1 Introduction

The possibility to store fish ova for extended periods of time would provide new perspectives for controlled reproduction in farmed fish, wild fish stock management and conservation of rare and endangered species. The availability of suitable cryopreservation techniques would permit indefinite storage and thus establishment of gene banks which, in turn, offer opportunities for conserving unique and/or desirable traits, particularly in threatened fish populations. Several salmonid species, particularly certain strains of trout and salmon, are rapidly gaining in importance as high-value "domesticated" fish in aquaculture. Hence, they are extensively being investigated for various aspects of their culture and reproductive biology. With regard to reproductive functions the preservation of gametes and embryos is extremely important. Cryopreservation is widely applied to store sperm in a number of fish species. However, methods for the cryopreservation of yolk-laden fish eggs and embryos remain elusive so far, mainly due to relatively large size, high water content, large yolk mass and the presence of enveloping membranes with different permeability properties. Chilling fertilized eggs supplying oxygen and removing wastes by a water recirculation system can prolong availability of eggs for hatching or sale. However, this system is not practical for maintaining the viability of eggs undergoing an extended period of transportation. Chilled storage of unfertilized eggs has been moderately successful in salmonids, with storage times of up to a few weeks, achievable through refrigeration of oxygenated eggs in coelomic fluid supplemented with antibiotics (Stoss, 1983; Jensen and Alderdice, 1984; Babiak and Dabrowski, 2003). However, to date no satisfactory technique has been developed for chilled storage of unfertilized rainbow trout eggs. The objective of the first part of this thesis was to explore optimal conditions for the establishment of a practicable technique for storing and shipping unfertilized rainbow trout eggs in a chilled state.

Fish possess a wide range of sex determination mechanisms, but commonly it is through primary genetic sex determination modulated by environmental factors. In rainbow trout sex is known to be determined by genetic system. However, until recently, no unequivocally sex-specific molecular marker was available to distinguish between male (XY) and female (XX) rainbow trout. In rainbow trout, males mature one year earlier than females. In males the onset of sexual maturation results in a reduction of meat quality, decrease in efficiency of feed conversion, and increase in disease susceptibility. This has effectuated the production of all-female fattening stock. The phenotypic identification of sex is only possible in adult fish during the spawning season. The availability of sex-linked marker would be very useful for identifying genetic sex long before fish reach sexual maturity. According to Ittura et al. (2001a) the *OmyP9* sequence (Sequence Characterized Amplified DNA Region, SCAR DNA) derived from a RAPD (Random Amplified Polymorphic DNA) marker is associated with the sex chromosomes in rainbow trout. Second part of this thesis encompasses an investigation designed to study the suitability of the *OmyP9* marker for identifying the sex in individual fish using DNA extracted from various tissues, such as blood, a fin clip, scales or slime from the skin surface. Samples from seven defined strain of rainbow trout were analyzed.

2 Chilled storage of unfertilized rainbow trout eggs

2.1 Literature Review

2.1.1 General information about rainbow trout

The rainbow trout belongs to the family of the *Salmonidae*, subfamily *Salmoninae* and represents the species *mykiss* in the genus *Oncorhynchus* (Smith and Stearley, 1989). The rainbow trout (*Oncorhynchus mykiss*) is a native species of the Pacific coast drainage of North America from Alaska to Baja California. Rainbow trout is a freshwater fish. The anadromous form of rainbow trout that hatches and spawns in streams but travels to the ocean - or large lake surrogates - to grow and live the majority of their life is called steelhead trout.

In the early 1880s the rainbow trout was transferred from North America to Europe where it became one of the preferred fish in aquaculture because it is relatively easy to rear. Under the natural conditions of the European rivers, rainbow trout can not reproduce. Yet, during summer, the eggs develop normally in the female. But in autumn when the spawning season arrives, the female can not spontaneously deliver them into the river and they remain inside its belly. Reproduction of rainbow trout has been strongly influenced by more than 100 years of rearing. Wild rainbow trout spawn in the spring. There are still some strains spawning at that time, but on most fish farms the rainbow trout spawn in winter or fall. In fact, there is a large variability in spawning time. Even trout of selected strains do not all mature at the same time and during the spawning season brood females must be sorted frequently (Billard, 1992).

Rainbow trout females are highly fecund, producing a single batch of 2000-30000 eggs per kilogram of body weight each year (Tyler et al., 1996). Synchronous growth of oocytes, all of which are ovulated at the same time, occurs in two ovaries (Tyler et al., 1996). Mature rainbow trout oocytes are expelled at ovulation into the coelomic cavity where they remain immersed in a semi-viscous fluid known as “ovarian” or “coelomic” fluid until spawning is

triggered by environmental and social stimuli. Under farming conditions, these stimuli are absent. Therefore, oocytes remain in the body cavity until manually stripped by man. If not collected, eggs degenerate and are progressively resorbed (Aegerter and Jalabert, 2004).

2.1.2 Eggs of rainbow trout

Ovulated eggs of rainbow trout are bounded by a series of membranes. The outer membrane, the chorion, is relatively thick, porous and elastic. It possesses a small funnel-shaped micropyle through which sperm enter to fertilize the egg (Leitritz and Lewis, 1976, Fig. 1 A). The yolk membrane, also termed vitelline membrane, which surrounds the yolk and holds it together, is a protoplasmic layer. This membrane is very thin and not porous. The rupture of this delicate membrane turns an egg white which causes most of the losses in hatcheries. Mechanical shock can rupture the membranes very easily. Fungus probably makes a chemical attack upon the membrane. Chemical injury to the yolk membrane could also occur on account of certain pollutants (Leitritz and Lewis, 1976).

In a trout egg there is a space between yolk membrane and chorion which is filled with perivitelline fluid. When an egg is freshly stripped from the female, there is no water in this space (Leitritz and Lewis, 1976, Fig. 1 A). Egg diameter is variable (3.5 to 5.5 mm) and depends on the age and size of the female and the feeding regime (Billard, 1992). The chemical composition of the eggs is also variable. Yazbeck-Chemayel (1974) found an energy value of 2.5 calories/g of egg and a composition (% of fresh weight) of proteins: 27%, lipids: 10%, ash: 0.7%, dry weight: 40%. Polysialoproteins, found in unfertilized rainbow trout eggs, become depolymerized after fertilization (Inoue and Inoue, 1986). After fertilization of the egg the outer shell, also called the vitelline envelope, transforms into the fertilization envelope. The vitelline and fertilization envelopes are able to protect the egg itself and the embryo, respectively, from bacterial infection in the internal or external environment. Extracts

obtained from the vitelline envelope and the fertilization envelope of rainbow trout eggs have the ability to exert a bactericidal effect on Gram-positive and Gram–negative bacteria, involving complete degradation of cell wall and plasma membrane of the bacteria (Kudo, 2000). The effect may be due to the presence of phospholipase D, lysozyme, proteinase and DNases as the extracts contain this enzyme activity (Kudo, 2000).

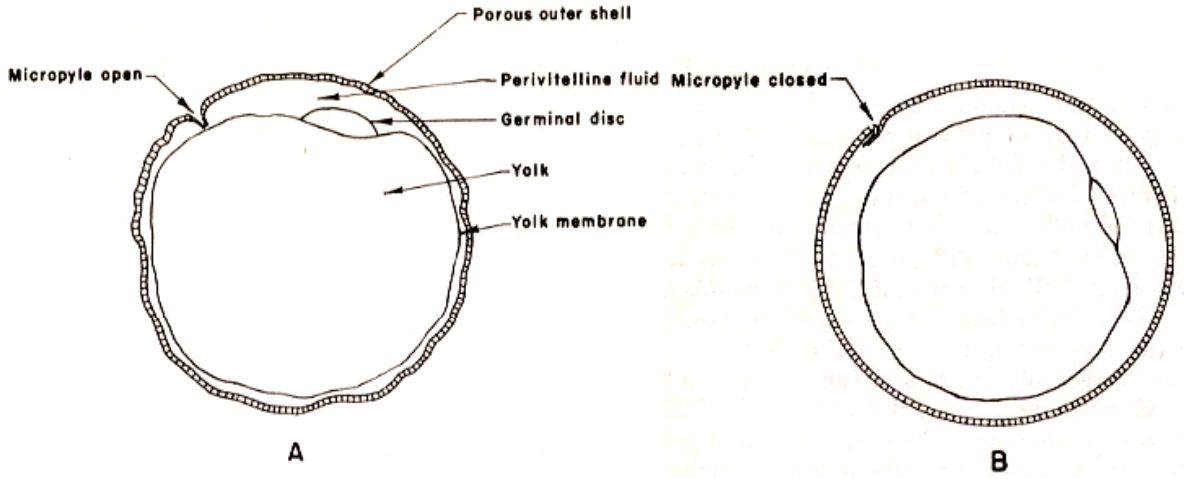

Fig. 1 A) newly taken steelhead trout egg, outer shell is not firm, egg feels soft and slightly adhesive; B) water-hardened trout egg, outer shell is drawn tight, mycropyle is closed, egg is firm and slick (Leitritz and Lewis, 1976).

The oxygen consumption of unfertilized and non-activated rainbow trout eggs is 1.29 ng/min per egg and does not change much after activation, fertilization and during the first hours of development (Czihak et al., 1979). The rate of respiration of eggs is increased during further developing of the embryo (Terner, 1968a, Table 1). After hatching, the increase continues at a slow rate, followed by a rapid rise after day 18 after hatching. Activated and fertilized eggs of trout are normally developing in oxygen rich water and show an increase in isocitric dehydrogenase activity while pyruvic acid kinase activity is higher in eggs from the coelomic cavity where oxygen concentration is lower (Czihak et al., 1979). Under oxygen deficiency the development of fertilized trout eggs slowed (Garside, 1966). The accumulated

oxygen deficit is matched after the addition of aerated tap water, by an increased uptake (Czihak et al., 1979).

Table 1. Endogenous respiration of rainbow trout eggs, embryos and larvae (Terner, 1968a).

Stage of development	O_2 uptake (µL/h per ovum or larva)
Unfertilized eggs	0.2 – 0.5
Embryos, 20-21 days[a]	2.5 – 2.7
Embryos, 28-32 days[a]	4.0 – 5.0
Larvae, 4th day[b]	11.7
Larvae, 11th day[b]	12.7
Larvae, 18th day[b]	19.0
Larvae, 25th day[b]	54.0

[a] Days after fertilization
[b] Day after hatching

Because of the absence of an exogenous supply of nutrients during the development in water, the eggs and embryos of invertebrates and lower vertebrates (e.g., trout eggs kept in running well water) support their growth by the endogenous reserves. The uptake of external material may be of minor, if any, nutritional importance (Terner, 1968a). Trout ova carry a store of free glucose. Glycogen is identified as a main storage form of free glucose (Terner, 1968b). Whereas unfertilized eggs contain only small amounts of free glucose, its amount increased at least tenfold by the time the embryo reaches the eyed stage (Terner, 1968a). Terner (1968b) observed that during incubation of cell-free extracts of trout embryos the level of free glucose increased following the rapid breakdown of ATP. When the ATP level is maintained, glucose declined with the appearance of lactate. The ATP level in embryos appears to control the maintenance of a pool of free glucose, too small to support the endogenous respiration, but adequate for brief outbursts of activity (Terner, 1968b). Calculations by Terner (1968b) showed that utilization of the total carbohydrate reserve would support the endogenous respiration for only a fraction of the time needed for development. The ability of the ovum to generate free glucose and to retain it appears to be

important for maintaining a source of metabolic energy which is readily available when sudden demands arise. This is exemplified by the sudden drop in glucose level during the vigorous lashing activity on hatching of the larvae (Terner, 1968a). The rapid increase in activity of gluconeogenic enzymes in trout eggs from unfertilized egg to the eyed stage indicates the increasing importance of gluconeogenesis with progressive development. The yolk glycerolipids appear to be the principal endogenous reserve conserving carbohydrate by preferential utilization of fatty acids and replacing it by conversion of glycerol to glucose (Terner, 1968b).

2.1.3 Ovarian fluid

Mature fish oocytes that have been released out of the follicles discharge into the ovarian cavity (Nagahama, 1983). While in most fish species the ovarian cavity is in continuity with the oviduct, in the *Salmonidae* the ovaries have no oviduct and mature oocytes must pass the body cavity to reach the genital papilla (Nagahama, 1983). The ovarian fluid in which the eggs are held until spawning could be of either ovarian or coelomic origin. The ovarian cavity containing the mature oocytes consists of wide fluid-filled spaces (Van den Hurk and Peute, 1979) and the cells lining the ovarian cavity were found to be secretory active in the medaka (*Oryzias latipes)* (Yamamoto, 1963). This suggest that ovarian fluid is a specific secretory product of the ovaries released together with the eggs (Lahnsteiner et al., 1995a). In contrast to this, the cells of the coelomic epithelium are small and cuboidal, do not have secretive function (Jew et al., 1971) and substantial amounts of ovarian fluid were never observed in the coelomic cavity (Lahnsteiner et al., 1995a).

The composition of the ovarian fluid of the *Salmonidae* is predestined for storage of eggs and for prolonging the fertilization period during natural spawning as well as during artificial fertilization conditions (Lahnsteiner et al., 1995a). The inorganic and organic composition of

ovarian fluid of salmonids is similar qualitatively but significantly different quantitatively among species with the exception of the pH (Lahnsteiner et al., 1995a). Fluctuations in organic components of the ovarian fluid generally are higher than in inorganic ones and may indicate dynamics in metabolism.

Rainbow trout ovarian fluid has a pH of 8.4 and contains the inorganic and organic compounds indicated in Table 2. The concentrations of cations and anions in coelomic fluid of rainbow trout are close to those in normal vertebrate blood serum (Czihak et al., 1979). Because of the low potassium levels and the alkaline pH, spermatozoa are activated when immersed in ovarian fluid (Billard et al., 1974; Holtz et al., 1977). As ovarian fluid is also iso-osmolar to the sperm cells the motility is prolonged in comparisons to fresh water (Billard et al., 1974). Fertilization is highest in alkaline media and is strongly decreased by acidification (Billard et al., 1974). The alkalinity of the ovarian fluid may be important to stabilize fertilization under natural conditions and especially in acidic water (Lahnsteiner et al., 1995a). Unfertilized and fertilized trout eggs are said to have a capacity of regulating the pH of the surrounding medium, probably by releasing buffering ions (Czihak et al., 1979).

The organic composition of the ovarian fluid of salmonids qualitatively and quantitatively differs from that of seminal fluid (Scott and Bayness, 1980; Lahnsteiner et al., 1993). In comparison to the seminal fluid the ovarian fluid has higher levels of protein, free amino acids, glucose, lactate, phospholipids and cholesterol, but triglycerides are absent. The composition of the ovarian fluid exerts a positive influence on sperm motility (Yoshida and Nomura, 1972). The proteins that are secreted into the ovarian fluid in large quantities by the trout ovary during and after ovulation, also called trout ovulatory proteins (TOPs). They may protect eggs from invading microbes. The TOPs inhibited the growth of Gram negative bacteria (Coffman et al., 2000). This group of ovulatory proteins acts as protease inhibitors and is responsible for maintaining oocytes in a nonactivated state after ovulation (Coffman

and Goetz, 1998). Ovarian fluid may also help eggs to fully mature prior to spawning. According to Springate et al. (1984) in rainbow trout fertilization rates increase from 88% immediately following ovulation to 100% after the eggs have been immersed in ovarian fluid in the coelomic cavity for 4-6 days.

Table 2. Composition of the ovarian fluid of rainbow trout (n=12) (Lahnteiner et al., 1995a).

Variable	Mean (SD)
pH	8.4 (0.1)
Osmolality (mOsm/kg)	291.6 (12.9)
Na^+ (mMl/L)	134.7 (7.4)
K^+ (mM/L)	2.7 (0.2)
Ca^{2+} (mM/L)	0.45 (0.04)
Mg^{2+} (mM/L)[a]	0.56
Cl- (mM/L)[a]	131
Glucose (µM/L)	1798 (505)
Fructose (µM/L)	53 (35)
Lactate (µM/L)	34 (15)
Triglycerides (µM/L)	0
Cholesterol (µM/L)	970 (780)
Phosphatidylcholine (µM/L)	0.25 (0.35)
Lysophosphatidylcholine (µM/L)	10 (100)
Choline (µM/L)	0
Alkaline phosphatase (µM/L/h)	1900 (900)
Acid phosphatase (µM/L/h)	100 (40)
Collagenase (µM tyrosin/L/h)	160 (105)
Gelatinase (µM tyrosin/L/h)	25 (15)
β-D-Glucuronidase (µM/L/h)	154 (53)
Lactate dehydrogenase (µM/L/h)	690 (190)
α- Glucosidases (µM/L/h)	0
Glucose-6-phosphate dehydrogenase (µM/L/h)	0
Protein (mg/100mL)	117.3 (20.4)
Fertilization rate	74.4 (5.1)

[a] Czihak et al., 1979.

No glycogen, which is the principal storage form of glucose, is present in the ovarian fluid as glucose levels are similar before and after incubation with amyloglucosidase (Lahnsteiner et al., 1995a). The absence of glucose-6-phosphate dehydrogenase activity also indicates that gluconeogenetic processes (generation of glucose from other organic molecules) do not occur in the ovarian fluid (Lahnsteiner et al., 1995a).