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Salmonid Embryo Development and Pathology

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Introduction

The development of each salmonid egg follows a predetermined course. In order for a salmonid to emerge from the redd gravel, all developmental steps from oogenesis, fertilization, embryonic development, hatching, yolk sac absorption, and emergence must be completed in the proper sequence. Knowing the developmental sequence between oocyte formation and fry emergence aids in live identification of species and determines the etiology of morbidity and mortality in these young fishes. This chapter provides an overview of fish gametogenesis, fertilization physiology and behavior, and documented embryo development stages. With this knowledge, it may be possible to collect developing or dead embryos from redds, identify developmental abnormalities, trace their origins, determine causes of death, predict survival rates, and/or back-calculate local spawning dates. Detailed comprehension of salmonid ontogeny helps fisheries biologists evaluate causes of mortality; provides pertinent information to project managers, engineers, and geomorphologists useful in assessing river salmon spawning habitats before and after construction projects; and improves quantitative assessment of spawning habitat remediation projects in disturbed habitats.

Collection and Examination of Fish Embryos

Proper collection techniques and thorough examination of fish embryos provides a great deal of information regarding their development. Historically, salmonid eggs have not been examined much prior to the eyed egg stage; unfortunately, this means many causes of infertility and early fry mortality get lumped together. Salmonid eggs are generally hardy enough to survive transport back to a laboratory for examination with adequate care to prevent desiccation and mechanical or thermal shock. Eggs can be removed from nests, or redds, with gentle suction and placed into a container of ambient water for live transport or can be placed into fixative for preservation. Oxygen consumption and waste elimination by the embryo is minimal, and live eggs kept in a few milliliters of cold water will survive several days without significant deterioration. Developing embryos change rapidly during early development, but changes are progressively slower as more detailed structures develop and differentiate. The most crucial consideration during the collection of live eggs is maintenance of incubation temperature. Eggs are sensitive to rapid changes in temperature, so they should be kept as close to their local temperature as practical. Changes in temperature also alter the developmental rate of the embryo. Increasing water temperature will cause embryos to develop more rapidly, while decreasing temperature will arrest development. If arresting the developmental stage is preferred, then eggs should be chilled to 4°C or killed by preservation in 10% pH neutral buffered formalin before examination. Freezing salmonid eggs generally ruptures the chorion and distorts the developing embryo; therefore, it is not a recommended method of preservation.

Eggs are also sensitive to mechanical

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shock; however, this sensitivity decreases as embryos mature (Jensen and Alderdice 1983, 1989; Crisp 1990). Krise (2001) documented the sensitivity of Atlantic salmon *Salmo salar* eggs during the first 6 h postfertilization. Sensitivity of trout eggs has been documented for both electrical and mechanical shock (Dwyer et al. 1993). Jensen and Collins (2003) evaluated the effect of mechanical shock on pink salmon *Oncorhynchus gorbuscha* and steelhead *O. mykiss* yolk coagulation. They concluded that lethal mechanical shock is apparent within 5 min of the event and readily detectable. Mechanical shock strong enough to kill the embryo caused a white opacity to appear in the yolk. They recommended that redds should not be disturbed for approximately 30 d after spawning to minimize the chance that sampling would cause a lethal mechanical shock to the developing embryo. Valuable information about egg fertility and early embryonic death may be lost if samples are not collected for 30 d postspawn; therefore, if early embryonic development samples are necessary, extra care should be taken not to damage the more delicate eggs. Carls et al. (2004) determined that observer classification of live, mechanically damaged, and dead pink salmon eggs was most accurate within 1 h of collection. The Canadian Department of Fisheries and Oceans has published a table of shock sensitivities for several salmonid species, which may be helpful with early embryonic sample collection (DFO 2003).

Often various opportunistic bacteria and saprogenic fungus will quickly infect dead or dying eggs (Chacko 1993; Czeczuga and Muszynska 1999). If eggs cannot be immediately examined, it is important to halt the decomposition between sample collection and sample examination in order to prevent loss of embryo quality and thus important details regarding development stage. Buffered neutral formalin preservation of decomposing eggs may not be sufficient to halt the microbial decomposition. Refrigeration plus additional antifungal or antimicrobial agents may temporarily reduce progressive decomposition; however, observing the eggs as soon as possible after collection is most advisable.

Eggs may be photographed when they are collected. Photographing eggs with a macrolens or close-up setting on a digital camera makes imaging the spherical eggs possible. Placing eggs into a Petri dish and covering them with water allows them to be photographed without the egg surface reflecting and distorting the image.

Eggs infected with fungus can also be evaluated (Xu et al. 1990; Kitancharoen and Hatai 1996; Hussein and Hatai 2002). *Saprolegnia* can grow about 1 mm/h, so a crude estimate of the infection's duration can be made retrospectively from the length of individual fungal hyphae and the extent of the infection. It will take typically about 3 d for fungus to completely encapsulate a salmonid egg at 10°C. It takes longer at lower temperatures, but the fungus will continue to develop even at 0°C as long as the water is not frozen. *Saprolegnia parasitica* is an aggressive pathogen of salmonid eggs, but other *Saprolegnia* species are primarily opportunistic pathogens attracted to nutrients leaking from damaged or dead eggs. Once one egg is infected and hyphae develop, they spread to adjacent healthy eggs (Figure 1).

The examination of dead eggs can provide a great deal of useful information about morbidity and mortality etiology. It is also important to determine if morbidity resulted from embryo death or chorion abnormalities. Normal healthy salmonid eggs will remain water-impermeable after water hardening for several weeks, even if the eggs were not successfully fertilized. The chorion can be transparent in some salmonid fishes, which makes observation of the developing embryo easy with a 4× magnification lens or dissection microscope. The blastodisc of unfertilized eggs will deteriorate in the days and weeks following water hardening. Fertilized eggs should, in contrast, become increasingly organized into developing embryos following water hardening (Figure 2). Eggs can be examined submerged under a thin layer of water so that the reflection of light sources does not interfere with observation of the embryo. Examining eggs submerged under a thin layer of water also allows fragile defects, hyphae

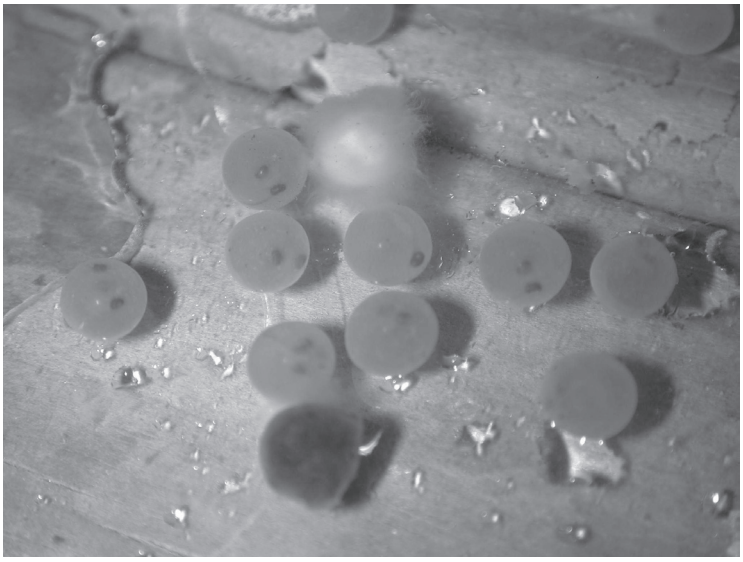

Figure 1. Eyed brook trout embryo. Dead egg at top of image heavily infected with *Saprolegnia* fungus; blue-purple egg at bottom of image infected with both *Chromobacteria* and *Saprolegnia*. Notice how *Saprolegnia* hypha extends from primary egg to adjacent eggs. The large red vitelline vein is visible in several individual eggs (Stage 22; G. R. Danner photo).

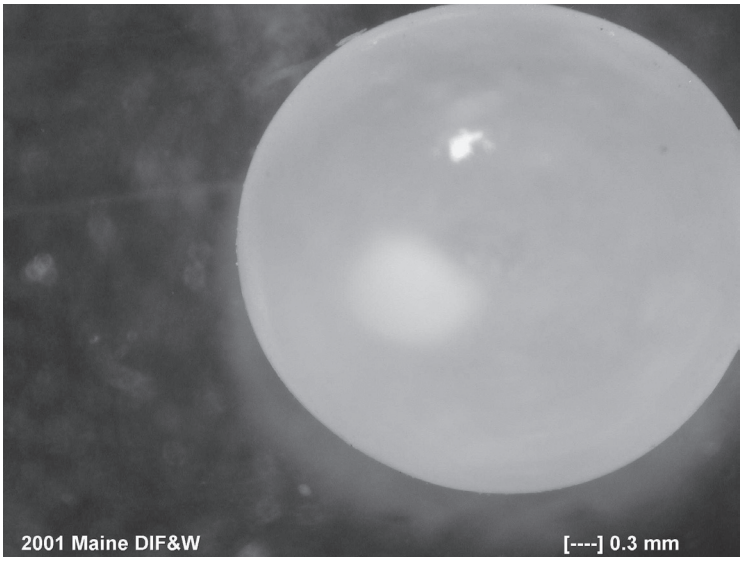

Figure 2. Unfertilized brook trout egg. Blastodisc visible through the chorion with Stockard solution used to help clear the chorion. The blastodisc will remain intact for about 10 d after incubation, after which it will look like the dead egg in the upper left corner of Figure 5. Notice the water-filled space between the chorion and the yolk on the bottom right side of the image. Usually, the chorion should not swell so much; however, in this instance, the Stockard solution is reducing the integrity of the chorion membrane and osmotic pressure draws water into the perivitelline space (G. R. Danner photo).

growth of aquatic fungi, and chorion texture patterns to be observed. Removed from the water, gravity affects delicate structures, collapsing them against the chorion obscuring their otherwise submerged appearance. Photographing submerged eggs is much easier than photographing exposed eggs because light sources do not interfere with the plane of focus. Eggs photographed out of water often have a big bright spot on their surface.

Eggs removed from redds should be separated into groups by development stage (Table 1). Totals and percentages from each development stage can be collected onto a datasheet (Table 2). Investigators should note whether all embryos died at the same

Table 1. Development stages of brook trout.

Development center	Daily temperature units	Approximate no. days incubated at 8°C	Description
Cleavage			
Stage 1	–	–	Cytoplasm concentrated in single mass called blastodisc at animal pole (Figure 2).
2	–	–	First meroblastic cleavage of blastodisc into two equal-size cells.
3	8	1	Four-cell embryo appears (second cleavage) (Figure 3).
4		1	Eight-cell embryo (second cleavage).
5	16		Sixteen-cell embryo.
6			Thirty-two cells.
7	24		Early morula; numerous visible small cells in a cluster. No increase in overall size of blastodisc.
8		5	Late morula; individual cells become too numerous and too small to individually identify.
9			Blastodisc expansion; morula flattens and spreads over animal pole.
10		8	Blastula; a crescent of cells accumulates on edge of animal pole. This is the start of the embryo's anterior. Start of the primary axis of embryo. Embryos become very susceptible to mechanical shock from this stage until stage 18.
Gastrulation			
11	80	10	Terminal caudal bud. Completion of the primary axis; development of the area that eventually becomes the tail (Figure 4).
12			Rough outline of embryo.
13			One third epiboly; embryo clearly visible. Cells begin to form tissues or germ ring, is preceded by the periblast, a clear layer of cells moving over the entire yolk.
14			One-half epiboly; embryo begins forming somites.
15			Two-thirds epiboly; embryo; periblast continues to move across yolk.
16	120	18	Three-fourths epiboly.
17			Blastula closed. After this point, embryos become much more resistant to mechanical shock. The blastopore closes near the embryo's tail.

Table 1. Continued.

Development center	Daily temperature units	Approximate no. days incubated at 8°C	Description
Organogenesis			
18			Caudal bud becomes free. At this point, the embryo can move its tail. Embryos may now appear as J-shaped. Prior to this embryos will appear I-shaped.
19			Parts of the brain become distinct. It is now possible to see a division between the hindbrain and midbrain. The retinal pigment becomes visible in eyes.
20	275	30	Heart begins to beat.
21	300		Early eyed embryo; eye pigment becomes more prominent; yolk veins cover one-fourth of yolk.
22			Early eyed embryo; eye pigment continues to deepen; yolk veins cover two-thirds of yolk.
23			Return of caudal blood supply via only the caudal vein.
24	350		Eyed egg; deep black eyes seen through chorion; yolk three-fourths vascularized (Figure 1).
25		45	Caudal flexing; anal fin visible.
26			Operculum covers part of the first branchial arch; dorsal fin visible.
27	400		Myotome buds in the dorsal fin, operculum covers first branchial arch.
28			Pelvic fin buds appear, indentation appears in caudal tail for fin rays.
29			Operculum covers the second branchial arch; rays in caudal fin differentiate.
Hatching			
30	470	60	Embryos begin to hatch; operculum covers all branchial arches (Figure 5).

development stage or a variety of stages; sometimes a histogram provides visual clues regarding embryo developmental problems (Figure 6). This data often give information regarding the time and cause of death. Investigators should be asking themselves,

- Are all of these embryos developing at the same rate?
- Are all of these embryos dead at the same time?
- Did these embryos die over the course of hours, days, or weeks?
- Are dead eggs about the same size as live eggs?
- Are dead eggs the same color, shape, or species as dead eggs?
- How many days did it take for the fungi to grow over the surface of the egg?
- Was the egg shell damaged before or after the embryo died?

A dichotomous key can help an investigator narrow the possible etiologies into specific developmental stages (Table 3).

Embryos can usually be examined within the chorion by making it more translucent. A mixture of Stockard solution improves the translucency of a fish chorion without distorting the embryo by osmotic pressure. One

Table 2. Datasheet for collecting data regarding development of salmonid eggs. Embryos from a location can be categorized broadly as dead or alive, and then those that are dead can be further identified by observations of bacteria, fungus, or a particular stage of development seen. After a group of embryos are examined, the percent of eggs in each category can be determined. Identifying why eggs are dying at a particular development stage can provide clues to the etiology of the underlying pathology.

							Development stage									
				Dead eggs			Cleavage			Gastrulation			Organogenesis			
Date	Location	Temp	Total no.	Fungus	Bacteria	Blanks	1–2–3	4–5–6	7–8–9	10–11–12	13–14–15	16–17–18	19–20–21–	22–23–24–	25–26–27	28–29–30
		Totals														
		Percent	100%													

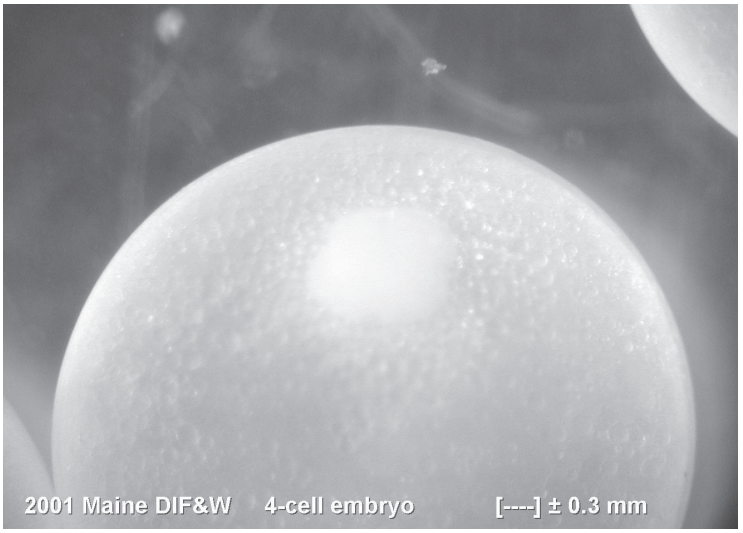

Figure 3. Four-cell brook trout embryo (Stage 3). Blastodisc divided into four distinct cells. The blastodisc does not change size as the number of cells increase. This image is looking through the chorion without any special treatment other than good lighting (G. R. Danner photo).

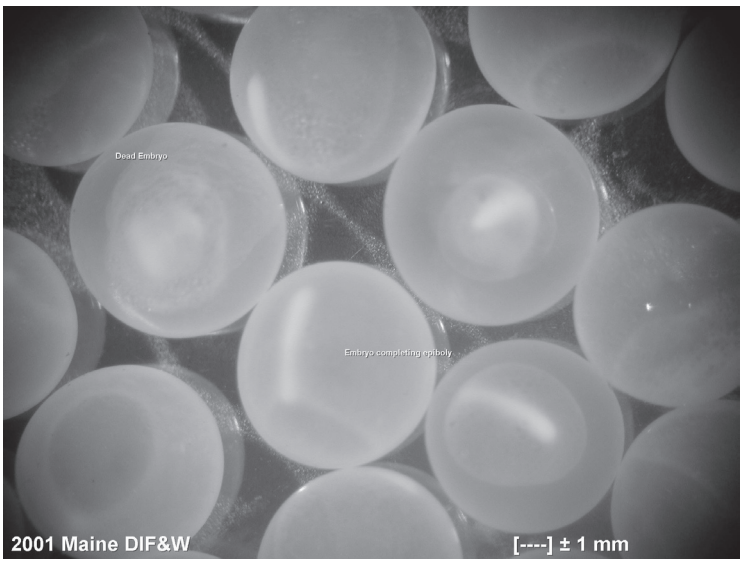

Figure 4. Developmental axis formed in developing brook trout during development stage 11. The egg in the bottom left shows the circular blastocoel before it closes. This will be the tail portion of the fish axis. The three eggs in the center and right side of the image show the developing head end of the fish. The image in the upper left corner is a dead egg; notice the decomposition of the blastodisc. This stage of development is as easy as counting ones and zeroes to determine the number of live and dead embryos (G. R. Danner photo).

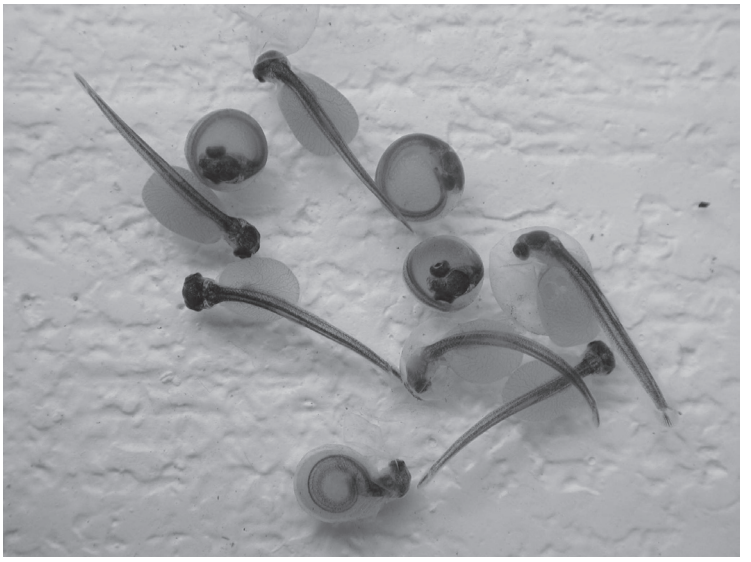

Figure 5. Hatching brook trout alevin. These embryos commonly emerge tail first; notice the well-pigmented bodies, the pigment on caudal fin rays, and the variety of yolk shades present. Embryo in center bottom of image has deformed tail curling back upon itself. Notice how other embryos have head and tail wrapping all the way around the yolk and past the head (G. R. Danner photo).

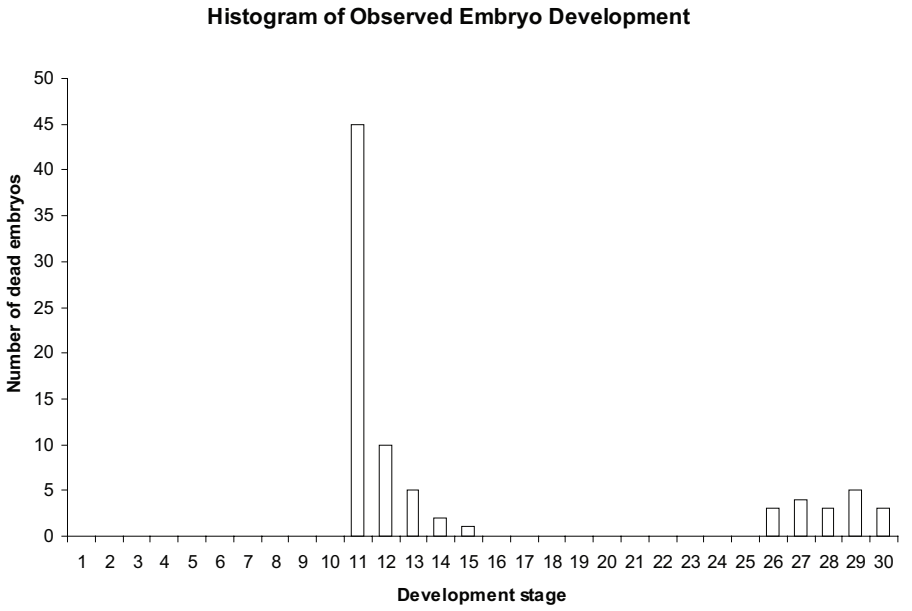

Figure 6. Histogram of observed embryo development by development stage. This is the pattern observed in brook trout experiencing embryo mortality as a result of maternal oocyte development in a low ambient dissolved calcium environment. Embryos develop until gastrulation where calcium dependent cells are unable to move across the yolk.

liter of Stockard solution can be made from 50 mL 37% formalin, 40 mL glacial acetic acid, 60 mL glycerin and 850 mL distilled water (Velsen 1980; Marancik and Danner 2002). Stockard solution works most effectively before the embryos have reached organogenesis, after which the chorion does not clear as effectively. It is also possible to preserve the

Table 3. Dichotomous key for determining cause of embryological pathologies in salmonid eggs.

Key	Dichotomous statements
1a	Most eggs show evidence of successful fertilization and development. GO TO 4a.
1b	Most eggs are not white but show no evidence of fertilization and development. GO TO 2a.
1c	Most eggs are white (opaque) and it is not possible to determine development. Return to collection site earlier in the season to collect eggs; these eggs died and turned opaque before useful information could be collected. If eggs turn white immediately upon entering the water, there is an oogenesis problem. END.
2a	Population structure contains proportional numbers of male and female fish. GO TO 3a.
2b	Population does not contain adequate numbers of male fish to properly fertilize eggs. Consider presence of sex changing hormones, higher mortality during life stage for males, escapement regulations that favor females, and diseases affecting only males. END
3a	Males have viable motile sperm greater then 90%. Check females for egg quality. Egg development may be affected by nutritional deficiency, genetic damage, or environmental contamination. END.
3b	Males have poor sperm motility. Check males for evidence of nutritional deficiency or environmental contamination. END.
4a	Eggs appear to be consistent size, and most eggs have reached the same development stage when examined. GO TO 5a.
4b	Eggs appear to be several different sizes, shapes, or colors. Developing embryos are at different development stages. Consider different spawning dates with redd superimposition, different species present, and spawning in the same location. If single species and single spawning date, then there is a developmental problem within the fertilized eggs. Consider toxin or pathogen with varying degree of intoxication or infection. END.
5a	Development stage is appropriate for the temperature units available in the stream. GO TO 6.
5b	Development stage is retarded behind available water temperature units available for development. Consider possible influence of cold hyporheic influence; otherwise, eggs may be retarded by nutritional abnormality or environmental toxicant. Will fry emerge at an appropriate time to feed on appropriate food items? GO TO 7.
5c	Development stage is beyond appropriate level for temperature units available in the stream. Consider possible influence of warmer hyporheic influence; consider if eggs are hybrid or different species than expected. Will fry emerge at an appropriate time to feed on appropriate food items? GO TO 8.
6a	Embryos have hatched to alevins. GO TO 9.
6b	Embryos have not hatched to alevins. Survival to this point is appropriately greater than 90% in wild fishes; recheck fish later in the season to determine if development continues. END.
7a	Embryos will hatch late and be inappropriately small, vulnerable to competitors and predators, and lack appropriate food items or poor water quality/quantity. Consider emergence timing as primary population problem. END.
7b	Embryos will hatch late, but they will have available food and no advantaged competitors or inappropriate water quality/quantity problems. GO TO 11.
8a	Embryos will hatch too early for appropriate food items, flow rates, or water quality. Consider emergence timing as a primary problem in population. Can water temperatures be altered to time emergence with appropriate water conditions, competitors, and food items? END.
8b	Embryos will hatch too early; however, appropriate food items, water flow and quality, or competitors are not a problem in this location. Recheck population later in season to confirm. END.

Table 3. Continued.

Key	Dichotomous statements
9a	Alevins are of equal size, have adequate egg yolks attached, and there is no opacity in egg yolks. GO TO 10.
9b	Alevins have variable sized egg yolks, vary in size, and yolk colors are different. Consider multiple species; consider toxin that affects pigmentation or growth. END.
9c	Alevins have variable-sized egg yolks, some filled with milky or blue liquid (hydrocoele embryonalis). Consider water quality problem in hyporheic water. END.
10a	Alevins have no mucus or small sand, gravel, or detritus attached to gills or mouth. GO TO 11.
10b	Alevins have mucous or small sand, gravel, or detritus attached to gills or mouth. Consider habitat selection by parents. Consider if appropriate cobble size is available and being used. Consider reasons parent fish are not using appropriate cobble for alevins, including access, hyporheic flow or contamination, unstable streambed, dewatering, ice formation, and so forth. END.
11a	Alevins will have appropriate food available for emergence as fry. Egg quality and development is not a likely cause for population failure. END.
11b	Alevins complete development but fry emerge into a habitat without appropriate conditions. Consider location of redd and emergence habitat for fry. END.

egg in formalin and then gently remove the chorion with a small needle and forceps. The tip of a small needle can be used as a blade to gently cut through the shell. Care must be taken not to damage the embryo in the process of removing the shell.

Egg Development (Oogenesis)

Egg development begins many months prior to spawning; maternal health status and egg quality can sometimes depend on factors unrelated to degraded spawning habitat. The effects of nutrition, pollution, water quality, and maternal stress have profound effects on egg quality and subsequent embryo development (Springate 1990; Brown et al. 1994; Campbell et al. 1994; Mann 2000; Danner 2004; Palace and Werner 2006). Salmonid fishes eating a high portion of Clupeidae forage subsequently suffer thiamine deficiency; eggs from affected females develop Cayuga syndrome, a condition characterized by embryo weakness and death (Fitzsimons et al. 2001; Amcoff et al. 2002; Honeyfield et al. 2005). This early mortality syndrome is reported in the Great Lakes and Baltic Sea, but may also occur in waters with similar forage bases. In the Baltic Sea, this condition is reported as M74 syndrome (Fitzsimons et al. 1999; Karlsson et al. 1999; Lundstroem et al. 1999). Vitamin A and E deficiencies lead to poor egg quality (Palace and Werner 2006). Several species of pathogenic aquatic bacteria can infect the inside of fish eggs. Eggs infected with bacteria develop until the infection overwhelms the developing embryo and kills it. These bacteria may be cultured from within eggs or presumptively identified by morphology. Cipriano and Holt (2005) described how *Flavobacterium psychrophilum* affects developing Atlantic salmon eggs. Pathogenic fish viruses may also be vertically passed from parents to eggs. These viruses kill embryos at different developmental stages and can be recovered from infected eggs if virus infection becomes a suspected cause of mortality (Wolf 1988; AFS–FHS 2004). The effects of acidic precipitation are becoming an increasing concern to salmonids. Changes in pH and low ambient calcium affect egg quality (Farmer et al. 1980; Höbe et al. 1984; Parker et al. 1985; Peterson and Martin-Robichaud 1986). Billard and Jensen (1996) provide an extensive review of salmonid egg development. Groot (1996) provides an extensive review of salmonid life history, including information regarding spawning behavior. Pepper and Crim (1996) reviewed broodstock genetics and husbandry conditions necessary for quality egg devel-

opment. Anecdotally, many people think that brightly colored eggs have better survival than pale eggs. There is no correlation between egg color and survival; however, since bright pigments of the blood, eyes, and skin give developing eggs a vibrant appearance just as they "eye-up." Egg color depends primarily on the parent's diet and secondarily on subtle color variations within species (Harris 1984).

Fertilization

Fertilized Egg Stage

Salmonid eggs are large for fish eggs, ranging from 3 to 7 mm depending on the age and species. The egg is surrounded by a thick multicellular membrane, the chorion, with a complex lamellar structure that becomes increasingly turgid in the hours immediately following water activation. The chorion contains a single location for fertilization called the micropile. In nature, salmonid eggs are fertilized not with the aid of, but in spite of, the presence of water (Terner 1986). Therefore, egg or milt quality decreases result in an increased fertilization time; the rapid initiation of water hardening may ultimately decrease fertility rates. In hatchery situations, in vitro spawning techniques can delay water hardening; however, in feral situations, lower fertility rates may occur when egg or milt quality decreases.

Another major distinction of the salmonid egg from many other vertebrates is that the cortical reaction (water hardening) is not induced by fertilization, but is induced by the osmotic gradient difference that occurs when the female expels the eggs into freshwater. Thus, the membrane hardening process is completely independent of fertilization and unfertilized eggs will remain water-hard for weeks within a redd. No embryo develops in these eggs; however, because they remain intact, they do not immediately serve as a nidus for opportunistic saprogenic bacteria and fungi. Water hardening is an eloquent physiological process initiated by the breakdown of cortical alveoli after a release of calcium from the oocyte (Yamamoto 1979). Dissolved calcium is also captured from the surrounding water and transported into the perivitelline space along with freshwater. (Khlebovich et al. 1977). Ninety-nine percent of the water drawn into the perivitelline space occurs within 1 h of the egg entering into freshwater. The increase in fluid expands the egg's total volume by approximately 20%. Over the next several days, the egg capsule continues to increase in elastic strength (Zotin 1958). The rates of water uptake and egg capsule strength in steelhead have been shown to be adversely affected by low pH (Rombough and Jensen 1985).

Redds containing eggs of varying shapes and sizes may indicate either multiple spawnings or water hardening pathology. Water quality samples can be taken and genetic tests of eggs can help separate the etiologies. Unsatisfactory water quality conditions can make eggs uncharacteristically adherent to one another (i.e., sticky).

Temperature

As poikilotherms, salmonid eggs are strongly influenced by temperature during embryonic development. Development slows when water temperatures fall to the minimal 2°C, and the rate of embryo development has been found to increase up to the maximum development rate at 11°C (Velsen 1980). Fertilization at temperatures above 10°C in landlocked Atlantic salmon *Salmo salar sebago* has been linked with greater than 50% mortality in embryos. Incubation of anadromous Atlantic salmon eggs above 12°C has been linked with congenital deformities in cardiac muscle and skeletal tissue (Baeverfjord 2000; Takle et al. 2005). Salmonid embryos continue to develop as long as temperatures remain above about 2°C. At temperatures between 0°C and 2°C, egg development is arrested. Developing embryos can tolerate a short period of arrested development, but extended periods can result in mortality.

Temperature extremes are more detrimental during earlier developmental stages than later in development. The number of accumulated temperature units (ATUs) is used as a guide for determining the time it

takes for embryos to develop and emerge. Accumulated temperature units define the midpoint in ranges of daily temperature units (DTUs) from fertilization to a specific biological development step (e.g., eye, 50% hatch, or emergence). Daily temperature units are daily measures of the ambient water temperature in degrees above 0°C. So if the water temperature is 7°C for a given day, then seven DTUs were gained by the embryo. Daily temperature units are summed throughout incubation to determine the ATUs. If water temperatures are taken more than once daily, then an average DTU is used. Accumulated temperature units have been studied in most salmonid species and are documented in a variety of resources (Velsen 1980; Crisp 1981, 1988; Jungwirth and Winkler 1984; Warner and Havey 1985; Elliott et al. 1987; Piper et al. 1988; Erdahl 1994; Billard and Jensen 1996; Wedemeyer 2001; Marancik and Danner 2002). The ATUs are good estimates of developmental times but are not absolute numbers for each species because there are intraspecific differences based on local adaptation. Additionally, the total number of ATUs necessary for development increases as incubation temperatures decrease. Local salmonid hatcheries and fisheries biologists will generally know the ATUs necessary for local salmonid stocks (Bonney 2005).

Incubation temperature affects not only the rate of development, but also determines some of the fish's meristic characteristics (Kwain 1975; MacGregor et al. 1977). Meristic traits such as numbers of fin rays, vertebrae, lateral scale rows, myotomes, and gill rakers are know to vary in relation to temperature (Kwain 1975; MacGregor et al. 1977). In many species, fin rays, vertebrae, or scale numbers increase with decreasing temperature; however, some species also see increasing meristic values as temperature increases above normal incubation ranges. Jordan's rule originally purported that meristic values increased with latitude; it is now understood that this relationship is due to incubation temperature rather than latitude. Brown trout have fewer meristic elements when incubated at intermediate temperatures and have increased numbers when incubated at either lower or higher temperatures. The actual quantitative difference between experimental groups raised at different temperatures is in the range of 0.1% to 3% difference in number of elements per Celsius degree of temperature. For example, a 1% per degree difference for an embryo incubated at 5°C above average would have an increased vertebral count of 105 rather than 100. Additionally, because of the developmental progression of embryos, this five degree change in incubation temperature does not necessarily need to persist throughout incubation. The development of the vertebral column occurs near the time of gastrulation, so an elevation in water temperature during that period of time may result in increased vertebral counts. Gill rakers develop much later so would not necessarily be affected by that temperature increase. Caudal fin rays develop as one of the final elements prior to hatch, so careful examination of groups of fishes reared in locations with temperature fluctuations might be able to carefully tease out information regarding when these temperature fluctuations occurred by comparing meristic elements in groups of embryonic fish that should otherwise be homogeneous. Additional information on meristic element development has been documented (Barlow 1961; Blaxter 1984; Lindsey 1988; Houde 1989; Helfman et al. 1997).

Egg Deposition

Egg Deposition Stage

Once fertilized and water hardened, eggs have been deposited within redds and abandoned by the broodfish, water quality and quantity determines the ultimate success or failure of their development (Bernier-Bourgault and Magnan 2002). The primary water quality considerations for developing salmonid embryos include temperature, dissolved oxygen, carbon dioxide and nitrogen levels, pH, mineral content, gravel size, and nitrogenous waste accumulation (Essington et al. 1998; Geist et al. 2002; Hall and Wissmar 2004; McHugh and Budy 2004). These are discussed below.

Dissolved Oxygen, Carbon Dioxide, and Nitrogen

Dissolved oxygen is essential for all developing salmonid embryos. A reduction in dissolved oxygen will slow embryonic development and result in reduced growth rate and yolk conversion efficiency. Low oxygen levels have been linked to deformed embryos and can cause death. Rombough (1986) and McLean et al. (1991) have developed oxygen consumption models for six Pacific salmon species. These models predict the oxygen consumption of developing embryos at different temperatures and different development stages.

Carbon dioxide is a normal waste product of aerobic metabolism. As salmonid embryos develop, carbon dioxide must be transported out of the egg. Because carbon dioxide is highly soluble in water, its toxic effect on developing embryos occurs as a consequence of decreases in ambient water pH. If the buffering capacity of the incubating water is low and insufficient water movement occurs across the eggs, pockets of acidified water can damage egg development (Ganaway 1980; Leivestad 1982; Peterson 1982; Eddy 1985; Grady 1988; Abrahamsen et al. 1989; Springate 1990; Gilmour et al. 1995; Besser et al. 2001; AFS 2003; Danner 2004).

Nitrogen gas is biologically inert to salmonids; however, nitrogen gas accumulation in tissues from supersaturation can upset buoyancy and damage fragile cell membranes and vasculature. Small salmonids suffering from gas supersaturation may swim irregularly; float vertically, head upwards in the water; and typically have visible gas emboli in gill and fin tissues. Gas supersaturation can occur as a consequence of rapid temperature change, air entrainment in water under pressure, or biological processes such as denitrification of nitrate by bacteria. While nitrogen is commonly the cause of gas supersaturation, oxygen and other gases have the potential to cause gas supersaturation in some circumstances.

Eggs are generally refractory to increasing gas pressure under most circumstances. Changes in buoyancy would result if gas became trapped within the chorion. Alevins, on the other hand, are very susceptible to gas pressure, and affected fishes will have gas emboli develop in highly vascularized tissues like swim bladders, gills, and retinal tissue.

Dissolved Nutrients

Dissolved macro- and micronutrients are very important to developing salmonid embryos. These elements are used to make many biological molecules. Iron is central to the construction of hemoglobin; calcium is essential to the development of bone, skin, and muscle tissues. Sodium and potassium are exchanged dby the gills for metabolic wastes (Lall 2002; Takei and Lorentz 2006). These macro- and micronutrients form the building blocks of developing embryos. Fish reared in waters with low mineral content or with unusual mineral content may develop abnormalities, use inappropriate minerals to build biological tissues, and have inappropriate minerals irreversibly bind to mineral uptake molecules, rendering them useless. For example, in acidified streams low in calcium, dissolved aluminum can bind to gill tissues. Salmonids do not have the ability to obtain sufficient calcium from their diet to meet biological needs. They require as much as 50% of their calcium to be acquired from dissolved calcium resources in the environment through their gills and skin (Evans 1998). Dissolved calcium is abundant in many freshwater environments; however, in parts of Europe and eastern North America, bedrock calcium is scarce, and not coincidently, salmonid stocks are imperiled. Much of the research on the effects of acid rain in these areas focuses on the change in ambient pH (Harriman and Morrison 1981; Somers and Harvey 1984; Lubieniecki and Steinberg 1985; Fryer et al. 1988; Mount et al. 1988a, 1988b, 1990; Tam et al. 1988; Tietge et al. 1988; Wood et al. 1988a, 1988b, 1988c; Hurley et al. 1989; Walker et al. 1989; Ingersoll et al. 1990; Hontela et al. 1991; Tam and Zhang 1996; Sharpe and Demchik 1998; Cole 1999; Lachance et al. 2000; Cole et al. 2001). Low pH has a negative

impact on egg fertilization, water hardening, fry growth, and smoltification; however, the lack of dissolved calcium available for biological uptake by the fishes may ultimately be the underlying nutritional etiology of their decline (Danner 2004; Helland et al. 2005). The degree of skeletal calcification can be assessed using alizarin red and methylene blue (Springer and Johnson 2000).

Developmental Stages

Development of salmonid eggs occurs in three distinct stages: cleavage, gastrulation, and then organogenesis. The discussion of more detailed developmental biology is covered exceptionally well in Gilbert et al. (2000) and Wolpert (2002). The stages of development described below follow the suggested developmental markers identified by Velsen (1980). The 30 developmental stages do not necessarily correspond to equivalent increments of ATUs, but they do provide a good illustrative guide to development (Table 1). Velsen (1980) photographed sockeye salmon *O. nerka* embryos. This document uses brook trout *Salvelinus fontinalis* embryos. These embryos are somewhat smaller than sockeye salmon embryos. Others have published keys to a variety of larval fishes (Auer 1982; Danner and Boucher 2005) and the online database FishBase (http://www.fishbase.org/) has an accompanying database specifically focusing on larval fishes, LarvalBase (http://www.larvalbase.org/).

Cleavage (Stages 1–10)

After successful fertilization, it takes several hours for the first signs of cleavage in the blastodisc to appear. The first phase of salmonid embryo development is a series of cleavage steps dividing the single fertilized cell into an increasing number of smaller and smaller cells. In the first stage of development (stage 1), the cytoplasm migrates over and concentrates in one location on the surface of the yolk. This area is called the animal pole. There, the cytoplasm rounds up and rises slightly to form the blastodisc, a hemispherical dome, which will be the area where the embryo will form (Figure 2). The blastodisc forms in both a fertilized and unfertilized egg. In an unfertilized egg, the blastodisc begins to collapse and deteriorate after water hardening. In a fertilized egg, it begins the first cleavage step. The salmonid embryo cleavage pattern is meroblastic, meaning that the blastodisc begins to divide but the underlying yolk does not. Contrast this with a mammal egg, where the entire blastodisc and yolk divide (holoblastic) into increasingly smaller parts throughout cleavage, or the lamprey eel, in which the blastodisc and about one-half of the yolk divide (semiholoblastic). In the fertilized egg, cell division begins with the first cleavage of the blastodisc to form two cells of equal size (stage 2). This stage can be easily seen through the egg with a hand 43 magnifying lens or dissecting microscope. Stage 2 is the first visual verification that fertilization of the egg has occurred. At stage 3, a four-cell embryo appears like a small four-leafed shamrock-shaped structure within the egg (Figure 3). The blastodisc continues to divide until individual cells can no longer been seen. The structure is now referred to as the morula. The appearance of the morula and an unfertilized egg are very similar; however, careful observation of the morula in a 1003 phase contrast wet mount will reveal numerous cells. The morula will also appear as a grouping of small cells if stained and examined at 1003 with conventional light microscopy. In contrast, the unfertilized blastodisc will appear as a single cell with its content ruptured onto the slide. The morula remains the same size as the original blastodisc despite the increase in cell numbers, which is why the two are hard to differentiate grossly. At stage 9, the morula begins to flatten out and spreads over the animal pole. The future location of the embryo, though yet to form, can be detected on one side of the disk as a thickening crescent edge. The proliferating cells form the crescent along one edge of the animal pole that will eventually become the anterior end of the embryo. This proliferation of cells ends the cleavage phase of development (Gilbert et al. 2000).

Gastrulation (Stages 11–17)

The axis of the embryo is formed next with cells synchronicly migrating over the surface of the yolk. The development of the primary axis is a significant accomplishment in the embryo's development. It is at this stage that deformities of the notochord axis occur: Siamese twinning, double heads, and extra eyes. Cells must migrate long distances across the yolk, and deficiencies in mineral content or amino acids often manifest at this development stage. Once the terminal caudal bud has formed, the major body axis is complete (stage 11). Development of the caudal bud area will eventually become the tail portion of the fish. This tail section is currently attached to the yolk sac. The tail section will not be free to move in the fish until stage 18. At this point, the embryos all appear perfectly straight when viewed from above (Figure 4). Cells begin to specialize to form tissues, and the body is formed. The overgrowing edge, or germ ring, is preceded by the periblast, a clear layer derived from the old cytoplasm. This migration of the germ ring over the yolk is called epiboly. The tissues formed by the germ ring will eventually envelop the yolk in what will become the yolk sac. At this time, the anterior organization of the embryo can be found at the original animal pole while the rudiment of the tail locates itself near the border of the germ ring.

Organogenesis (Stages 18–30)

Next, the caudal portion of the fish is freed from the yolk and able to move laterally (stage 18). Usually, the first indication that the fish has reached this stage is the sudden J-shape the embryo's body can form. Prior to this stage, the fish are fixed in an I-shape. This makes stage 18 an easy stage to visually inspect the developing embryos. Parts of the brain become distinct (e.g., metencephalon and myelencephalon). The metencephalon and myelencephalon are part of the rhombencephalon or hind brain. The myelencephalon eventually becomes the medulla oblongata, whose neurons generate the nerves that regulate the respiratory, gastrointestinal, and cardiovascular movements. The more anterior metencephalon gives rise to the cerebellum, the part of the brain responsible for coordinating movements, posture, and balance. Now more complicated organs begin to develop, including the skin and its pigmentation. Eyes develop and the retina becomes pigmented (stage 21–24). Eye developments are important steps in salmonid aquaculture because eggs have become increasingly resistant to mechanical shock and can be cleaned, shipped, and counted. Blood forms, giving the egg a redder or orange appearance; fins begin to form; and finally the operculum begins to cover the four branchial arches. Once fins have begun to form and the operculum has developed (stage 30), embryos begin to hatch. This ends the egg stage of salmonid larval development. Much development of the fins and gastrointestinal tract has yet to form; this forms during the next development stage as an alevin.

Pathology

Genetic Pathologies

Genetic diseases occur in fish populations, much as in other taxa, which could influence the interpretation of the cause of mortality. As such, affected embryos should show familial patterns of disease. Some redds should contain large proportions of affected embryos while other redds contain few or none. Genetic pathologies may be in somatic cell lines or germ lines of salmonid embryos. Somatic cell genetic pathologies will manifest themselves in the physiology or morphology of the embryo sometime during its life cycle. Germ line genetic damage will be passed from one generation to the next. Germ line genetic damage persists in populations of salmonids for many more generations than for other vertebrates because of the tetraploidy found in salmonids. Polyploidy is both beneficial and detrimental to salmonids. While the genetic damage will persist for more generations, the multiplicity of many loci can mask the deleterious alleles for many generations. If genetic damage is suspected in a population of salmonids, genetic samples should be collected from developing embryos and archived for

future genetic evaluation. Embryos can be preserved in 95% ethanol and frozen (–20°C) indefinitely.

Genetic pathologies can also result in neoplastic diseases (e.g., tumors, cancers, and deformities) in salmonids. Like other vertebrates, few embryos are affected by neoplastic diseases. Examination of returning adult fish may provide clues regarding neoplastic diseases resulting from genetic damage. Smith (1993) provides an overview of common neoplastic diseases of salmonids.

Nutritional Pathologies

Incubating salmonid eggs can be considered to be self-contained nutrient packages. The materials necessary to develop a single embryo are packaged within the egg prior to fertilization. Little nutritional pathology should be observed in developing embryos that are not directly traceable to parent nutrition during oogenesis. The exception may be mineral deficiencies like calcium found in extremely soft waters. Calcium deficiency can result in skeletal deformities especially around the head and mouth or vertebra developing as aggregate blocks or hemivertebra. Roberts (2002) provides a comprehensive overview of general salmonid nutritional pathology.

Aquatic Pollutants and Environmental Pathologies

Pollutants exhibit a variety of effects on developing embryos, including acute mortality, developmental delays, developmental alterations, chronic mortality, and even permanent genetic mutations. For example Bue et al. (1998) investigated the long-term effects of the *Exxon Valdez* oil spill on pink salmon embryo survival and demonstrated that increased embryo mortality was apparent in oil-contaminated lineages generations after the spill and occurred in both contaminated streams and in lineages moved to uncontaminated streams. This provides evidence that the contamination permanently affected the fish's germ line. Long-term studies on aquatic pollution are necessary to establish if pollutants are having detrimental effects to germ lines, causing physiological effects that result in functional sterility, or if other environmental changes affect fish survival. Past studies have demonstrated that embryonic fish take up polycyclic aromatic hydrocarbons (PAHs; Moles et al. 1987). Numerous studies have demonstrated that PAHs are also capable of inducing chromosomal lesions in laboratory experiments and field observations (Bickham et al. 1998; Hose and Brown 1998; Farmer et al. 2003; Chen and White 2004; Goanvec et al. 2004; Reynaud et al. 2004). Numerous studies have demonstrated that PAHs are capable of influencing endocrine function in laboratory and field observations (Hontela et al. 1992; Hose and Brown 1998; Evanson and Van Der Kraak 2001; Farmer et al. 2003; Fossi et al. 2004; Teles et al. 2005; Wong et al. 2005; Gesto et al. 2006; Hinfray et al. 2006).

The list of aquatic pollutants is extensive and new substances are being investigated and discovered annually. Many aquatic substances are only toxic under certain environmental conditions such as acidification (Leivestad 1982; Richardson et al. 1995; Myllynen et al. 1997; Gesto et al. 2006). Some environmental pollutants are toxic only at certain developmental stages. It is beyond the scope of this chapter to discuss developmental toxicology in detail, and most spawning habitat remediation efforts are in streams where this is not an issue. However, toxicity should always be ruled out before initiating physical spawning habitat remediation efforts. Sorensen (2000) gives a good overview of the effects of metals on developing embryos. Water quality testing will usually reveal when metals or other sources of acute or chronic toxicity are suspected, in which case remediation efforts will generally address all life stages.

A variety of environmental diseases occur in salmonids, including diseases caused by increased solids, changes in water quality, and indirect causal factors. Klontz (1993) provides a good review of these factors in cultured salmonids, and discussion is directly applicable to salmonid embryos.

Infectious Diseases

The infectious diseases of salmonids are well documented and procedures are standardized for detection of these pathogens. Embryos collected in the field can be tested according to standardized procedures for locally prevalent as well as exotic diseases (USFWS and AFS–FHS 2004). Some diseases have common symptoms, for example, embryonic trout with black tails may be infected with infectious pancreatic necrosis virus (Plumb 1999). Many infectious pathogens take advantage of poor water quality conditions or high densities of vulnerable organisms; therefore, care should be taken to determine if observed pathogens are primary pathogens attacking salmonid embryos or simply opportunistic commensals taking advantage of a disadvantaged salmonid embryo. Coordination of sampling and observation with a fish pathologist will help identify infectious etiologies of embryo mortality.

Summary

The development of salmonid eggs follows a predetermined course. Successful emergence of alevins from redds is predictable and repeatable but requires the sequential execution of specific biological processes beginning with proper parental health and nutrition, maternal egg development, proper behavioral interaction between male and female adult salmonids, successful deposition of fertilized eggs into a suitable hyporheic environment, and an appropriate amount of time in that habitat within a narrow temperature and water quality range. Forensic examination of developing embryos will allow an investigator to separate a myriad of different causes of embryo mortality into meaningful categories and time frames. Pinpointing the time frame responsible for the largest mortality of embryos will coincide with the biological factors having the greatest negative impact on the fish's reproductive success. For example, embryos emerging from the redd before natural food resources are available will die of starvation. They may be incubating at accelerated temperatures due to habitat degradation. On the other hand, embryos that are dying before they have developed a notochord may be suffering from nutritional deficiencies acquired from their mother before fertilization. Table 1 provides a summary of developmental stages to help investigators identify the stage of embryos they examine. Table 2 provides an example of a data sheet an investigator can use to summary this information. And Table 3 provides a dichotomous key that an investigator can use to narrow potential causes of embryonic death. Degradation of salmonid spawning habitat is only one of many potential causes of salmonid embryo mortality that needs much future research. Investigators who take the time to pinpoint when embryos are dying will pave the way to improving salmonid reproduction.

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