Ethical Considerations for the Application of Cryopreservation to Aquatic Species

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"All things considered it looks as though Utopia were far closer to us than anyone ... could have imagined ... Indeed, unless we choose to decentralize and to use applied science, not as the end to which human beings are to be made the means, but as the means to producing a race of free individuals, we have only two alternatives to choose from: either a number of national, militarized totalitarianisms, having as their root the terror of the atomic bomb and as their consequence the destruction of civilization (or, if the warfare is limited, the perpetuation of militarism); ... or else one supra-national totaitarianism, called into existence by the social chaos resulting from rapid technological progress in general and the atomic bomb in particular, and developing, under the need for efficiency and stability, into the welfare-tyranny of Utopia. You pays your money and you takes your chances."

Aldous Huxley (1894-1963)
Foreword to Brave New World (1946)

Introduction

This chapter could have been placed at the beginning of the volume because the points raised are central to the application of cryopreservation. We chose to place it at the end, however, as a summary, and to provide a connection to the future and to those who will thaw and thereby inherit what we cryopreserve.

During the last half of the twentieth century, we have witnessed a series of remarkable technologic advances in the exploration of space; a highlight of that exploration was the Appolo moon landing of July 1969. Now, as our unmanned probes rove the surfaces of distant planets, we contemplate the completion of space stations under construction and the personal exploration of Mars and the more distant planets. Yet for all our enthusiasm, we are bound essentially to our own solar system, held back by the vast reaches between universes and the limitations of speed and energy. Even if we could attain the speed of light, a round trip to our nearest neighbor, the star Alpha Centuri, would take almost 9 yr.

We have witnessed, too, a series of remarkable technical advances in the physical sciences, especially genetics. We have deciphered the genetic code and developed the means for gene splicing and cloning. We have transferred genes from one organism to another and in so-doing we have created novel life forms. But unlike the situation with space travel, genetic exploration is virtually without limit. We are developing the techniques for gene therapy, for the repair or replacement of mutant genes, and even as these words are written, the Human Genome Project is underway -- a massive and comprehensive approach to the determination of the entire genetic sequence of a human being. Although the project may take several years, there is no reason to doubt that it will be completed. Indeed some 25 prokaryotic genomes have already been sequenced (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov).

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As for the genetics of reproduction, we have learned how to make infertile couples fertile. We can treat women such that they produce many eggs in a single ovulatory cycle. We can use the eggs to produce embryos in test tubes. We can cut the embryos in half to make twins, we can fuse two to make one, we can clone one to make four, we can freeze the embryos and store them and we can predetermine their sex. We can transfer them into surrogate mothers who will carry them to birth.

Given the rate of scientific discovery, it would seem wise to consider standards of scientific behavior, for there is little consensus concerning the legitimacy or the morality of our novel technologies, and we are manipulating the very elements of the generative process. Even the idea that Man cannot make life, that life is uniquely a creation of the Divine, can now reasonably be challenged. Yet the available and other, developing, techniques could be used to protect and conserve any of several endangered species, mammalian and non-mammalian. When particular environments are threatened, for example, germ cells and embryos of the species at risk -- even intact individuals -- might be cryopreserved, with a view to the thawing and "seeding" of favorable environments in the future. But these novel methods raise novel questions -- the question of *multigenerational inbreeding*, for instance, the inadvertent mating of individual animals with their descendants, the question of *temporal exotics*, species out of synchrony with their environment because of changes occurring during their period of cryopreservation, and so forth.

In this chapter, we discuss reproductive methods that have been developed in mammals and their potential application to conservation of the non-mammalian species -- fishes in particular. We conclude with a consideration of some of the ethical and religious questions germane to the application of these methods.

Assisted Reproductive Technologies (ART)

Assisted Reproductive Technologies refers to any of several methods used to enhance the reproductive potential of individuals and includes such techniques as *in vitro* fertilization (IVF), cryopreservation, twinning and cloning, embryo transfer, and surrogate motherhood, and, in the human and domestic mammals, pre-implantation and prenatal diagnosis and sex selection. Many of these methods were developed in laboratory rodents and in the larger domestic species. Pregnancy resulting from embryo transfer in cattle was first reported in 1951 (Seidel 1981); mice born from frozen embryos were first described in 1972, and cattle produced from frozen embryos were first reported in 1973 (Hafez 1992).

Children born as a result of IVF are called "test-tube babies." The first such was Louise Brown, born in England in 1978. Nearly 15,000 test tube babies were born in the United States and Canada in 1994 and 1995, the last 2 yr for which figures are available (Society for Assisted Reproductive Technology and the American Society for Reproductive Medicine 1994, 1998) and over 300,000 children have been produced by IVF Worldwide (Institute for Science, Law, and Technology Working Group 1998). Following hormonal stimulation in women undergoing IVF, as many as 20 (or more) embryos can be produced, but only a few are transferred. The rest are often cryopreserved.

The first child resulting from transfer of a cryopreserved embryo was born in 1984. Since then the technique has been widely used. More than 7,000 frozen human embryos were thawed and transferred in the United States and Canada in 1994 and again in 1995. At present there are tens, perhaps hundreds of thousands of human embryos frozen in liquid nitrogen (LN_2), awaiting transfer or other disposition.

Cryopreservation of human embryos has generated extraordinary circumstances that few would have contemplated even in the recent past. An example involves two embryos frozen in Australia after being produced by IVF with sperm from an anonymous donor. The parents-to-be were killed in an air crash and the question arose whether the embryos should be thawed and transferred into a surrogate mother. Should those embryos be given the chance for survival? And if they survived should they inherit their parents' estate, valued at seven million dollars? A committee was formed to provide guidelines. The committee ruled that couples enrolled in IVF programs should make provisions concerning the disposition of frozen embryos, that in the absence of such provisions, embryos should be destroyed. But the Victoria Parliament ruled that all embryos should have the chance to be transferred and born, even in the absence of provisions (Davis 1990).

Conservation by ART in the Non-human Mammals

As implied above, cryopreservation and other technologies raise fundamental issues about the legal status of, and our moral obligations to, the developing human, but they also provide the opportunity to rescue species whose numbers have been critically depleted by deforestation, pollution or overharvest. Cryopreservation in particular would enable storage and safeguarding of germ cells and embryos for development at a time and place favorable to the survival and well-being of the species. Once thawed, embryos could be cloned (Willadsen 1986). Given a favorable environment, the combination of cryopreservation and cloning could enable rapid re-population.

Cryopreservation is straightforward. The embryos or germ cells are placed in a cryoprotectant such as glycerol to protect against the formation of lethal ice crystals, and the temperature of the solution is reduced gradually, often in a programmable cooling machine. Crystallization of the medium is induced and the embryos are plunged into LN_2 for extended storage. For thawing, the embryos are merely removed from the LN_2 and allowed to warm quickly in air.

This or similar techniques could work well with fish and other taxa in which embryos develop in the open environment, but it may be more difficult to implement rescue of mammalian species such as the cheetah, in which uterine gestation is a necessary component of development. This difficulty could be circumvented by use of surrogate mothers from related species but this is a development for the future. Where germ cells are available instead of embryos, the embryos could be produced by IVF. This would enable preimplantation diagnosis of the embryos, and thereby selection of gender and other desired traits.

The Ethics of ART: Religious and Secular Considerations

Considerable discussion has been generated by the development and application of ART in humans and domestic mammals. The recent cloning of a sheep was widely reported in the press and electronic media, for example, and as might be expected, it created a stir in certain religious circles. In a news article in Nature (Masood 1997), Jeremy Rifkin, President of the Foundation on Economic Trends in Washington was described as heading a "coalition of 300 religious and ethics organizations" calling for a worldwide ban on human cloning and suggesting that human cloning should carry a penalty "on a par with rape, child abuse and murder." Yet the cloning of human embryos had already been performed 4 yr earlier (Hall et al. 1993).

The reaction to the applications of ART in conservation of the domestic species would be more subdued, but the questions raised are no less compelling. Is there really a need for species preservation? How are the effort and expense of preservation justified? Are we morally obliged to preserve endangered species? Which species should be selected for rescue and which should not? Should techniques such as cloning be banned?

In the book, *Principles of Conservation Biology*, Callicott (1994) identifies "four categories of the instructional value of biodiversity:" 1) goods, such as foods, fuels, materials for clothing, drugs; 2) services, such as pollination, nitrogen fixation, etc.; 3) information, such as that concerned with genetic engineering and pure and applied science, and 4) the psycho-spiritual, such as that concerned with aesthetic beauty and religious awe. The author notes that the drug, vincristine, obtained from the Madagascar periwinkle, is used in the standard treatment of childhood leukemia, and that the plant grows in an area which is threatened with "wholesale destruction." It is asked whether other yet undiscovered species could provide additional benefits, and whether these potentially valuable species are being destroyed routinely by careless exploitation and destruction of habitat.

In considering the potential loss of information the author compares what he refers to as "mindless destruction of biodiversity" with book burning. From the perspective of aesthetics and religion, he compares it with "vandalizing an art gallery or desecrating a church." Later the author points out that there are religious precedents or conservation and in particular for the protection of endangered species. Indeed the Bible and commentaries are full of references implying the stewardship of Man over the animals, and his responsibility to protect them:

Rav Judah said in the name of Rav: a man is forbidden to eat before he gives food to his beast, because it says (Deuteronomy 11:15) 'and I will give grass in your fields for your cattle' and then 'you will eat and be satisfied.'

Babylonian Talmud: Berachot 40a

In it (the Sabbath day) you shall not do any manner of work, you nor your son nor your daughter, not your man-servant nor your maid-servant, nor your ox, nor your ass, nor any of your beasts...

Deuteronomy 5:14; see also Exodus 20:10)

In this connection, it is interesting that the forms of work that are otherwise forbidden on the Sabbath (unloading for example) must be done to reduce animal suffering (Babylonian Talmud: Shabbat 128b). Thus, in imitation of the Divine, Man is cast in the role of the caretaker obliged to protect the various species in his stewardship. Given the novel technologies discussed above, this would imply an obligation to apply those and other developing technologies to the protection of all species, and in particular endangered species.

Is there guidance on how to proceed in this obligation? Perhaps an example comes from the response of the Buddha to a monk seeking answers to the questions of higher philosophy.

"*A man was shot by a poisoned arrow. With the arrowhead still embedded within him, his relatives raced to find a doctor. As the doctor was preparing to cut out the arrow-head, the man said, "Wait! I will not let you take out this arrowhead until you tell me the name of the man who shot me. Where did he live? What caste was he? What kind of arrow did he use? Did he use a bow or a cross-bow? What was the arrow made of? Of what was the bow made? Of what was the bow-string made? What kind of feather was attached to the end of the arrow? Until I find out the answers to these questions, I will not let you take this arrow out."*

The Buddha was teaching that it is important to relieve human suffering and to avoid wasting time in vain pursuits and debates. In this light, do we need to know all of the details associated with cryopreservation of sperm of endangered species before we engage in the work? How do we decide that we know enough?

Conservation by Cryopreservation in Aquatic Species

While many areas of human bioethics are well codified, and others are still being framed, the ethics of cryopreservation of gametes and embryos of aquatic species are unexplored. Although certain questions can be addressed by looking to established areas such as conservation ethics (Callicott 1994), ethical concerns for cryopreservation pose unique problems. In this section we address several examples of ethical questions involving application of cryopreservation to threatened and endangered (T & E) species of fishes. This is relevant because fully one third of all fish species are now threatened with extinction (Tuxill and Bright 1998). These questions relate to ethical problems in other arenas as well. The four primary examples are: 1) use and disposal of samples; 2) multigenerational inbreeding; 3) temporal exotic species, and 4) eco-temporal effects.

Use and Disposal of Samples

Cryopreservation is actually a form of time travel, because frozen samples will likely remain viable well beyond our lifetimes. The social, biological, and political contexts that exist when we freeze samples will most probably be irrelevant given passage of enough time before thawing. Thus we are freezing samples for potentially unknown uses in the future.

Samples frozen today for research such as optimization of cryoprotectants or cooling rates may eventually need to be used for breeding purposes. Or, samples frozen for use in breeding may only be minimally viable due to use of unrefined techniques. In this first case, we may not be able to gain maximum benefit for breeding purposes from the sample because insufficient biological information on the genetic characteristics of the donor fish was recorded (the samples were not intended for breeding use). Should we dispose of the samples to ensure genetic integrity of populations? In the second case, we have poor albeit well-documented samples occupying potentially limited space that could be occupied by more valuable (viable) samples. Even if space were not limiting, potentially fertilizable eggs could be lost by use of inferior sperm. Should we keep the samples for some other use (e. g. genetic analysis) or should we dispose of them?

In each of these cases we must weigh the costs and benefits of using thawed samples for purposes different than those at the time of freezing. Neither case is unlikely -- almost certainly each will occur repeatedly in the future. The ethical dilemma would also be intensified given the survival status of the species. Flawed samples become more valuable based on simple supply and demand. It should be noted that the market and non-market values have yet to be established for such samples. A new economic discipline, environmental or ecological economics, is emerging to deal with issues in valuation of natural resources (e.g. Costanza et al. 1997). At present, the methodologies are controversial (Pimm 1997) and have not been applied to cryopreservation in aquatic species.

Are there solutions? Perhaps systematic record-keeping and a centralized data base should accompany any sample cryopreserved for any reason (though costly today, this may prove to be a bargain in the future). Perhaps analysis of DNA from a sample of frozen material could resolve problems associated with genetic husbandry. The problems of poor quality samples and poor quality control are more difficult. Perhaps technology such as microinjection of sperm nuclei or intracytoplasmic sperm injection could make even poor samples useful.

Multigenerational Inbreeding

This is another question of usage. When working with T & E species it is often difficult to obtain samples in a uniform and systematic fashion. Fish may congregate only in specific areas during spawning season (e. g. over gravel substrates) and only certain males may yield copious quantities of high quality semen. In short, a few males from a few populations may provide disproportionate excesses of the total volume of sperm cryopreserved. This is not unlikely given that the doctrine of “it’s all we have so we better freeze it” seems quite reasonable when faced with the imminent collapse of a species.

Let us consider use of these samples. With frequent thawing and breeding over time, it is possible to create a situation wherein sperm from a single over-represented male are used to fertilize eggs produced by his offspring for successive generations -- multigenerational inbreeding by overuse of a single sire (also an extreme version of recurrent founder effect). This is, like our first examples, not an unlikely event, or one without precedent. The overuse of cryopreserved sperm of a single Holstein bull (named “Elevation”). His sperm produced more than 2 million offspring. Of course, while inbreeding in domesticated animals may be desirable to some extent to fix desired traits, it is not desirable for natural populations of T & E species.

Is there a solution to this problem? Yes, but not without ethical considerations. Limits could be set *a priori* on the volume of sperm frozen from any one male regardless of the population level of a species. There is another relevant concept here. Frozen samples could be viewed as a means to reconstitute genetic variation in the form of fish. The frozen lifetime of sperm could be set (again *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 a few males in this scheme (the females would be useful too, of course), which would then provide sperm for production of fishes and for cryopreservation using newer improved techniques.

Temporal Exotic Species

Our first two ethical questions have dealt with problems essentially of inventory and use, which is the domain of fish production. Our next two questions address what happens to the fish that are produced from cryopreserved material. For these questions we can focus on application of a hypothetical method for cryopreservation of fish embryos (to be developed in the future).

The concept and consequences of exotic species are familiar in fisheries, and this topic was the subject of a book recently published by the American Fisheries Society (Schramm and Piper 1995). When a species is introduced outside its natural range it can cause significant alteration in the new habitat. Often this is detrimental to the resident organisms. Inasmuch as cryopreservation is a form of time travel, another type of exotic species is therefore possible: the temporal exotic -- a species 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. As an extreme example, what if the extinct placoderm fish, *Dunkleosteus* (Romer 1966), were re-established in its former range? This 10-m long predator (extinct for some 350 million yr) presumably capable of shearing telephone poles in its jaw plates could have dramatic effects on its reclaimed territories (Figure 1). Though not as dramatic, the re-establishment of an extirpated large predator could affect waterways in the future. The Colorado squawfish *Ptychocheilus lucius* which attains sizes of 2 m and 100 Kg is currently listed as endangered and would be a candidate for reintroduction in the Colorado River system in the future.

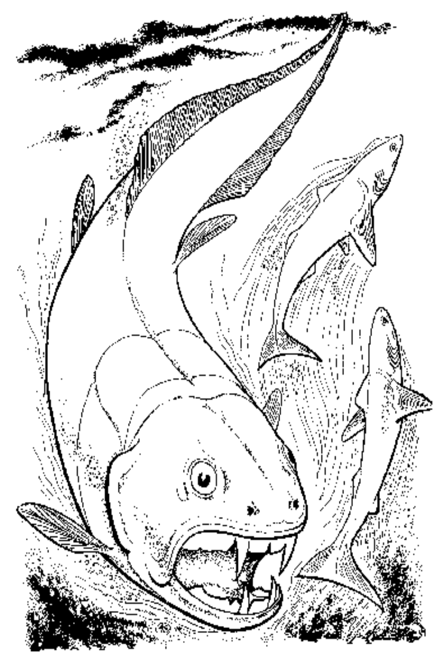

Figure 1. The Devonian predator *Dunkleosteus* depicted menacing primitive sharks. Re-establishment of this 10-meter long extinct fish into formerly occupied territories would be an extreme example of a temporal exotic species. Reproduced with permission of Spizzirri Publishing Company from "*An Educational Coloring Book of Prehistoric Fish*."

Other examples of temporal exotics could include commensal organisms such as innocuous bacterial flora, that when reintroduced, say 50 yr after extirpation, could assume new roles as pathogens. The inadvertent transfer of today's pathogens to the future could also be dramatic. We need only look at clinical examples of human disease provided by the reemergence of tuberculosis and polio after immunization programs were curtailed. Thus we must consider the possibility, if not the likelihood, that the organisms and germ cells we freeze today can have detrimental effects in the future, even if the organisms and germ cells are innocuous in the present.

Eco-temporal Effects

All of the preceding examples have addressed implicity the "emergency response mode" that is currently operational with T & E species. Given the precipitous declines now occuring, we often provide attention to the most endangered of species first and presume that anything done is better than doing nothing. Unfortunately there is often little time to plan, for some species such as the humpback chub *Gila cypha* have only a few hundred representatives left in the wild. This has lead to an "act first, think later" approach. The result is a collection of single-species programs, each addressing a dying population. However, species seldom go extinct by themselves. In reality it is specific habitats and communities that are endangered and become extinct. In view of this precept of conservation biology, is it reasonable to preserve embryos of a particular fish and not to preserve embryos of other members of the aquatic community? It is beneficial to thaw fish embryos 50 yr from now for introduction into suitable habitat if the food sources (for example) are gone? Should we thus cryopreserve insects, plants, microbes and other prominent members of aquatic systems such as "keystone species" (New 1995) to ensure the future success of embryos frozen today? This is a particularly thorny ethical question.

The four preceding questions were used to introduce a range of ethical problems related to the use of cryopreservation with aquatic species. Clearly there are other ethical issues. For example, how will resources be allocated? Do we need to employ a triage system? Should we cryopreserve only those species that cannot be saved by present-day management practices (the same approach as freezing the bodies of people with diseases that are incurable today)?

There are further questions raised related to the social impacts of cryopreservation. Does cryopreservation lead to decreased protection of sensitive habitats? It has been argued that frozen sperm can open the path for future development and habitat destruction by reducing the perception that species are actually imperiled. It could also be argued that freezing of sperm or embryos is a legitimate form of mitigation to compensate for destruction of habitat (we can build a new dam if we "protect" the displaced species by cryopreservation). Precautions for the use of cryopreservation with T & E species need to be discussed (Table 1).

Table 1. Caveats for application of cryopreservation to threatened and endangered species.

1)	Technology is seductive and not always necessary.
2)	The techiques of cryopreservation are unrefined or non-existent for most species.
3)	Cryopreservation is a tool, not a final solution.
4)	Cryopreservation does not protect habitat.
5)	Cryopreservation does not replace existing management plans.
6)	Cryopreservation can buy time or waste resources.
7)	There are few ethical guidelines for the application of cryopreservation to species conservation.

Other questions pertain to the market value of the sperm of an endangered species or the frozen embryos of an extinct species. Given that value is related to supply and demand, what is the dollar value of the last remaining embryos on Earth of a particular extinct species? Would cryopreservation of T & E species be best served by private sector involvement? It could be argued that private businesses (with government regulation) are capable of development and management of germplasm or embryo repositories for T & E species. This leads to the concept of resource privatization (Figure 2), where the market value of natural resources can in part be used to protect the resources (Vogel 1994, Macilwain 1998).

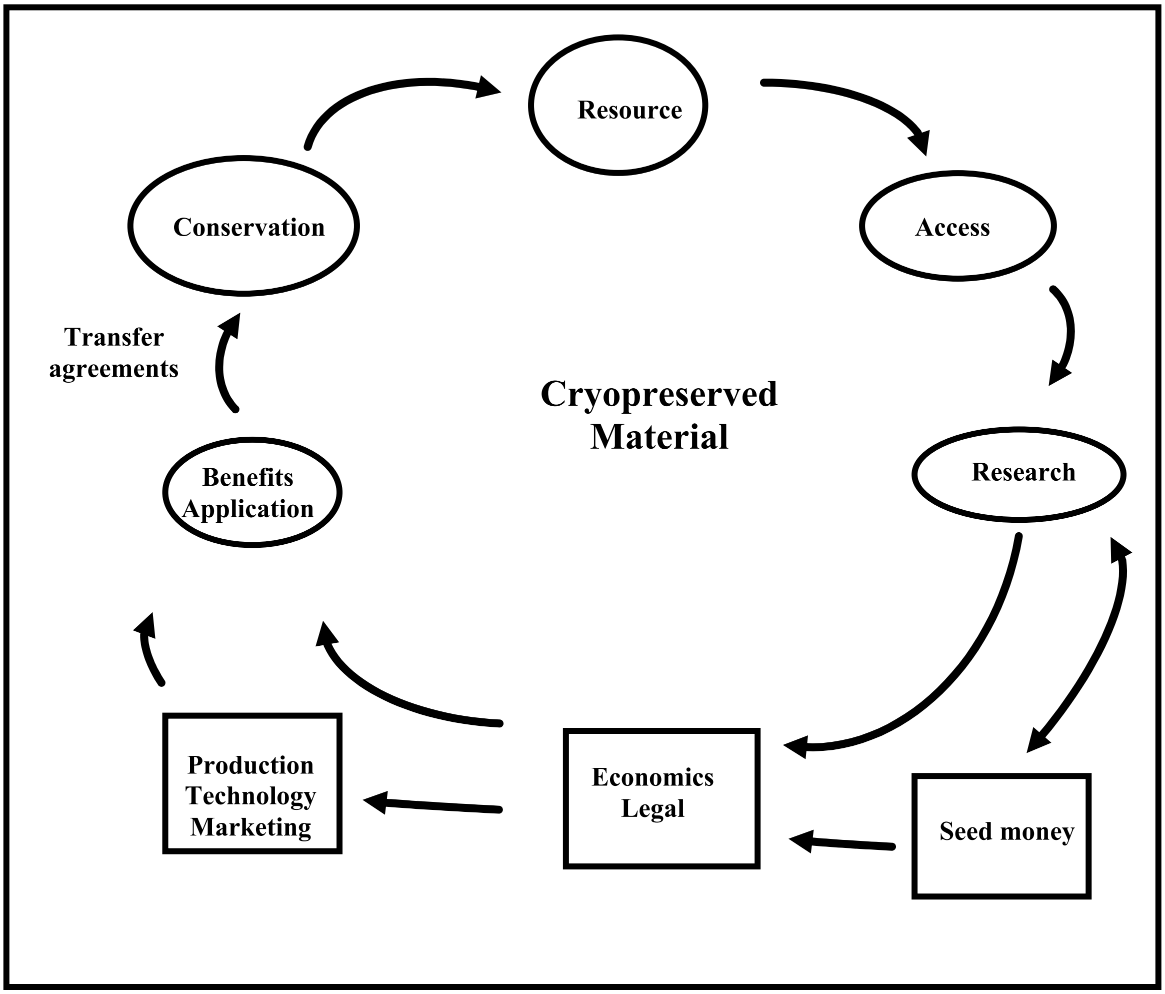

Figure 2. A scheme for utilizing market value of natural resources to generate funding for protection of the resource. This scheme would involve commercialization by the private sector (in rectangles) of public resources (ovals) and would involve resolution of legal, economic, social, biological, and ethical questions before introduction.

As a final example of potential questions, we should consider the extent to which technology should be applied. Consider the use of androgenesis. This is a process (with natural examples) allowing production of fish with nuclear DNA originating from sperm only. When used as a technique for genetic manipulation, fish can be produced from cryopreserved sperm and irradiated eggs (in which the nuclear DNA has been destroyed). This has been demonstrated in rainbow trout *Oncorhynchus mykiss* for example (Thorgard et al., pp. 305-309, this volume). In a worst-case scenario, cryopreserved sperm from an extinct species could be used to provide the nuclear DNA for use with eggs from a closely related "donor species." The resulting fish would combine nuclear and mitochondrial (cytoplasmic) inheritance from two species. Is this a suitable substitute for the original extinct species?

Clearly the ethical considerations are numerous and important (Table 2). At present, we not only lack good answers, we also lack good questions. In order to give relevance to our actions today, we are obliged to develop an appreciation for the scope and nature of the problems of the future. Application of cryopreservation to aquatic species, especially T & E species, is an unexplored realm of bioethics that will increase in importance with time. Perhaps by asking the question "What will they be thinking 50 yr from now?" We can avoid having those in the future ask "Why didn't they think about this 50 yr ago?"

Table 2. Points for discussion of ethical considerations for the application of cryopreservation to aquatic species.

1) Cryopreservation is a form of time travel.
2) We cannot be certain of how frozen samples will be used in the future.
3) Use and disposal of cryopreserved samples will require rules.
4) Quality control and quality assurance will be required.
5) The value of frozen samples is determined by record-keeping and database management.
6) We should consider the use of cryopreserved material for genetic reconstitution instead of production.
7) Genetic management can be intentional or unintentional.
8) Organisms considered to be desirable or innocuous today, may not be so in the future.
9) Diseases of today could be worse when reintroduced in the future.
10) We cannot predict the interactions of today's organisms with those of the future.
11) Species do not live in isolation: it takes a community.
12) Resource allocation is necessary for cryopreservation.
13) Cryopreservation could be viewed as mitigation for development activity.
14) Private sector involvement is possible for cryopreservation of T & E species.
15) Saving a species can transform it.

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