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PRESERVATION OF RAINBOW TROUT EGGS AND EMBRYOS USING A PERFLUOROCHEMICAL

A thesis

Submitted

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the Department of Health Management

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ABSTRACT

The objective of this study was to maintain viability of unfertilized, fertilized and eyed eggs of rainbow trout during storage using fluorocarbon (FC) as an oxygen carrier, to allow more flexibility in the reproductive management of salmonids.

For the unfertilized eggs, we were able to differentiate between water-hardened eggs and those that were not water-hardened ($P < 0.001$) and, therefore, we used this method to estimate the viability of unfertilized eggs during storage.

In order to select a storage medium for unfertilized eggs that does not trigger water-hardening before fertilization but promotes maximal fertility, eggs were tested in: D-532, Fish Ringer's, Stoss's solution or water. Overall, eggs in Stoss's solution were found to have the lowest water-hardening and the highest fertility percentages; therefore, Stoss's solution was used in subsequent trials.

Unfertilized eggs were stored in: FC, FC plus mineral oil, FC plus Stoss's solution (with 0.6, 1.0, 1.2 or 2.0 g pluronic F-68 or 1.0 g lecithin), Stoss's solution, mineral oil or water. The eggs were stored in a moisture-saturated atmosphere at one of three temperatures for up to four weeks. There was no consistent beneficial effect on egg viability from the addition of pluronic F-68 or lecithin. Fluorocarbon plus Stoss's solution was the most promising medium to maintain viability in eggs stored at 1.0 or 3.0°C. The water-hardening results from these experiments suggest that it is possible to store unfertilized eggs for up to four weeks.

Fertilized, non-water-hardened eggs and fertilized, water-hardened eggs were stored in a moisture saturated atmosphere at 1.0°C or -3.3°C in: FC, FC plus mineral oil, FC plus Stoss's solution (with 1.0 g or 1.2 g pluronic F-68, or 1.0 g lecithin), Stoss's solution, mineral oil or water. When fertility was assessed, there was no evidence of embryonic development for any treatment or temperature. This was probably due to intolerance of the early embryos to low temperatures.

Eyed eggs at several stages of development were stored in FC, FC plus PBS, FC plus 0.3 M glucose, FC plus mineral oil, FC plus water, or water at 1.0°C in a moisture-saturated atmosphere. After five weeks, eggs stored in FC plus water resulted in the highest hatching percentages with high percentages of normal alevins.

We have demonstrated that the use of oxygenated FC can benefit the aquaculture industry by maintaining the viability of rainbow trout eggs during storage or shipment.

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DEDICATION

To Bob and Dianne Gardiner

Who have always been there to support me in all my endeavors.

Thanks, Dad and Mom

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I. GENERAL INTRODUCTION

1.1 Aquaculture Industry

1.1.1 History

The term "aquaculture" is defined as the art of increasing and rearing aquatic animals and plants (Barnabé, 1994). A public misconception is that aquaculture has been introduced into the world market recently, but, in reality, man has produced aquatic organisms for centuries. The ancient peoples of Rome and Gaul cultured oysters, and the legendary Fan-li of the fifth century B.C. is said to have reared carp in ponds (Bardach *et al.*, 1972). The Romans were able to maintain and raise fish in brackish water along the Italian coast. It is believed that they learned the methods from the Etruscans, who learned it from the Phoenicians (Brown, 1977). Wen Fang is attributed with the development of aquaculture in China. He was confined to an estate for 13 years by the Shang Dynasty ruler (1135-1122 B.C.) and he made the first recorded references of fish behavior and growth. Much of the early skill in rearing fish is said to have originated with him and his workers (Brown, 1977). Scientists have speculated that aquaculture may have had its beginnings in the highly organized ancient water-oriented civilizations, in which fish was an important dietary component (Bardach *et al.*, 1972).

Canada's aquaculture industry began in Quebec in 1857, raising Atlantic salmon and speckled trout. A hatchery was established in Ontario by the Canadian government in 1866 (Brown, 1977). Aquaculture in Canada evolved with federal and provincial hatcheries, commercial hatcheries, and operations using ponds and sloughs situated in the prairie provinces. There are also a number of small private hatcheries, principally in eastern

Canada, which are operated primarily by fishing clubs for stocking their own waters (Brown, 1977). Since this time Canada has become a major producer of salmonids such as trout, salmon and Arctic char.

1.1.2 Importance of Aquaculture

The focus of aquaculture in different countries will depend upon the dietary needs of the people and specialty requirements together with their economic circumstances. Industrialized countries want to produce more high-value products for the consumer than are being supplied by the commercial fishery. With the depletion of the cod stocks and other commercial fisheries, it is evident that aquaculture is needed in Canada. In 1990, the weight of Atlantic salmon produced in countries bordering on the Atlantic Ocean was almost 50 times the weight of Atlantic salmon caught in the wild by all methods (Laird, 1996). The commercial fishery for salmon has declined due to the decline in price because of the increased availability of farmed salmon. It could be argued that salmon farming has reduced pressure on the wild stocks (Laird, 1996).

In developing countries, the objective is to produce enough animal protein to supply the dietary requirements of the ever-increasing population. Climate change in many of these countries has caused increasing desertification resulting in less land for terrestrial agriculture. A new approach to food production such as aquaculture was needed to replace and augment the traditional protein sources. Furthermore, these countries hope to produce high-value products for export. Thus the incorporation of aquaculture into these countries could help increase food production (Barnabé, 1994).

1.2 Reproduction in Salmonids

1.2.1 Oogenesis

Wallace and Selman (1981) identified four main stages of oocyte growth: (1) primary growth (previtellogenesis), (2) "yolk" vesicle (exogenous vitellogenesis, which is the production of lipids for the yolk from sources outside the ovary), (3) true vitellogenesis and (4) maturation (hydration/ovulation).

Egg development occurs within a follicle in the fish's ovary. The primary growth stage of the egg is characterized by an increase in the size of the nucleus, the multiplication of the nucleoli and by a complex accumulation of RNA, and is thought to be gonadotropin hormone (GnRH)-independent (Billard, 1992).

The second stage which is GnRH-dependent is characterized by the formation of vesicles that eventually form the cortical alveoli which are located in the chorion (shell). The cortical alveoli release colloids which are involved in the formation of the perivitelline space (Yamamoto, 1961; Nosek, 1984).

Vitellogenesis is characterized by a considerable increase in the cytoplasmic volume due to the incorporation of vitellogenin into the yolk (Appendix A, Figure 1, Gordon *et al.*, 1987). The vitellogenin is a lipophospho-protein-calcium complex synthesized by the liver (Tyler *et al.*, 1987; Riazi and Fremont, 1988) involving lipids mobilized from the visceral fat (Takashima *et al.*, 1971). During this stage there is an increase in the thickness of the zona radiata, development of rough endoplasmic reticulum in the granulosa and thecal cells (Appendix A, Figure 1, Gordon *et al.*, 1987), and a progressive migration of the nucleus from the centre of the oocyte toward the periphery. At this time, the yolk globules integrate

mainly in the centre of the cell (Billard, 1992).

The nucleus of the egg which contains the genetic material eventually migrates to the plasma membrane close to the micropyle, the pathway for sperm penetration. The nuclear envelope ruptures, signifying the final stage of oogenesis. Ovulation occurs shortly after oocyte maturation and results from the rupture of the follicular wall; at the same time, the micropyle opens so that spermatozoa can penetrate the chorion (Billard, 1992).

1.2.2 Control of Reproduction

1.2.2.1 External Factors

Environmental factors affecting reproduction in fish are termed: proximate factors, which stimulate gonadal development and breeding activities such as temperature and photoperiod; zeitgebers which control endogenous rhythms, such as a change in tide or sudden changes in the environment; and ultimate factors such as sufficient food (Barton, 1996).

Temperature can play an indirect or a direct role on gametogenesis. Salmonids are considered 'cold water' fish and their thermal optima and preferences are reflected by their distribution in nature. Because salmonids are poikilotherms, their metabolic rate is controlled directly by the ambient temperature (Barton, 1996). Warm water increases metabolism and therefore a greater amount of feed is consumed allowing reproduction to proceed. In addition to these indirect effects, gametogenesis only takes place within a given temperature range for each species (Barnabé, 1994).

Changes in temperature, either increasing for some species or decreasing for others, stimulate the neuro-endocrine system which determines when spawning will take place.

Temperature has a direct effect on the production of GnRH by the hypothalamo-hypophyseal complex (Barnabé, 1994) which is described in the next section (1.2.2.2).

Photoperiod is known to affect reproduction in fish (Bromage *et al.*, 1995). Photoperiod affects gonadal growth and maturation (Bromage, 1995). When gametogenesis in trout was stimulated by an artificially decreased photoperiod, ovulation occurred earlier than in untreated fish (Billard, 1990). This action is exerted via photoreceptors in the eye and pineal gland then through the brain to the hypothalamo-hypophyseal axis (Barnabé, 1994).

In the tropics where temperature and photoperiod only change slightly, it is believed that humic acids washed out of the soil from the mountains in the rainy season act as a signal for the start of the spawning season (Barnabé, 1994).

Fecundity is enhanced by feeding high energy diets or by increasing daily rations (Springate *et al.*, 1985), whereas periods of diet restriction or starvation have been shown to reduce fecundity (Baiz, 1978). Other factors which are thought to influence gametogenesis and spawning are: low salinity (Zanuy and Carillo, 1984), seasonal variation, water currents, pH, barometric pressure and social factors such as high density and stress (Barton, 1996).

1.2.2.2 Neuro-endocrine Control of Oogenesis

Maturation of fish and the development of eggs are environmentally controlled via the hypothalamic-pituitary-ovarian axis (Fostier *et al.*, 1983; Scott and Sumpter, 1983). The stimulus of the external factors determines the activity of the hypothalamus. The hypothalamus exerts both a stimulatory (via GnRH-releasing hormone) or inhibitory (via dopamine) influence on the pituitary gland. In the stimulatory situation, the hypothalamus

releases GnRH-releasing hormone which causes the pituitary to produce and release GnRH. Gonadotropins are the major hormones involved in egg maturation and release. Two types of GnRH have been found, one rich in carbohydrate (Gnh) and the other low in carbohydrate (Gnl) (Breton *et al.*, 1976; Ng and Idler, 1978). Both Gnh and Gnl are required for the process of vitellogenesis (Idler and Ng, 1983).

The Gnh stimulates the production of estradiol 17-β by thecal and granulosa cells of the follicles (Nagahama, *et al.*, 1982) (Appendix A, Figure 1, Gordon *et al.*, 1987). Estradiol 17-β then acts directly on the liver to initiate and maintain the synthesis of vitellogenin. The Gnl molecule (Appendix A, Figure 1, Gordon *et al.*, 1987) enhances incorporation of vitellogenin in the egg. Estradiol 17-β is also thought to initiate external signs of sexual maturation (Gordon *et al.*, 1987).

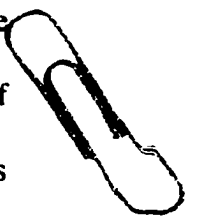
Increasing levels of Gnh then stimulate the thecal cells to produce 17α, 20β-dihydroxyprogesterone (DHP) (Appendix A, Figure 1, Gordon *et al.*, 1987), which is required for the final nuclear and hydration changes in the egg before ovulation. Corticosteroids, produced in the inter-renal cells in the kidney, act together with 17α, 20β-DHP to produce these changes (Gordon *et al.*, 1987).

Ovulation is mediated by proteases (Appendix A, Figure 1, Gordon *et al.*, 1987), which create holes in the follicle though which the eggs will be ejected. Final ejection may be mediated by prostaglandin $F_{2\alpha}$ which causes contractions of the smooth muscles interspersed between the thecal cells of the follicle (Jalabert and Szöllösi, 1975; Goetz, 1982; Goetz, 1983). Production of these mediators is stimulated by Gnh. The eggs are released into a mesenteric fold within the abdomen of the fish.

1.2.3 Reproductive Seasons of Salmonids

The native range of salmonids is off the coasts and in the rivers and lakes of the northern temperate zone. This range has been artificially extended in the last hundred years to the southern temperate zone and to the highland regions of the tropical zone. All salmonids breed in freshwater. Most migrate to the sea when they are young to feed and grow before returning to freshwater to spawn (to produce or deposit eggs or semen) (Scott, 1990).

Time of spawning is a heritable trait in salmonids (Scott, 1990). Spawning season is precise within each strain and normally occurs once per year. It usually occurs at the same time every year and lasts two to six weeks, depending on the species. In the majority of salmonids, including most stocks of brown trout and Atlantic salmon, spawning occurs between September and December (Scott, 1990).



In winter spawners (January - early February), gonadal development occurs between spring and the onset of spawning. Spawning in winter is thought to be synchronised but not regulated by the natural decrease in photoperiod (Scott, 1990). Late-winter spawners (late February - March) are not common among salmonids. Late-winter spawners are usually domesticated rainbow trout stocks which have been deliberately selected for this trait (Scott, 1990). Autumn-spawning salmonids (October - November) are affected by decreasing photoperiods which accelerate maturation in these species.

Spring (April - June) and summer (July - September) spawning is a normal occurrence in salmonids living in more northern latitudes. The reason for this spawning pattern is due to the low temperatures these fish experience during winter. The temperature

retards and inhibits the effects on gonadal development and these effects appear to override the photoperiod effects (Scott, 1990).

For most of the salmonid species, spawning seasons are manipulated by the external factors that have been mentioned previously. These factors are put into use by the aquaculture industry to control the time of spawning.

1.2.4 Water-hardening and Incubation of Eggs

1.2.4.1 Water-hardening

Water-hardening is a process that produces the hard shell of salmonid eggs which protects them from mechanical damage and microbial attack (Gilkey, 1981). The process of water-hardening begins when salmonid eggs are first spawned and exposed to water. Fertilization must take place before this process is completed. Water-hardening results from water uptake by the egg followed by the formation of a perivitelline space (Appendix A, Figure 2, Piper *et al.*, 1982) which fills with fluid, after which the chorion hardens (Rall, 1993). Water-hardening induces a rapid increase in the strength of the chorion which occurs over two to three hours (Winnicki and Bartel, 1967). There is also a release of colloids by the cortical alveoli into the perivitelline space. The perivitelline fluid, which occupies the perivitelline space, is produced when water flows across the chorion in response to an osmotic pressure gradient between the external water and the colloids (Rall, 1993). Lectins are released into the perivitelline space to block the micropyle, preventing entry of spermatozoa and polyspermy (Szöllösi and Billard, 1974; Krajhanzl *et al.*, 1984). There are different hypotheses on the causes of water-hardening. One is that the eggs of fresh-water fish are normally spawned in a hypotonic medium which causes the egg to water-harden due

to osmotic shock (Billard, 1992).

In contrast, Gilkey (1981) believed a zone of high free calcium occurs at the point of sperm entry and crosses the egg as a narrow band. During water-hardening, free calcium rose by 300 fold in the cytoplasm of the egg (Gilkey, 1981). The activating stimulus, such as water, induces a local increase in free calcium to a threshold level, stimulating the release of calcium from local stores and producing the calcium wave. The released calcium diffuses into neighbouring regions, bringing free calcium there above threshold, thus propagating the wave. The calcium wave is followed by fusion of the cortical vesicles with the plasma membrane in a wave-like fashion. Upon fusion, the cortical alveoli from the cortical vesicles discharge colloids into the perivitelline space between the plasma membrane and the chorion. A major component of these colloids is an acidic mucopolysaccharide which is too large to penetrate the chorion (Tesoriero, 1978). Since the chorion is permeable to water and small electrolytes, the mucopolysaccharide increases the osmolarity of the perivitelline space relative to the natural spawning medium. The resulting inflow of external medium increases the hydrostatic pressure in the perivitelline space, forcing the elastic chorion to expand away from the vitelline membrane. Consequently, the chorion loses its elasticity and becomes tough and rigid. Uptake of released calcium by local sinks then returns free calcium to the resting level. The identity of the calcium sources and sinks involved in water-hardening are unknown. Apparently, it is not calcium from the external environment because when eggs are immersed in a medium with less than 10^{-9} M free calcium, the wave still occurs. The yolk, mitochondria and endoplasmic reticulum are thought to be possible sources of the calcium. Free calcium gradually returns to the resting level when water-hardening is

complete (Gilkey, 1981).

Determination of water-hardening has been used as a method to assess the viability of unfertilized eggs (Harvey and Ashwood-Smith, 1982). Hardening occurs even if the egg is not fertilized and begins within five minutes after exposure to water. Damage to eggs, such as that produced by heating or freezing of the cytoplasm can result in the inability of the eggs to water-harden. If an egg is able to water-harden, then it is still physiologically active, i.e. viable (Harvey and Ashwood-Smith, 1982).

1.2.4.2 Spawning and Incubation of Eggs in Aquaculture

In a hatchery setting, fish are anaesthetized and then wiped dry to prevent water contamination, and eggs and semen are collected separately by abdominal massage. Eggs are put into a bowl and semen is added. A small amount of water is then added to activate the sperm and mix with the eggs. A large amount of water is then added to water-harden the eggs and to wash away excess semen. The eggs are then stored in incubators until they hatch. Water is circulated through the incubators to eliminate wastes and carry oxygen to the eggs. Dead eggs, which are white, are removed to prevent fungal outbreaks.

The different species of salmonids vary in the time for eggs to hatch. Rainbow trout eggs incubated at 10°C take 32.2 days from fertilization to hatch, Atlantic salmon 47.7 days (Gordon *et al.*, 1987), brown trout 41 days and brook trout 44 days (Piper *et al.*, 1982). Since development of fertilized eggs is influenced by temperature, the duration of incubation is measured in degree days which is the temperature of the water in the incubator in degrees Celsius multiplied by the number of days since fertilization.

1.3 Preservation of Gametes and Embryos

1.3.1 Why Preserve Eggs and Embryos?

It would be beneficial to preserve the eggs and embryos of fish for the following reasons: 1) to enable the shipment of genetic material across long distances, 2) to maintain the desirable genetic material of ova or embryos, 3) to conserve a broad-based gene pool and 4) to reduce the effects of seasonality on hatchery production.

It is important to preserve embryos for periods of time when shipping long distances, or when delays, such as customs or misdirection occur. Salmonids spawn once per year in a relatively short period leaving aquaculture hatcheries empty for most of the year. If eggs could be stored, then the opportunity would exist to hatch them at various times during the year. For example, if a system failure occurred and all the fry for that year were destroyed, there would be an opportunity to obtain more fry that year, which would reduce the substantial financial loss which would have occurred. If both the eggs and embryos could be preserved, fry at different stages of development would also be available at various times throughout the year.

1.3.2 Preservation of Eggs and Embryos of Mammals

The first successful transfer of a cryopreserved bovine embryo occurred over twenty years ago (Wilmut and Rowson, 1973). Both the dairy and beef cattle industries presently use embryo transfer extensively. Frozen embryos which have been thawed and transferred to the uteri of recipients have produced live offspring in many species including human beings, sheep, horses and cats (Pomp and Critser, 1988).

Oocytes of mammalian species are more difficult to cryopreserve successfully than

embryos (Shaw *et al.*, 2000). For example, Martino *et al.* (1996) found that cooling bovine oocytes to 0°C greatly reduced their capacity to cleave and develop after fertilization.

1.3.3 Developmental Stages of Fish Embryos

The stage of development of fertilized eggs to be preserved is important because temperature tolerance (Pullin and Bailey, 1981; Sasaki *et al.*, 1988) and water permeability (Harvey and Chamberlain, 1982) change as the embryo develops (Pullin and Bailey, 1981).

Embryonic development can be divided into three stages: 1) the green stage, 2) the tender stage and 3) the eyed stage (Gordon *et al.*, 1987). The green stage ends 36-48 hours after fertilization and the eggs are fairly resistant to damage and can be shipped during this stage (Gordon *et al.*, 1987). The tender stage extends from the end of the green stage until the eyed stage. During the tender stage, eggs are extremely sensitive to damage, and handling should be kept to a minimum (Gordon *et al.*, 1987). The eyed stage starts when the lightly pigmented eyes of the embryo are visible through the eggs' shell (Gordon *et al.*, 1987). The eyed stage for rainbow trout begins approximately halfway through incubation, from fertilization to hatch, at 155 to 170 degree days (Gordon *et al.*, 1987). Usually eggs are sold and transported during the eyed stage because they are relatively resistant to damage from handling (Gordon *et al.*, 1987). A more detailed description of the stages of embryo development according to Gordon *et al.* (1987) and Ballard (1973) is presented in Appendix A, Table 1.

1.3.4 Preservation of Fish Eggs and Embryos

Scientists have tried to preserve unfertilized eggs, fertilized eggs and eyed eggs of fish with limited success. Most of the research to date has concentrated on the cryopreservation of fertilized fish eggs in trout, carp and zebrafish (Harvey and Ashwood-Smith, 1982; Harvey, 1983; Rothbard *et al.*, 1996). There are many reasons why researchers have experienced difficulty cryopreserving unfertilized and fertilized eggs of fish (Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983). The fish eggs' complex membrane system is a barrier for the penetration of cryoprotectants which protect the eggs from intracellular damage during freezing. Freezing can induce fatal damage due to ice crystal formation or osmotic pressure (Hammerstedt *et al.*, 1990). Cryoprotectants reduce the temperature for intracellular freezing and ice formation (Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983). Unfortunately, cryoprotectants can also be toxic to embryos, especially at the high concentrations that might be used to increase penetration (Suzuki *et al.*, 1995).

In addition to membrane barriers, the large size of the egg and the presence of the yolk hinder the permeability of the cryoprotectants (Horton and Ott, 1976). One of the reasons that the freezing of bovine embryos has been so successful is because the embryo is so small. A salmonid egg is 7 mm in diameter for rainbow trout or 11 mm for chinook salmon (Gordon *et al.*, 1987). By contrast, a bovine embryo is approximately 170 μm in diameter (Mapletoft, 1986). Therefore, the diameter of a rainbow trout egg is approximately 40 times larger than that of a bovine embryo. These factors that hinder cryoprotectant penetration have led to studies of freezing embryos with the chorion removed, with the yolk

removed, or freezing individual cells.

Rainbow trout and zebrafish embryonic cells without the chorion, with or without the yolk have been frozen to determine if there is an increase in viability after freezing compared with intact embryos results after freezing intact embryos (Harvey, 1983; Calvi and Maisse, 1998). Harvey (1983) froze individual embryonic cells of zebrafish blastoderms (the part of a fertilized egg that gives rise to the germinal disc), clumps of cells from blastoderms and blastoderms in and out of the chorion to -196°C in a two-step procedure and also used a slow freezing procedure to -25°C for intact blastoderms. The cells were thawed in air at 25°C. Viability of the clumps of cells was measured using a stain where cells or blastoderms that were living remained unstained. The dechorionated blastoderm was assessed using six developmental grades. The cryoprotectant used for the two-step procedure was DMSO. Glycerol or DMSO were used for the slow freezing experiment. It was found that the cells of the blastoderm survived at a higher percentage (84.8%) than the clumps of cells (26.2%) or the blastoderms (2.1%). The intact blastoderms frozen by the slow freezing method, using glycerol as a cryoprotectant, had a higher percentage of development (48%) than the blastoderms frozen using DMSO (32%). In a study by Calvi and Maisse (1998), rainbow trout blastomeres at stages 6A, 6B and 6C (Ballard, 1973; Appendix A, Table 1) were frozen to -196°C using 1,2-propanediol. Survival rates were significantly higher at the later 6C stage (95%) compared to blastomeres stored at stages 6A (53%) or 6B (88%).

There have been a limited number of reports on the cryopreservation of unfertilized fish eggs of various species. Unfertilized eggs of rainbow trout were frozen to -20°C and had a 66% fertility rate after being frozen for less than five minutes (Zell, 1978). These

results could not be repeated by Erdahl and Graham (1980) leading to the suggestion that the eggs did not actually freeze but were supercooled (Harvey and Ashwood-Smith, 1982). Supercooling occurs when the temperature of a solution is below its freezing point but it does not freeze.

Other researchers have used chilling to preserve eggs instead of freezing. When eggs are chilled they still need oxygen and produce metabolic wastes (Maddock, 1974). Therefore, a system is needed to contribute oxygen and remove metabolic wastes similar to what would be provided by a continuous supply of water in a hatchery.

A medium is also needed to prevent dehydration when preserving eggs by chilling. Many different extenders have been used. Some researchers have used ovarian fluid to store eggs (Rothbard *et al.*, 1996), while others have developed extenders that will not water-harden eggs so that they may be fertilized later (Billard, 1977; Harvey and Ashwood-Smith, 1982; Stoss and Donaldson, 1983). The extenders were, for the most part, salt solutions and included Stoss's solution (Stoss and Donaldson, 1983), D-532 (Billard, 1977) and Fish Ringer solution (Harvey and Ashwood-Smith, 1982). Water has also been used to store fertilized trout eggs (Maddock, 1974).

A study on the short-term preservation of 94 degree-day-old brown trout eggs was conducted by Maddock (1974) by placing the eggs in troughs with recirculated water at 1.4°C from a reservoir. Oxygen was introduced into the system by aeration into the reservoir water with compressed air. The hatching rate was 95% after 123 days of incubation.

In contrast, Stonecypher *et al.* (1994) reduced the temperature of fertilized trout eggs from 7°C to 2°C or 4°C. Eggs were also incubated at 7°C. When eggs held at reduced

temperatures reached the eyed stage they were allowed to acclimate to 7°C at a rate of 1°C/hour and then were maintained at 7°C until hatching. The lower incubation temperatures decreased the survival rates of eggs with the normal incubation temperature of 7°C having the highest survival rate.

The fertilized eggs of chum and pink salmon were stored at 2°C at five stages of development: 16-cell stage, blastula stage, completion of epiboly (Appendix A, Figure 3, Piper *et al.*, 1982), early eye pigmentation and the late eyed stage (Beacham and Murray, 1987). Eggs were fertilized at 8°C and kept in an incubator at 8°C for 24 h to reach the 16-cell stage, 72 h to reach the blastula stage, 17 days for completion of epiboly, 24 days for early eye pigmentation and 47 days to reach the late eyed stage. Eggs in their containers were placed in an 8°C water bath in which the temperature was lowered to 2°C. All pink salmon eggs at the blastula stage or earlier died after transfer to 2°C, whereas 50% of the chum salmon eggs survived until hatching. Eggs at epiboly and later stages of development had an average of 75% survival with chum salmon eggs having higher survival rates than pink salmon eggs at all stages of development (Beacham and Murray, 1987).

Rothbard *et al.* (1996) compared the hatching rates of unfertilized carp eggs stored for 6 to 7 hours at 6°C to 9°C then fertilized at 21°C and incubated at 21°C, to those of control eggs fertilized directly after spawning at 21°C. The hatching rate of the cooled eggs ranged from 1 to 2 % compared to the control rate of 57.3%.

Cloud *et al.* (1988) found that the tolerance of fathead minnow embryos to cooling depended upon the stage of development. For their first experiment they used fertilized eggs in the early and late blastula stages. A difference of two hours in incubation at 23°C

distinguishes the early blastula stage from the late blastula stage. The embryos were held for 90 minutes at 5 °C after which they were returned to room temperature. Survival and retinal pigmentation of these embryos were used as the end points in these experiments; under normal conditions at room temperature, the retina was clearly pigmented after three days of incubation. All late blastulas survived a reduction in temperature to 5 °C while only 50% of the early blastulas survived. In the second experiment, eggs were allowed to develop in Fish Ringer solution until one hour before the experiment and then transferred at the early blastula, late blastula or gastrula stage into either Fish Ringer solution diluted to 10% with distilled water or undiluted Fish Ringer solution. After 16 to 18 hours at 0 °C, the fertilized eggs were returned to room temperature to resume development and were assessed using the same method as above. All early blastulas failed to survive the 16 to 18 hours at 0 °C in either concentration of Fish Ringer solution. The late gastrula stage had a 98.8% survival in either solution. The late blastula stage had survival rates of 54.7% and 93.3% for the 10% and 100% Fish Ringer solution, respectively. These results indicate that the survival of fertilized eggs increased as the stage of development increased.

1.4 Fluorocarbon

Fluorocarbons (perfluorochemicals) are liquid perfluorinated carbon compounds that are produced by replacing the hydrogen atoms of hydrocarbons with fluorine atoms. They are stable, chemically and biologically inert compounds, clearly distinct from the fluorochlorocarbon anaesthetics and propellants implicated in atmospheric ozone depletion. The high solubility of gases in fluorocarbon has led to interest in these compounds as vehicles for respiratory gas transport (Thurston *et al.*, 1993). Clark and Gollan (1966) first

demonstrated that mammals could remain alive while breathing liquid fluorocarbon saturated with oxygen. The lack of strong interactions between fluorocarbon molecules enables gases such as oxygen to enter the spaces between them (Ju and Armiger, 1992). Gas solubility increases as temperature decreases which makes fluorocarbon even more attractive for the preservation of gametes that require oxygen in a cool environment (Reiss, 1994).

Fluorocarbon has been used to deliver oxygen to and transport carbon dioxide away from animal, plant and microbial cells (Hamamoto, 1987; King *et al.*, 1989; Thurston *et al.*, 1993; McNiven *et al.*, 1993; Anthony, 1997). Fluorocarbon has been used to deliver oxygen and reduce the mechanical damage to cells from aeration (Hamamoto, 1987; Lowe *et al.*, 1997a). Oxygen delivery from fluorocarbon was similar to the aeration method, but the use of fluorocarbon decreased damage to mouse hybridoma cells caused by aeration (Hamamoto, 1987). Cryopreserved rice cells were thawed and placed in a culture medium containing fluorocarbon. The fresh weight gain after 30 days in the fluorocarbon culture medium was significantly ($P < 0.05$) higher than for the control cells without fluorocarbon (Anthony *et al.*, 1997).

Sloviter and Kamimoto (1967) were able to overcome the problem of insolubility of compounds in fluorocarbon by preparing emulsions of fluorocarbon and bovine serum albumin for use in perfusion of isolated organs. Formulation of stable fluorocarbon emulsions has allowed for their clinical development as vehicles for gas transport, and they have many other potential uses in medicine, biology and research (Lowe, 1991).

Fluorocarbon, containing oxygen, has been used with extenders to store turkey and rainbow trout semen (Thurston *et al.*, 1993; McNiven *et al.*, 1993; Thurston *et al.*, 1994).

Thurston *et al.* (1994) showed that storage of turkey semen for 24 hours in an emulsified extender/aerated fluorocarbon mixture at 12 to 13°C improved the fertilizing capacity relative to semen that was stored without fluorocarbon. McNiven *et al.* (1993) stored rainbow trout semen in oxygenated fluorocarbon in a moisture-saturated atmosphere. This semen maintained a fertility level of 81.1% for 37 days compared with 0% when fluorocarbon was not used to store semen.

Unfertilized and non-water-hardened fertilized eggs consumed 5.4 X 10^{-2} μl of O_2/hour/egg at 10°C (Boulekbache *et al.*, 1969). After fertilization and water-hardening there was a 50% increase in O_2 consumption by rainbow trout eggs. During gastrulation (Appendix A, Figure 3, Piper *et al.*, 1982), O_2 consumption increased from 10 X 10^{-2} μl of O_2/hour/egg up to 30 X 10^{-2} μl of O_2/hour/egg. At the end of epiboly (Appendix A, Figure 3, Piper *et al.*, 1982), O_2 consumption increased to 1.2 μl/hour/egg (Boulekbache *et al.*, 1969). This demonstrates that eggs and embryos require oxygen in order to survive; therefore, fluorocarbon has great potential as an oxygen source for the storage of eggs and embryos.

1.5 Objectives

The objectives of these experiments were:

1) to verify a method to detect the viability of unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) based on water-hardening,

2) to select a storage medium that would maintain the viability of unfertilized eggs without inducing water-hardening,

3) to select a medium, with or without fluorocarbon as an oxygen carrier, and a

temperature, that would maintain the viability of unfertilized eggs or fertilized water-hardened or non-water-hardened eggs during storage,

4) to conduct storage trials of unfertilized eggs using water-hardening and fertility as an assessment of viability,

5) to determine if there is a relationship between the results of water-hardening and fertility as methods of assessment of viability, and

6) to maintain the viability of eyed rainbow trout eggs using fluorocarbon as an oxygen carrier during storage.

II. ESTIMATING VIABILITY OF RAINBOW TROUT EGGS, SELECTING STORAGE MEDIA FOR THE EGGS, AND THE STORAGE OF UNFERTILIZED AND FERTILIZED EGGS

2.1 Introduction

There has been considerable success in the cryopreservation of mammalian and fish spermatozoa (Foote, 1986; Billiard, 1992; Purdom, 1993). In 1951, the first calf was produced from frozen semen (Foote, 1986). Semen cryopreservation in the bovine species has been so successful that most dairy cows are now inseminated by artificial means (Foote, 1986). This development has led to a general increase in potential for milk production per cow because farmers now have greater access to semen from high producing genetic lines.

Semen from various aquatic species has been preserved at temperatures ranging from 4°C to -196°C with varying levels of success (McNiven *et al.*, 1993; Babiak *et al.*, 1995; Lahnsteiner *et al.*, 1996). The industry would benefit if eggs or embryos of salmonids could be preserved, since most salmonids spawn only once per year (Scott, 1990). This technology would extend the time period in which eggs could be fertilized and in which the fertilized and unfertilized eggs and embryos could be shipped. Improved preservation techniques would also benefit the long distance transport of embryos and eggs. Preservation of both eggs and sperm would reduce the effect that the one spawning per year has on the reproduction of fish.

Embryos of some mammalian species can be successfully cryopreserved and there have been successful attempts to cryopreserve mammalian oocytes (Pomp and Critser, 1988; Niemann, 1991; Schiewe, 1991; Parks and Ruffing, 1992; Rall, 1993 ; Dobrinsky, 1996),

but the embryos and oocytes of fish have been more difficult to cryopreserve than those of mammals (Billard, 1992; Rall, 1993). In past attempts, unfertilized and fertilized fish eggs have been stored for 5 to 10 minutes at temperatures ranging from just above 0 °C to -80°C (Zell, 1978; Harvey and Ashwood-Smith, 1982). The viability of fish eggs and embryos after freezing or chilling has been variable and low (Whittingham and Rosenthal, 1978; Zell, 1978; Rothbard *et al.*, 1996).

Instead of freezing gametes using cryoprotectants, some researchers have used chilling in combination with fluorocarbon (FC) (McNiven *et al.*, 1993; Thurston *et al.*, 1993; Thurston *et al.*, 1994). The storage life of turkey semen (Thurston *et al.*, 1993; Thurston *et al.*, 1994) and rainbow trout semen (McNiven *et al.*, 1993) was extended using FC and extenders. To extend the life of turkey semen, Thurston *et al.* (1993) used pluronic F-68 to emulsify the FC/extender solution so that the exchange of oxygen could occur over a greater surface area (Thurston *et al.*, 1993). McNiven *et al.* (1993) reported that rainbow trout semen, which had been stored for 37 days in a moisture-saturated atmosphere, at a 1:1 ratio of semen and FC, had fertility levels of 81.1%; whereas undiluted semen or semen stored in extender without FC had 0% fertility.

Fluorocarbons are useful in regulating the oxygen supply to cells to increase growth and productivity. When used in conjunction with surfactants, e.g., pluronic F-68 or lecithin, they are known to protect insect and mammalian cells in culture. The effect of FC is enhanced by the interaction of the surfactants with components of cytoplasmic membranes (Lowe *et al.*, 1997b).

Fluorocarbon can be used to deliver oxygen at a higher rate than water. The

solubility of oxygen (O_2) in FC (FC-77) is 56 ml of O_2/100 ml of FC, whereas the solubility of O_2 in water is only 3.2 ml of O_2/100 ml of water (3M Laboratory Materials, St. Paul, MN) . Oxygen is needed by unfertilized and fertilized eggs for survival as mentioned in Chapter 1 (Boulekbache *et al.*, 1969).

Most researchers use fertility trials to measure the viability of eggs after storage, but this is time-consuming and costly. It could take up to several weeks, depending upon temperature, before the unmistakable presence of an embryo can be seen. Detection of water-hardening has been used as a method to determine the viability of unfertilized eggs because eggs that are dead or damaged cannot water-harden (Harvey and Ashwood-Smith, 1982). Water-hardening is a process that hardens the chorion of the salmonid egg. The process of water-hardening begins when a salmonid egg is first spawned. At this time, there is a release of cortical granules and colloidal materials from the cortical alveoli into the future perivitelline space. These materials create an osmotic pressure, drawing water into the egg, resulting in the formation of the perivitelline space (Figure 1, Gordon *et al.*, 1987) followed by hardening of the chorion (Billard, 1992). Water-hardening occurs over two to three hours and results in a rapid increase in the strength of the chorion (Winnicki and Bartel, 1967). Hardening begins within five minutes after being placed in water and occurs even if the egg is not fertilized. Once the egg is water-hardened, the micropyle becomes blocked, preventing entry of spermatozoa and polyspermy (Ginsburg, 1961; Szöllösi and Billard, 1974). Eggs that have sustained damage, such as that produced by heating or freezing of the cytoplasm, do not water-harden (Harvey and Ashwood-Smith, 1982). Therefore, we used assessment of water-hardening as a simple and quick method to assess the viability of

unfertilized eggs after storage in our experiments.

To store unfertilized eggs for an indefinite period of time, a storage medium is needed that will not water-harden the eggs in order to allow fertilization to take place after the storage period. Ovarian fluid released with the eggs has been shown to possess some physiological functions such as sperm activation, prolongation of motility, preservation of unfertilized eggs (Ginsburg, 1972), and it does not water-harden the eggs. However, only a limited amount of ovarian fluid is released with the eggs and the quantity is usually insufficient for storage. Therefore, an artificial medium is required for egg storage. In addition, consideration must be given to prevent dehydration of the eggs during storage. Mineral oil has been used to prevent dehydration of mammalian embryos during storage (Cseh *et al.*, 1997).

The objectives of this study were a) to verify a method to detect the viability of unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) based on water-hardening, b) to select a storage medium that would maintain the viability of unfertilized eggs without inducing water-hardening, c) to select a medium, with or without fluorocarbon as an oxygen carrier, and a temperature, that would maintain the viability of unfertilized eggs or fertilized water-hardened or non-water-hardened eggs during storage, and d) to determine if there was a relationship between the results of water-hardening and fertility as methods to assess viability.

2.2 Materials and Methods

2.2.1 Source and Transport of Eggs and Semen

Because of limited availability, unfertilized eggs and semen were obtained from several sources (Table I). Eggs in ovarian fluid with oxygen were transported in plastic bags on ice. Semen with oxygen was shipped in sealed plastic containers on ice.

2.2.2 Verification of Water-hardening Detection

The purpose of this experiment was to verify that water-hardening could be detected by our method. Three replicates of 100 eggs each were placed in petri dishes containing either 50 ml of tap water or ovarian fluid. After 90 minutes, an 18-gauge hypodermic needle was used to attempt to penetrate each of the eggs. We assumed that water-hardening occurred when it was difficult to penetrate the egg with the needle.

2.2.3. Selection of a Non-Water-Hardening Storage Medium for Unfertilized Eggs that Maximizes Fertility

The purpose of this experiment was to select a storage medium for unfertilized eggs that would not trigger water-hardening before fertilization but would promote maximal fertility. Media, selected from similar work in the literature, were: D-532 (Billard, 1977), Stoss's solution (Stoss and Donaldson, 1983), Fish Ringer (Harvey and Ashwood-Smith, 1982) and water. The composition, pH and osmolality of D-532, Stoss's solution and Fish Ringer solution are listed in Table II.

Lots of 100 unfertilized eggs in three replicates were placed in petri dishes containing 50 ml of each medium. After 90 minutes, the eggs were evaluated for water-hardening in a blind trial by one operator.

For the fertility trial, lots of 100 eggs in three replicates were stored in each medium or ovarian fluid in beakers in a 7°C water bath for 90 minutes. The semen sample used to fertilize the eggs in this experiment was evaluated to assure high motility (>60%) (Gallant, 1990), and concentration was determined (Gallant, 1990). Forty-five milliliters of the medium was then drained from each petri dish and the eggs were fertilized with 1 ml of semen (3.44×10^7 sperm/egg), gently swirled and left for two minutes. Two ml of 10°C water was then added to the eggs and gently swirled. After two minutes the eggs were rinsed twice with 50 ml of 10°C water and each replicate was placed randomly in a compartment of a Heath incubator tray in which the water temperature was maintained at 10°C. The incubator trays each had twelve compartments that were separated by plastic dividers sealed with silicone. Opaque eggs, which were unfertilized or dead, were removed periodically and placed in a clearing solution consisting of methanol, acetic acid and distilled water (1:1:1) (Baynes and Scott, 1987). The eggs were examined using a dissecting microscope at 9 x magnification to determine if embryos were present.

After twelve days in incubator, eggs from each compartment were removed from the incubator and placed in a petri dish containing 50 ml of water. The eggs were then examined for the presence of embryos. The fertility percentage was calculated from the number of embryos divided by the total number of eggs for each medium.

2.2.4 Storage of Unfertilized Eggs

The purpose of these three experiments was to select a medium and temperature that would enhance the viability of unfertilized eggs during storage.

The first experiment evaluated the viability, by detection of water-hardening, of

unfertilized eggs stored in one of six media at 1.0°C or 3.0°C in 190 ml containers. The media were: 1) FC (Florinert-77, 3M, St. Paul, MN, USA), 2) FC plus mineral oil (1:1) (Rhone Merieux, Victoriaville, PQ), 3) FC plus Stoss's solution (1:1) (Stoss and Donaldson, 1983) with 2.0 g of pluronic F-68 (Sigma, St. Louis, MO, USA) (McNiven *et al.*, 1993), 4) Stoss's solution, 5) mineral oil, and 6) water. Before allotment to treatment, the FC was oxygenated for two minutes with 100% oxygen. Medium 3 was homogenized on a Brinkmann Homogenizer Pt 10/35 for one and a half minutes. Lots of 100 eggs with three replicates for each treatment and sampling time were stored in 50 ml of medium.

Eggs were stored on ice at 1.0°C or at 3.0°C in a 3°C cold room. The temperatures of the media were recorded for 24 hours on Monday and Thursday of each week with an electric thermometer (Fluke, Model 52, Fluke, Irvine, CA, USA). Containers were placed in a 98% moisture-saturated atmosphere. The humidity was measured by a CR10 data logger with a HMP35C humidity sensor (Campbell Scientific, Edmonton, AB, Canada). The rack on which the containers were stored was covered in plastic and two air pumps circulated air through water to maintain a moisture-saturated atmosphere.

The eggs were sampled on Day 0 (before storage), Day 4, Day 7, Day 11 and Day 17. The eggs were removed from their respective media and put into water for 90 minutes and assessed for water-hardening.

From the results of the first experiment, we decided to evaluate the effect of level of pluronic F-68 as well as other promising treatments on egg viability after storage. The media were: 1) FC, 2) FC plus mineral oil, 3) FC plus Stoss's solution with 1.2 g of pluronic F-68, 4) FC plus Stoss's with 0.6 g of pluronic F-68, 5) FC plus Stoss's solution, 6) Stoss's

solution, 7) mineral oil, and 8) water. Preparation of media was the same as above. Because of limited availability of eggs, 50 eggs per container, two replicates for each medium at each temperature and sampling time were used. An additional temperature of -3.3 °C was obtained by storing eggs on ice in a modified chest freezer. Moisture was added to the atmosphere of eggs stored at 1.0°C and 3.0°C as described above. Eggs were sampled before storage (Day 0), and once every seven days for four weeks and assessed for water-hardening.

Because of the potential of surfactants to improve egg viability in our egg storage system, we decided to investigate another surfactant at the optimal temperature from the previous experiment. Five media were used: 1) FC, 2) FC and Stoss's solution (1:1) with 1.0 g of pluronic F-68 and 0.5 g antifoam C (Thurston, 1993), 3) FC and Stoss's solution (1:1) with 1.0 g of lecithin (emulsifier) (Sigma, St. Louis, MO) and 0.5 g antifoam C (Sigma, St. Louis, MO), 4) FC and Stoss's solution (1:1), and 5) Stoss's solution. Media 2 and 3 were emulsified for three minutes with a Brinkman Homogenizer Pt 10/35. Each medium containing FC was oxygenated with 100% oxygen for one minute.

One hundred eggs were placed in each container and stored at 1.0°C. There were two replicates for each medium and sampling time. Moisture was added to the atmosphere as described above. The eggs were sampled on Day 0 (before treatment) of the experiment and then every seven days for three weeks and checked for water-hardening. Data for the water-hardening assessments were expressed as a percentage of water-hardening results on Day 0 (Table III).

In addition to the water-hardening assessment, a fertility trial was conducted. There were two replicates for each medium and sampling time. At the weekly sampling time,

samples were fertilized as described above and incubated for twelve days, after which they were observed for the presence of embryos. Opaque eggs were removed periodically during the incubation period and put into a 1:1:1 methanol, acetic acid and distilled water solution (Baynes and Scott, 1987) to determine if embryos were present.

2.2.5 Storage of Fertilized Eggs

Four experiments were conducted to select a medium and temperature that would enhance the viability of water-hardened or non-water-hardened fertilized eggs during storage. The following media were used for each type of egg: 1) FC, 2) FC plus mineral oil (1:1), 3) FC plus Stoss's solution (1:1) with 1.2 g of pluronic F-68, 4) Stoss's solution, 5) mineral oil and 6) water. In two further trials 1) FC, 2) FC plus Stoss's solution (1:1) with 1.0 g of Pluronic F-68 and 0.5 g antifoam C, 3) FC plus Stoss's solution (1:1) with 1.0 g of lecithin and 0.5 g antifoam C, 4) Stoss's solution and 5) water were used.

For all trials, the FC was oxygenated with 100% oxygen for one minute. All containers contained a total of 50 ml of medium. Media which contained pluronic F-68 or lecithin were homogenized with a Brinkman Homogenizer Pt 10/35 for one minute and thirty seconds.

Semen motility and concentration were determined using the methods described by Gallant (1990). The semen sample for experiments using the first set of media had a motility of 60%. Eggs in these two experiments were fertilized using 8.56×10^5 sperm/egg. Semen for the experiments using the second set of media had a motility rating of 65%. Eggs in these two experiments were fertilized with 1.49×10^6 sperm/egg.

For the non-water-hardened egg trials, semen was added to the eggs in the petri

dishes, and the eggs and sperm were gently swirled and left for 1.5 minutes and then put into storage containers on ice at 1.0°C in a 3°C cold room or on ice at -3.3°C in a modified chest freezer. Each week, 0.5 ml of 7°C water was added to the fertilized eggs being sampled to trigger water-hardening, and then the eggs were placed randomly into compartments in the incubator.

For the water-hardened egg trials, the eggs were fertilized as described above. Eggs were left for 45 minutes to water harden and then 100 eggs were used for each medium replicate and stored at 1.0°C or -3.3°C. Each week, sampled eggs were placed randomly into compartments in the incubator.

In all the fertilized egg trials, the eggs were placed in 190 ml containers which contained the media. Moisture was added to the atmosphere of eggs stored at 1.0°C and the temperatures were recorded as described previously. There were two replicates for each medium at each temperature and sampling time in all the fertilized egg trials. The eggs were sampled on Day 0 (before treatment) and once per week for four weeks. Opaque eggs were removed periodically from the incubator tray to prevent the growth of fungus and these dead eggs were incorporated into the results for fertility. After twelve days of incubation, eggs from each medium were removed and observed under a microscope for the presence of embryos.

2.2.6 Statistical Analysis

The results from the experiments testing water-hardening, water-hardening of eggs in different storage media, fertility percentages of eggs when fertilized in the storage media and Day 0 fertility of each experiment were individually analyzed using the Statistical

Analysis System (SAS, 1995) by a one-way analysis of variance with medium as the main effect. Percentages were transformed into arcsine square root before analysis.

The results for the unfertilized egg experiments were analyzed using the Statistical Analysis System (SAS, 1995), by a two-way analysis of variance with medium and temperature as main effects. Significance for all statistical analysis was determined at the $P < 0.05$ level. The fertility percentages of eggs fertilized on Day 0 are listed in Table III.

2.3 Results

2.3.1 Verification of Water-hardening Detection

We detected that 1.8% of the eggs stored in ovarian fluid showed evidence of water-hardening whereas we detected water-hardening in 85.1% of eggs stored in water ($P < 0.001$) (Table IV).

2.3.2 Selection of a Non-Water-Hardening Storage Medium for Unfertilized Eggs that Maximizes Fertility

Unfertilized eggs that were stored in Stoss's solution had a significantly lower ($P < 0.001$) water-hardening percentage than eggs stored in Fish Ringer solution or water, but results for eggs in Stoss's solution were not significantly different from those of eggs stored in D-532 (Table V). Unfertilized eggs stored in water for 90 minutes had a significantly higher ($P < 0.001$) percentage of water-hardening than eggs stored in any of the other media.

There were significant differences in egg fertility depending upon the medium in which the eggs were stored and fertilized (Table V). Eggs stored in Stoss's solution and then fertilized showed a significantly ($P < 0.01$) higher fertility percentage than unfertilized eggs stored in Fish Ringer solution, D-532, ovarian fluid or water. Eggs stored in water and then

fertilized displayed a significantly lower fertility percentage (0%) than eggs stored in any of the other media.

2.3.3 Storage of Unfertilized Eggs

After four days in storage, eggs stored in Stoss's solution at 1.0 ± 0.3°C (SEM) or in mineral oil at 1.0°C or 3.0 ± 0.2°C (SEM) had significantly ($P < 0.0001$) higher percentages of water hardening than eggs stored in FC plus Stoss's solution with 2.0 g of pluronic F-68 at 1.0 or 3.0°C or water at 1.0°C (Table VI).

When unfertilized eggs were stored for seven days, only the media affected water-hardening results. Eggs stored in FC plus Stoss's with 2.0 g pluronic had significantly lower percentages of water-hardening than eggs in all the other media.

After 11 days of storage, the water-hardening results were again only affected by the media. Eggs stored in Stoss's solution alone had a significantly higher percentage of water-hardening then eggs stored in any of the other media except for eggs stored in FC alone.

After 17 days, the eggs that were stored in FC, FC plus mineral oil, Stoss's solution or in mineral oil at 1.0°C and eggs stored in FC, FC plus mineral oil, Stoss's solution or in water at 3.0°C exhibited a significantly higher ($P < 0.05$) percentage of water-hardening than eggs stored in the emulsified FC and Stoss's solution at either temperature, water at 1.0°C, or mineral oil at 3.0°C. Over time, eggs stored in water at 1.0°C and FC plus Stoss's at 1.0°C or 3.0°C and FC plus mineral oil at 3°C had lower percentages of water hardening.

In the second trial, less than half of the eggs stored at -3.3 ± 1.5°C (SEM) in FC plus mineral oil, mineral oil or in FC plus Stoss's solution were able to water-harden after one week (Table VII); eggs stored in the other media at this temperature failed to water-harden.

After one week and two weeks of storage, there significant differences in water-hardening between eggs stored at different temperatures. In both cases, eggs stored at -3.3°C had significantly lower percentages of water-hardening than eggs stored at the other temperatures. After the second week, there were also significant differences in water-hardening between eggs stored in each medium. Eggs stored in FC plus mineral oil, FC plus Stoss's solution with 0.6 g pluronic, FC plus Stoss's solution and Stoss's solution had significantly higher water-hardening percentages than eggs stored in water and FC. After the third week of storage, all eggs that were stored at -3.3°C were unable to water-harden.

Eggs stored for four weeks at 1.0°C in FC plus Stoss's solution with 0.6 g of pluronic F-68 had significantly ($P < 0.0001$) higher water-hardening percentages than eggs stored in any other media. However, eggs stored for four weeks in FC plus Stoss's solution at 1.0°C or 3.0°C had 30.2% and 35.8% water-hardening, respectively, compared with 0% for the other treatments, except as mentioned above.

For the third trial, after two weeks of storage, eggs in Stoss's solution had significantly ($P < 0.0001$) higher water-hardening percentages than eggs stored in any other media (Table VIII). After three weeks, unfertilized eggs stored in FC plus Stoss's solution had a numerically higher percentage of water-hardening than eggs stored in the other media ($P=0.077$).

A water-hardening assessment and a fertility trial were conducted simultaneously to compare the two methods of assessment. The water-hardening method obtained higher viability results (Table VIII) than the fertility trial. The fertility results for this batch of eggs at Day 0 were already very low (Table III) and by Week 1, the fertility was zero for all

treatments.

2.3.4 Storage of Fertilized Non-Water-Hardened or Water-Hardened Eggs

Four experiments were conducted to select a medium and temperature that would enhance the viability of fertilized water-hardened or non-water-hardened eggs during storage. Unfortunately, there was no evidence of embryonic development after the stored eggs were incubated for twelve days. The fertility percentages of eggs on Day 0 for these trials are presented in Table III. The two lots of eggs purchased from the Cardigan Salmonid Enhancement Centre had lower Day 0 fertility than eggs purchased from other companies ($P< 0.01$).

2.4 Discussion

2.4.1 Verification of Water-hardening Detection

Water-hardening is a process that hardens the outer shell of fish eggs when they come in contact with water. Researchers have been able to use this physiological process to detect the viability of eggs after storage (Harvey and Ashwood-Smith, 1982). We were able to detect water-hardening in 85% of the eggs that we water-hardened for the trial compared with 1.8% for the eggs that were not water-hardened ($P < 0.001$) (Table IV). The results of the water-hardened eggs were comparable to those of Harvey and Ashwood-Smith (1982) who found that water-hardening did not occur in eggs stored in Fish Ringer solution but did occur when the eggs were transferred to fresh water although fertilization had not taken place (Harvey and Ashwood-Smith, 1982).

Since water-hardening of rainbow trout eggs can be detected to estimate their viability, it offers a simple option that can be used as a preliminary assessment before

conducting fertility trials. Water-hardening is an accurate test to determine if an egg has been damaged, but is not completely accurate to determine if eggs can still be fertilized. When we compared fertility results to water-hardening results, fertility levels were zero for each treatment and at each sampling time, but we were still able to detect water-hardening (Table VIII). Poor quality eggs at arrival may have played a role in the low fertility, but the results demonstrate that viability determined only by water-hardening assessment should be used mainly in preliminary trials to determine the most promising treatments to evaluate with fertility trials. Water-hardening takes less time and facilities than a fertility trial to assess eggs for viability after storage, but a fertility trial should be conducted to confirm fertilizability.

2.4.2 Selection of a Non-Water-Hardening Storage Medium for Unfertilized Eggs that Maximizes Fertility

The purpose of this experiment was to select a storage medium for unfertilized eggs that would not trigger water-hardening before fertilization but would promote maximal fertility. Harvey and Ashwood-Smith (1982) demonstrated that after five days of storage in ovarian fluid, egg fertility decreased, and since there is often a limited quantity of ovarian fluid, another medium capable of storing eggs for an extended period of time was needed. In this trial, we evaluated several media to determine if water-hardening would occur during storage and we also conducted a fertility trial. Water-hardening during storage would be detrimental to fertility if the micropyle was already closed before fertilization. The eggs that had been stored in Stoss's solution had a significantly lower ($P < 0.001$) water-hardening percentage than eggs stored in Fish Ringer solution or water but results for eggs in Stoss's

solution were not significantly different from those of eggs stored in D-532 (Table V). Eggs stored in water for 90 minutes had a significantly higher ($P < 0.001$) percentage of water-hardening than eggs stored in any of the other media but could not be expected to be fertilized because the micropyle was already closed.

Egg fertility decreased sharply 30 seconds after exposure to freshwater (Szöllösi and Billard, 1974), because water-hardening and the closure of the micropyle had begun. Eggs that were fertilized in Stoss's solution had a significantly higher fertility percentage ($P < 0.001$) than eggs fertilized in the other three media (Table V). The osmolality of ovarian fluid of rainbow trout is 292 mmol/kg of fluid (Lahnsteiner *et al.*, 1995). Stoss's solution had an osmolality of 323 mmol/kg which was the closest of the artificial media to that of ovarian fluid. The osmolality of D-532 was 402 mmol/kg. This was considerably higher than the osmolality of ovarian fluid and could have interfered with the fertilization of the eggs.

Baynes *et al.* (1981) found that diluents with a neutral pH inhibited the activation of sperm. This could have affected the fertilization potential of the sperm that fertilized the eggs stored in Fish Ringer solution because the pH was 7.5. According to Stoss and Donaldson (1983), the pH of Stoss's solution was adjusted to 8.4 using citric acid. Ovarian fluid has a pH of 8.4 which is the physiological pH at which fertilization usually occurs (Lahnsteiner *et al.*, 1995). Ginsburg (1972) chilled eggs in Fish Ringer solution or ovarian fluid to 0.4 to 1.0°C. Eggs were fertilized after four, eight, ten or fifteen days in storage. The fertility of the eggs stored in Fish Ringer decreased from 100% to 0 after twelve days, whereas the fertility of eggs stored in ovarian fluid decreased from 100% to 50%. It was not

mentioned if these differences were significant. In the present trial, when eggs were fertilized in Fish Ringer solution or ovarian fluid (Table V), there was no significant difference between the fertility percentages of the eggs. However, the eggs were stored for only 90 minutes at 7.0°C and a change was not detected until after four days of storage in Ginzburg's (1972) study.

Based on the results from the water-hardening and fertility trials, we used Stoss's solution in subsequent storage trials because eggs in this solution did not water-harden during storage and had high fertility after fertilization.

2.4.3 Storage of Unfertilized Eggs

The 2.0 g of pluronic F-68 per 25 ml of FC (80 mg/ml) was calculated from a study on the chilling of rainbow trout semen by McNiven *et al.* (1993). Thurston *et al.* (1993) used 40 mg of pluronic F-68 per ml of FC (1.0 g pluronic F-68/25 ml FC) for an emulsion that was used for the chilling of fresh turkey semen. Since these two sources used different levels, there is the possibility that there was an excess of pluronic F-68 in the medium using the method of McNiven *et al.* (1993). This could have caused a decrease in the viability of eggs in this medium. In two later experiments, reduced amounts of pluronic F-68 produced higher water-hardening percentages over a longer period of time (Tables VII and VIII). This result demonstrates that using less pluronic F-68 in the medium had a beneficial effect on the water-hardening percentages of eggs, and therefore, increased viability. In fact, there was no clear advantage to the addition of pluronic to the storage media (Tables VI, VII and VIII).

Eggs stored in mineral oil at 3.0°C had a significantly lower ($P < 0.05$) water-hardening percentage than eggs stored in FC plus mineral oil, water, Stoss's solution or FC

at the same temperature after 17 days of storage (Table VI). Mineral oil could have had a detrimental effect on the unfertilized eggs by hindering the exchange of gases. Eggs that were stored in mineral oil at 1.0°C would have had a slower rate of metabolism and waste production than the eggs at 3.0°C, thus accounting for the higher viability of eggs stored at 1.0°C. Mori *et al.* (1983), found that mineral oil, when used with sea water, reduced the hatching percentages of sea bass and Japanese parrotfish eggs.

After the third week of storage in the second trial (Table VII), all the eggs that were stored at -3.3°C were unable to water-harden. This was probably due to the fact that there was intracellular ice formation that caused freezing damage to the egg thus preventing water-hardening (Harvey and Ashwood-Smith, 1982).

Eggs stored in FC plus Stoss's solution at 1.0°C or 3.0°C or in FC plus Stoss's solution with 0.6 g of pluronic F-68 at 1.0°C were the only ones to water-harden after four weeks in storage (Table VII). The eggs stored at 1.0°C for four weeks in FC plus Stoss's solution with 0.6 g pluronic F-68 had a significantly higher ($P < 0.0001$) percentage of water-hardening than eggs stored in the other two media which had survival to four weeks. This could have been due to the emulsification of this medium where the FC molecules were distributed throughout the medium, which provided the eggs with greater access to oxygen via the FC, instead of having the eggs rest on the surface of the FC as they did in the FC plus Stoss's medium.

The eggs stored in FC plus Stoss's at 1.0°C or 3.0°C (Table VII) or in FC plus Stoss's solution with 0.6 g of Pluronic F-68 at 1.0°C could have survived because they were stored at suitable osmolality and oxygen was supplied by the FC.

In the third trial, after three weeks of storage, the eggs in FC plus Stoss's solution had a numerically higher viability than eggs in any of the other media (P=0.077), although the differences were not significant (Table VIII). An increased number of replicates might have allowed detection of significant differences. One gram of the surfactants Pluronic-F68 or lecithin per 25 ml of FC was used to correspond with the amount of Pluronic F-68 recommended by Thurston *et al.* (1993). The emulsification of FC and Stoss's with 1.0 g of lecithin per 25 ml FC also resulted in eggs that water-hardened after the third week of storage but the combination of FC plus Stoss's solution with no surfactant provided a higher percentage of water-hardened eggs (Table VIII). In our study, FC provided oxygen and Stoss's solution provided the appropriate conditions (pH, osmolality, etc.) needed by the egg. There was no consistent beneficial effect on egg viabililty from the addition of the surfactants pluronic F-68 or lecithin.

The failure of the fertility trial was probably due to the poor quality eggs as evidenced by the low Day 0 fertility values (Table III). All the egg lots obtained from the Cardigan Salmonid Enhancement Centre were of poor quality, but unfortunately, this was not detectable until after fertility trials were conducted.

2.4.4 Storage of Fertilized Non-Water-Hardened Eggs or Water-Hardened Eggs

The presence of an embryo could not be detected for any medium at either temperature, in any week for fertilized eggs, either water-hardened or non-water-hardened.

Beacham and Murray (1987) and Sasaki *et al.* (1988) found that the amount of damage that occurred after low temperature incubation depended on the stage of development of the fertilized eggs. Beacham and Murray (1987) decreased the incubation

temperature for fertilized eggs of pink and chum salmon, to 2°C from 8°C. They found that at 2°C, the fertilized eggs, which had been water-hardened for two hours, showed substantial embryo mortality compared to those at 8°C. Sasaki *et al.* (1988) used red sea bream and multicolorfin rainbowfish to determine the chilling sensitivity of fertilized eggs at different stages of development. The normal incubation temperatures for eggs of these species are 17.0°C and 27.0°C, respectively. The eggs of these fish were held in 4°C water for 30 minutes. They found that the earlier the stage of development of the eggs when chilled, the less chance they had for survival. The cleavage stage, which starts 30 seconds after fertilization, was the most sensitive to low temperatures. This could have been the cause of the failure of fertilized eggs to develop in our trials.

2.5 Conclusions

The water-hardening assessment of viability is a useful tool to detect storage damage as a preliminary test of media for egg preservation. However, results of viability determined only by water-hardening assessment should be used cautiously. Water-hardening takes less time and facilities than a fertility trial to assess eggs for viability after storage, but a fertility trial should be conducted to confirm fertilizability. We were unable to draw conclusions from a comparison of viability by water-hardening assessment or fertility trial because of the poor quality of the unfertilized eggs upon arrival.

The unfertilized eggs that had been fertilized in Stoss's solution had significantly higher ($P < 0.01$) fertility than eggs fertilized in the other media. Also, eggs stored in Stoss's solution for 90 minutes had a low water-hardening percentage. The ideal storage medium for eggs should not cause water-hardening, so that after storage the sperm can still penetrate

and fertilize the eggs. Because Stoss's solution met these requirements for a storage medium, it was selected as a storage medium for further trials.

The water-hardening results from these experiments suggest that it is possible to store unfertilized eggs for up to four weeks at 1.0 or 3.0°C. Fluorocarbon plus Stoss's solution was the most promising medium to maintain high water-hardening percentages (or viability) in stored eggs. There was no consistent beneficial effect on egg viability from the addition of surfactants to the media containing FC. Unfertilized eggs stored at -3.3°C perished after three weeks. This was probably due to intracellular freezing which caused damage to the eggs and they failed to survive (Harvey and Ashwood-Smith, 1982).

The storage of fertilized water-hardened eggs and non-water-hardened eggs was unsuccessful in each medium and at each temperature. The early stage of embryonic development when they were stored probably increased the sensitivity of the eggs to damage during storage.

There was success in maintaining the viability, as measured by water-hardening capacity of unfertilized eggs for four weeks. If future fertility results using these media indicate a similar duration of viability, then the reproductive season of rainbow trout could be extended, but this needs to be confirmed by further research. This technology could give producers a wider time frame in which to fertilize eggs, as well as facilitating the transport of eggs. The use of fluorocarbons could help achieve the goal of delaying the time of fertilization (and hatching) by delivering oxygen to unfertilized eggs that are kept at low temperatures, thus maintaining their viability.

2.6. Tables

Table I. Sources of unfertilized eggs and semen

Experiment	Source of Eggs and Semen
Water hardening detection	Troutlodge Inc.,WA[1]
Medium for storage with low water-hardening	Troutlodge Inc., WA
Medium for storage with high fertility	Troutlodge Inc., WA
Unfertilized eggs stored for 17 days	Troutlodge Inc., WA
Unfertilized eggs stored for 4 weeks	Pisciculture des Alleghanys Inc., PQ[1]
Unfertilized eggs stored for 3 weeks	Cardigan Salmonid Enhancement Centre, PE[2]
Storage of fertilized non-water-hardened eggs	Pisciculture des Alleghanys Inc., PQ
Storage of fertilized non-water-hardened eggs	Cardigan Salmonid Enhancement Centre, PE
Storage of fertilized water-hardened eggs	Pisciculture des Alleghanys Inc., PQ
Storage of fertilized water-hardened eggs	Cardigan Salmonid Enhancement Centre, PE

[1]Air shipment
[2]Ground transport

Table II. List of components , pH and osmolality of storage media.

Component	Stoss[1]	Fish Ringer[2]	D-532[3]
NaCl (M)	0.1454	0.1112	0.154
KCl (M)	0.0034	0.0034	-
$CaCl_2*2H_2O$ (M)	0.003	0.002	-
$MgSO_4$ (M)	0.0005	-	-
Tris (M)	0.02	-	-
Bovine Serum Albumin	10 mg/ml of solution	-	-
Glycine (M)	-	-	0.025
$NaHCO_3$ (M)	-	0.0024	0.05
Citric Acid	until pH 8.4	-	-
0.2 M Tris	-	-	until pH 9.0
pH	8.4	7.5	9
Osmolality[4]	323	241	402

[1]Stoss (Stoss and Donaldson, 1983)
[2]Fish Ringer (Harvey and Ashwood-Smith, 1982)
[3]D-532 (Billard, 1977)
[4]measured by a Micro Osmometer (Precision Systems, Inc., Natick, MA)

Table III. Results for mean viability assessed by water-hardening and mean fertility percentages for the Day 0 eggs in each trial.

Experiment	Viability (%)	Fertility (%)
Unfertilized eggs stored for 17 days	96.9[a]	
Unfertilized eggs stored for 4 weeks	86.4[b]	
Unfertilized eggs stored for 3 weeks	81.4[b]	29.5[b]
Storage of fertilized non-water-hardened eggs		86.6[a]
Storage of fertilized non-water-hardened eggs		44.2[b]
Storage of fertilized water-hardened eggs		82.9[a]
Storage of fertilized water-hardened eggs		42.5[b]
P value	0.0119	0.0021
(SEM[1])	(0.04)	(0.06)

[a,b]Means within column without a common superscript are significantly different ($P < 0.05$)
[1]standard error of the mean

Table IV. Water-hardening detection when eggs were placed in water or ovarian fluid (n=3).

Medium	Mean Water-hardening (%)
Water	85.1[a]
Ovarian fluid	1.8[b]
P value	0.0001
(SEM[1])	(0.51)

[a,b]Means without a common superscript are significantly different ($P < 0.05$)
[1]standard error of the mean

Table V. Water-hardening percentage after eggs had been placed in storage media for 90 minutes (n=3) and fertility percentage after eggs were fertilized in one of the storage media, incubated for 12 days, then observed for the presence of an embryo (n=3).

Medium	Mean Water-hardening (%)	Mean Fertility (%)
Stoss's solution[1]	2.0^{c}	66.8^{a}
Fish Ringer[2]	5.2^{b}	35.9^{b}
D-532[3]	2.6^{bc}	49.6^{b}
Ovarian fluid	-	38.2^{b}
Water	88.3^{a}	0.0^{c}
P value	0.0001	0.002
(SEM[4])	(0.91)	(0.30)

[a,b,c] Means in column without common superscripts are significantly different ($P < 0.05$)
[1]Stoss's solution (Stoss and Donaldson, 1983)
[2]Fish Ringer (Harvey and Ashwood-Smith, 1982)
[3]D-532 (Billard, 1977)
[4]standard error of the mean

Table VI. Mean percentages of unfertilized eggs that water-hardened after storage at one of two temperatures and in one of six media, as a percentage of Day 0 values (n=3).

	Day 4	Day 7	Day 11	Day 17
Temperature (± SE)				
1.0 ± 0.3 °C	89.2[b]	82.8	57.6	70.6
3.0 ± 0.2 °C	93.8[a]	80.4	64.9	68.5
Medium				
Fluorocarbon (FC)	93.9[ab]	79.7[a]	75.7[ab]	88.0[ab]
FC + Mineral oil	92.7[ab]	93.9[a]	58.7[b]	93.0[a]
FC + Stoss's solution[1] (2.0 g pluronic)	77.6[c]	52.6[b]	0.0[c]	0.0[d]
Stoss's solution	97.4[a]	91.2[a]	92.7[a]	93.6[a]
Mineral oil	97.0[a]	86.6[a]	70.3[b]	72.9[bc]
Water	90.4[b]	85.6[a]	70.3[b]	69.9[c]
Temperature X Medium				
1.0 FC	95.0[ab]	88.2	77.3	83.3[a]
1.0 FC + Mineral oil	90.5[ab]	91.6	39.7	98.7[a]
1.0 FC + Stoss's solution (2.0 g pluronic)	68.0[c]	49.0	0.0	0.0[c]
1.0 Stoss's solution	98.7[a]	92.9	95.2	93.8[a]
1.0 Mineral oil	97.7[a]	92.1	72.7	90.8[a]
1.0 Water	85.9[b]	83.2	61.0	57.3[b]
3.0 FC	93.3[ab]	71.2	74.1	92.7[a]
3.0 FC + Mineral oil	94.9[ab]	96.3	77.7	87.3[a]
3.0 F C + Stoss's solution[1] (2.0 g	87.3[b]	56.2	0.0	0.0[c]
3.0 Stoss's solution	95.3[ab]	89.5	90.2	93.4[a]
3.0 Mineral oil	97.0[a]	81.1	67.9	55.1[b]
3.0 Water	94.9[ab]	88.0	80.0	82.5[a]
P value & (SEM)				
Temperature	0.0132	0.6234	0.1532	0.6684
	(1.20)	(3.51)	(3.50)	(3.46)
Medium	0.00001	0.00001	0.00001	0.00001
	(2.07)	(6.08)	(6.05)	(0.99)
Temp X Medium	0.00001	0.6638	0.1023	0.0332
	(2.93)	(8.60)	(8.56)	(8.47)

[a,b,c,d] Means within section in a column without a common superscript are significantly different ($P < 0.05$)

[1]Stoss's solution (Stoss and Donaldson, 1983)

Table VII. Mean percentages of unfertilized eggs that water-hardened after storage at one of three temperatures in one of eight media, as a percentage of Day 0 values (n=2).

Temperature ± SEM	Week 1	Week 2	Week 3	Week 4
1.0 ± 0.3°C	87.0[a]	78.4[a]	36.9[a]	10.4[a]
3.0 ± 0.2°C	96.4[a]	80.0[a]	41.9[a]	4.5[b]
-3.3 ± 1.5°C	16.0[b]	20.8[b]	0.0[b]	0.0[c]
Medium				
Fluorocarbon (FC)	42.9	21.1[b]	0.0[c]	0.0[c]
FC + Mineral oil	80.6	73.4[a]	15.4[bc]	0.0[c]
FC + Stoss's solution[1] (1.2 g pluronic)	62.4	60.2[ab]	43.3[a]	0.0[c]
FC + Stoss's solution (0.6 g pluronic)	66.2	63.5[a]	56.6[a]	17.6[b]
FC + Stoss's solution	78.8	85.8[a]	56.7[a]	22.0[a]
Stoss's solution	65.4	76.9[a]	24.2[b]	0.0[c]
Mineral oil	70.6	61.1[ab]	8.4[bc]	0.0[c]
Water	65.0	35.8[b]	4.8[c]	0.0[c]
Temp X Medium				
1.0 FC	45.4	19.3	0.0[c]	0.0[d]
1.0 FC + Mineral oil	86.6	58.0	0.0[c]	0.0[d]
1.0 FC + Stoss's solution (1.2 g pluronic)	94.1	85.0	49.6[b]	0.0[d]
1.0 FC + Stoss's solution (0.6 g pluronic)	100.5	101.3	87.0[a]	52.7[a]
1.0 FC + Stoss's solution	102.0	100.7	82.3[a]	30.2[c]
1.0 Stoss's solution	95.5	92.8	40.8[b]	0.0[d]
1.0 Mineral oil	79.0	89.2	19.4[bc]	0.0[d]
1.0 Water	92.8	80.6	14.5[c]	0.0[d]
3.0 FC	83.2	43.9	0.0[c]	0.0[d]
3.0 FC + Mineral oil	108.4	91.8	46.3[b]	0.0[d]
3.0 FC + Stoss's solution (1.2 g pluronic)	93.1	95.6	80.4[a]	0.0[d]
3.0 FC + Stoss's solution (0.6 g pluronic)	98.1	89.2	82.8[a]	0.0[d]
3.0 FC + Stoss's solution	103.1	96.5	87.8[a]	35.8[b]
3.0 Stoss's solution	100.6	101.7	31.8[b]	0.0[d]
3.0 Mineral oil	87.9	94.3	5.8[c]	0.0[d]
3.0 Water	102.4	26.7	0.0[c]	0.0[d]
-3.3 FC	0.0	0.0	0.0[c]	00[d]
-3.3 FC + Mineral oil	46.8	70.3	0.0[c]	0.0[d]
-3.3 FC + Stoss's solution (1.2 g pluronic)	0.0	0.0	0.0[c]	0.0[d]
-3.3 FC + Stoss's solution (0.6 g pluronic)	0.0	0.0	0.0[c]	0.0[d]
-3.3 FC + Stoss's solution	36.2	60.3	0.0[c]	0.0[d]
-3.3 Stoss's solution	0.0	36.2	0.0[c]	0.0[d]
-3.3 Mineral oil	44.8	0.0	0.0[c]	0.0[d]
-3.3 Water	0.0	0.0	0.0[c]	0.0[d]
P value & (SEM)				
Temperature	0.00001 (5.81)	0.00001 (5.75)	0.00001 (3.70)	0.00001 (0.35)
Medium	0.2148 (9.49)	0.001 (9.39)	0.00001 (6.05)	0.00001 (0.53)
Temp X Medium	0.5585 (16.43)	0.0609 (16.26)	0.00001 (10.47)	0.00001 (1.00)

[a,b,c]Means within section without a common subscript are significantly different ($P < 0.05$)

[1]Stoss's solution (Stoss and Donaldson, 1983)

Table VIII. Mean percentages of unfertilized eggs that water-hardened after storage at 1.0 ± 0.3°C (SE) in one of five media, as a percentage of Day 0 values (n=2).

Medium	Week 1	Week 2	Week 3
Fluorocarbon (FC)	4.7	0.0[c]	0.0
FC + Stoss's solution[1] (1.0 g pluronic)	14.0	49.1[b]	0.0
FC + Stoss's solution (1.0 g of lecithin)	17.3	4.3[c]	23.0
FC + Stoss's solution	57.6	49.9[b]	60.9
Stoss's solution	76.0	66.1[a]	12.0
P value	0.0721	0.00001	0.0776
(SEM[2])	(15.10)	(3.84)	(12.50)

[a,b,c]Means in each column without a common superscript are significantly different ($P < 0.05$)

[1]Stoss's solution (Stoss and Donaldson, 1983)

[2]standard error of the mean

III. PRESERVATION OF RAINBOW TROUT EYED EGGS

3.1 Introduction

There are many reasons why the aquaculture industry would benefit if eyed eggs could be preserved for an extended period of time. Advantages would include increasing the length of time for marketing eyed eggs, reducing the effects of seasonality, and increasing the viability of shipped eggs, especially if a shipment is prolonged. Reproductive seasonality occurs in salmonids which means that spawning occurs only once per year (Schreck *et al.*, 1995). Spawning once per year also creates the problem of having all stock of the same approximate age in the hatchery. Equipment failure in the hatchery could lead to a large loss of offspring, leaving the hatchery without means to produce more.

Freezing unfertilized eggs, fertilized eggs and eyed eggs of fish is difficult due to the fact that the eggs of most fish species are 1) relatively large, 2) contain a large amount of yolk, 3) have many layers within the egg, the layers being the chorion (two layers), perivitelline space, yolk, and vitelline membrane (Figure 1, Gordon *et al.*, 1987), and 4) have low permeability (Harvey and Ashwood-Smith, 1982; Zhang *et al.*, 1993). Cryoprotectants help prevent ice formation (Horton and Ott, 1976) and the previously mentioned factors all hinder the permeability of cryoprotectants into the egg.

The freezing point of a rainbow trout egg is -1.7°C. As mentioned previously, researchers have used supercooling to preserve the eggs of rainbow trout and other fish species below this temperature. Another method that has been used to extend the life of eggs is chilling. This is used to slow down the process of development of fertilized eggs without freezing (Maddock, 1974). Chilling has been used for preservation of fertilized brown trout

eggs by Maddock (1974) with considerable success. A temperature-controlled recirculation system was used to store the eggs of brown trout that were 91 degree days at the beginning of the experiment (degree days are the number of days from fertilization multiplied by the temperature (°C) of the water in which they are incubated). This method used physical means, via a recirculation system, to deliver oxygen and eliminate the metabolic wastes of eyed eggs (Maddock, 1974). The temperature of the water was 7.6°C for 12 days and was then reduced to 1.4°C for 63 days (Group A) and finally increased to 10°C for 16 days until hatch. Groups B and C remained in 1.4°C water for 123 days and then the temperature was raised in two 24 hour stages to 9.0°C and the eggs hatched 3 days later. The hatching percentages for groups A, B and C were 90, 95 and 95, respectively (Maddock, 1974). This method extended the normal available period of brown trout fry from four months to eight months.

Other researchers have used a chemical called fluorocarbon (FC) to deliver oxygen and transport wastes away from animal and plant cells in culture (Hamamoto, 1987; Thurston *et al.*, 1993; McNiven *et al.*, 1993; Anthony, 1997). Fluorocarbons are liquids that are perfluorinated carbon compounds which are produced by replacing the hydrogen atoms of the hydrocarbons with fluorine atoms. Fluorocarbons dissolve and transport large quantities of oxygen and other gases, are stable and non-toxic, and release oxygen at a greater rate than hemoglobin (Reiss, 1994). Gas solubility increases as temperature decreases (Reiss, 1994) which makes it even more attractive for supplying oxygen to gametes at low temperatures.

Using FC to deliver oxygen can eliminate the mechanical damage to cells from conventional aeration (Lowe *et al.*, 1997b). Lowe *et al.* (1997b) found that oxygenated FC

enabled mouse hybridoma cells to reach the maximum cell density. This technique was not superior to the aeration method, but made it possible to culture delicate cells that would suffer serious damage from aeration (Hamamoto *et al.*, 1987). Anthony *et al.* (1997) thawed cryopreserved rice cells and put them into a culture medium containing oxygenated FC. The fresh weight gain after 30 days in the FC culture medium was significantly ($P < 0.05$) higher than for the control (Anthony *et al.*, 1997).

Semen from various species has also been preserved using oxygenated FC. The storage life of turkey and rainbow trout semen was prolonged using FC and extenders (Thurston *et al.*, 1993; McNiven *et al.*, 1993; Thurston *et al.*, 1994). The eggs of turkeys that were fertilized with semen stored in a FC diluent emulsion for three hours had significantly higher fertility and hatching percentages than eggs fertilized with semen stored for three hours in diluent only (Thurston *et al.*, 1993). Rainbow trout semen that had been stored in FC in a moisture saturated atmosphere had 81.1% fertility after 37 days compared with 0% fertility for semen stored without FC (McNiven *et al.*, 1993).

The solubility of oxygen (O_2) in FC (FC-77) is 56 ml of O_2/100 ml of FC while the solubility of O_2 in water is only 3.2 ml of O_2/100 ml of water (3M Laboratory Materials, St. Paul, MN). Oxygen is needed by both unfertilized and fertilized eggs. Unfertilized and non-water-hardened fertilized eggs consume 5.4×10^{-2} μl of O_2/hour/egg at 10°C (Boulekbache *et al.*, 1969). After fertilization and activation, there is a 50% increase in O_2 consumption by rainbow trout eggs. During gastrulation, O_2 consumption increases from 10×10^{-2} μl of O_2/hour/egg to 30×10^{-2} μl of O_2/hour/egg. At the end of epiboly (blastopore closure, or end of gastrulation), O_2 consumption increases to 1.2 μl/hour/egg (Boulekbache *et al.*, 1969).

The objective of these trials was to maintain the viability of eyed rainbow trout eggs using fluorocarbon as an oxygen carrier during storage.

3.2 Materials and Methods

3.2.1 Eyed Egg Collection

All eggs used in the following five trials were shipped at separate times in plastic bags filled with water on ice. The eggs were at approximately 0°C so further development during shipping was minimal. Eggs at stages of development of 147, 200 and 217 degree days (Appendix A, Table 1) at the start of each trial were obtained from Troutlodge, Inc., Sumner, WA, USA where the eggs were treated with peroxide (200-250 ppm) for five minutes immediately after fertilization to control fungal growth. Eyed eggs at 161 degree days were obtained from the Cardigan Salmonid Enhancement Centre. These eggs had been fertilized, placed in water and three hours later were treated with ovadine at 100 ppm for five minutes to control fungal growth. Eyed eggs at 180 degree days were shipped by air from Pisciculture Alleghanys Inc. in Quebec to the laboratory in Charlottetown. These eggs were not treated to reduce fungal growth.

3.2.2 Preservation Experiments

There were six media used for each of the five trials: 1) FC (Fluorinert-77, 3M, St. Paul, MN, USA), 2) FC plus 0.3 M glucose (osmolality 423 mmol/kg) (1:1) (Sigma, St. Louis, MO, USA), 3) FC plus phosphate-buffered saline (PBS) (osmolality 332 mmol/kg) (1:1), 4) FC plus mineral oil (1:1) (Rhone Merieux, Victoriaville, PQ, Canada), 5) FC plus water (1:1) and 6) water (osmolality 5 mmol/kg).

Before eggs were allocated to media, all opaque (dead) eyed eggs were removed.

Two liters of FC was oxygenated with 100% oxygen for 20 minutes using diffusion stones. Lots of 100 eggs with two replicates per treatment and sampling time (three replicates for 217 degree days) were stored in 50 ml of medium. All the trials had sampling times at three weeks and five weeks in order to assess the viability of the eggs during storage.

In each experiment, the 190 ml plastic containers which held the eggs were placed in styrofoam boxes filled with ice and placed in a 3°C cool room. The shelves in the cool room were enclosed in plastic and two aquarium pumps circulated air through water to maintain a 98% moisture-saturated atmosphere (CR10 data logger, HMP35C relative humidity sensor, Campbell Scientific, Edmonton, AB, Canada). Temperature was monitored periodically by a type-T thermocouple wire attached to a Rustrak Ranger II data logger (Gulton-Rustrak, East Greenwich, RI, USA). The temperature of the media in the storage containers in ice in the cold room was 1.0°C.

Treatments in all trials were oxygenated for a minimum of two minutes per week for the duration of each trial. At the end of the storage period, containers were removed and placed in a 7°C water bath for transport to the incubator room. Each lot of eggs was gently poured onto a fine net to remove the medium and then randomly placed in a compartment of a Heath incubator in which the water temperature was maintained at 10°C. The incubator trays each had twelve compartments that were separated by plastic dividers sealed with silicone. The eggs remained in the incubator until hatch. The numbers that hatched from each treatment were used to calculate the hatching percentages and the percentages of normal alevins (sac-fry) produced. For each trial, the hatching percentage of eyed eggs in each medium was calculated as a percentage of eggs that hatched from eggs incubated on Day 0.

The percentage of normal alevins that hatched was calculated as a percentage of hatched eggs. Deformed alevins were detected visually upon hatch; they had crooked backs, two heads, two bodies or two yolk sacs. The alevins were euthanized by placing them for one hour in 2.5 L of water containing 1 ml of benzocaine.

3.2.3 Statistical Analysis

The Day 0 hatching percentages [(no. of hatched/no. of eyed eggs)100] for all trials (Table IX) were analyzed by the Statistical Analysis System (SAS, 1995) using a one-way analysis of variance with experiment as the main effect. Percentages were transformed into arcsine square root for analysis. For eyed egg trials using 161, 180, and 217 degree day old eggs, the hatching percentage results were analyzed using the Statistical Analysis System (SAS, 1995) in a two-way analysis of variance with media and stage of development of the eyed eggs as the main effects. The percentage of normal alevins were analyzed using the Statistical Analysis System (SAS, 1995) in a one-way analysis of variance with media as the main effect. For eggs stored at 147 degree days the hatching percentages and percentages of normal alevins were analyzed using the Statistical Analysis System (SAS, 1995) in a one-way analysis of variance with media as main effect. Significance for all statistical analysis was determined at the $P < 0.05$ level.

3.3 Results

Eyed eggs at 200 degree days were of poor quality after a delay in shipping and resulted in low hatchability (Table IX). As a result of low hatching rates in this trial (most of the stored eggs in this experiment had a 0% hatching rate) they are not included in the results.

All eyed eggs were stored at 1.0 ± 0.3 °C (SEM). Table IX contains the percentages of hatching and the percentages of normal alevins when incubated on Day 0. Eyed eggs incubated at 200 degree days had significantly lower ($P < 0.05$) hatching percentages and significantly lower percentages of normal alevins ($P < 0.05$) that hatched than eyed eggs incubated at the other stages of development, which were not different from each other.

Table X illustrates the hatching percentage of the eyed eggs after storage for three weeks. There was an interaction between treatment and stage of development for hatching percentages after three weeks of storage. Thus the hatching percentages of eggs in specific media depended upon the stage when they were put into storage. Except for the poor results of the eggs stored in water alone, there were no great differences in the hatching percentages of eggs stored in any medium or at any stage of development with the range of hatching from 75 to 106 % of Day 0 values.

For the percentages of normal alevins, in instances where eggs did not hatch and therefore, there was no result, the percentages of normal alevins were assigned missing values. Since the incidence of eggs not hatching was high in some media, many treatments were assigned missing values and interaction could not be calculated. After three weeks, eyed eggs stored at 161 degree days in water alone had a significantly lower percentage of ($P < 0.05$) normal alevins hatching than eggs stored in any of the other media except for eggs stored in FC plus glucose (Table XI). There was no significant difference in the percentage of normal alevins for the eyed eggs stored at 180 or 217 degree days.

After five weeks of storage, interaction was also apparent in the hatching percentages. Eyed eggs stored at 161 degree days in FC plus PBS or FC plus water and eggs stored at 217

degree days in FC or FC plus water had significantly higher ($P < 0.05$) hatching percentages than eggs stored in any of the other treatments (Table XII). Eggs stored at 161 degree days in FC plus water had significantly higher ($P < 0.05$) percentages of normal alevins hatching than eggs in FC plus PBS (Table XIII). Eggs stored at 217 degree days in FC plus glucose had significantly lower ($P < 0.05$) percentages of normal alevins than eggs in any other media while eggs stored in FC plus mineral oil or in water did not hatch. There were no significant difference in the percentage of normal alevins for the eggs stored in any treatment at 180 degree days.

The eggs obtained when they were 147 degree days were still at the tender stage of development rather than at the eyed stage. Because these eggs were not at a similar stage of development as the other eggs, the results (Table XIV) are shown separately from the others (Tables X, XI, XII and XIII). The eggs stored at 147 degree days for 3 weeks in FC or in FC plus PBS had a significantly higher hatching percentage ($P < 0.05$) than the eggs in the other treatments (Table XIV). There were no significant differences in the percentages of normal alevins that hatched in this trial according to treatment. After 5 weeks of storage (Table XIV) eggs in FC had a hatching percentage of 4.3 while no eggs hatched after storage in the other media.

3.4 Discussion

The reason for the low hatching percentages of eggs stored in water may have been because the oxygen carrying capacity of the water was too low to meet the requirements of the eggs since eggs stored in FC and water had a high percentage of hatching (Tables X and XII). Oxygen consumption of eggs increases as embryonic development progresses

(Boulekbache *et al.*, 1969) and the addition of FC to the water was able to provide enough oxygen to enable the embryos to survive. After five weeks of storage, eyed eggs stored at 161 degree days in FC plus water, or in FC plus PBS, or at 217 degree days in FC plus water had hatching percentages greater than 60% (Table XII). Aver'yanova *et al.* (1983) found that eggs at the eyed stage of development suffered no adverse effects when stored in aerated water for seven days. Also, Maddock (1974) found similar results when chilling eyed eggs in aerated water for 123 days.

The osmolality of glucose and PBS solutions was over 300, whereas water had an osmolality of 5. This could have interfered with the osmoregulation of the eggs stored in FC plus glucose or FC plus PBS, and adversely affected hatching percentages. It has been shown that an osmolality of 300 can be detrimental to fertilized eggs of the fathead minnow (Cloud *et al.*, 1988). The high osmolality of the storage media may have had less effect on the more advanced 217 degree day embryos, resulting in a high hatching percentage of 217 degree day embryos stored in FC plus PBS or in FC plus glucose for three weeks (Table X). In contrast, eyed eggs stored at 161 degree days in FC plus PBS had a relatively high hatching percentage after five weeks of storage (Table XII).

After three weeks storage in FC plus mineral oil, the hatching percentages of 161 and 180 degree day groups were not significantly lower than those of eggs stored at the same stage in the other FC media (Table X). However, 217 degree day eggs stored in FC plus mineral oil had a lower hatching percentage than eggs stored in the other FC media, except for eggs stored in FC plus glucose. This is in agreement with a study that demonstrated that when cod eggs were exposed to crude oil (from fertilization to 20 days after fertilization),

there were no adverse effects on the eggs or the hatching percentages (Mangor-Jensen and Fyhn, 1985). However, Mori *et al.*, (1983) found that mineral oil reduced the hatching rates in eggs of the Japanese parrotfish and sea bass. In our experiment when eggs were stored in mineral oil at a later stage, the hatching percentage significantly decreased ($P < 0.01$). Mineral oil could have inhibited the exchange of oxygen and waste gases between the FC and the eggs. Oxygen consumption increases as embryonic development progresses (Boulekbache *et al.*, 1969) and the reduced amount of gases exchanged between eggs immersed in mineral oil and the FC may have reduced survival for eggs in later developmental stages or in eggs stored for five weeks.

The hatching percentages after three weeks of storage (Table X) in this study were similar to those obtained by Maddock (1974) . He used a recirculation system with aerated hatchery water in which brown trout eggs at 91 degree days were kept at 1.4°C for 63 days or 123 days, at which time the eggs were placed in water at 10°C or 9°C, respectively, until hatch. Total incubation days for the groups were 90 and 139 days and the hatching rates were 90 and 95%, respectively. In Maddock's experiment, incubation time was extended by the low temperature and the oxygen delivery to the eggs via the aerated water. In the present study, incubation of eyed eggs was extended using a low temperature and FC to deliver oxygen. Few eggs stored for three or five weeks without FC hatched (Tables X and XII). Eggs stored for three weeks at 217 degree days in FC plus water, FC plus PBS or in FC alone had significantly higher ($P < 0.001$) hatching percentages than eggs stored in water or FC plus mineral oil (Table X), emphasizing the necessity of optimal gaseous exchange at this stage. The eggs stored for five weeks at 161 degree days in FC plus PBS and FC plus water

or eggs stored at 217 degree days in FC plus water or FC alone had significantly higher ($P < 0.005$) hatching percentages than eggs stored in any other media or at 180 degree days (Table XII).

The difference between each trial within this study was the stage of development of the eyed eggs when put into storage and also the source from which the eyed eggs were obtained. Researchers in the past have suggested that tolerance to chilling increases with progressive embryonic development (Roubaud *et al.*, 1985; Calvi and Maisse, 1998; Dinnyés *et al.*, 1998). Dinnyés *et al.* (1998) stored 86,000 morulae (Appendix A, Table 1), half-epiboly (blastopore closure) (Appendix A, Figure 3, Piper *et al.*, 1982), and heartbeat-stage eyed eggs of carp at 0, 4 or 24°C for five minutes or one hour. The fertilized eggs were then put in a 24°C water bath to assess the hatching rates. The results suggested that as eggs develop, they are less sensitive to chilling. Dinnyés *et al.* (1998) felt that these findings may be related to changes in cell and tissue types, number of cells, effectiveness of repair mechanisms and enzymatic reactions. Changes in the size and structure of the yolk compartment and membrane structures might be among the key factors involved.

The age of the eyed eggs obtained for these experiments was at the discretion of the company from which they were obtained. Because of time and space constraints, eggs at 147 degree days (Table XIV) were used although they were not at the eyed stage (Gordon *et al.*, 1987). These eggs were in the tender stage and were sensitive to handling, although the hatching percentage of these eggs incubated on Day 0 was high. Although the eggs stored in FC or FC plus PBS for three weeks did have high hatching percentage, after five weeks, the only eggs to survive were in FC and the hatching percentage was low (Table XIV).

These results support other findings that earlier stages of embryonic development are more sensitive to chilling than the later stages.

For these trials, a low hatching percentage was often accompanied by an increase in abnormalities. If the embryos have developed abnormally, then the probability is increased that they will not hatch at all.

3.5 Conclusions

The use of FC plus water at 1.0°C could benefit the aquaculture industry by extending the season of eyed eggs by five weeks, thus giving a longer opportunity to market these eggs. Overall, eggs in FC plus water resulted in the highest hatching percentages with high percentages of normal alevins. Fluorocarbon could also be used in other species of salmonids, especially those that are not responsive to the manipulation of photoperiod to extend spawning seasons. Fluorocarbon could also be used when shipping eyed eggs to aquaculture facilities, because the oxygen that is soluble in FC could enhance the viability of eyed eggs that arrive at the destination. We have demonstrated that oxygenated FC can be used to increase the storage time of rainbow trout eyed eggs.

3.6 Tables

Table IX. Mean hatching percentages of eggs incubated on Day 0 at different stages of development, and the mean percentages of normal alevins hatched from these eggs.

Stages	Hatching percentage[1]	Percentage of Normal Alevins[2]
147 degree days (n=2)	91.0[a]	85.3[a]
161 degree days (n=2)	92.3[a]	85.6[a]
180 degree days (n=2)	95.1[a]	91.7[a]
200 degree days (n=2)	57.1[b]	36.9[b]
217 degree days (n=3)	84.9[a]	74.4[a]
P value & (SEM[3])		
Experiment	0.0145	0.0316
	(0.09)	(0.07)

[a,b] Means within each column with same superscripts are not significantly different ($P < 0.05$)

[1](no. of hatched alevins/no. of eyed eggs)100

[2][(no. of hatched alevins - no. of deformed alevins)/no. of hatched eggs]100

[3]standard error of the mean

Table X. Mean hatching percentages of eyed eggs stored at different stages of development for 3 weeks, as a percentage of Day 0 results.

Treatments	161 degree days	180 degree days	217 degree days
FC[1]	80.0^{ef}	95.2^{abcd}	106.4^{a}
FC + PBS[2]	85.8^{bcdef}	82.9^{cdef}	105.6^{a}
FC + glucose (0.3M)	82.7^{cdef}	75.1^{f}	101.3^{ab}
FC + mineral oil	96.5^{abc}	82.0^{def}	92.2^{bcde}
FC + water	101.3^{ab}	90.2^{bcde}	106.1^{a}
water	14.3^{g}	0.0^{g}	0.0^{g}
P value & (SEM[3])			
Stage X Treatment		0.0081	
		(6.88)	

[a,b,c,d,e,f,g] Means with same superscripts are not significantly different ($P < 0.05$)

hatching percentage = [(no. of hatched alevins/no. of eyed eggs)/hatching rate (%) at Day 0]100

[1]Fluorocarbon

[2]Phosphate buffered saline

[3]standard error of the mean

Table XI. Mean percentages of normal alevins that hatched from the eggs stored for 3 weeks.

Treatment	161 degree days	180 degree days	217 degree days
FC[1]	83.3[ab]	77.8	93.6
FC + PBS[2]	67.4[ab]	65.2	82.0
FC + glucose (0.3M)	57.7[bc]	65.6	96.6
FC + mineral oil	84.9[ab]	69.1	91.7
FC + water	93.4[a]	82.1	94.0
water	28.6[c]	-	-
P value & (SEM[3])			
Treatment	0.0425	0.3015	0.071
	(14.90)	(6.00)	(3.20)

[a,b,c] Means within each column with same superscripts are not significantly different ($P < 0.05$)
percentage of normal alevins = [(no. of hatched alevins - no. of deformed alevins)/no. of hatched eggs]100
[1]Fluorocarbon
[2]Phosphate buffered saline
[3]standard error of the mean

Table XII. Mean hatching percentages of eyed eggs stored at different stages of development for 5 weeks, as a percentage of Day 0 results.

Treatment	161 degree days	180 degree days	217 degree days
FC[1]	0.0[b]	20.2[b]	50.2[a]
FC + PBS[2]	65.4[a]	2.0[b]	15.0[b]
FC + glucose	0.0[b]	0.0[b]	2.1b[b]
FC + mineral oil	0.0[b]	11.9[b]	0.0[b]
FC + water	73.1[a]	10.7[b]	62.9[a]
water	0.0[b]	0.0[b]	0.0[b]
P value & (SEM[3])			
Stage X Treatment		0.0013	
		(15.11)	

[a,b] Means same superscripts are not significantly different ($P < 0.05$)

hatching percentages = [(no. of hatched alevins/no. of eyed eggs)/hatching rate (%) at Day 0]100

[1]Fluorocarbon

[2]Phosphate buffered saline

[3]standard error of the mean

Table XIII. Mean percentages of normal alevins that hatched from the eggs stored for five weeks.

Treatment	161 degree days	180 degree days	217 degree days
FC[1]	-	71.1	93.5[a]
FC + PBS[2]	41.7[b]	75.0	91.9[a]
FC + glucose	-	-	60.0[b]
FC + mineral oil	-	38.0	-
FC + water	59.1[a]	37.5	90.5[a]
water	-	-	-
P value & (SEM[3])			
Treatment	0.0284	0.8382	0.0207
	(0.60)	(49.60)	(6.60)

[a,b,c] Means within each column with same superscripts are not significantly different ($P < 0.05$)
percentage of normal alevins = [(no. of hatched alevins-no.of deformed alevins)/no. of hatched eggs]100
[1]Fluorocarbon
[2]Phosphate buffered saline
[3]standard error of the mean

Table XIV. Mean hatching percentages of eggs stored at 147 degree days for three or five weeks, as a percentage of Day 0 results, and the mean percentages of normal alevins from the hatched eggs.

Treatment	3 weeks		5 weeks	
	Hatch[1]	Normal[2]	Hatch[1]	Normal[2]
FC[3]	99.7[a]	99.0	4.3[a]	80.0
FC + PBS[4]	81.1[a]	98.8	0.0[b]	-
FC + glucose (0.3M)	0.0[b]	-	0.0[b]	-
FC + mineral oil	19.3[b]	92.3	0.0[b]	-
FC + water	27.3[b]	91.7	0.0[b]	-
water	0.0[b]	-	0.0[b]	-
P value & (SEM[5])				
Treatment	0.019	0.9108	0.001	-
	(14.40)	(11.40)	(-)	(0.20)

[a,b] Means within each column with same superscripts are not significantly different ($P < 0.05$)

[1]([(no. of hatched alevins/no. of eggs)/hatching rate (%) at Day 0]100

[2][(no. of hatched alevins-no. of deformed alevins)/no. of hatched eggs]100

[3]Fluorocarbon

[4]Phosphate buffered saline

[5]standard error of the mean

IV. FINAL CONCLUSIONS

The purpose of this study was to devise a method to improve the viability of rainbow trout eggs during storage using fluorocarbon (FC) as an oxygen carrier. This would be beneficial for the following reasons: 1) to maintain the desirable genetic material of valuable ova or embryos, 2) to improve the viability of eggs shipped over long distances or if unforseen problems occur during transport, and 3) to reduce the effects of seasonality on hatchery production. There have not been many successful attempts to achieve these goals.

Unfertilized eggs are not often shipped but shipping them would provide the opportunity to buy eggs and fertilize with local semen. Furthermore, if female fish spawn too many eggs for the amount of semen available, the excess eggs could be stored and fertilized later. Finally, in order to make selective crosses when the female might spawn before the male, her eggs could be stored until the semen is available.

Eggs that have been fertilized for less than forty-eight hours are less sensitive to handling than eggs at later stages, up to the eyed stage (Gordon *et al.*, 1987). Improving the viability of transported eggs that have just been fertilized would allow eggs to be shipped at an earlier stage, reducing pressure on the seller's incubator space.

Eyed eggs are at the most common stage of development to be sold and shipped. Improving the viability of stored eggs at this stage would certainly be of benefit to the aquaculture industry.

The main objectives of this study were: 1) to verify a method to detect the viability of unfertilized eggs of rainbow trout based on water-hardening, 2) to select a storage medium that would maintain the viability of unfertilized eggs without inducing water-hardening, 3) to select

a medium, with or without fluorocarbon as an oxygen carrier, and a temperature, that would enhance the viability of unfertilized eggs or fertilized water-hardened or non-water-hardened eggs during storage, 4) to increase the viability of eyed rainbow trout eggs using fluorocarbon as an oxygen carrier during storage.

A simple method of egg viability assessment is required because fertility trials are time-consuming and expensive, and incubator space is usually limited. Harvey and Ashwood-Smith (1982) developed a method to estimate the viability of unfertilized eggs using an assessment of water-hardening. The first objective was to verify that water-hardening can be detected reliably. We were able to distinguish water-hardened eggs in water from non-water-hardened eggs in ovarian fluid ($P < 0.001$) (Table IV).

The water-hardening assessment of viability is a useful tool to detect storage damage as a preliminary test of media for egg preservation. However, viability determined only by water-hardening assessment should be used cautiously. Water-hardening can be used in preliminary trials because it takes less time and facility space than fertility trials to assess eggs for viability after storage. The most promising treatments can then be used in a fertility trial to confirm fertilizability. However, fertility trials may not always provide an accurate measurement of egg viability; for example, if semen quality is poor then the resulting fertility rates will be low. We were unable to draw conclusions from a comparison of viability by water-hardening assessment to fertility because of the poor quality of the unfertilized eggs upon arrival (Table III). The relationship between egg viability based on water-hardening and egg fertility requires further investigation.

Ovarian fluid is not available in sufficient quantities for storage, and because eggs

dehydrate if not covered with fluid, a suitable storage/fertilization medium is required. The following media were compared: D-532, Stoss's solution, ovarian fluid and Fish Ringer solution.

The unfertilized eggs that were fertilized in Stoss's solution had significantly higher ($P < 0.01$) fertility than eggs fertilized in the other media (Table V). Also, eggs stored in Stoss's solution for 90 minutes had low water-hardening percentages. The ideal storage medium for eggs should not water-harden the eggs, so that after storage the sperm can still penetrate and fertilize the eggs. Because Stoss's solution met these requirements for a storage medium, it was selected as a storage medium for further trials.

Unfertilized eggs were stored at one of three temperatures in FC, FC plus mineral oil, FC plus Stoss's solution (with 0, 0.6, 1.2 or 2.0 g pluronic F-68 or with 1.0 g lecithin and antifoam C or 1.0 g pluronic F-68 and antifoam C), Stoss's solution, mineral oil or water. When sampling was done after four weeks of storage, the eggs that were stored at 1.0°C in FC plus Stoss's solution with 0.6 g of pluronic F-68 had significantly higher ($P < 0.0001$) water-hardening percentages than eggs in all the other treatments (Table VII). Eggs that were stored in FC plus Stoss's solution at 1.0°C or 3.0°C also survived after 4 weeks (Table VII). The reasons that unfertilized eggs had higher water-hardening percentages when stored in FC plus Stoss's solution were that the FC contributed the oxygen needed by the eggs and Stoss's solution supplied the appropriate environment for storage and fertilization. Unfertilized eggs stored at -3.3°C did not survive. This was probably due to intracellular freezing which caused damage to the egg (Harvey and Ashwood-Smith, 1982). Fluorocarbon and Stoss's solution without pluronic F-68 appears to be a suitable medium for storage of unfertilized eggs for four

weeks at either 1.0°C or 3.0°C and does not require emulsification.

Fertilized, non-water-hardened and fertilized, water-hardened eggs were stored at 1.0°C or -3.3°C in FC, FC plus mineral oil, FC plus Stoss's solution (with 1.2 g pluronic F-68 or with 1.0 g pluronic F-68 and antifoam C, or with 1.0 g lecithin and antifoam C), Stoss's solution, mineral oil or water. The presence of an embryo was not apparent in any of the fertilized water-hardened or fertilized non-water-hardened eggs stored at either temperature after they were taken out of storage and incubated for twelve days. The early stage of embryonic development when they were stored probably increased the sensitivity of the eggs to damage during storage.

There was success in maintaining the viability of unfertilized eggs for four weeks. If future fertility results using these media indicate a similar duration of viability, then the reproductive season of rainbow trout can be extended, but this needs to be confirmed by further research. This technology can give producers a wider time frame in which to fertilize eggs, as well as facilitating the transport of eggs. The use of fluorocarbons could help achieve the goal of delaying the time of fertilization (and hatching) by delivering oxygen to unfertilized eggs that are kept at low temperatures, thus maintaining their viability.

In Chapter 3, the objective of the trials was to increase the viability during storage of eyed eggs for transport or other uses. In order to accomplish this goal, several types of media and several stages of development of eyed eggs were evaluated for egg storage. The media used were: FC, FC plus PBS, FC plus 0.3 M glucose, FC plus mineral oil, FC plus water and water alone. The stages of development were 147, 161, 180, 200 and 217 degree days. At three and five weeks of storage, eggs were evaluated for percentage hatched and percentage

of normal alevins.

The eggs that were the most sensitive to storage were those stored at 147 degree days. According to Appendix A , Table 1, eggs at this stage are classified as the tender stage, which occurs before the eyed stage. They were more sensitive to handling and storage than the eyed eggs stored at later stages of development. This confirms the findings of many researchers (Rouboud *et al.*, 1985; Beacham and Murray, 1987; Cloud *et al.*, 1988; Sasaki *et al.*, 1988; Calvi and Maisse, 1988; Dinnyés *et al.*, 1998) who found that eggs stored at a later stage of development were more resilient than those stored at an earlier stage. Except for eggs stored at 147 degree days, after three weeks of storage, there were no large differences in the hatchability of eyed eggs stored in any of the FC media (Table X). For that reason, the results after five weeks of storage will be discussed primarily, since differences in results of storage in media were much more apparent at that time (Table XII and XIII). With respect to the optimal medium for storage, eyed eggs stored in FC and water most consistently resulted in the highest percentage of hatch and the lowest percentage of abnormal alevins. This treatment delivered the oxygen that was needed at this stage of development. Water provided the normal environment for these eggs. In contrast results with eggs stored in FC, are the adverse effects on the eggs stored in water without FC. After three weeks of storage very few eggs hatched, demonstrating the benefit of the addition of FC to deliver oxygen to developing eggs. Although the storage temperature of 1.0°C should reduce the rate of embryonic development, the embryo continues to develop at a slower rate requiring oxygen at a reduced level. In the study by Maddock (1974), where eggs were stored in water at a reduced temperature, oxygen was supplied by recirculated water which had been reoxygenated. An added benefit of FC would

be the possibility to charge small quantities of FC with oxygen to be included in the shipping container of eggs for transport. Recirculation of aerated water is not possible for transported eggs.

The use of FC could assist the aquaculture industry by increasing the viability of eyed eggs especially when shipping to remote areas where transportation time may be extended.

In conclusion, these studies:

1) have verified that water-hardening can be detected reliably and can be used to determine if eggs are damaged. However, it remains to be determined how reliable water-hardening is as a test for fertilizability.

2) have selected a storage medium that would maintain the viability of unfertilized eggs without inducing water-hardening. The solution in which eggs had a low water-hardening percentage and a high fertility percentage was Stoss's solution.

3) have demonstrated that by using FC plus Stoss's solution and storing the eggs at a low temperature, the viability of unfertilized eggs can be maintained for four weeks. It does not appear possible to maintain the viability of newly fertilized eggs during storage at a low temperature.

4) have demonstrated that the viability of eyed eggs can be maintained during storage by providing oxygen to the eggs using FC plus water and storing the eggs at a low temperature.

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VI. APPENDIX A.

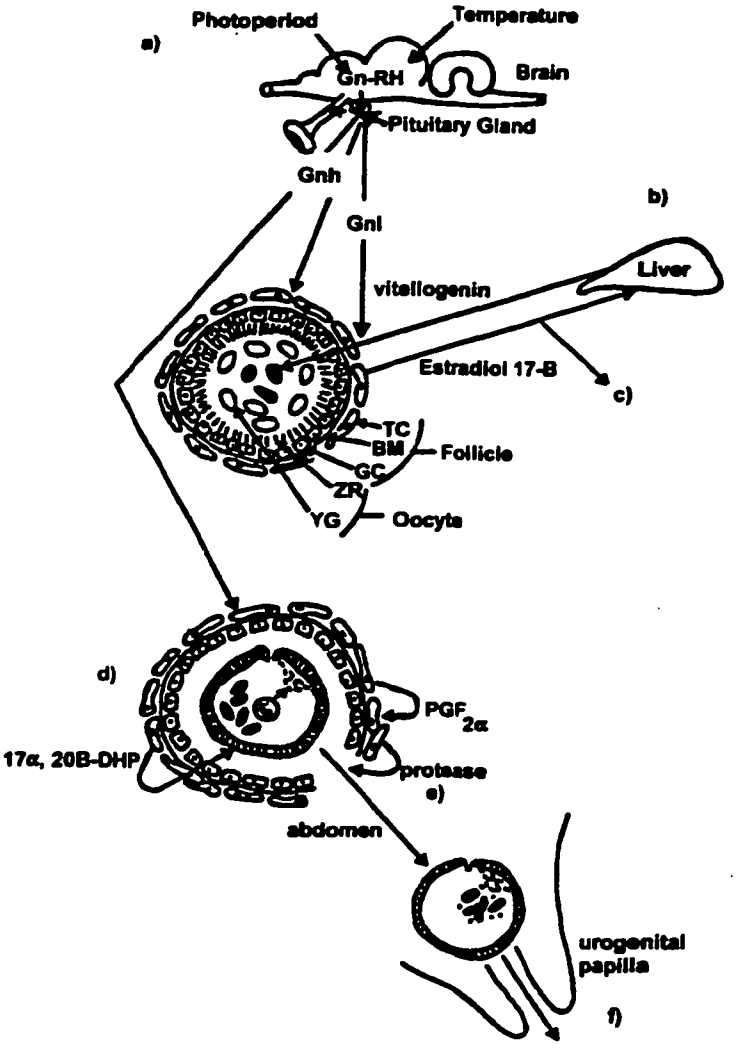

Figure 1: A general overview of the female salmonid reproductive cycle. a) Initiation of gonadal maturation, b) exogenous vitellogenesis, c) secondary sex characteristics, d) final oocyte maturation, e) ovulation and f) spawning. Key: TC, thecal cell; BM, basement membrane; GC, granulosa cell; ZR, zona radiata; YG, yolk granule; Gnh, gonadotropin high in carbohydrate; Gnl, gonadotropin low in carbohydrate, 17α, 20B-DHP; 17α, 20B-dihydroxyprogesterone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$. (Modified from Gordon, M.R., Klotins, K.C., Campbell, V.M. and Cooper, M.M. 1987. Farmed Salmon Broodstock Management. Ministry of Environment, Victoria, B.C. Industrial Research Assistance Program, National Research Council of Canada: 7-17.)

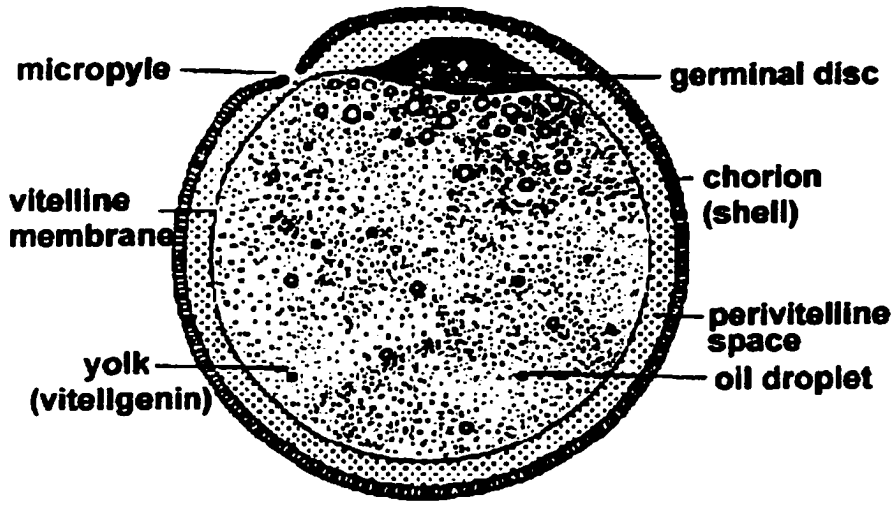

Figure 2. Diagram of a fertilized egg. (Modified from Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. and Leonard, J.R. 1982. Fish Hatchery Management (pp 131-207). United States Government Printing Office, Washington, D.C.)

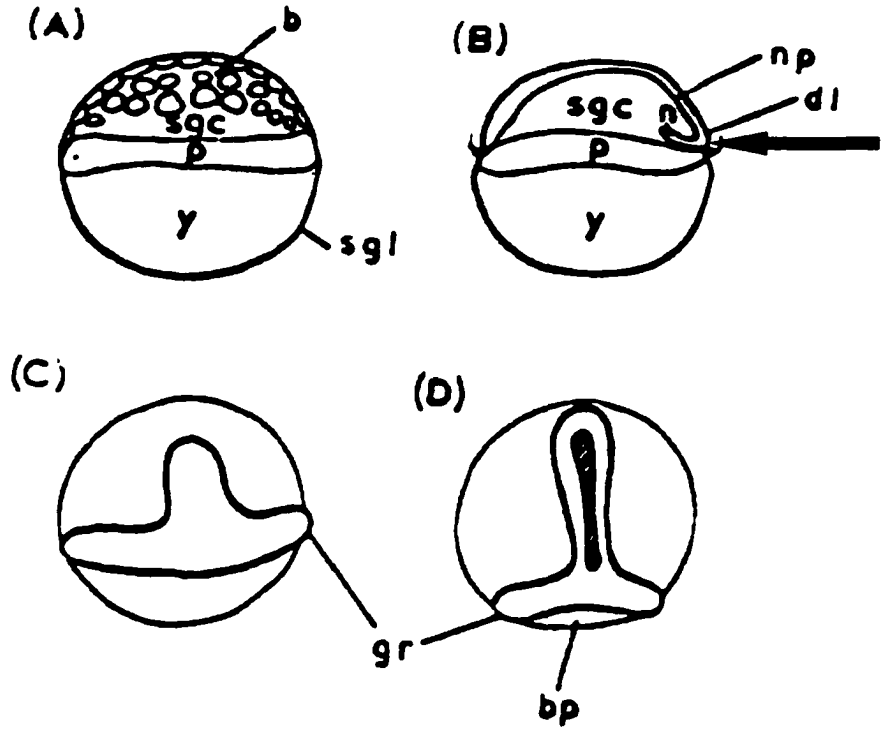

Figure 3. (a) Transverse section of early blastula showing adhesion of blastomeres to the surface gel layer and attachment of blastoderm to the periblast at the periphery only. (b) Sagittal section of later blastula showing gastrulation; epiboly shown by arrow. (c) and (d) Surface view of eggs in later stages of gastrulation. Key: b, blastoderm; bp, blastopore; dl, dorsal lip of blastopore; gr, germ ring; n, notochord; np, neural plate; p, periblast; sgc, subgerminal cavity; sgl, surface gel layer and y, yolk. (Modified from Piper, R.G., McElwain, I.B., Orme, L.E., McCaren, J.P., Fowler, L.G. and Leonard, J.R. 1982. Fish Hatchery Management (pp 131-207). United States Government Printing Office, Washington, D.C.)

Table 1. Stages of Development for rainbow trout

Stages[1]	Periods[2]	Degree Days[2]	Development[2]
Green	1	0	from fertilization to the first cleavage
	2-5	2.5	blastomeres form and cleave synchronously until at least the 9th round of divisions, though irregularities in their arrangement begin to appear at the eight cell stage.
	0.25	20	100 blastomeres - those on surface rounded, majority are internal, those of the lowest tier not completely segregated from each other or from the yolk
Tender	6B	25	the blastodisc is a high mound with a cobbled surface of large cells arranged in a cuboidal epithilium
	6C	30	The blastodisc mound has more gentle slopes, with a faintly pebbled or smooth surface. A whitish corona or periblast (Figure 3) extends beyond the rim of the blastodisc. Internal blastomeres in the sagittal section clump together. The process of consolidation spreads out from this quadrant which becomes the posterior pole of the embryonic shield.
	7A	35	The blastodisc has a roughly pitted lower surface caused by the release of rounded cells from it. These released cells gather at the posterior "nubbin" which is a virtual cavity separating the blastodisc from the central periblast. Some also form a fluid filled subgerminal cavity (Figure 3) between the disc and periblast at one quadrant. Most of these loose round cells remain adherent to the blastodisc but some stick to the periblast syncytium.
	8A	45	The terminal node becomes visible. The inner or anterior border of the embryonic shield is a sharply defined transverse line slightly curved. The central part of the blastodisc floor becomes clear of most of the loose cells and it is now 4 cell layers thick and a shallow fluid-filled cavity separates it from the yolk syncytium. A thin split (they are still touching) between a definitive epiblast and a hypoblast occurs in the lateral shield areas, but not farther out in the germ ring.

Stages[1]	Periods[2]	Degree Days[2]	Development[2]
Tender cont...	9	57.5	The germ ring has extended two-thirds the distance toward the equator of the yolk sphere, 60° down from the animal pole. The narrowed embryonic shield shows a neural groove which is a sign that the prospective central nervous tissue is in active convergence toward the midline. Lateral wings of mesoderm have become visible by this movement and are widening toward the germ ring. Stage 9-13 cover the period of epiboly (closure of the blastopore).
	10A	60	The germ ring has almost reached the equator. The segregating brain mass, pressing toward the midline, has projected downward against the yolk syncytium as neural keel. Somites begin to form, a rate of one pair per hour at 10°C. These somites do not separate at this stage
	10B	62.5	This has acquired 10 pairs of somites. Kupffer's vesicle has formed in the posterior endodermal sheet. Optic primordia are separating from the rest of the forebrain. The prospective head mesoderm forms a prominent horseshoe-shaped band wrapped under and anterior to the forebrain.
	11	70	The germ ring is about halfway down from the equator to the yolk sphere toward the vegetal pole. There are more than 10 pairs of somites but less than 20. The optic alagen and tree brain vesicles are clearly defined. The split between epiblast and hypoblast has now extended to all sectors of the germ ring.
	12	80	The germ ring is intruding upon the vegetal pole, the diameter of the exposed yolk is approximately equal to the width of the head. There are between 15 and 25 pairs of somites. Between these and the lateral plates the primitive kidney ducts are segregating.

Stages[1]	Periods[2]	Degree Days[2]	Development[2]
Tender cont...	14	100	The trunk-tail mound has been raised up but not undercut. Up to 30 pairs of somites are present. In some, the pericardial cavity is inflated and the heart becomes visible. The hindbrain ventricle starts to inflate.
	15A	110	The trunk-tail bud protrudes beyond the yolksac. The length of the bud is no greater than the forebrain plus the midbrain. The heart starts beating when there are about 35 pairs of somites visible.
	16	130	The free portion of the trunk-tail bud as long as the whole brain, if not longer. Mounds of pectoral mesenchyme are evident but lack externally projecting ridges. There are 10 or more pairs of post-anal somites. The ventral part of the tailfin is emerging and the anus is free from the yolk. Spontaneous trunk movements now occur, and the brisk heartbeat is driving blood though the first pair of ortic arches.
	17	135	The free trunk-tail portion is shorter than the attached part of the trunk but longer than the brain and the pectoral-level spinal cord. Each pectoral mound shows a slight ridge on its surface. The heart is bent strongly to the left side. Up to 20 pairs of postnatal somites have formed.
Eyed	18	155	The previously mentioned ridges have expanded to narrow disc-like rims. The free portion of the trunk-tail is equal in length to the part of the body attached to the yolk sac. The head has become undercut as far back as the eyes. The tail has straightened and contains 25-30 pairs of post-anal somites. The first eye pigment appears.
	19	160	The pectoral fins have become vertical and nearly circular discs, with narrow rims. Their diameter is smaller than the eye. There are 30 pairs of post-anal somites, but the tail tip is still unsegmented. The head is undercut as far as the upper jaw but the mouth is not yet open.

	Periods[2]	Degree Days[2]	Development[2]
Eyed cont...	21A	180	The first trunk pigment appears. The pectoral fins have a diameter equal to the eye, but they are not yet rhythmically motile. The operculum overhangs the first branchial segment. First the caudal fin, then the anal fin appear as concentrations of mesoderm.
	21B	200	The pectoral fins can twitch but are not rhythmically motile, yellow bile is in the intestine mesenchyme concentrations successfully mark the dorsal fin and the paired pelvic fins.
	22A	220	The pectoral fins begin to wave rhythmically. The pelvic fins are, at this time, elongated plaques. Finrays are forming in the heterocercal tailfin. The operculum extends back over the second branchial segment. The yolk mass begins to lose its spherical shape. The head has been undercut as far as the bulging pericardial cavity.
	22B	230	The pelvic fins acquire surface ridges, the jaws can twitch(but not rhythmically), the liver mass exceeds the eye in diameter, and the operculum covers the rest of the branchial segments.
Hatch	23	250	Jaws and hyobranchial apparatus are involved in rhythmic breathing movements. The pelvic fins become projecting lobes. Finrays appear in the anal and dorsal fins. Gill filaments make their appearance while the yolk mass is elongating and shrinking.

[1]All information in this column is from: Gordon, M.R., Klotins, K.C., Campbell, V.M. and Cooper, M.M. 1987. Farmed Salmon Broodstock Management. Ministry of Environment, Victoria, B.C. Industrial Research Assistance Program National Research Council of Canada. pp 1-9 to 7-17.

[2]All information in this column is from: Ballard, W.B. 1973. Normal Embryonic Stages for Salmonid Fishes, based on *Salmo gairdneri* Richardson and *Salvelinus fontinalis* (Mitchill). Journal of Experimental Zoology; 184: 7-26.