONCLUSIONS

The increase in global transport of agricultural species and their products in the last few decades has exacerbated the spread of disease agents. The proactive development of biosecurity measures for aquaculture is a sensible approach to address these concerns (Tiersch and Hargreaves 2002). Worldwide fisheries and aquaculture interests agree about the need for better cooperation, unification, and integration of regulatory guidelines and authorities for preventing the importation of non-endemic infectious disease agents. A biosecurity program for aquatic species cryopreservation will decrease disease transmission between entities and display global resource stewardship. All components of cryopreservation programs should be reviewed by the involved parties, including international, federal, state, university, and private-sector alliances.

The types of problems and precautionary procedures presented in this paper are intended to serve as examples for biosecurity considerations related to cryopreservation in aquatic species. None of these problems, by themselves, would be likely to lead to the failure of an industry, extinction of a species, or collapse of an ecosystem, but each does represent potential negative effects that in combination with other detrimental activities could impair animal or ecosystem health. Because cryopreservation is only beginning to be applied to aquatic species (Caffey and Tiersch 2000b), aquaculturists are in a position to avert problems before they occur. This effort could benefit greatly from the fifty years of experience available from the application of cryopreservation to animal livestock industries (e.g., Johnson 2000).

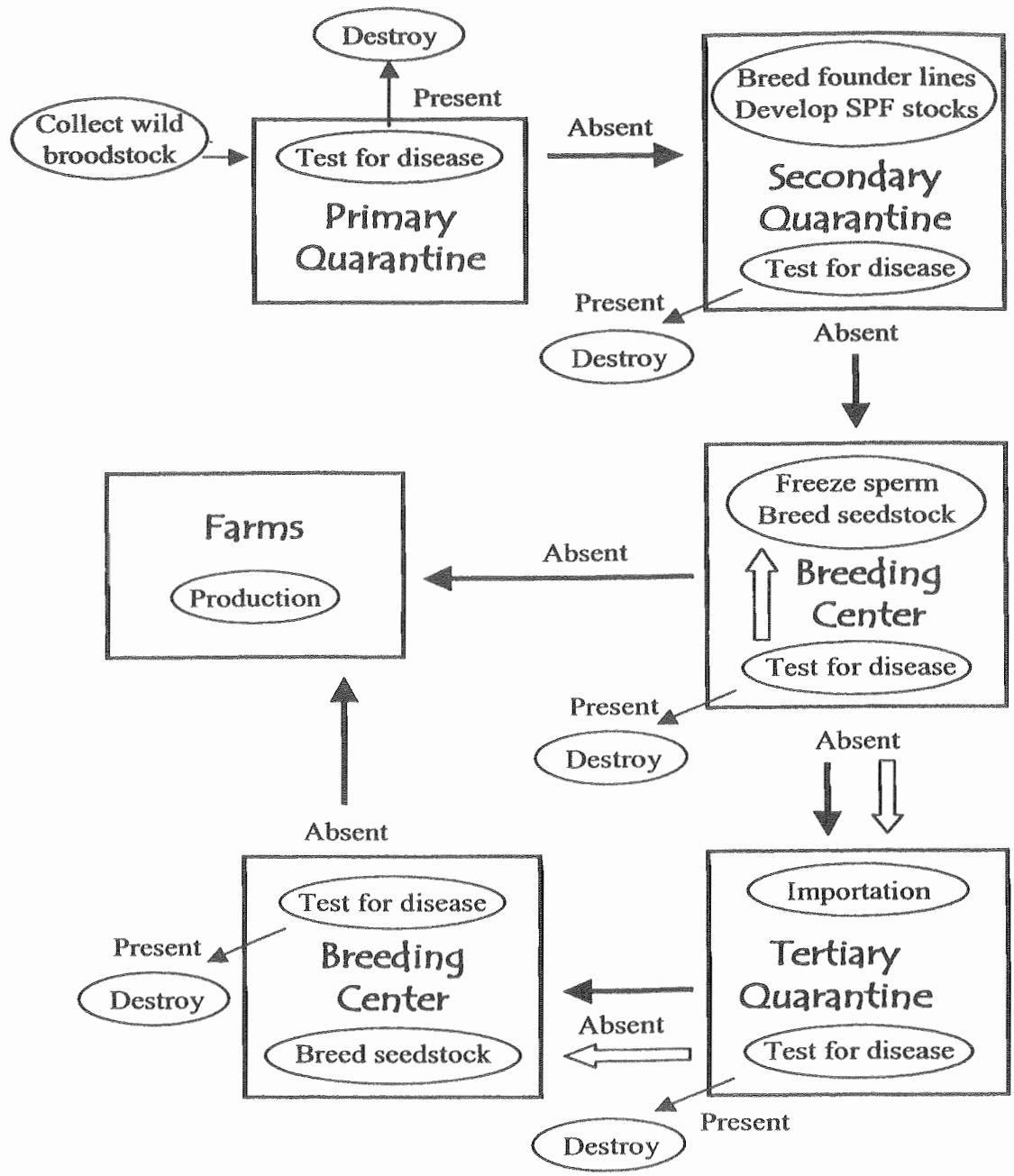

Figure 4. A scheme for the commercial application of cryopreservation by integration with existing activities. In this example, a biosecurity system used to develop and distribute specific pathogen-free (SPF) lines of shrimp from candidate populations of wild broodstock (adapted from Lightner 2002) can provide a foundation for application of cryopreserved sperm. Facilities are indicated by rectangles; activities are shown in ovals. Black arrows indicate movement of animals; white arrows indicate movement of cryopreserved sperm samples; "present" or "absent" refers to detection of specific disease. Tertiary quarantine would be useful in an importing country to verify the SPF status of samples.

ACKNOWLEDGMENTS

This work was supported in part by funding from the United States Department of Agriculture, the Louisiana Sea Grant Program, and the Louisiana Catfish Promotion and Research Board. We thank J. Chandler, J. Cloud, J. Hargreaves, P. Lang, L. Johnson, S. Leibo, K. Riley, C. Paniagua-Chavez, G. Thorgaard, W. Wayman, and W. Wolters for discussion. This manuscript was approved by the Director of the Louisiana Agricultural Experiment Station as number 02-66-0037.

LITERATURE CITED

ABRAC (Agricultural Biotechnology Research Advisory Committee, United States Department of Agriculture). 1995. Performance standards for safely conducting research with genetically modified fish and shellfish. Document No. 95-01, United States Department of Agriculture, Washington, D.C., USA.

Ackefors, H. and O. V. Lindqvist. 1994. Cultivation of freshwater crayfishes in Europe. Pages 157-204 *in* J. V. Huner, editor. Freshwater Crayfish Aquaculture in North America, Europe, and Australia: Families Astacidae, Cambaridae, and Parastacidae. Food Products Press, The Haworth Press, Inc., New York, New York, USA.

AFS (American Fisheries Society Fish Health Section). 1997. Bluebook: Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. American Fisheries Society, Bethesda, Maryland, USA.

Avault, J. W. 1996. Maintaining health of a culture species. Pages 459-548 *in* Fundamentals of Aquaculture: A Step-by-Step Guide to Commercial Aquaculture. AVA Publishing Co., Baton Rouge, Louisiana, USA.

Bartlett, D. E. 1991. Bull semen: "Specific microorganisms" in disease control in semen and embryos. FAO (Food and Agriculture Organization of the United Nations) Animal Health and Production 2:29-48.

Bates, M. C., W. R. Wayman, and T. R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. Transactions of the American Fisheries Society 125:798-802.

Blaxter, J. H. 2000. Seminal studies in cryopreservation of fish sperm. Pages 380-382 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Busack, C. A. and K. P. Currens. 1995. Genetic risks and hazards in hatchery operations: Fundamental concepts and issues. Pages 71-80 *in* H. L. Schramm and R. G. Piper, editors. 15th International Symposium and Workshop on Uses and Effects of Cultured Fishes in Aquatic Systems. Albuquerque, New Mexico, USA, 12-17 March 1994. American Fisheries Society, Bethesda, Maryland, USA.

Caffey, R. H. and T. R. Tiersch. 2000a. Cost analysis for integration of sperm cryopreservation into an existing fish hatchery. Journal of the World Aquaculture Society 31:51-58.

Caffey, R. H. and T. R. Tiersch. 2000b. Economics and marketing of cryopreserved fish sperm. Pages 388-408 *in* T. R. Tiersch and P. M. Mazik, P. M., editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Carlton, J. T. 1996. Marine bioinvasions: The alteration of marine ecosystems by nonindigenous species. Oceanography 9:36-43.

Carlton, J. T. and J. B. Geller. 1993. Ecological roulette: The global transport of nonindigenous marine organisms. Science 261:78-82.

Cunningham, A. A. 1996. Disease risks of wildlife translocations. Conservatiuon Biology 10:349 353.

Epstein, P. R., T. E. Ford, C. Puccia, and C. Possas. 1994. Marine ecosystem health: Implications for public health. Pages 13-25 *in* M. E. Wilson, R. Levins, and A. Spielman, editors. Disease in Evolution: Global Changes and Emergence of Infectious Disease. The New York Academy of Sciences, New York, New York, USA.

Ferguson, N. M., C. A. Donnelly, and R. M. Anderson. 2001. The foot-and-mouth epidemic in Great Britain: Pattern of spread and impact of interventions. Science 292:1155-1160.

Foote, R. H. 1996. Review: Dairy cattle reproductive physiology research and management - past progress and future prospects. Journal of Dairy Science 79:980-990.

Friedman, T. L. 1999. The Lexus and the Olive Tree. Farrar, Straus and Giroux, New York, New York, USA.

Fryer, J. L. and J. L. Bartholomew. 1996. Established and emerging infectious diseases of fish: As fish move, infections move with them. American Society for Microbiology News 62:592-594.

Fryer, J. L. and M. J. Mauel. 1997. The Rickettsia: An emerging group of pathogens in fish. Emerging Infectious Diseases 3:137-144.

Ganzhorn, J., J. S. Rohovec, and J. L. Fryer. 1992. Dissemination of microbial pathogens through introductions of transfers of finfish. Pages 175-192 *in* A. Rosenfield and R. Mann, editors. Dispersal of Living Organisms into Aquatic Ecosystems. Maryland Sea Grant, College Park, Maryland, USA.

Gibbs, E .P. J. 1981. Persistent viral infections of food animals: Their relevance to the international movement of livestock and germ plasm. Advances in Veterinary Science and Comparative Medicine 25:71-100.

Gorman, O. T. 2000. Ecological and genetic considerations for collection of gametes from wild fishes. Pages 319-322 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Hagedorn, M. and F. W. Kleinhans. 2000. Problems and prospects in cryopreservation of fish embryos. Pages 161-178 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Hagedorn, M., E. Hsu, F. W. Kleinhans, and D. E. Wildt. 1997. New approaches for studying the permeability of fish embryos: Towards successful cryopreservation. Cryobiology 34:335-347.

Hallerman, E. and A. Kapuscinski. 1993. Potential impacts of transgenic and genetically manipulated fish on natural populations: Addressing the uncertainties through field testing. Pages 93-112 *in* J. G. Cloud and G. H. Thorgaard, editors. Genetic Conservation of Salmonid Fishes. Plenum Press, New York, New York, USA.

Hare, W. C. D. 1985. Diseases Transmissible by Semen and Embryo Transfer Techniques. Office International des Epizooties, Paris, France.

Heckmann, R. A. 1993. Parasites of salmonid fishes. Pages 408-428 *in* M. K. Stoskopf, editor. Fish Medicine. W. B. Saunders Co., Philadelphia, Pennsylvania, USA.

Hnath, J. G., editor. 1993. Great Lakes fish disease control policy and model program. Special Publication, Great Lakes Fishery Commission, No. 93-1:1-38.

Horner, R. W. and R. L. Eshenroder. 1993. Protocol to minimize the risk of introducing emergency disease agents with importation of salmonid fishes from enzootic areas. Special Publication, Great Lakes Fishery Commission 93-1:39-54.

Hughes, R. M. and R. F. Noss. 1992. Biological diversity and biological integrity: Current concerns for lakes and streams. Fisheries 17:11-19.

ICES (The International Council for the Exploration of the Sea). 1995. ICES code of practice on the introductions and transfers of marine organisms 1994/ Code de conduite du CIEM pour les introductions et transferts d'organismes marins 1994. Amended version of ICES Cooperative Research Report No. 204. [On-line]. Available: http://www.ices.dk/pubs/pubs.htm).

Jenkins, J. A. 2000a. Infectious disease and quality assurance considerations for the transfer of cryopreserved fish gametes. Pages 343-363 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Jenkins, J. A. 2000b. Regulatory considerations for the global transfer of cryopreserved fish gametes. Pages 364-379 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Jenkins, J. A. 2000c. Minimizing microbial contamination of sperm samples. Pages 276-279 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Jenkins, J. A. and T. R. Tiersch. 1997. A preliminary bacteriological study of refrigerated channel catfish sperm. Journal of the World Aquaculture Society 28:282-288.

Johnson, L. A. 2000. Lessons from the cryopreservation of livestock sperm. Pages 383-387 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Kincaid, H. L. 2000. Development of databases for germplasm repositories. Pages 323-331 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Kincaid, H. L., M. Gray, L. Mengel, and S. Brimm. 1998. National Fish Strain Registry - Trout, NFSR-T Database (CD-ROM) and Operating Manual. United States Fish and Wildlife Service Resource Publication, Washington, D.C., USA.

Leary, R. F., F. W. Allendorf, and G. K. Sage. 1995. Hybridization and introgression between introduced and native fish. Pages 91-101 *in* H. L. Schramm and R. G. Piper, editors. 15th International Symposium and Workshop on Uses and Effects of Cultured Fishes in Aquatic Systems. Albuquerque, New Mexico, USA, 12-17 March 1994. American Fisheries Society, Bethesda, Maryland, USA.

Lightner, D. V. 2002. Exclusion of specific pathogens for disease prevention in a penaeid shrimp biosecurity program. Pages 81-116 in C.-S. Lee and P. J. O'Bryen, editors. Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables. World Aquaculture Society, Baton Rouge, Louisiana, USA. (in press).

Lutz, C. G. 2001a. Inbreeding, crossbreeding and hybridization. Pages 93-119 *in* Practical Genetics for Aquaculture. Iowa State University Press, Blackwell Science Ltd., Ames, Iowa, USA.

Lutz, C. G., 2001b. Genetic threats to wild stocks and ecosystems. Pages 225-228 *in* Practical Genetics for Aquaculture. Iowa State University Press, Blackwell Science Ltd., Ames, Iowa, USA.

NAAB (National Association of Animal Breeders). 1995. Certified Semen Services minimum requirements for disease control of semen produced for artificial insemination. Columbia, Missouri, USA.

NARA (United States National Archives and Records Administration), Office of the Federal Register. Code of Federal Regulations. 50CFR 16.13. 1997. Title 50 – United States Wildlife and Fisheries. Chapter 1. Part 16 – Injurious Wildlife. Importation of live or dead fish, mollusks, and crustaceans, or their eggs. United States Government Printing Office, Washington, D.C., USA.

Nash, J. M. 2000. Grains of hope. Time 156: 38-46.

OIE (Office International des Epizooties). 1986. International Zoo-sanitary Code: Rules Recommended for Trade in Animals and Animal Products, 5th edition. Office International des Epizooties, Paris, France.

OIE (Office International des Epizooties) Fish Diseases Commission. 1997a. Diagnostic Manual for Aquatic Animal Diseases, 2nd edition. Office International des Epizooties, Paris, France.

OIE (Office International des Epizooties) Fish Diseases Commission. 1997b. International Aquatic Animal Health Code: Fish, Molluscs and Crustaceans. Recommendations for International Trade in Aquatic Animals and Aquatic Animal Products, 2nd edition. Office International des Epizooties, Paris, France.

OIE (Office International des Epizooties) Fish Diseases Commission. 2000. Diagnostic Manual for Aquatic Animal Diseases, 3rd edition. Office International des Epizooties, Paris, France.

OIE (Office International des Epizooties) Fish Diseases Commission. 2001. International Aquatic Animal Health Code: Fish, Molluscs and Crustaceans, 4th edition. Office International des Epizooties, Paris, France.

Olive, P. J. W. and W. B. Wang. 1997. Cryopreservation of *Nereis virens* (Polychaeta, Annelida) larvae: The mechanism of cryopreservation of a differentiated metazoan. Cryobiology 34:284-294.

Paniagua-Chavez, C. G., J. Buchanan, J. E. Supan, and T. R. Tiersch. 1998. Settlement and growth of eastern oysters produced from cryopreserved larvae. Cryo-Letters 19:283-292.

Paniagua-Chavez, C. G., J. T. Buchanan, J. E. Supan, and T. R. Tiersch. 2000. Cryopreservation of sperm and larvae of the eastern oyster. Pages 230-239 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Philpott, M. 1993. The dangers of disease transmission by artificial insemination and embryo transfer. British Veterinary Journal 149:339-369.

Pittman-Cooley, L. and T. R. Tiersch. 2000. Isolation of DNA from cryopreserved fish sperm. Pages 310-313 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Rana, K. J. 1995. Cryopreservation of fish spermatozoa. Pages 1-165 *in* J. G. Day and M. R. McLellan, editors. Cryopreservation and Freeze-drying Protocols. Humana Press, Inc., Totowa, New Jersey, USA.

Reichhardt, T. 2000. Will souped up salmon sink or swim? Nature 406:10-12.

Russell, P. H., V. H. Lyaruu, J. D. Millar, M. R. Curry, and P. F. Watson. 1997. The potential transmission of infectious agents by semen packaging during storage for artificial insemination. Animal Reproduction Science 47:337 342.

Schramm, H. L. and R. G. Piper, editors. 1995. 15th International Symposium and Workshop on Uses and Effects of Cultured Fishes in Aquatic Systems. Albuquerque, New Mexico, USA, 12-17 March 1994. American Fisheries Society, Bethesda, Maryland, USA.

Segovia, M., J. A. Jenkins, C. Paniagua-Chavez, and T. R. Tiersch. 2000. Flow cytometric evaluation of antibiotic effects on viability and mitochondrial function of refrigerated sperm of Nile tilapia. Theriogeneology 53:1489-1499.

Sherman, J. K., T. L. Hostetler, K. McHenry, and J. J. Daly. 1991. Cryosurvival of *Trichomonas vaginalis* during cryopreservation of human semen. Cryobiology 28:246-250.

Symula, J., J. Meade, J. C. Skea, L. Cummings, and J. R. Colquhoun. 1999. Blue-sac disease in Lake Ontario lake trout. Journal of Great Lakes Research 16:41-52.

Tave, D. 1993. Genetics for Fish Hatchery Managers. Van Nostrand Reinhold, New York, New York, USA.

Thacker, B. J., R. E. Larsen, H. S. Joo, and A. D. Leman. 1984. Swine diseases transmissible with artificial insemination. Journal of the American Veterinary Medical Association 185:511-516.

Tiersch, T. R. 2001. Cryopreservation in aquarium fishes. Marine Biotechnology 3:S212-S223.

Tiersch, T. R. and J. A. Hargreaves. 2002. Contending with criticism: Sensible responses in an age of advocacy. Pages 355-371 *in* R. R. Stickney and J. P. McVey, editors. Responsible Marine Aquaculture. CABI Publishing, London, UK.

Toledo, J. D. and H. Kurokura. 1990. Cryopreservation of the euryhaline rotifer *Brachionus plicatilis* embryos. Aquaculture 91:385-394.

Tylewska-Wierzbanowska, S. K. and D. Kruszewska. 1990. Q fever—sexually transmitted infection. Journal of Infectious Diseases 161:368-369.

USFWS (United States Fish and Wildlife Service). 1995. Fish health policy. United States Fish and Wildlife Service Manual No. FWM 170. United States Fish and Wildlife Service, Washington, D. C., USA.

Velasquez, C. G. 1980. Health constraints to integrated animal-fish farming in the Philippines. Pages 103-111 *in* R. S. V. Pullin and Z. H. Shehadeh, editors. Integrated Agriculture-Aquaculture Farming Systems. Proceedings of the 1979 International Center for Living Aquatic Resources Management (ICLARM)-Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARC) Conference on Integrated Agriculture-Aquaculture Farming Systems, Manila, Philippines. International Center for Living Aquatic Resources Management, Manila, Philippines.

Wachtel, S. S. and T. R. Tiersch. 2000. Ethical considerations in the application of cryopreservation to aquatic species. Pages 416-426 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Wayman, W. R. and T. R. Tiersch. 2000. Research methods for cryopreservation of sperm. Pages 264-275 *in* T. R. Tiersch and P. M. Mazik, editors. Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Windsor, M. L. and P. Hutchinson. 1994. International management of Atlantic salmon, *Salmo salar* L., by the North Atlantic Salmon Conservation Organization, 1984-1994. Fish Management and Ecology 1:31-44.