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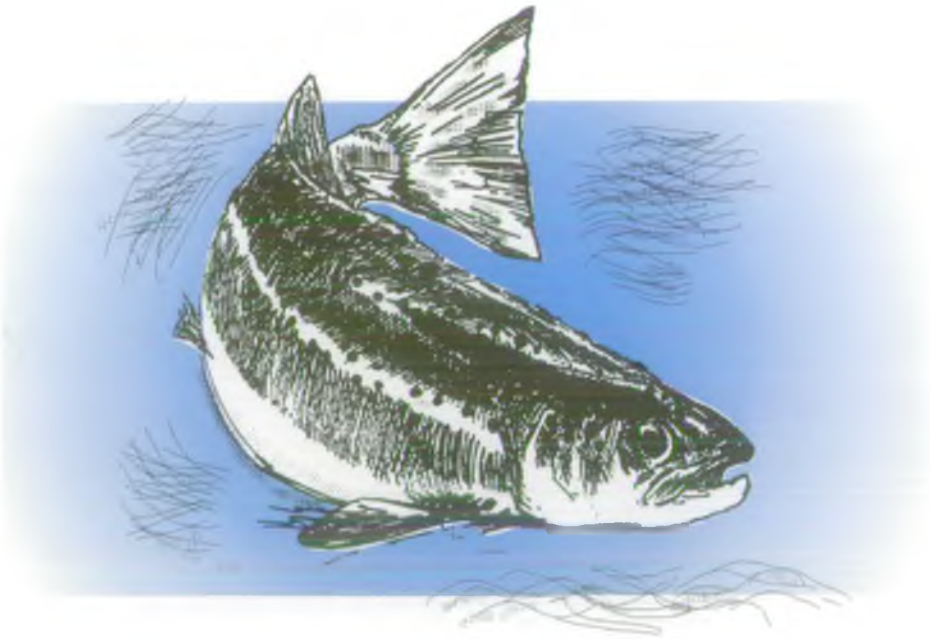
SEA TROUT
GENE BANKS



FISHERIES TECHNICAL REPORT

4

Fisheries Technical Reports

1. Sea Trout in England and Wales.
2. Analysis of Sea Trout Catch Statistics for England and Wales.
3. Sea Trout Literature Review and Bibliography.
4. The Feasibility of Developing and Utilising Gene Banks for Sea Trout (Salmo Trutta) Conservation.

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FISHERIES TECHNICAL REPORT NO.4

THE FEASIBILITY OF DEVELOPING AND UTILISING GENE BANKS FOR SEA TROUT (*SALMO TRUTTA*) CONSERVATION

This report has been compiled by the Department of Zoology, University College Cork, under contract to the NRA.

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EXECUTIVE SUMMARY

1. This report on preservation and rehabilation of sea trout (*Salmo trutta*) stocks was commissioned by the National Rivers Authority, because of their concern about the decline of sea trout in parts of Britain and Ireland.

2. Methods of preservation are reviewed and it is concluded that only two are presently feasible for sea trout, viz. cryopreservation of milt and the use of living gene banks (specialised rearing stations), where both sexes can be maintained. Both of these techniques should be used together, since cryopreservation in fishes is currently feasible only with male gametes. The backgrounds and details of these techniques are described, as are the costs involved with each. Established gene banks for Atlantic salmon and other fish species are described.

3. Preservation of sea trout stocks are complicated by the ecology and genetics of the species, in particular by details such as: a) sea trout and conspecific, freshwater-resident, brown trout co-exist in most river systems and may interbreed. Thus, cohabiting brown trout must be considered in the planning of sea trout conservation; b) juveniles of the two forms are morphologically and genetically indistinguishable (though the entire genome has not been investigated) and it is not known what triggers smoltification in some individuals.

4. Since all efforts at preservation and rehabilitation must contain some element of rearing, it is important that this process can be carried out satisfactorily. This has not always been the case with sea trout and the reasons for variations in success must be investigated. In addition, stock structure (with each river system containing at least one genetically distinct population) and the use of adequate numbers of broodstock to avoid inbreeding, must be considered in planning preservation.

5. Ideally, preservation should proceed using native sea trout, either as spawners or smolts, if sufficient numbers still occur in a system. Failing this, several options exists, viz. use of co-habiting natal brown trout, or use of the nearest ecologically and genetically "suitable" sea trout (or brown trout) stock or stocks. If multiple neighbouring stocks are being used, they can either be marked specifically and individual stock performance assessed or they can be mixed to increase variability, on the assumption that less adaptive genotypes will be eliminated by natural selection. All of this implies a great deal more investigation of ecology and genetics of trout populations.

6. It will not usually be possible, because of logistical considerations, to preserve all of the sea trout stocks in a particular area. Therefore, management decisions will have to be made as to which are most "valuable" and criteria on which these decisions might be made are discussed. Assuming the cause of the present sea trout decline in Britain and Ireland is identified and rectified, rehabilitation can then proceed.

7. For rehabilitation to be successful, using either cryopreserved or living material, the optimal method of sea trout enhancement must be applied (which has yet to be fully established). Use of cage rearing and ranching to speed recovery is recommended, but biotechnological techniques such as androgenesis, are not considered appropriate, at present. Detailed ecological and genetic monitoring of all rehabilitation exercises is recommended.

1 INTRODUCTION

The European trout, *Salmo trutta* L., provides valuable commercial and sports fisheries in England and Wales and sea trout, the anadromous[1] form, is an important resource for tourist angling in the West of Ireland, Wales and in Scotland. An indication of the value of sea trout stocks is given by Elliot (1989) based on commercial and rod catches of sea trout in England and Wales. By averaging total catches from all Water Authority (now National Rivers Authority) sectors, it was estimated that 110,547 fish were caught per year from 1983 to 1986, with an approximate value of £55 million.

Recently, populations of sea trout have declined dramatically, particularly in the west of Ireland (Mills *et al.*, 1990a). The best evidence for this comes from the Burrishoole fishery, where traps are in operation to record returning salmon and sea trout, whereas other systems with only net and rod fisheries, depend on catch returns which, by their nature, are less accurate. In the Burrishoole fishery a decline in stock was reported in 1987 and by 1989 there was a finnock (post smolt) collapse with smolts returning very early. The early return of smolts appears, in the west of Ireland at least, to be a symptom of the overall problem. These fish often had a sealice infestation and occasionally looked thin and emaciated (Dr. K. Whelan, Salmon Research Agency of Ireland (SRAI) - Sea trout Action Group (STAG), pers. comm.). The sea trout situation in the west of Ireland has not improved and in some well known fisheries, no sea trout were caught in 1990 (Sea Trout Action Group, 1991). Consequently, in 1990, emergency bye-laws were introduced for the west of Ireland, making it illegal to kill or sell sea trout. The 1991 smolt run in the west of Ireland was again accompanied by early returning fish. Smolts that were marked by Pan-Jet tattooing in the Erriff fishery returned within 5 days of marking and it was judged that these fish had a suprisingly high lice burden for the time spent at sea (Dr. P. Gargan, Central Fisheries Board, Dublin, pers. comm.).

The status of sea trout in Scotland, England and Wales was discussed at a recent workshop held in Westport, Co. Mayo, Ireland, by A. Walker (Scottish Office of Agriculture and Fisheries Department (S.O.A.F.D.)), D. Jordan (N.R.A. England) and A. Winstone (N.R.A. Welsh region). The data presented indicate that there has been a decline in sea trout in parts of both Scotland (in particular, the west coast) and Wales, but no apparent overall decline in England. There were regional declines in some parts of England and Wales but these were thought to be as a result of drought. Data presented by Rasmussen (Denmark) suggested that there was no problem in the Danish sea trout populations. There have been no reports of a decline in sea trout populations in western Norway (Dr. L.P. Hansen, Norwegian Directorate for Nature Management (NINA), pers. comm.).

The reason(s) for the decline or collapse of the sea trout populations in the west of Ireland has been the subject of considerable debate and speculation. Numerous factors have been suggested and are currently being investigated. These include afforestation and alteration in land use within catchments leading to alteration in run-off water quality, especially changes in pH, which may result in poor smoltification (Raite and Staurnes, 1987). Other possible reasons being explored include warmer sea temperatures, oceanographic changes indicated by the presence of *Phaeocystis* sp. and sea lice in the marine environment and disease, drainage, gravel removal and low water levels in fresh water (Whelan, S.R.A.I., pers. comm.). As yet, there is no concrete evidence as to which of these reasons (if any) cause the decline.

It was against this background that the N.R.A. commissioned this report on the feasibility of establishing a gene bank for the preservation of sea trout. The major part of this report consists of four sections. Section 2 provides an overview of some of the aspects of the complex biology of the sea trout pertinent to the application of gene banking. Section 3 consists of a detailed review of gene banking, covering the theory, development and

[1] An anadromous fish is one which spawns in fresh water but where the progeny later move to the sea to feed.

applications of the techniques, with descriptions of the methodologies and protocols. Section 4 examines the practicalities of applying these technologies to the preservation and rehabilation of sea trout, assessing both theoretical and practical limitations and putting forward tentative working models. In addition, other alternatives for use in rehabilation such as androgenesis and gynogenesis are evaluated. Section 5 is a concluding section, which contains suggestions for future work.

2 ASPECTS OF THE BIOLOGY OF *S. TRUTTA*

2.1 Present distribution and post-glacial origins of *S. trutta* populations

The natural distribution of the trout, *S. trutta* extends from northern Norway and the White Sea area of the USSR south to the Atlas Mountains region of North Africa and from Iceland in the west to the Aral Sea in Afghanistan and Pakistan in the east (Behnke, 1986 quoted in Hamilton *et al.*, 1989). Sea trout, the anadromous form is absent southwards from latitude 42° North. Ferguson (1989) and Hamiliton *et al.*, (1989) suggests that at the end of the last Ice Age, the fresh waters of northern Europe were colonised, via the sea, successively by two races of trout. Both of these types have given rise to brown trout that no longer migrate to sea. Using enzyme electrophoresis, Ferguson and Fleming (1983) proposed the form of the polymorphic, eye specific, lactate dehydrogenase enzyme that is coded for by the *Ldh - 5(105)* allele, (now termed *(100)*), as a marker for the ancestral race of trout, while the later form is characterised by the presence of the *Ldh - 5(100)* allele (now termed *(90)*). The former race was later replaced by, or introgressed with, the later - arriving "modern" race characterised by the *Ldh - 5(90)* allele, except where physical barriers prevented colonization by the latter form.

2.2 The sea trout life-cycle in Britain and Ireland

Spawning occurs in fresh water in winter and early juvenile life occurs there. In the Burrishoole system in western Ireland, spawning occurs over a period of up to 7 weeks. Major spawning activities were found to coincide with flood conditions and although males spend longer on the redds than females, few fish spend more than one week in the spawning area. The eggs hatch into alveins which have a yolk sac attached and live among the gravel. In time, the yolk sac is absorbed and the fish now called fry, emerge from the gravel and begin to feed. After a few months the young trout are referred to as parr. It is not possible to morphologically distinguish anadromous trout from freshwater residents as juveniles, until smoltification begins in the former. Indeed, since sea trout and coexisting brown trout may freely interbreed, no difference may exist (see section 2.3) and some presently unknown ecological trigger may cause smoltification and subsequent migration (see section 2.5). In some systems, parr destined to become anadromous may complete their freshwater life and descend to brackish or salt water in the autumn months, mainly as 1+ or 2+ fish. The remainder of each sea trout stock smoltifies the following spring (or the entire stock if autumn trout do not occur), usually as 2 or 3 year olds. Sea-run trout can thus be derived either from silvered sea trout smolts which migrate out of fresh water in spring or from autumn migrants which have a brown trout - like appearance (Mills and Piggins, 1988). Smolt numbers appear to be the more important determinant of the relative strength of a particular year class than autumn trout. In the Burrishoole system, for example, the majority of smolts migrate as 2+ fish (68%), the remainder at 3+ (32%). Autumn trout tend to be younger than smolts but range from 0+ to 3+ (Mills *et al.*, 1990a). However, autumn migration is variable and can sometimes be as large or larger than the smolt run. The mean smolt age and size in different catchments appears to be linked to latitude and thus climatic factors (L'Abee Lund *et al.*, 1989). Hoar (1976) concluded that the more rapidly growing individuals in a population will reach a size at which successful migration is feasible in a shorter time than the slower growing members of the population. The fastest growing fish, although smaller than smolts that took extra years in fresh water before migration, will generally be the largest of their own age class and will show greatest success in terms of survival through the fresh water/sea water transition. It has been suggested that resident behaviour may be associated with slow growth during the young (parr) stage (Dr. K. Hindar, NINA, pers. comm.).

As described by Mills and Piggins (1988), from the time the fish reach salt water until the end of their first post-migration winter, they are known as post-smolts. Various other terms are used to describe the fish which return to freshwater in the summer following migration, finnock and whitling being common terms. Alternatively, the fish may take one or more years to reach maturity. At the end of their first post-migration winter they are known as sea trout and, until they have spawned for their first time are known as maiden sea trout. After

spawning, such trout are known as kelts and if they survive, leave fresh water the following spring. In the sea, kelts recover rapidly after a period of intensive feeding, going through a period of recovery after which they are ready to spawn the following autumn.

Sea trout are multiple spawners. In the Burrishoole system, for example, the population is dominated by post smolts (approximately 50% of the population in higher reaches and 70% or greater in lower reaches of tidal loughs). Finnock that enter fresh water are mostly immature (approximately 75%) and their total contribution to the total egg deposition is only about 10%. There is evidence that non-spawning finnock continue to grow in fresh water. The main contributors to spawning are 1 sea winter (SW) and 2SW maiden sea trout which have been estimated to contribute up to 50% of the total sea trout egg deposition (Mills *et al.*, 1990a).

This life - cycle complicates the collection of sea trout for preservation. Firstly, sea trout cannot be distinguished from freshwater residents until the smolt stage. Also, while there is genetic evidence for accurate natal homing of spawning sea trout (see section 2.4), this does not seem to be the case for non - spawning post smolts. Therefore, the only time other than as smolts, when fish can be definatively identified as belonging to a particular sea trout population is just before, during or after spawning (as upstream migrating or spawning 1+ or older maidens or previous spawners or as kelts of one of these forms).

2.3 Brown trout and sea trout

Many authors have looked at the genetical composition of trout to try to establish the relationship between brown and sea trout. Jonsson (1982) tested the possibility that, in trout populations separated from the sea by waterfalls impassable to ascending fish, selection would favour non-diadromous genotypes above falls and diadromous[2] genotypes below barriers. This was done by transposing trout from each of two lakes on the same system, where the trout were a) from a lake above a waterfall and b) from a lower lake in which diadromous trout occurred. It was concluded that the trout from the lower lake were more vagrant than those from the lake above the impassable barrier and that this was due to genetics. Ferguson and Fleming (1983) have shown electrophoretically that in the majority of cases, Northern Irish sea trout did not differ genetically from brown trout that were present in the same system. This suggests interbreeding between sea trout and cohabiting coastal brown trout. Hindar *et al.* (1991) examining the genetic relationships among landlocked, resident and anadromous brown trout found no genetic difference between co-existing resident and anadromous types, both of which used the same locality and time for spawning on two tributaries of the same river in Norway. However, significant genetic differences were found between brown trout spawning in geographically separate localities and "particularily large differences" were found between landlocked brown trout and those from localities accessible from the sea. Cross *et al.* (in press) found large differences in gene frequencies between two populations of trout from above impassable barriers and also when comparing samples from above and below such barriers. No differences were evident in two sampling years between anadromous trout and cohabitating freshwater resident trout and it is suggested that interbreeding may occur. Consequently, Cross *et al.* (*loc. cit.*) suggest that should restoration measures be needed after the demise of sea trout, then cohabitating brown trout would be most similar genetically to sea trout from the same system or sub-system.

2.4 Sea trout population structure

In contrast to the similarity between brown trout and sea trout from the same areas, genetic differences are evident between sea trout populations from neighbouring river systems or even tributaries of the same system (Ferguson and Fleming, 1983; Ferguson, 1989; Skaala and Naevdal, 1989; Ferguson and Taggart, 1991). This indicates accurate homing to natal

[2] A diadromous fish is one which spends part of its life cycle in fresh water and the remainder in the sea.

spawning areas. Furthermore, almost complete reproductive isolation between populations must exist (and probably has existed for hundreds of generations) for such differences to occur. However, the existence of these differences does not prove local adaption, since forces other than natural selection, such as genetic drift, can alter gene frequencies. So, we currently do not know the importance of population structure. Until this is determined, a conservative approach of not eroding population structure during enhancement or rehabilitation is suggested.

2.5 Smoltification

Thus, as outlined in 2.2, there are migratory and non-migratory forms of the one species, *S. trutta*. However, it may be possible that all trout (or at least those living below impassable falls) undergo or have the genetic capability to undergo the physiological changes associated with migration. The exact reason that a proportion of trout migrate to sea has not yet been fully determined. Hoar (1976) states that while light and temperature are involved in the process of smoltification, it is the increase in day-length in the spring (photoperiod) acting as the synchroniser of the endogenous rhythm, which is the environmental factor most influencing the onset of parr to smolt transformation. Work has been carried out on some of the environmental factors which may influence migration and survival of smolts. Working on the Burrishoole system, Galvin *et al.* (1990) found that migration did not commence until the freshwater temperature reached a threshold level of approximately 9°C. Smolt migration was found to be adversely affected by the occurrence of temperatures of 13°C or higher before smolt development had taken place. More generally, Zaugg (1981, 1982), (quoted in Galvin, *et al.* (*loc. cit.*)) showed that if the threshold temperature is exceeded, this causes a premature decrease in euryhalinity, or a desmoltification in salmonids. Water levels are also of importance. Low levels tend to inhibit or at least deter smolt migration by impeding passage. Many workers feel that in most rivers trout below impassable waterfalls have the potential to migrate, but this option is not always exercised, suggesting that environmental pressure is the trigger. In landlocked situations, areas above impassable waterfalls, for example, there is a strong tendency for migratory fish to be selected against. It may still take up to 100 or more generations to select against migration, depending on heritability (Prof. A. Ferguson, Q.U.B., pers. comm.). Heritability decreases as selection progresses, but this decrease is not linear.

2.6 Sex-ratios

The proportion of females in the sea run population has been found to be greater than 50% by all investigators (Le Cren, 1985). It has been known for a long time that in some rivers only female trout migrate, the males remaining in fresh water. In other rivers both sexes may migrate but, among the migrants, females predominate, probably for evolutionary reasons, since migratory females will grow larger and hence produce greater numbers of eggs (Le Cren, 1985).

3 METHODS OF PRESERVATION: GENE BANKS

A gene bank, in essence, is a repository of genetic material or germplasm which is maintained to facilitate the re-establishment or reconstitution of a species or population. There are two major types of gene bank: a) frozen gene banks and b) living or captive gene banks. However, in practice, these two approaches are usually used interactively.

3.1 Frozen Gene Banks

Preservation of genetic material in a gene bank requires a method of long-term storage which will prevent alteration of the stored material (NASCO, 1989, 1990). The principle technology employed in frozen gene banks is cryopreservation.

3.1.1 Cryobiology

The responses of living cells to ice formation and the freezing of biological systems is thoroughly reviewed by Mazur (1970) and more recently by Grout *et al.* (1990) and Rall (in press). Freezing and thawing are severe stresses to cells during the entire cryopreservation process. In summary, when cells are subjected to subzero temperatures, they initially supercool. The manner in which they regain equilibrium depends mainly on the rate at which they are cooled and their permeability to water. If they are cooled slowly or if their permeability to water is high, they will equilibrate by dehydration i.e. loss of intracellular water to ice. If they are cooled rapidly or if their water permeability is low, they will equilibrate by intracellular freezing, i.e. the formation of intracellular crystals, which results in lethal injury. Either way, water is lost and the concentration of intra- or extracellular solutes increases. Therefore, the rate of cooling is very important in cryopreservation and optimal rates of cooling are calculated for each cell/organ. By modifying the chemical environments of the cell, i.e. by adding protective reagents such as glycerol, methanol or dimethyl sulfoxide (DMSO) cryo-injury may also be reduced. To be effective, cryoprotectants must be cell permeable and non-toxic. Prior to freezing, the cells are incubated in as high a concentration of cryoprotectant as is feasible while mitigating toxic effects. The effectiveness of the various protectants varies with cell type and cell shape and the degree of permeation is temperature dependent. After the cell suspension has been frozen using controlled cooling rates, usually to -120°C, it can be stored, in theory indefinitely, at -196°C in liquid nitrogen. There is very little quantitative evidence on the effects of long-term storage on cells but it has been estimated, for example, that a frozen cell population would have to be exposed to background radiation for 32,000 years to reduce survival of the material to 10% (Ashwood-Smith and Grant, 1977, quoted in Grout *et al.*, 1990).

Post-thaw survival may be influenced by the thawing process. The cell suspension is usually warmed and thawed rapidly under controlled conditions. This is a very important stage as drastic osmoregulatory consequences can occur if incorrectly carried out (Rall, in press). Depending on the molarity of the cryoprotectant, cells are diluted in water, which rehydrates the cells and causes swelling, and the cryoprotectant is removed by serial dilution. Cells are then returned to normal physiological conditions.

3.1.2 Plant Gene Banks

The concept of gene banks was probably first realised with the establishment of seed banks for crops. In 1947 four major germplams stations were established in USA, in response to the growing concerns of crop inbreeding and the realisation that few grain species were indigenous. In 1958, a national, long-term seed storage facility was opened in Fort Collins, Colorado, U.S.A. (Bass, 1979). The national seed bank in Colorado acts as a repository for all plant seeds and when received, each sample is given a serial number, a genus and species number and tested for viability. If germination is satisfactory the seeds are stored. Most seeds are stored at 4°C and 35% relative humidity in cans, while others are dried to between 5 and 7% moisture content and stored at -10 to -12°C in hermetically sealed cans. However,

longevity and type of storage conditions are species specific. A more recent paper states that at Fort Collins 240,000 samples are stored in sealed containers at -18°C (Clarke, in press). All acquistions are logged into a germplasm resources information network (GRIN) with data on parameters such as number of plants sampled, habitat, location and elevation. All foreign material is quarantined (Clarke, in press). An international germplasm bank is located in Rome, termed the International Board of Plant Genetic Resources (IBPGR). This bank has close associations with the Food and Agricultural Organisation of the United Nations (FAO).

3.1.3 Frozen banks for Animal Tissues and Organs

Developments in cryobiology technology have led to the establishment of many tissue banks for organ transplants, including erythocytes, bone marrow, skin, corneas, bone, gametes and embryos. Since Polge *et al.* (1949) reported a protective effect of glycerol, which in combination with low rates of cooling, resulted in high survivals after freezing and thawing of spermatozoa of several species, the breeding of domesticated animals with frozen semen and artifical insemination has become widespread. This is reviewed by Watson (1979). In 1971, sperm banks for frozen human semen were opened as commercial ventures in several American cities (Francoeur, 1975). Since the early 1980s sperm cryopreservation techniques have been developed and extended to some of the more endangered species of the world, with research being carried out on primates, ungulates and felids, among other species. In a recent review of the subject, Holt and Moore (1988) examined the feasibility of semen banking for endangered species and proposed that an international repository be established for frozen semen of endangered species. However, there are still technical difficulties associated with cryo-freezing, as established at a recent conference in London Zoo (4 and 5 Sept., 1990) entitled "Biotechnology and the Conservation of Genetic Diversity". The main problems arise from the large variations in types of mammalian sperm and the marked physiological differences in reproductive mechanisms, even in closely related species (Howard *et al.*, 1986; Wildt *et al.*, in press). Other difficulties include assessment of the timing of ovulation and subsequent timing of insemination. Wildt *et al.* (*loc. cit.*) suggest that the ability to use biotechnology for practical wildlife conservation will require that many species receive attention, to elucidate basic, individual, biological problems. Success with cryobiological techniques has been reported, for example, with the re-establishment of the black-footed ferret *Mustela nigripes* in USA (Wildt, in press).

Cryopreservation of mammalian embryos is reviewed by Whittingham (1980), Schneider and Mazur (1986) and Pomp and Critser (1988). Live offspring have been produced in the mouse, rabbit, rat, goat, horse, antelope, human and baboon (Leibo, 1986, quoted in Pomp and Critser, 1988). In the case of the mouse, there are now over 15 embryo banks around the world which are used for research purposes. The application of artifical insemination, *in vitro* fertilisation or embryo transfer can improve the reproductive contribution of an individual. Similarily, the long-term cryopreservation of germplasm of an individual, with periodic exchange of germplasm with living populations, extends an individuals reproductive contribution long after its death (Ballon and Cooper, in press).

3.1.4 Cryopreservation of Fish Spermatozoa

For over a hundred years the short-term storage of fish spermatozoa has been possible (Truscott *et al.*, 1968) but in the last 20 - 30 years research has concentrated on developing long-term cryopreservation techniques. The majority of work undertaken in this field is for commercially important fish species, in particular salmonids. Scott and Baynes (1980) reviewed the biology, handling and storage of salmonid spermatozoa. Table 1 shows some of the fish species in which sperm have been successfully cryopreserved. General review papers on cryobiology and the storage of teleost sperm have been written by Mazur (1970), Polge (1980), Harvey (1982, 1986), and Stoss (1983). However, the standard of fertilization achieved using cryopreserved fish milt is highly variable and until the cause of this variability is understood and controlled, cryopreservation is unlikely to be fully exploited by the aquaculture industry (Baynes and Scott, 1987).

Table 1 Some fish species in which spermatozoa have been successfully cryopreserved

Species	Common Name	Reference
Coregonus muksun	Whitefish	1
Salmo salar	Atlantic salmon	2, 3, 4, 5, 6
S. trutta	Brown trout	7, 8, 2
Oncorhynchus mykiss	Steelhead trout	7, 8, 9, 10, 11, 12, 13
O. tschawytscha	Chinook salmon	14
O. kisutch	Coho salmon	14
Salvelinus fontinalis	Brook charr	7
Hucho hucho	Huchen	15
Thymallus thymallus	Grayling	15
Clupea harengus	Herring	17
Esox lucius	Pike	15
Cyprinus carpio	Common carp	15
Hypophthalmichthys molitrix	Silver carp	22
Labeo rohita	Indian carp	25
Aristichthys nobilus	Bighead carp	22, 25
Ctenopharyngodon idella	Grass carp	27
Pangasius sutchi	Catfish	25
Ictalurus punctatus	Channel catfish	30
Gadus morhua	Cod	18, 6
Oreochromis niloticus	Tilapia	16, 5
Lates calcifer	Barramundi	20
Sparus aurata	Gilthead seabream	21
Epinephelus tauvina	Grouper	26
Brachydanio rerio	Zebrafish	29
Mugil cephalus	Grey mullet	23, 24
Chanos chanos	Milkfish	28
Pleuronectes platessa	Plaice	19

1 Piironen (1987), **2** Stoss and Refstie (1983), **3** Alderson and MacNeil (1984), **4** Truscott and Idler (1969), **5** Rana (1988), **6** Mounib (1978), **7** Erdahl and Graham (1978), **8** Erdahl and Graham (1980), **9** Holtz *et al.* (1979), **10** Ott and Horton (1971b), **11** Baynes and Scott (1987), **12** Cloud *et al.* (1990), **13** Wheeler and Thorgaard (1991), **14** Ott and Horton (1971a), **15** Stein and Bayrle (1978), **16** Rana *et al.* (1990), **17** Blaxter (1953), **18** Mounib *et al.* (1968), **19** Pullin (1972), **20** Leung (1987), **21** Chambeyon and Zohar (1990), **22** Sin (1974), **23** Chao *et al.* (1975), **24** Chao (1982), **25** Withler (1982), **26** Withler and Lim (1982), **27** Durbin *et al.* (1982), **28** Hara *et al.* (1982), **29** Harvey *et al.* (1982), **30** Guest *et al.* (1976).

3.1.5 Methodologies for Cryopreservation of fish spermatozoa

Published methods for sperm cryopreservation are similar. There are two stages. Initially, the milt is diluted with an extender solution which prevents activation of the sperm and then a cryoprotectant, such as DMSO, is added. In many cases albumin, lecithin or whole hen's egg yolk may also be added to give further protection against freeze/thaw damage. Milt is either frozen in droplets on solid CO_2 and stored in vials over liquid nitrogen or frozen in plastic straws and stored over liquid nitrogen.

There is considerable variation in post-thaw fertility and one of the factors causing this variation may be sperm motility (Baynes and Scott, 1987; Wheeler and Thorgaard, 1991). Generally, the motility and fertilisation ability of salmonid sperm is lost completely within 1 or 2 minutes after dilution with fresh water. Both water temperature and age of fish have a marked effect on the period of motility of sperm. Rainbow and brown trout sperm have a motility time only half as long as that of many other freshwater fishes. Hey (1939, 1946, quoted in Scott and Bayne, 1980) found, for example, that several trout produced non-viable sperm. Wheeler and Thorgaard (1991), who determined sperm motility by visual inspection after activation, found that motility varied from 0 to 95% among samples. With such a range of motility, only semen samples with 50% or more sperm motility were frozen.

Undiluted sperm, with or without a cryoprotectant directly added, is not suitable for freezing. Semen must be diluted with a suitable extender solution i.e. a solution of salts, sometimes including organic compounds, which helps maintain the viability of cells. A good extender is "one that will allow dilution of cells without their activation on the theory that the activation of a salmonid sperm is a once-only action and the cell cannot be re-activated" (Truscott and Idler, 1969).

The main requirements for a cryoprotectant are good solubility in water and non toxicity. There are a variety of cryoprotectants used. Dimethyl sulfoxide (DMSO), in particular, is used for salmonids. Ethylene glycol and propylene glycol have also been used with limited success. Glycerol is used as a cryoprotectant with mammalian spermatozoa, but this has been found to be ineffective for salmonid sperm (Erdahl and Graham, 1980).

3.1.6 Cryopreservation of *S. trutta* spermatozoa

Cryopreservation of spermatozoa of brown trout and sea trout (*S. trutta*) has been reported by Stein and Lamina (1976), Stein and Bayrle (1978), Stein (1979), Erdahl and Graham (1980) and Stoss and Refstie (1983). The findings of these authors are summarised in Table 2. The first two papers reported post-thaw fertilisation rates of between 41 and 77% using different types of extenders and Stein (1979) looked at different cryoprotectants and found that using DMSO gave a 71% post-thaw fertilisation rate, glycerine 47% and ethylene glycol (EG) 5% (see table 2). After storage at -79°C, post-freeze spermatozoa of both brown trout and rainbow trout resulted in fertilisation rates in excess of 80% when introduced to fresh ova, but this varied depending on the extender and cryoprotectant used (Erdahl and Graham, 1980). These authors also found that higher fertility was obtained using the straw rather than the pellet method for freezing. Stoss and Refstie (1983) looked at short-term storage and cryopreservation of milt from Atlantic salmon (*S. salar*) and sea trout (*S. trutta*). Results from fertilisation using cryopreserved sea trout sperm ranged from 38.6 - 54.9% eyed eggs which is lower than those reported by Stein (1979) and Erdahl and Graham (1980) for "trout". However, milt collection for sea trout was towards the end of the spawning season and these authors, quoting Legendre and Billard (1980); Bueyuekhatipoglui (1977) and Sanchez - Rodriquez *et al.* (1978), suggest that "the ability of spermatozoa to tolerate the stress of freezing and thawing may be altered during the course of the spawning season as changes occur in the physiologocal characteristics of semen in some teleosts".

Table 2 Cryopreservation of *Salmo trutta* sperm (adapted from Scott and Baynes, 1980).

Author	Species	Handling Temp. °C	Final Temp. °C	Storage time	Technique	Volume µl	Extender	Dilution ratio
Stein & Lamina (1976)	*S. trutta*	4	-196	7 days	Pellet	200	V2	1 : 3
Stein & Bayrle (1978)	*S. trutta*	4	-196	7 days	Pellet	200	V2e	1 : 3
Stein (1979)	*S. trutta*	4	-196		Pellet or straw	200	V2e	1 : 3
Erdahl & Graham (1980)	*S. trutta*	4	-79	1 hr.	Pellet	100	# 6	1 : 1
			-79	1 hr.	Straw	250	# 6	1 : 1
Stoss & Refstie (1983)	*S. trutta* (sea trout)		-196	2 days	Pellet	500	A	
							A	
							0.3M glucose	
							0.3M glucose	

Exdender composition:

V2 (g/l): 7.5 NaCl, 0.38 KCL, 0.46 $CaCl_2$ ($2H_2O$), 2.0 $NaHCO_3$, 0.23 $MgSO_4$.($7H_2O$), 0.53 Na_2HPO_4, 5.0 glycine, 1.0 glucose, 20ml egg yolk.

V2e (g/l) 7.5 NaCl, 0.38 KCL, 2.0 NaHCO3, 1.0 glucose, 20ml egg yolk.

Equilibration time	Cryoprot. %	Freezing rate	Thaw temp. ℃	Fertilisation rate frozen sperm	Fertilisation rate fresh sperm	Fertilisation technique
None	DMSO 10	30°C/min to -79°C	1	41	90	3 pellets thawed in 10ml 1% $NaHCO_3$ Added to 500 eggs
15 min.	DMSO 10	30°C/min to -79°C	1	77	90	As above
	DMSO (10) Glycerine(20) E.G. (10)	10° - 30°C / min	0	71 47 5	82	As above
10 min	DMSO (7) E.G.	-85°C/min to -30°C	5	10 15	94	Pellets thawed in 2ml extender, stored at 5°C; no. of eggs varied.
10 min	DMSO (7) E.G.	-85°C/min between -5 and -30°C	5	60 60	90	Straws thawed by immersion in water, stored at 5°C, no. of eggs varied.
	DMSO 10		10	55	73.9 - 98.3	Dissolved 10 pellets (each 0.05ml) in 10ml $NaHCO_3$ solution (0.12M, 10°C). Suspension added to approx. 147 eggs.
	DMSO 10		10	52	"	As above
	DMSO 10		10	55	"	As above
	DMSO 10		10	39	"	As above

#6 (g/2l)	0.205 $CaCl_2$. $2H_20$, 0.440 $MgCl_2.6H_2O$, 0.530 Na_2HPO_4, 5.115 KCL, 11.682 NaCl, 0.2 citric acid, 20.0 glucose, 20ml KOH, 40ml Bicine.
A	101.1mM NaCl, 23.1mM KCL, 5.4mM $CaCl_2$, 1.3mM $MgSO_4$, 200mM Tris, citric acid, 1 % bovine albumin.

3.1.7 Possible Future Developments in Cryobiology

3.1.7.1 Cryopreservation of Fish Ova and Embryos

While cryopreservation of spermatozoa is relatively successful for many fish species, many major problems still exist for the cryopreservation of fish ova and embryos. Zell (1978) found that *Salmo gairdneri* eggs and embryos survived freezing to -55°C when frozen immediately after fertilisation or after eyeing. However, many authors (Erdahl and Graham, 1980; Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983) were unable to achieve similar results and it is suggested that the ova and embryos were only supercooled not frozen.

Several factors interfere with the removal of intracellular water during cooling: 1) the large egg volume, 2) the presence of two different membanes (the outer capsule and the perivitelline membrane which surrounds the yolk) and 3) the differential water permeability of both membranes (Stoss, 1983). Penetration of cryoprotectants such as glycerol, DMSO and methanol has been found to be extremely slow in unactivated ova and cooling rates as low as 0.01°C/min are still too high to prevent intracellular freezing (Harvey and Ashwood-Smith, 1982; Harvey, 1982). Harvey *et al.* (1983) examined cryoprotectant permeability (using ^{14}C - DMSO and ^{3}H glycerol) of zebra fish embryos (*Brachydanio rerio*) and found that glycerol was a better penetrant than DMSO and that the chorion retards the exchange of solutes. DMSO was found to be toxic at concentrations of 1.5 and 2.0M at 23°C after one hour. However, although glycerol appears to be a better cryoprotectant than DMSO, there are problems in removing it, relating to cellular disruption and the removal of cellular barriers (Harvey, 1983). Suprisingly little has been published on cryopreservation of fish ova and embryos since that time although anecdotal reports suggest that some success has been achieved in the cryopreservation of embryos of grass carp from China (Dr. M. Mc Lellan, ex. Cell Systems, now British Technology Group, pers. comm.). There is also evidence from Australia that fish ova have been cryopreserved. Jamieson and Marshall (in preparation) quoted in Leung and Jamieson (1990) reported "modest but significant fertilization rates for eggs of the Australian bass (*Macquaria novemaculeata*) after cryofreezing". Cryopreservation was achieved using a two - step freezing process to -196°C in a DMSO based cryoprotectant solution, following equilibration in a vacuum. It is reported that the vacuum equilibration method shortens the equilibration time required, while augmenting penetration of the cryoprotectant and thus minimizing toxicity. It also seems possible that the use of a vacuum facilitates removal of the cryoprotectant on addition with normal medium which, in the last stages of thawing, might alleviate toxic cryoprotectant effects (Leung and Jamieson, 1990). At a recent meeting entitled "Genetic Conservation of Salmonid Fishes" in Moscow, Idaho and Pullman, Washington, July 1991, the general concensus of participants was that fish ova and embryo cryopreservation is still at a very early stage of development.

Some work is being carried out in Norway to extract the nucleus and thus the nuclear DNA of fish ova and then to try and cryopreserve this smaller organelle (Dr. D. Gausen, NINA, pers. comm.). The rationale behind this work is that this frozen nuclear material could be tranplanted back into a suitable recipient ovum. Work being carried out on other species, in particular the Fruit fly *Drosophila*, may provide an alternative technique to standard cryopreservation techniques, namely vitrification.

3.1.7.2 Vitrification

Vitrification can be defined as the solidification of a liquid brought about not by crystallization (as in freezing), but by an extreme elevation in viscosity during cooling. This solidification, described as 'glass formation' differs physically from the type of solidification resulting from ice crystal formation (Pomp and Critser, 1988 and references quoted therein). Vitrification eliminates the need for controlled freezing, which is one of the major factors affecting ova/embryo cryopreservation and thus prevents intracellular freezing and mechanical damage, and allows embryos to be cryopreserved by direct plunging into liquid nitrogen. The procedure is slightly different than that of normal cryopreservation but the precautions taken are

similar. Cells are equilibrated and dehydrated in concentrated but non toxic solutions of vitrifying solutes and the suspension is vitrified by cooling to -120°C and subsequently stored at -196°C. The factors affecting cell survival are similar to those associated with using cryoprotectants. The vitrifying solution must be physiologically compatible with intracellular fluid. Generally, the concentration of the solution is very high (5.5 - 8.5 Molar) and may therefore be toxic. Toxicity may be decreased if combinations such as acetamide with DMSO or non-permeable solutions e.g. polyethylene glycol or bovine serum albumin with other cryoprotectants, are used (Rall, in press). Vitrification has been carried out on some mammalian embryos, including mouse, cattle, rabbit, sheep and cat and on human monoctyes. This process has been used with some success on *Drosophila* embryos, which like fish embryos are large, with low surface to volume ratio, have membrane systems which limit permeability of cryoprotectants and cellular dehydration and have high sensitivity to chilling injury (usually associated with high lipid/yolk content). So far, survival of embryos is estimated at 18%, with a maximum survival of 25% (Rall, in press). Future develpoment of this technique may help in elucidating a method for cryopreserving fish embryos (Rall, in press; Harvey, in press).

3.2 Living Gene Banks

Living gene banks involve the maintenance of specimens as broodstock in a secure artificial environment such that their offspring are available for re-stocking into the natural environment. Captive breeding of endangered species is a familar and long-established concept. For example, the Dexter National Fish Hatchery, near Dexter, New Mexico has, since 1974, been used to rear a variety of threatened and endangered fishes of the American Southwest, some of which are extinct in the wild and only exist in captivity (Meffe, 1987). The idea of zoos aiding wildlife conservation was first suggested as long ago as 1895 by Samuel P. Johnson who, in attempting to establish the Smithsonian National Zoological Park in Washington, D.C., emphasised the concept of breeding threatened species for reintroduction to the wild. Captive breeding should be the aim of all zoos and most have developed specialities in techniques for successful breeding of at least one species. Nonetheless, captive breeding is only half the battle. Animals must be bred over generations and viable breeding groups built up for eventual reintroduction into the wild. This task is fraught with difficulties (Woodroffe, 1981). The first problem is that curators must ensure that captive populations remain fit for the wild, by retaining enough natural behaviour for survival. Secondly, genetic diversity must be maintained. Since no zoo has enough space to keep sufficient numbers of animals to maintain genetic diversity, zoos operate an international network which allows transfer of animals for breeding purposes. To this end, zoos maintain studbooks for many species containing data on which animal is decended from which parents. These studbooks are used with the aid of computer programs, to plan pairings in breeding programmes, or what Tudge (1988) euphemistically refers to as "breeding by numbers". In the past five years IUCN/SSC's Captive Breeding Specialist Group has become increasingly involved in these breeding programmes as a co-ordinating/umbrella organization (De Boer, in press) and biotechnology (e.g. improved techniques in artificial reproduction and cryopreservation) is playing an increasingly important role.

Examples of salmonid gene banks are described in a NASCO (1989) report and include the banks run by the Directorate for Nature Management (NINA), Trondheim, Norway; the Institute of Aquaculture, University of Stirling, Scotland and Cells Systems Ltd., Cambridge, England. Many of these programmes successfully combine biotechnology, in the form of sperm cryopreservation and captive breeding as in the case of the Norwegian Atlantic salmon gene bank and to a lesser extent, Stirling University. Details of the first two of these ventures are given in Appendix 1 and 2 to demonstrate the principles and complexities involved. Cells Systems Ltd. is at present undergoing re-organisation and details of their operation are unavailable.

4 PRACTICAL CONSIDERATIONS IN RELATION TO SEA TROUT GENE BANKING

4.1 The feasibility of establishing a sea trout gene bank

Anadromous salmonid species consist of populations that originate from a specific watershed as juveniles and generally return to their natal streams to spawn. Unlike many salmonids, *S. trutta* appears to exist in two forms: a migratory and a non-migratory form. As stated earlier, no genetic differences have been found between sea trout and co-existing brown trout (Hindar *et al.*, 1991, Cross *et al.*, in press) but differences between river systems and even tributaries on the same river have been found (Fleming, 1983, Ferguson and Fleming, 1983, D. Bembo, University of Wales, pers. comm.). Even with sympatric populations of brown trout, as in Lough Melvin, genetic differences have been reported (Ferguson, 1989, Ferguson and Taggart, 1991). *S. trutta* is therefore a highly polytypic species. The high genetic variability between stocks, combined with the as yet unresolved reason or factor(s) which triggers some, most and in some cases all, trout in certain rivers to smoltify and migrate makes their conservation and management problematical, particularily when the migratory form appears to be at risk. That is to say, certain philosophical questions or problems have to be addressed and answered, not least among them is what should be conserved: species; genetic diversity; individual stocks? Entirely different management/conservation strategies might be implemented depending on which is chosen.

Currently, international conservation efforts recognise the species as a unit of concern, as within the context of the Endangered Species Act (ESA) in the USA (Meffe, 1987). The application of this act to sub-species and stocks is therefore tenuous. However, Nehlsen *et al.* (1991) argue that individual stocks (as defined by hereditary differences, i.e. fish that spawn in a particular river system at a particular season and do not interbreed with any group spawning in a different place, or at the same place by a different season) should qualify for protection under the ESA.

Ideally, the entire range of genetic diversity available within a species should be retained, but this may be impractical (Meffe, 1987). This problem is especially acute for sea trout as any one population contains only part of the genetic diversity of the species (Ferguson, 1989). Meffe (*loc. cit.*) discusses the philosophical biases and assumptions made regarding conservation of genetic resources and concludes that by adopting the conservative approach (assuming that as much genetic diversity should be retained as possible) the probability of inflicting irreparable harm upon systems which clearly are not fully understood is minimised. This approach has also been taken by Cross (1989) in recommending enhancement of depleted salmon rivers and will also be taken in this report.

4.2 Models for Sea Trout Gene Banks

Most conservation or management plans assess the targeted population, examining the intrinsic biological characteristics such as age at maturity, age at smoltification, time of migration, sex-ratios, habitat and factors which may limit abundance, e.g. high temperatures, low water levels and natural barriers such as waterfalls. Depending on the severity of the problem and assuming that some fish still remain in the system being investigated three basic strategies can be employed:

a) sanctuaries can be established, preventing stocking or fishing. Specifically, this can be implemented in areas where habitat improvement has been carried out or where the habitat is recovering, thus enabling the population to recover without man's interference;
b) hatcheries can be used to supplement or enhance depleted stocks;
c) both cryopreserved and living gene banks can be established.

The following scheme, designed for rehabilitation of a depleted sea trout stock, incorporates strategies b) and c) above. It is similar to the scheme operating in Norway for the conservation of Atlantic salmon (see Appendix 1). Three basic assumptions were made when formulating this plan;

1) that it would run for a limited period of time to prevent possible domestication;
2) that since the interrelationship between the two forms of trout is not fully understood, it would be better to use sea trout as broodstock where possible;
3) that the cause of the problem affecting areas, such as the west of Ireland, would be identified and rectified.

The plan, diagramatically represented in Figure 1, can for convienence be divided into two sections i) preservation and ii) rehabilation.

4.2.1 Preservation: the establishment and utilisation of gene banks

The gene bank should incorporate both frozen and living elements. Frozen gene banks are probably the last insurance policy against extinction of a stock, population or a species. However, at present only half the genome, in the form of frozen sperm can be preserved. Unlike other species, particularly mammals, where ova or embryos can be frozen, cryopreservation of fish sperm usually necessitates a corresponding living or captive gene bank to supply the other half of the genome.

4.2.1.1 Preservation in situations where sea trout occur

Frozen gene banks:

Cryobiological techniques, while still developing, are well established for the cryopreservation of salmonid sperm, as described in sections 3.1.4 and 3.1.5. Sea trout returning to fresh water, can be captured, either in traps or by electofishing or netting, tagged and, depending on their maturation status can either be stripped or kept in holding tanks until mature. If possible, milt should be extracted using a catheter (as used in Stirling University, see Appendix 2) and should be cryopreserved using one of the standard protocols developed for salmonids, as described in sections 3.1.5 and 3.1.6. Either the pellet or straw method could be used and samples should be numbered corresponding to the adult tag number. Although Erdahl and Graham (1980) found that the straw method had a higher post-thaw fertilisation rate than the pellet method, most workers have found no difference (Dr. B. Harvey, MTL Biotech, Victoria, B.C., Canada, pers. comm.). Straws, as well as being more widely used, may take up less storage space than pellets stored in vials. If possible, the permanent storage facility should be located in a building where cryopreservation techniques are already established, like a farm animal artifical insemination station (as with the Norwegian gene bank, see Appendix 1) and where staff are trained to look after liquid nitrogen containers. Ideally, two permanent storage units should be in operation, one as duty and the other as a back-up. It has been estimated that different age classes contribute in various degrees to sea trout egg deposition (see section 2.2), but male contribution has not been estimated on the basis of age or size. It is therefore important that when collecting the milt, samples from as broad a year class selection as possible should be taken and throughout the whole spawning season. Milt from at least 50 individuals from each population should be collected as a minimum number. Even though 50 of each sex is the effective population size calculated to minimize loss of variance and fitness through inbreeding (Cross, 1989), cryopreserved milt has never as high a fertility rate as fresh sperm, on which the estimates were made.

Living gene banks:

In parallel with the development of frozen gene banks for specific populations, living gene banks should be established taking precautions to minimize inbreeding and directional genetic change. Strict anti-disease measures will need to be applied and it is suggested on theoretical grounds, that only a few generations be maintained in captivity to minimize domestication. All of this presupposes that rearing of sea trout can be effected successfully, which has not always been the case (Mills *et al.*, 1990a). The setting up and running of living gene banks is likely to be costly but is necessary as a adjunct to cryopreservation of milt.

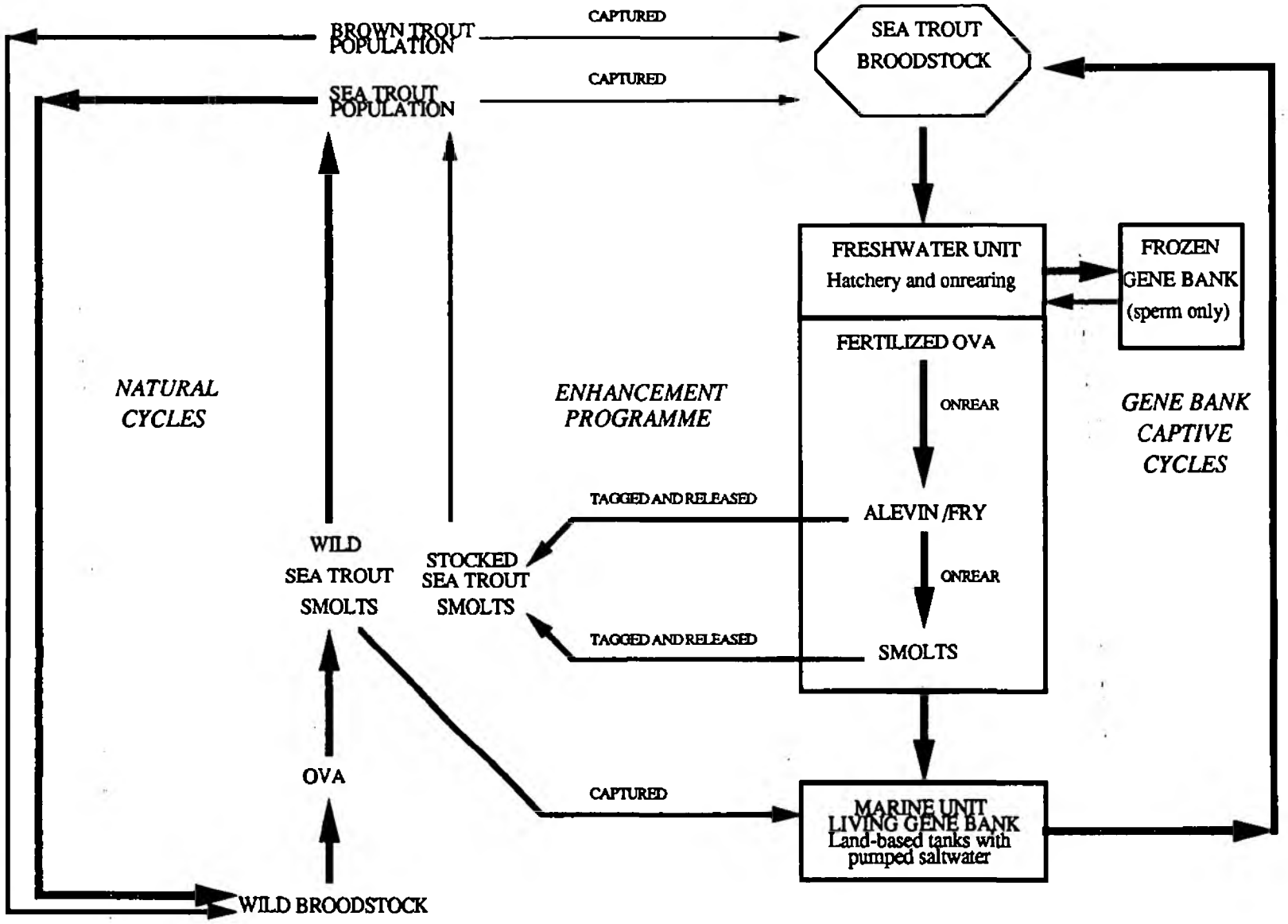

Figure 1 **A possible model for sea trout preservation and rehabilitation**

4.2.1.2 Preservation in situations where there are few returning sea trout adults but smolts still occur

If the situation has arisen, as in the west of Ireland, where the sea trout run has collapsed, but smolts are still being produced, smolts (and autumn trout, where they occur) could be captured in traps or netted, tagged and held in cages or land-based units with pumped sea water or possibly fresh water. Land-based units are probably better in that disease outbreaks are easier to control and sea-lice infestations can be avoided. These fish may then be on-grown to maturity.

As an example of this, in 1991, along with a concentrated research programme, the Salmon Research Agency in western Ireland captured smolts (and kelts) from the Burrishoole system and have retained them in a cage in a partially saline (10‰) lake, and are feeding the fish maggots and a moist/wet food diet with an oil supplement, eventually moving onto a dry diet. These fish will be kept for at least 2 years and if they mature will be stripped and probably crossed with some cohabiting brown trout (R. Poole, SRAI, pers. comm.). To further increase numbers of females, brown trout will also be used to produce sex-reversed milt which, after the first generation may be crossed with eggs stripped from the caged sea trout, to produce an all-female progeny. The Irish Department of the Marine has also set up a captive programme, capturing fish which re-entered fresh water in a number of rivers in the hope of growing these fish to maturity and breeding from them.

Frozen gene banks:

As with the collection of milt from adult sea trout, it is important to randomly take a large sample of smolts to minimise inbreeding. Also, sampling should be spread throughout the run. It is also important, during the subsequent growth of the fish, that no operational selection should be implemented (e.g. fish with poor growth culled out) and a rearing protocol should be established that provides more natural rearing conditions than those normally associated with intensive aquaculture production. Since little is known of the behavioural habits of sea trout in the marine environment, it is difficult to suggest a suitable stocking density, since ideally densities should mimic natural conditions. However, a low stocking density would probably reduce stress and reduce risks of disease outbreaks. As sexual maturity approaches, the fish should be transferred to fresh water. The transition from saline to fresh water should be done gradually, again to mimic the natural situation and to reduce possible physiological stress. Fish can then be held until mature and milt stripped and cryopreserved, as described above.

Cryopreserved sperm, if stored properly should remain viable for many generations. It can either be used occasionally to introduce new genetic material to the captive breeding programme or can be used to reconstitute a population. In a situation where no anadromous females are available, reconstitution of a depleted sea trout stock might be achieved by repeated back-crossing with co-habiting non-migratory brown trout from the same river (Thorgaard and Cloud, in press). In the second generation trout would have a 50% sea trout and 50%brown trout genome. However, if females of these hybrids were back crossed with sea trout milt the progeny would have a 75% sea trout genome (i.e. the brown trout contribution would reduce by 50% in each generation). In such a situation, however, inbreeding is very likely to occur.

Living gene banks:

For use in a living gene bank programme, sufficient numbers of smolts should be caught, to yield at least 50 mature individuals of each sex, tagged and held as described above. Then at maturity they should be stripped and both ova and sperm brought to a hatchery and ova disinfected. Broodstock can also be removed from the returning sea trout if any appear and stripped if mature or kept in tanks until ripe. At the hatchery where fertilisation is carried out, some cohabiting brown trout could also be used if sea trout numbers were low (providing further work confirms that the two forms interbreed). As sea trout are multiple spawners, kelts

should be maintained in freshwater tanks until they have recovered and then stripped again in the following year. Autopsies should be carried out on any mortalities to guard against disease transmission.

4.2.1.3 Strategies in situations where mature sea trout and smolts no longer exist

The above sections on preservation pertain to river systems where a certain proportion of the original sea trout population still exists. If too few sea trout remain in a river for rehabilitation it may be possible, as Cross *et al.* (in press) suggests to use the remaining brown trout population to enhance sea trout production. This would involve capturing brown trout and holding them in a hatchery for two years. In "crowded" hatchery conditions, silvering up may occur (even if this occurs in the first year, fish should be held and released after silvering up in the second year (Mills *et al.*, 1990b)). As with sea trout progeny, a proportion of these fish could be kept in a sea cage or pumped sea water unit to monitor growth and survival. If survival in the cages or tanks is good, these could be used in conjunction with ranched returns as broodstock for the next generation.

Alternatively, or in combination with the above approach, sea trout from the nearest "suitable" river might be chosen for rehabilitation in the target river. Suitability might be based on a number of criteria, such as:

1) geographical location of possible donor stocks;
2) genetic information;
3) environmental compatibility of river systems.

This sort of choice is difficult to make and is discussed, in detail, in relation to Atlantic salmon in Cross (1989).

Another way of re-establishing an anadromous run in a system where no sea trout remain, might be to introduce several sea trout stocks from other rivers, with suitable parallels to the river being restocked and to allow interbreeding and natural selection to operate, thereby establishing a new population (Ferguson, pers. comm.). Alternatively, the different stocks could be marked uniquely and assessment of the most suitable donor stock could be made on return from the sea, using total trapping facilities. The most suitable stock might then be used in future. Such a scheme has been successful in Atlantic salmon rehabilitation in Norway (Dr. L.P. Hansen, NINA, pers. comm.).

4.2.2 Rehabilation: reintroduction of preserved material into the wild

As with maintaining sea trout smolts to maturation or maintaining a living gene bank, hatchery rearing of eyed ova to time of release must be carried out under as natural conditions as possible. This is particularly important since "the objective is to mimic important rearing conditions (such as temperature) as much as possible except for providing a more abundant food supply and eliminating predation" (Backman, in press). Mills *et al.* (1990a) reported many difficulties in the hatchery rearing of sea trout. However, Rasmussen (in press) reported on hatchery rearing of sea trout in Denmark, which worked well and led to an effective enhancement scheme. As in the Norwegian system for salmon (Appendix 1), initially the progeny of single pair matings should be kept separately, to allow individual marking, since only fish larger than 5cm can be easily marked. This is also a way of minimising the spread of disease. When the fish are big enough to be marked, either by Pan-Jetting or tagging, families from the same population should be held together. Since the production of the migratory form is the principle aim of the plan, it is important to regulate the increase in "daylight" carefully and food should also be given careful consideration. As stated in section 2.2, slow growth to parr stage may be associated with non-migratory forms and fast initial growth with smolts. In contrast, since competition or lack of food has also been associated with the production of sea trout, it may be prudent to reduce the food availability of fish being reared to smolts.

One of the prerequisites of a successful enhancement scheme is that the habitat must be in a condition that makes the restoration of the target species feasible. Anadromous trout require different habitats throughout their life-cycle. While habitat assessment is relatively easy in the freshwater phase (e.g., adequate spawning habitat is easily assessed based on gravel size, water quality, flow rates, water temperature, carrying capacity and adequate food supply for juveniles), it is difficult to assess the problems associated with habitat in the marine phase. This is partly due to ignorance of the behaviour of trout once they enter the sea. Extensive tagging carried out by Potter and Solomon (quoted in LeCren, 1985) on the trout from the Rivers Tweed and Yorkshire Esk, found that smolts migrated south towards East Anglia and then in an anti-clockwise direction round the southern North Sea. A similar migration was found for kelts, but with more variation. Smolts and kelts tagged on the North Esk showed less distant movements and there is some evidence to suggest that smolts, from the River Axe, that migrated on any one day had a similar pattern of return, suggesting that batches of smolts stayed together at sea (Le Cren, 1985). In areas such as the west of Ireland and Scotland, it is thought that sea trout stay close to the coast, not far from their natal rivers. This hugh variation in migratory behaviour is a strong arguement, in addition to the genetic population differences described in section 2.3, for using the same stock in enhancement. Migratory direction and distance appears to be under genetic control in other salmonids (Brannon, in press).

A suitable release strategy should be implemented to ensure success of the enhancement scheme. Decisions to be taken include numbers of fish stocked, life stage at which to stock, timing of release and area of release. Sufficient numbers of fish should be stocked and matched to the biological productivity of the habitat, thereby not exceeding the carrying capacity of the system, to "ensure an adequate level of seeding" (Backman, in press). It is also important not to swamp the existing natural population, and numbers stocked should probably be equivalent to, but not greater than, the natural populations (Ferguson, pers. comm.).

When deciding the life-stage to use in stocking, certain factors should be taken into consideration. These include imprinting on natal streams, survival rates, subsequent monitoring and evaluation. Successful imprinting to the chemical cues associated with a particular river usually occurs in salmonids in the period prior to smoltification. Despite this, it is argued by Backman (in press) that stocking with eggs should provide better imprinting to the stream compared to stocking fry and smolts. Also, monitoring of subsequent survival could not be accurately carried out if eggs or fry were stocked, since individual marking is currently impossible at these stages. Le Cren (1961) quoted in Mills (1971) reported natural survival rates of 94% from egg to fry stage but only a 2.7% survival during the fry stage. Therefore, stocking should possibly be carried out after the fry stage with marked (Pan-Jetted or tagged) parr or smolts. The fish should be reared in a hatchery using the same water as the natal stream in which they are going to be released. Thus problems with imprinting should not occur.

To minimise potential adverse interactions (unequal competitive advantage, predation between wild and hatchery fish) it is advisable that fish released be of a compatible size and age to those fish in the receiving stream or river. To an extent, size can be controlled in the hatchery using suitable temperatures and feeding regimes.

Timing of release from hatcheries into rivers is a major determinant of success (Backman, in press; Brannon, in press; Rasmussen, in press). In the Danish sea trout enhancement programme, fry are restocked during April/May; half-yearlings in September, 1+ fish in April/May and older fish/smolts in June/July (Rasmussen, in press). Many factors are taken into account, including fish size, degree of smoltification, water flows, depth and width of stream, water temperature, food availability and predator abundance. Different sized fish are not liberated in the same area. Fry, for example, should not be stocked where older fish are to be released. River depth and width are judged as important criteria for releases in Denmark, with fry being released in a minimum of 10cm of water, usually at a stream width of 1m; half-yearlings in 20cm depth, 1.5m width; 1+ fish in 30cm depth, 2.5m width and older fish in 60cm depth, 4m wide. Extensive habitat grading is carried out prior to release and each

potential area for release is graded from 0 - 5 on what is known as a "Biotroph scale" and depending on grade, suitable stocking densities are calculated for that area (Rasmussen, in press). Release of fish into such areas of adequate, although specific, water depth and stream/river width is important as hatchery reared fish, initially at any rate, will probably still behave like small tributary fish, rather than trout from a section of a large river (Brannon, in press).

If fish cannot be reared in the same river water as they will be released in, acclimation may be necessary to reduce stress in fish which, on release, could lead to mortality. Sudden changes in water quality parameters, in particular temperature should be avoided and handling should be kept to a minimum. Imprinting will also be a problem in that case.

It is vital that after the fish are released, the rehabilitation scheme should be monitored to determine survival of different life-history stages and the subsequent ratio of wild to hatchery fish, in particular spawners. This has rarely been done in past enhancement schemes. Genetic marking might also be considered to monitor survival of reared or hybrid progeny, though this introduces an element of selection (Cross, in press).

4.2.3 Practicalities of establishing facilities for cryopreservation of sea trout milt and specialised rearing stations to act as living gene banks

As shown from the literature review in section 3, it appears feasible to establish both cryopreservation facilities for sea trout milt and living gene banks where both sexes are held. This is further borne out by consideration of the Norwegian experience with the closely related Atlantic salmon (Appendix 1). The practicalities of the establishment and maintenance of such facilities are discussed below.

One biologist, preferably with relevant experience, should be appointed as full - time co-ordinator to establish and run preservation facilities. This individual would initially interact with senior management to identify target stocks, bearing in mind the various genetic and practical considerations discussed above. The co-ordinator would then, in consultation with field staff, produce a detailed plan for the preservation of each stock.

In the case of cryopreservation, advice and possibly practical assistance should be initially sought from specialists. Methods of fish collection and equipment preparation; of milt collection (whether by standard stripping, catheter or syringe); the holding method (whether as pellets or in straws); the method and rate of cooling and the storage method, are all aspects that must be optimised for sea trout. The variation in methodology used previously is obvious from Table 2 and makes the postulation of optimal methods without comparative trials inappropriate. In the case of long term storage, it is best to hold the material in a facility such as an agricultural gene bank, where staff are familiar with maintaining samples in liquid nitrogen. It is also sensible to have a back - up facility at another location, to guard against catastrophic loss. The approximate costs for this sort of operation are given in Appendix 3. These are derived from discussions with Dr. D. Gausen, NINA and Dr. K. Rana, Institute of Aquaculture, Stirling, both of whom operate gene banks, as described in Appendices 1 and 2.

The design of living gene banks for salmonid fishes does not appear to have been optimised. The most relevant model example for sea trout is perhaps the Norwegian Atlantic salmon preservation facility (Appendix 1) and discussion with the designer of this unit is suggested. Requirements for *S. trutta* include a facility where survival is optimised, but not at the expense of subsequent "normal" behaviour on release to the wild e.g. smoltification and migratory behaviour. To achieve this will require considerable experimentation on aspects such as holding facilities (tanks and/or cages), stocking densities and feeding regimes. Risks of contracting diseases within the facility must also be minimised by entry restrictions, sterilisation of equipment and separation of year classes and stocks. Obviously, the personnel employed in such a facility must be well trained and conscientious. It is also unlikely that

existing rearing stations can be adapted to this role without substantial modification, so new facilities may need to be built. One way of estimating the cost of constructing and running such a facility might be to add 50% to the cost of a standard trout enhancement rearing facility (to cover additional specialized requirements). Approximate costs for the Norwegian salmon living gene bank are given in Appendix 3.

4.2.4 General aspects of Genetic Conservation

It is important that genetically unique stocks be identified and conserved. To do this, a comprehensive examination should be undertaken of the genetic make-up of trout in all systems. To date, the most extensive study in Britain and Ireland was carried out by Fleming (1983), who looked at the population biology and genetic diversity of many anadromous trout stocks. Fleming (1983) identified two trout rivers in Northern Ireland where trout had unique rare alleles and it is argued that these populations should be conserved (Ferguson, pers. comm.). Ferguson (1989) also suggests that protection of Lough Melvin brown trout (where three sympatric populations occur) would result in the conservation of 43% of the variant alleles known to date. He further suggests that ancestral stocks (see section 2.1) should also be conserved because they are currently being lost by introgression with the modern form. Finally, sea trout from the Waterville system in south west Ireland, because of their unique growth characteristics and longevity, should also be conserved. This system frequently produces specimen-weight sea trout and the large size of these fish does not appear to be related to marine growth but to longevity (Ferguson, pers. comm.). There are probably many other examples of sea trout stocks with unique and "valuable" characteristics in Britain and Ireland and these need to be urgently identified.

One way of preserving a unique stock in a "natural" environment would be to choose a small stream, of little angling and genetic significance, but similar to the conditions found in the habitat of the target stock, remove by rotenoning the existing trout population and after a time restock with the target species (Ferguson, pers. comm.). This would confer the "natural" advantage and avoid any possible hatchery effects.

Since trout appear to exist in two forms, migratory and non-migratory, it should be possible to take fish with the appropriate migratory behaviour, e.g. trout which migrate to lakes and use these to rehabilitate a depleted population (Ferguson, pers. comm.). This is being done on an experimental basis in Ireland where fingerlings of sonaghan lake trout from Lough Melvin, are being transferred to a small coastal river in Connemara. Provided that directional behaviour and other biological and physical features are compatible, this choice of fish is ideal because:

1) all fish migrate to the lake in Melvin;
2) these fish have a unique gene marker (*GPI-2*135*), so they can be traced and subsequent performance can be followed up (Ferguson, pers. comm.).

Brown trout have been successfully introduced into at least twenty four countries outside Europe (Elliott, 1989), some of which have sea trout runs. When this is considered in combination with the extensive restocking (successful or otherwise) by government and angling bodies, it could be argued that trout from hatcheries could be added to rivers where stocks are low and that natural selection would operate to mould an "ideal" population. However, indiscriminate stocking would result in interbreeding between reared and wild trout, thereby possibly causing loss of co-adapted gene complexes (Ferguson, 1989). Taggart and Ferguson (1986), for example, have shown using electrophoresis that, after extensive restocking of the Erne - Macnean system in Northern Ireland, interbreeding did occur between the 'ancestral' race fish and the restocked fish.

4.2.5 Alternative Strategies for Rehabilitation

4.2.5.1 Androgenesis

Stoss (1983) suggested that androgenesis i.e. inseminating sterile (irradiated) eggs with viable sperm cells as a way round the inability to use cryopreserved eggs. These 'eggs', once inseminated are shocked to produce diploid organisms (see Figure 2) since haploids (N) have a very low survival rate. These diploids are either XX females or YY "supermales". Scheerer *et al.* (1986) produced androgenetic rainbow trout by fertilising ^{60}Co gamma-irradiated eggs with untreated sperm and then blocking the first cleavage division with hydrostatic pressure. However, survival of androgens is low and a high degree of homozygosity or inbreeding results using this technique (Prof. N. Wilkins, U.C.G., pers. comm.) and may therefore have limited application in gene banking. Occasionally, some fertility effects are also found with the homozygous females, including phenotypically expressing different (male) sex to the genotype (suggesting reduced developmental stability) and the sperm from resulting homozygous males are not as viable as sperm from normal males (Thorgaard and Cloud, in press). In theory, androgenesis would not allow the mitochondrial genome to be reconstituted. However, the mitochondrial genome is small and present in multiple copies in the cell and gamma - radiation does not appear to effect all copies (Dr. G. Thorgaard, W.S.U., pers. comm).

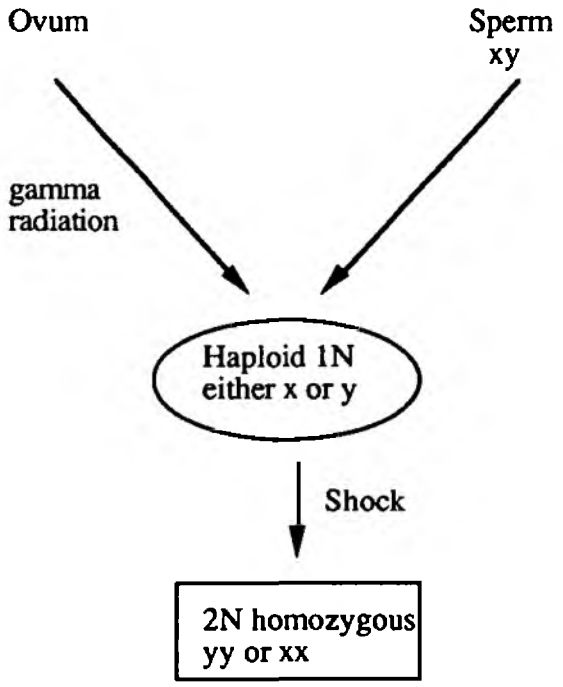

Figure 2 Diagramatic representation of androgenesis

The impact of genetically altered fish on the environment is an area where very little work has been done further limiting the use of androgens in a gene banking programme aimed at conservation as opposed to producing unique strains for research.

4.2.5.2 Gynogenesis and hormonal methods of producing all female progeny

Gynogenesis is a technique employed to produce all female progeny. This is done in several ways but the technique employed is the same for the different types, with differences in the timing of the treatment. Sperm are irradiated to inactivate the genetic material, while still allowing mobility and thus are capable of initiating egg division and embryonic development. The resulting haploid gynogen is heat or pressure shocked (as haploids have very low survival) to produce a diploid female. Depending on whether the shock is applied early or late to the ova the resultant gynogen is either a diploid meiotic gynogen or a mitotic gynogen (Cross, 1989). Meiotic gynogens are easy to produce but are not fully (90%) inbred (Wilkins, 1987; Cross, 1989). Mitotic gynogens, on the other hand are fully inbred clones but are difficult to produce. As with androgens, gynogens are only of limited use in gene banking, because of the high degree of inbreeding. However, the technique might be useful on a one-off basis, as part of a rehabilitation programme to maximize the number of females available and thus egg production. A second way of producing all female progeny is achieved by feeding synthetic male hormones to fry. This results in the production of stock in which all individuals are phenotypically male (see Figure 3, modified from Wilkins, 1987). When the phenotypically male, but chromosonally female individuals are crossed with normal females, the resulting offspring, in species where the female is the homogametic sex, will all be female. Wilkins (1987) suggests that gynogenetic lines derived from wild females could be sex-inverted and the resultant sperm stored. However, mtDNA, which is inherited in the maternal line, would not be present in sex reversed spermatozoa. Furthermore, there are technical difficulties in cryopreserving sex-reversed milt (Rana, pers. comm).

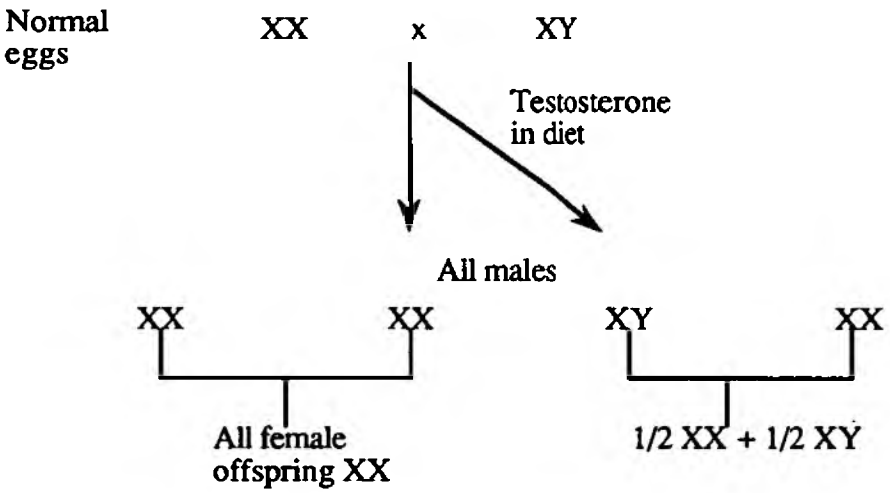

Figure 3 **Diagramatic representation of the production of an all female stock (modified from Wilkins, 1987)**

5 CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

1. The dramatic decline in sea trout (the anadromous form of *S. trutta*) escapement in the west of Ireland (specifically, reduced survival from smolt to returning adult) has prompted this investigation of methods of preserving and rehabilitating sea trout, should a similar problem occur in England and Wales. It is noted that rehabilitation should not be attempted unless the cause of the sea trout decline is identified and rectified. It is also stressed that a decline in catch may not mean a reduction in stock and that the latter should be quantified.

2. Consideration of sea trout conservation is complicated by the fact that in fresh water, the anadromous form coexists and may interbreed with conspecific resident brown trout. Direct observation, sex ratio information and evidence from genetic studies using protein electrophoresis imply a degree of interbreeding. The amount may be quantified in the near future by genetic techniques utilising hypervariable nuclear DNA analysis. Meanwhile, other non genetic methods of establishing the extent of interbreeding should be investigated, as should the ecology of freshwater resident brown trout which cohabit with sea trout. In the latter context, the rearing of these brown trout and their efficacy in re-establishing sea trout runs should be investigated.

3. Preservation of sea trout genetic material may be undertaken in two ways, cryopreservation or by the use of living gene banks. Cryopreservation of milt is technically feasible (the background, methodology and costs are described in detail). It should also be noted that a cryopreservation facility should not be regarded as a static entity, but rather as a unit to which new material can be added or material used for rehabilation as time progresses. However, trout ova or embryonic tissues cannot yet be successfully cryopreserved and this method may not be technically feasible in the near future. Living gene banks are specialised rearing stations where sea trout are held for a number of generations in either fresh water or fresh water/sea water systems. It is noted, that while successful rearing of anadromous sea trout has been achieved in Denmark, for example, it has not always been successful in Britain and Ireland. The reasons for the variable success require investigation. With living gene banks both sexes can be maintained but for a much shorter time due to fears of artifical selection and possible disease risks. Either method is costly and requires specialised personnel. Examples of the Norwegian gene bank for Atlantic salmon and the University of Stirling efforts in relation to cultured fish species in general, where both cryopreservation and rearing stations are used, are described (see Appendix 1 and 2).

4. Whichever method of preservation is employed, it is important to sample an adequate number of wild parents from a single population (see 5 below), so as to avoid inbreeding (a minimum of 50 females and an equal number of males taken at random should be used). It is also important to sample all the more common year classes and to take individuals from throughout the upstream run or spawning period. The relationship with cohabiting brown trout must also be considered. Detailed breeding records should be kept and the progress of preservation exercises monitored genetically using protein electrophoresis or DNA analysis (mitochondrial (mt) DNA, because of its mode of inheritence, is particularily sensitive in detecting inbreeding).

5. Of vital importance in designing preservation and rehabilitation methods for sea trout is consideration of population structure. Protein electrophoresis studies have shown that sea trout populations of each river system or even tributaries within a system are genetically distinct. Populations are partially or entirely reproductively isolated; the population structure being maintained by accurate homing to natal spawning areas. It should be emphasised that this evidence does not prove local adaptation, though there is additional evidence from ecological studies that this type of adaptation occurs. Ideally, native sea trout should be used when seeking to preserve a population (as determined by genetic investigation). In areas where sea

trout have declined drastically or have disappeared, this will not be possible. Here a decision will have to be made as to whether to choose the genetically and ecologically most similar non - native sea trout population or to utilise native co-habiting brown trout (see 2 above). Choosing a donor sea trout population is complicated because genetic difference is not positively related to geographic distance, while use of native co-habiting brown trout is also problematic since the degree of interbreeding with sea trout is unknown.

6. It will usually be impossible, because of economic considerations, to preserve all of the sea trout populations in an area. Thus, a choice will have to be made as to which populations are considered most "valuable". Whatever populations are chosen, it is recommended that they be preserved using both available techniques (cryostorage and living gene bank) since the former technique will provide milt only.

7. For rehabilitation using preserved material to be successful, several criteria must be considered. Two situations can be envisaged, where sea trout remain and where they become extinct. In the former case preserved material from native stock can be used to speed recovery, possibly by a mixture of conservation, ranching and judicous use of sea cages. The latter case will be considered below.

8. All rehabilitation efforts will contain some element of rearing and care must be taken to ensure that this rearing does not reduce the propensity for sea trout smolts to go to sea. The use of such manipulative techniques as androgenesis or gynogenesis has been considered in detail, but is not recommended, since at present, survival from these techniques is low and result in inbred fish, which are considered inappropriate for rehabilation. It should be stressed, that the optimal method of re-establishing or enhancing sea trout runs by stocking has not yet been established in Britain. This area needs urgent investigation, if rehabilitation is to be successful.

9. If there is a shortage of sea trout females for rehabilation in a particular situation, native co-habitating brown trout females might be used (but see point 2 above). Repeated backcrossing using cryopreserved sea trout milt would reduce the brown trout genetic input from 50% by half in each subsequent generation, but inbreeding would be a problem with such a scheme unless milt from a large number of males had been preserved in the first instance.

10. In rehabilitating a system where sea trout originally existed, but where insufficient numbers were available for preservation, the choice of donor stock(s) is difficult. If the conservative approach of assuming a high degree of local adaptation is taken, then restocking with the progeny of native co-habitating brown trout would be suggested (assuming future experimental work established that sea trout and co-existing brown trout freely interbreed). Alternative strategies would be:
(i) to choose the "ideal" donor stock based on genetic and ecological considerations;
(ii) to take the experimental approach of introducing a number of stocks marked individually and see which performs best;
(iii) to introduce a single highly variable mixture of populations and to allow natural selection to eliminate less fit genotypes.
Approaches (i) and (ii) have been used successfully with other salmonids, whereas approach (iii) has been suggested on theoretical grounds.

11. In conclusion, it should be stated that, as shown in this report, preservation of sea trout genetic material is feasible using cryopreservation and living gene banks. However, attempts at rehabilation may be hampered by lack of knowledge of sea trout genetics, ecology, culture and restocking. Since preservation by cryostorage is possible without utilising any rearing, then this might be the more appropriate method to use initially.

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APPENDIX 1: THE NORWEGIAN ATLANTIC SALMON GENE BANK

Many of the practicalities of setting up and running a salmonid gene bank may be gleaned from the Norwegian experience. Norway was visited by one of us (E.R.) in June 1991 as part of compiling this report. What follows is derived from detailed discussion with Dr. Dagfinn Gausen, chairman of the Nordic Committee for Gene Banks and from direct observation. The gene bank operated by NINA is the only salmon gene bank operated on a national level, although Iceland is also in the process of developing a national gene bank for salmon (Dr. D. Gausen, NINA, pers. comm). In 1986, on the recommendation of the Advisory Committee of the Sea Ranching Programme, NINA began a sperm bank programme, as a result of concern in Norway about the extinction of local, indigenous salmon stocks and the erosion of genetic resources. Threats to the Norwegian wild salmon include overfishing, acid rain, escaped farm salmon and the parasite *Gyrodactylus salaris*. The latter threats are particularily important in the Norwegian situation.

Escaped farm salmon:

Based on morphology and scale analysis the proportion of reared fish, in commercial fisheries in Norwegian homewaters, has increased from about 10% in 1986 to about 30% in 1989 (Egidius *et al.*, 1991), mostly from escapes from farms. If not caught in the fisheries, the adult salmon enter rivers to spawn (Hansen *et al.*, 1987) and, since most salmon populations in Norway are small (more than half the rivers have fewer than 100 spawning fish annually, Ståhl and Hinder, 1988, quoted in Gausen and Moen, 1991), these populations are highly vulnerable to genetic deterioration (Gausen and Moen, 1991). Using data from rod and net catches, Gausen and Moen (*loc. cit*) found farmed salmon in 70% (38) of the rivers sampled, accounting for 15% of the total salmon caught in southern Norway and 1% in northern Norway (reflecting location and volume of salmon farms). These authors consider that the "potential for large-scale introgression of exogenous genetic material into native populations exists and that escaped farmed salmon may carry diseases like furunculosis into rivers where it has not been previously reported".

Gyrodactylus salaris :

The monogean trematode *Gyrodactylus salaris*, has been spread to 32 rivers through fish being stocked from infected hatcheries (Egidius *et al.*, 1991). This parasite is thought to have been originally introduced into Norwegian rivers by restocking with Atlantic salmon brought in from the Baltic. Baltic *S. salar* appear to be more resistent to *Gyrodactylus* than Atlantic *S. salar* (Bakke *et al.*, 1990). This parasite attacks salmon parr causing heavy mortalities and has recently caused a yearly loss in production of between 250 - 350 tonnes. This situation is futher exacerbated as a result of acidification in southern Norway where approximately 25 stocks of Atlantic salmon have been lost and 15 more are severely reduced (NASCO, 1989; Hesthagen & Hansen, 1991).

These impacts are expected to have further detrimental effects before they can be controlled so, it was against this background that NINA established a gene bank for salmon, initially with emphasis on sperm banking but more recently expanding to incorporate a living gene bank. Rivers to be sampled are selected by an Advisory committee with additional advice from fisheries officers from the counties. Priority is given to threatened stocks. In the absence of genetic information about a river, each river is treated as a separate stock and major tributaries are sampled separately. Sampling usually takes place over a 2-year period thereby ensuring representatives from different year classes. A minimum of 50 individuals is the target for the cryopreservation of each stock. This is based on a sampling strategy proposed by Allendorf and Phelps (1981) (Gausen, in press).

All sampling for the Norwegian gene bank takes place at Sport Fishing Association hatcheries of which there are 128. Rivers are electrofished and salmon approaching maturity are held at these hatcheries or in large riverside circular covered tanks until mature. The fish are anaesthetised and stripped and the milt is collected in a dry beaker (because salmonid sperm become motile upon contact with water). All equipment in contact with the sperm is kept at 4°C. On collection of the milt, about 1.5ml is taken from each fish and mixed with 3.4ml DMSO/glucose freezing medium. It is estimated that 1.5ml frozen sperm is capable of fertilising 1000 eggs (Gausen, pers. comm.). Drops of the extended semen are then frozen in previously drilled shallow holes in a frozen CO_2 (dry ice) block. The pellets are left in place for about 3 minutes and are placed in two labelled polypropylene vials and sealed prior to transfer to liquid nitrogen. Approximately 120 pellets are divided between two tubes from each fish. On return from the field, samples are transferred to the permanent storage facilities housed at the Breeding Station for Norwegian cattle just outside Trondheim. The sperm bank comprises a 530l liquid Nitrogen tank capable of holding samples from 3000 fish. To date, sperm from 2798 fish have been frozen (Gausen, in press). The post-thaw fertility of salmon sperm cryopreserved in this manner is estimated at 60% when compared with fresh milt.

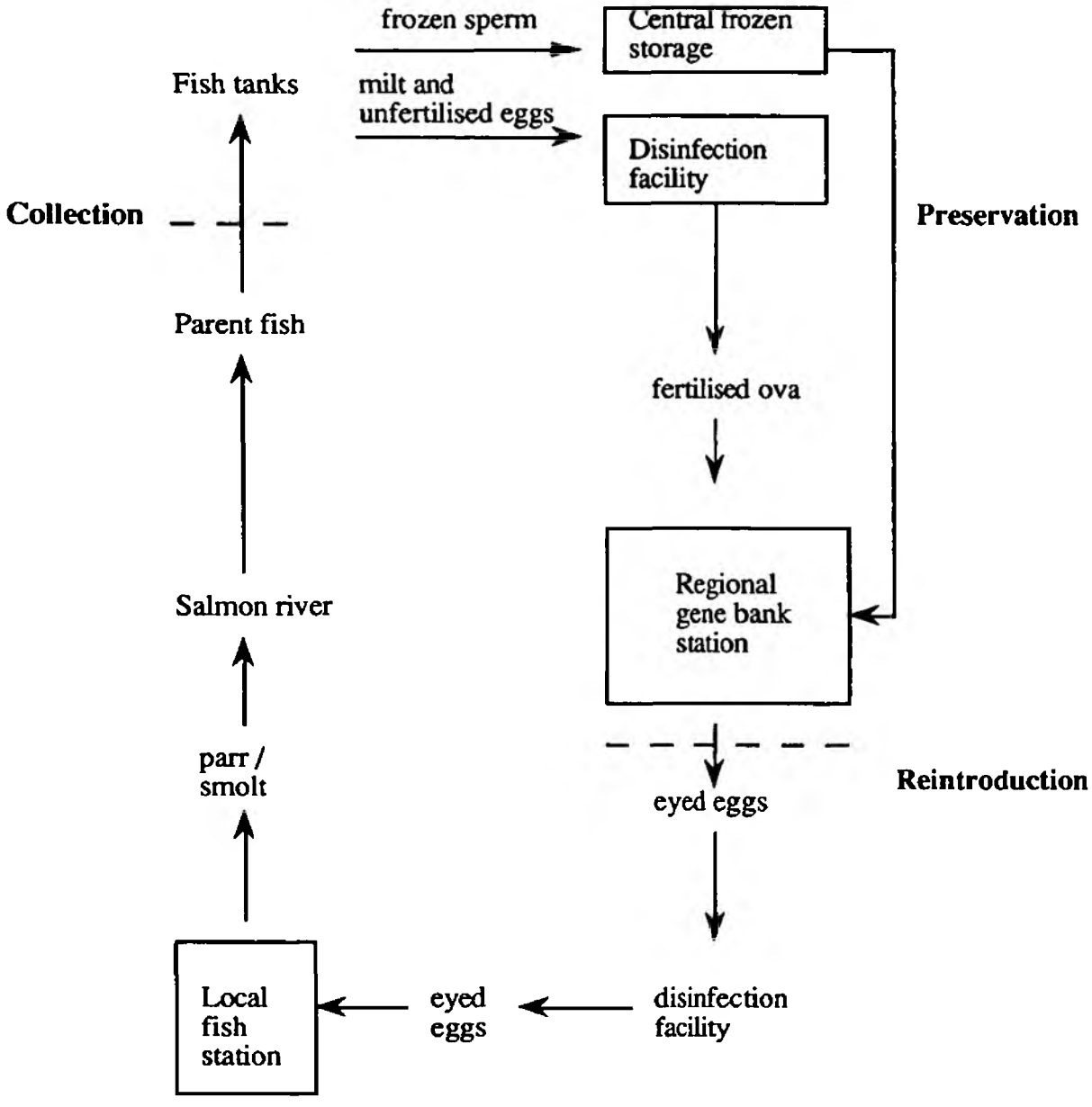
The donor fish are sacrificed and tissues preserved for enzyme electrophoresis. Great care is taken not to select farmed fish for the gene bank and scale samples from each fish are also taken. The scales are examined and plastic impressions made which are maintained with the above information as a record for each fish.

In conjunction with the large scale collection of cryopreserved sperm NINA has also established a "living" gene bank, located in the middle region of Norway which has the capacity to maintain 20 distinct riverine or tributary stocks. A second station, to be located in southern Norway is being built (Gausen, pers. comm.). These "living" banks are planned to operate for 10 years or approximately two salmon generations, because of worries of domestication. The present station contains fish from 14 stocks.

As with the collection of sperm for cryopreservation, a large emphasis in the "living" gene bank is placed on hygiene and preventing disease. Fish are caught and kept locally in holding tanks until mature. Fish are then stripped and fresh milt and ova are transported in plastic bags on ice to a station where disinfection takes place. The eggs are disinfected after fertilisation with a buffered iodine-based disinfectant and then transported to the regional station where fertilised eggs (progeny of one male X one female) are kept in separate hatching cylinders until such time as the disease status of the broodstock is determined. Disease certification is carried out by a local veterinary surgeon. Eggs are removed if either of the parents have transmissible bacterial or viral diseases. Otherwise, family groups are kept in separate tanks until the fish can be marked. This requires an elaborate hatchery facility with each cylinder/tank having its own separate water supply. After marking, each stock is kept in separate tanks throughout its life-cycle. The station is divided into closed sections based on various stages of the life-cycle, particularly between year classes, with no inter-connections and access to these sections by staff operators is again only after rigorous hygiene controls, e.g., separate footbaths, new overalls and boots for each section. As a precaution against the spread of furunculosis, which can be easily acquired in the marine environment, the fish are going to be reared in fresh water for their entire life-cycle (Gausen, in press). At present fish from 14 distinct stocks are at various stages of being reared, the oldest being smolts. To date, no problems associated with rearing have been recorded.

To date, sperm from 90 stocks have been cryopreserved. A large proportion of the cost involves transportation to and from the sites on the long Norwegian coastline (Gausen, pers. comm.). A schematic representation of the Norwegian gene bank system is given in figure 4 (from Gausen, in press).

Figure 4 **Schematic representation of the Norwegian Atlantic salmon gene bank strategy (modified from Gausen, in press)**



APPENDIX 2: INSTITUTE OF AQUACULTURE, STIRLING UNIVERSITY

The Institute of Aquaculture, University of Stirling, Scotland are also involved in gene banking, but on a smaller scale than the Norwegian system. This work involves cryopreserving sperm of Tilapia spp. and a number of salmonid species, namely *Salmo salar*, *S. trutta* and *Oncorhynchus mykiss* for the aquaculture industry and owners of private fisheries. Research work includes developing techniques for cryobiology, e.g., egg/embryo supercooling, androgenesis, cryofreezing of sex-reversed milt. Protocols for cryopreserving sperm of 13 species of Tilapia have been developed, both for commercial exploitation and/or conservation. A small living gene bank for Tilapia spp. is also maintained. Unlike the Norwegian system, which uses pellets as a means of cryopreserving sperm, Stirling personnel, along with many other workers use the straw method. Depending on the amount of sperm required to be frozen, different size straws are used; 5ml straws for salmon, 0.5ml straws for brown trout and 0.25 or 0.5ml straws for Tilapia spp.

Fertilization rates of 94% have been recorded using 5ml straws of extended milt from *S. salar* and each straw fertilises between 3000 - 4000 eggs (Dr. K. Rana, Inst. of Aquaculture, Stirling, pers. comm.). On collection of milt, data as to the method, location, date of collection are catalogued using a cross-referencing system. Samples are checked for motility before the extender is added, after extender is added and also before freezing. In the case of farmed salmon, sperm are stored but not frozen for a period of up to one month to allow disease screening for notifiable diseases, e.g., IPN, to be carried out. Up to three straws are used for motility testing just after freezing to -196°C. Cooling is achieved using a programmable cooling chamber, capable of cooling rates from -0.01°C/min to -200°C/min before transfer to liquid nitrogen (Rana, 1988). With brown trout, a 0.5ml straw fertilises between 200 - 300 eggs. Work with sex-reversed rainbow trout has been less successful with only 70 eggs being fertilised using a 0.5ml straw. However, little work has been done in this technically difficult area. For the Tilapia spp., 400 - 800 eggs can be fertilised using a 0.5ml straw, depending on which of 13 species is being used.

APPENDIX 3: COSTS OF ESTABLISHING A GENE BANK

The cost of establishing a gene bank will vary according to the sampling costs, storage system required and the techniques adopted. Obviously, costs will vary from country to country. Some indication of costs (in STG £s) may be obtained from examining the costings calculated by D. Gausen, NINA in Norway and K. Rana, Institute of Aquaculture, Stirling for their respective operations.

Norwegian Atlantic salmon milt cryostorage:

Portable field equipment, for example, dry ice, liquid nitrogen, Dewar flasks and disinfectants, are estimated to cost a maximum of £1000. Sample collection is one of the main costs of the cryopreservation programme in Norway (Gausen, pers. comm.). The Directorate has three field crews which operate in different parts of Norway and the estimated cost of 800 NOK (£72) per sperm sample is based on the whole cryopreservation process, including cost of renting vans, administration of programme, labour, equipment and disposable supplies. Disease analysis costs, which are estimated by Stirling personnel to be £160 per fish, are not included. Thus total cost is likely to exceed £230 per fish. One cryopreserved sperm sample is sufficient to fertilise only 1000 eggs. The cost of cryopreservation of sperm from 50 fish (one "population") would therefore be 40,000 NOK (£3,600), without disease analysis. The cost of maintaining samples in liquid nitrogen at the cattle breeding station near Trondheim is 30,000 NOK/year (£2,700). This price includes maintainance of the entire tank with the capacity to hold samples from approximately 3000 fish (60 populations) and is included in the unit cost of £72 per sample.

Institute of Aquaculture, Stirling salmonid milt cryostorage:

K. Rana estimated the costs of cryopreserving Atlantic salmon sperm samples with the two different sized straws used at the Institute of Aquaculture, Stirling. The descision to use 0.5ml or 5ml straws is based on the ultimate use of the frozen milt, i.e. if the milt was being used on a once off basis, where many eggs were being fertilised, then a 5ml straw would be used, whereas, if smaller amounts of milt were required to fertilise different batches of eggs over a longer time scale, smaller straws could be used. For a sample preserved using a 0.5ml straw, the cost of collection and storage for one year is £30, while using a 5ml straw the cost is £45. The figures are calculated on a minimum number basis, i.e. one thousand five hundred 0.5ml straws (thirty 0.5ml straws/fish for fifty individuals) or five hundred 5ml straws (ten 5ml straws/fish for 50 individuals) being frozen (Rana, pers. comm.). The above costs include viral disease screening (cost of which is estimated as £160/fish), disposable sampling equipment, storage costs for one year, collection, chemicals and labour. So under this system the cost/fish is £900 or £450 depending on straw size but the number of eggs that can be fertilised is 6,000 or 25,000, respectively, which is considerably greater than the 1,000 eggs fertilisible under the Norwegian system. Use of a smaller number of straws/fish might reduce these costs but this option would need to be discussed directly with Stirling personnel. Straws are then held in Dewar flasks which can hold approximately two thousand 0.5ml straws or five hundred 5ml straws at 75 - 80% capacity. Maintainance costs for the period following the first year are approximately £1600/container per annum.

The capital cost of setting up the cryo-laboratory at Stirling was £0.25 million. However, this laboratory is used for research into other areas of biotechnology and cryobiology and the cost for setting up a laboratory solely designed for sperm cryopreservation may be closer to £100,000 (Rana, pers. comm.). This price includes equipment such as a programmable cooler (£11,000), a liquid nitrogen back-up facility and Dewar flasks .

The Norwegian living gene bank:

The total cost of an individual living gene bank station in full operation in Norway has been estimated to be about 3 million NOK (£270,000)/year or 150,000 NOK (£13,500)/stock. This cost has been calculated from renting the facility at a cost of 1.3m NOK (£117,000)/year and on annual operating costs of 1.7m NOK (£153,000) (Gausen, pers. comm.). Here a stock consists of at least 100 fish (50 males and 50 females) which are used as broodstock for the first generation of the progeny.

APPENDIX 4: LIST OF RELEVANT CONTACTS/ORGANISATIONS

Address	Area of expertise	Contact person
Columbia River Inter-Tribal Fish Commission, 975 SE Sandy Blvd., Portland, OR 97214, USA.	Salmonid enhancement/ restocking	T. Backman
School of Pure and Applied Biology, University of Wales, College of Cardiff, P.O. Box 15, Cardiff CF1 3TL, Wales.	Trout population genetics	D. Bembo
School of Biology and Biochemistry, Queen's University, David Keir Building, Stranmillis Road, Belfast, BT9 5AG, Northern Ireland.	Trout population genetics	A. Ferguson
Directorate for Nature Management, Tungasletta, 2, N - 7004 Trondheim, Norway.	Cryopreservation and salmonid gene bank.	D. Gausen
MTL BioTech, P.O. Box 5760, Station B, Victoria, B.C., Canada, V8R 6S8.	Cryopreservation	B. Harvey
Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotalnd.	Cryopreservation	K. Rana
DITMAR, Inland Fisheries Lab., Lysbrogade 52, DK8600 Silkeborg Denmark.	Salmonid restocking/ enhancement	G. Rasmussen
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