Effect of Diluent Type, Diluent:Sperm Ratio and Extender Use on Rainbow and Cutthroat Trout Egg Fertilization

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Abstract

Premature sperm activation can reduce fertilization. Sperm extenders are a potential remedy. In Test 1, rainbow trout (*Oncorhynchus mykiss)* sperm motility and motility duration were compared among three diluent types, three milt:diluent dilutions and between extended and un-extended milt. Dilutions ≥ 1:1 were sufficient for complete activation of un-extended rainbow trout sperm with all three diluents. For extended milt, complete activation was observed in 4 of 5 replicates at 1:2 and all replicates of 1:3, but not at 1:1. Sperm motility lasted from 21 to 52 s and was unaffected by extender, diluent type, or dilution. In another test using extended and un-extended sperm to fertilize eggs at high female to male ratios (4:1), no significant difference in percent fertilization was observed between 4:1 and 1:1 ratios or between extended and control sperm treatments. For cutthroat trout (*O. clarkii pleuriticus*) eggs fertilized with extended sperm, there was no significant difference in survival to eye-up. The data indicated extender requires three-fold dilution, but did not negatively affect fertilization or duration of motility when common activating solutions were used.

Key words: fertilization, diluent, extender, spermatozoa, *Oncorhynchus*

Introduction

Several subspecies of cutthroat trout (*Oncorhynchus clarkii*) are endemic to Utah. The Utah Division of Wildlife Resources annually collects eggs from wild cutthroat trout at various locations around the state for the purpose of propagation and stocking for both sport fish enhancement and for re-establishing populations within their historic range. A review of the historical production at these wild traps in Utah was made recently in response to concerns about egg survival (Wagner and Oplinger 2013). A data meta-analysis of egg survival among all the trap sites suggested that although site specific variables such as travel distance (trap to hatchery) and reservoir size were significant, within-site variance in egg survival to the eyed stage (0 to 100%) indicated other variables that were not measured were likely affecting egg survival. These variables include weather, air temperature, gamete quality and operational variables such as personnel and gamete handling (despite uniform spawning protocols), which may vary at a given trap site. Chief among variables biologists can control, are those associated with gamete handling.

Sperm motility is a critical factor for fertilization and is easily compromised. It is known that water dripping into the milt from the fish or a spawning glove can lead to premature activation (Piper et al. 1982). Blood or urine contamination of the milt may also reduce fertilization by prematurely activating sperm (Poupard et al. 1998, Linhart et al. 1999, Ingermann et al. 2010). Ovarian fluid can also stimulate premature activation of sperm (Rucker et al. 1960, Billard 1983, Ingermann et al. 2010), which can be a problem when several males are

added sequentially to a pool of eggs. Poor milt production by males can contribute to poor fertilization via reduced sperm numbers (Stockley et al. 1997). Although not usually measured, the volume of milt produced per male spawned at the wild traps has typically been lower and more variable than in captive broods.

The primary goals of our research were to determine whether sperm extenders can be used to prevent premature activation of sperm and to determine the best dilution ratios and diluents for the activation of extended sperm. The wild cutthroat trout collected by the Utah Division of Wildlife Resources typically produce a low volume of sperm (< 1 mL) and we were concerned that this limited volume exacerbates issues of water, urine, or blood contamination. Sperm extenders are solutions that maintain physiological conditions similar to seminal fluid (Henderson and Dewar 1959, Baynes et al. 1981) and prevent flagella activation until a diluent (activation) solution is added. Sperm extenders developed for salmonids typically have high potassium ion (K+) concentrations (Morisawa et al. 1983a). Short term sperm storage in extender solutions has been described (e.g., Henderson and Dewar 1959, Baynes et al. 1981) but the use of extenders during the spawning process has not been described to our knowledge. Theoretically, stripping sperm directly into an extender solution could offset contamination by water, urine, or blood and could prevent premature flagella activation and thus increase fertilization rates. However, to our knowledge, no research on the optimal solution (diluent) or dilution ratio for the activation of extended sperm has been performed. Water is the natural diluent, activating sperm as K+ concentrations drop, but solutions have been developed that significantly extend the duration of motility of salmonid sperm (Billard 1985). A saline solution called D532, buffered to pH 9 and containing 20 mM Tris and 50 mM glycine, is known to increase motility duration of salmonids and extend the time that the micropyle remains open for receiving sperm (Billard 1985). Steyn et al. (1989) found that a Borax-boric acid buffer diluent resulted in higher fertilization percentages than water, saline, or Tris-glycine buffer. However, sodium chloride alone has proven to be as effective as sodium chloride solutions containing other ingredients (Petit et al. 1973, Scott and Baynes 1980, Krise et al. 1995). Rock salt, although comprised primarily of NaCl (>99.4%), contains other cations and anions in small amounts and is cheaper than purified NaCl. A rock salt solution of 0.5-0.7% has been used routinely at the Utah wild trap sites and Utah brood hatcheries as a diluent.

In addition to preventing premature flagella activation, sperm extenders could be used to improve sperm distribution during fertilization. Increasing the volume of sperm by producing a dilute sperm+extender solution could improve the coverage of eggs and increase fertilization. Poon and Johnson (1970) showed that dilution of sperm at the time of fertilization improved percent fertilization (72-80% versus 39-40% in undiluted controls). Similar results were observed by Plosila et al. (1972) for brook trout (*Salvelinus fontinalis*) eggs. These studies diluted sperm with water, but we are not aware of similar studies being conducted diluting sperm with extender solutions. One potential advantage to diluting sperm with extender solutions rather than activating solutions (diluents) is that extenders do not activate the sperm; i.e., the dilution occurs before activation, providing a few more seconds of motility in a situation where flagella are only active for a limited period (Billard and Cosson 1992).

We hypothesized that improvements could be made in fertilization success (or at least no negative impact), in both trap and hatchery settings, by collecting sperm in an extender solution that would buffer against premature activation. We also hypothesized that when milt quantity is low, as is typical when spawning wild males, dilution in extender solution better distributes the sperm to the eggs, leading to higher percent fertilization. Prior to implementing the use of extender solutions at the wild traps, we

conducted some preliminary tests. Three objectives were targeted in these tests: 1) determine the dilutions needed to activate extender solutions with common diluents, 2) determine the effectiveness of extender solutions for fertilizing under low milt:egg scenarios and 3) evaluate extender use in the field for cutthroat trout in a wild trap setting. We conducted this research with the goal of improving the fertilization and hatch rates of eggs from cutthroat trout, but since availability of wild brood fish is limited and fisheries managers were unwilling to risk using extenders without some preliminary testing, two experiments were performed with hatchery rainbow trout as a surrogate. One field experiment did evaluate extender use with cutthroat trout.

Methods

We conducted two tests to determine the effect of extenders and various fertilization variables on egg survival of rainbow trout (4 year-old females and 3 year-old males) from the Mantua State Fish Hatchery, Mantua, Utah. A third test evaluated extender use for cutthroat trout collected from Lake Canyon Lake, Duchesne County, Utah. The extender solution used in each test was derived from Negus (2008) and was comprised of 6.02 g/L NaCl , 2.98 g/L KCl, 4.77 g/L HEPES; It was mixed with the sperm at a 1:1 (v/v) ratio.

For data analysis, we used NCSS Version 2007 (J. Hintze, Kaysville, Utah) or R (Hornik 2015). We conducterd normality tests with the Martinez-Iglewicz and Kolmogorov-Smirnov tests (Hintze 1995). These tests were followed by appropriate tests (GLM, ANOVA, or t-tests) detailed within each experimental test section below. A two-tailed probability of <0.05 was considered significant. We used Scheffé's test for mean separation (Scheffé 1959).

Test 1:Effects of Dilution, Diluent Type and Extender use on Sperm Motility and Duration

We evaluated the effect on sperm motility and motility duration of three different dilution ratios (1:1, 1:2, 1:3; v/v, sperm:diluent) and three diluent types (0.75% rock salt[Solar Salt, Western Sun, Salt Lake City, Utah; label states sodium chloride content >99.4%], 0.75% NaCl and Fisheries Experiment Station [FES] well water) using both extended and un-extended milt (control). The FES well water had a hardness of 222 mg/L as $CaCO_3$, total alkalinity of 222 mg/L and a pH of 7.6 and the rock salt and NaCl diluents were prepared in de-ionized water. We collected milt on 20 October 2014 by hand stripping the milt from males into a Styrofoam cup using a metal sieve (1.6 mm mesh) to prevent feces from entering the cup. By holding the cup at an angle away from the fish, only expressed milt reached the cup, avoiding dripping water from the fish and gloved hand. A total of three pools of milt were made, with three males in each. The pools were placed into 50 mL centrifuge tubes and kept in a cooler for transport to FES (30 min drive). Extender was not added to the sperm until motility tests were initiated.

We checked motility 3-5 h after sperm collection. There were three replicates of each treatment. Each pool of sperm was divided into two even volume portions and an equal volume of extender was added to one of the portions whereas no extender was added to the other portion. The sperm was activated by adding the appropriate amount of sperm and then 100 µL of diluent in 1.7 mL microcentrifuge tubes. For the 1:1, 1:2, 1:3 dilutions, 100, 50, or 33.3 µL of milt or the sperm + extender mixture was added, respectively. After dilution, we mixed each solution with a pipette by drawing it in and out twice and then 10-20 µL of the mixture was placed onto a microscope slide and viewed at 100x magnification (10x objective). An estimate of the percent motility was made as quickly as possible (usually about 7-10 sec after activation) by visual inspection and the time (sec) until sperm motion ceased was recorded. Motion was defined as >2 sperm body (head + tail) lengths/sec. The sperm + extender mixture was allowed to sit for 15 min prior to estimating motility. We attempted to

keep milt and diluent solutions at the same temperature (14.1°C) during the experiment. We analyzed duration of motility with a general linear model; diluent type, extender use and dilution ratio were considered fixed factors in the model.

Test 2: Effects of Extender With Low Sperm:Egg Ratios

In this experiment, the effect of high egg numbers relative to the amount of sperm was evaluated, with or without the use of extender. We hypothesized extender would help dilute the sperm and improve fertilization. Rainbow trout of the West Virginia strain were used for the experiment.

We divided a pool of eggs from 10 females into 4 lots: two with 1/10 of the eggs each and 2 with 4/10 of the eggs each. One male was used to fertilize one 1/10 lot and another male was used to fertilize a 4/10 lot. The remaining two lots were also fertilized by one male each, but the milt had been diluted in 10 mL of sperm extender (about 1:1 v/v). After egg pooling, this approximates male: female ratios of 1:1 and 1:4, each fertilized by either extended sperm or an un-extended control. A separate Styrofoam cup was used to collect sperm from each male. The four lots were fertilized at the same time and 0.75% rock salt solution was used to initiate fertilization, pouring enough solution to cover the eggs within a small pail. The eggs were rinsed after 2 min and left to water harden in hatchery well water at 9°C. We repeated this process five times to generate 5 replicates per treatment.

A subsample of 50 mL of eggs from each treatment was retained for the extender experiment and the rest were given back to the hatchery for production needs. Each subsample was placed into a 1 L plastic beaker and water hardened for 1-1.5 hr. After hardening, the eggs were disinfected for 10 min in 100 mg/L of iodine. The subsamples were randomly assigned to one of 20 egg incubation trays supplied with about 15 L/min of hatchery well water (pH = 7.8, total hardness = 185 mg/L, total alkalinity = 179 mg/L).

Survival to hatch was determined by hand counting and removing dead eggs on three separate dates and expressed as a percentage of total eggs at the start (also hand counted). The number of deformed fry was also determined and expressed as a percentage of the number of live fry at hatch. Data were analyzed with two-way ANOVA using R (Hornik 2015). Extender use (yes, no) and milt:egg ratio (1:1, 1:4) were considered fixed variables.

Test 3: Effect of Extender on Cutthroat Trout

On 27 May 2015, we captured Colorado River cutthroat trout from Lake Canyon Lake, Duchesne County, Utah, for the annual spawning operation. See Wagner and Oplinger (2013) for more details on the site and its history. For the experiment, eggs from each female were divided into two separate plastic bowls. Eggs from a second female were similarly split into two roughly equal aliquots that were added to the bowls from the first female, creating two groups with the same genetic composition. We stripped milt from two males sequentially onto eggs within one of the bowls, which served as a control. Milt from two other males was collected in a 50 mL centrifuge tube with 10 ml of extender solution (Negus 2008). The milt-extender solution was added to the second bowl of eggs. Efforts were made to keep the bottle of extender solution at the same temperature (11-13°C) as the lake water and spawning fish. A rock salt diluent (0.75% in hatchery well water), also kept at lake temperature, was used to initiate fertilization in both bowls, adding enough to cover the eggs (about 100-200 mL). After 5 min, we rinsed the bowls with fresh hatchery well water to remove excess sperm and dead eggs. Eggs were added to coolers with hatchery well water, one cooler for the "Extender" treatment and second cooler for "Control" treatment. We repeated this process four more times to acquire eggs from a total of 10 females (5 fertilization groups per treatment). This was considered a single replicate. A total of three replicates were obtained per treatment, each

contained in a separate cooler. This resulted in a total of 30 females and 60 males being used.

After transport to the isolation station at Fountain Green State Fish Hatchery, Fountain Green, Utah, we treated eggs in each cooler with 100 mg/L iodine for 10 min. Eggs were put into six separate egg incubation jars, one for each replicate. Eggs were treated daily with 1,667 mg/L formalin until reaching the eyed stage. Upon reaching the eyed stage, the egg survival was determined based on the proportion of the volume of live eggs over total volume of live + dead eggs. Live and dead eggs were separated using a commercial egg sorter. Percent survival of eggs in the treatment and control was evaluated for significance using a t-test.

Results

Test 1:Effects of Dilution, Diluent Type and Extender Use on Sperm Motility and Duration

The dilution ratio needed to obtain complete activation of sperm in extender solution was 1:2 when water was used as an activator and 1:3 when the rock salt diluent was used (Table 1). Un-extended sperm needed less dilution, activating at dilutions of ≥ 1:1 for all three diluent types.

Duration of sperm motility ranged from 21 to 52 sec among individual replicates and mean values ranged from 29.3 to 42.3 s among treatments (Table 1). Use of sperm extender had a significant ($P = 0.04$, F = 5.0, d.f. = 1), positive effect on the duration of motility (mean of 35.9 s versus 30.6 s

Table 1. Comparison of the percent motility and duration of motility (mean ± SD, N = 3) among different dilution ratios and diluents for extended or un-extended rainbow trout sperm.

Extender treatment	Diluent Type	Dilution Ratio	Motility (%)	Duration of motility (sec)
Extended	Water	1:1	0.0 ± 0.0	-
		1:2	100.0 ± 0.0	42.3 ± 9.1
		1:3	100.0 ± 0.0	33.3 ± 0.6
	0.75% NaCl	1:1	100.0 ± 0.0	37.3 ± 1.5
		1:2	100.0 ± 0.0	34.0 ± 1.0
		1:3	100.0 ± 0.0	29.3 ± 6.7
	0.75% rock	1:1	63.3 ± 28.9	32.7 ± 4.0
	salt	1:2	73.3 ± 46.2	27.3 ± 5.5
		1:3	100.0 ± 0.0	39.3 ± 10.6
Un-extended	Water	1:1	100.0 ± 0.0	35.7 ± 2.5
		1:2	100.0 ± 0.0	29.3 ± 3.2
	0.75% NaCl	1:1	00.0 ± 0.0	35.0 ± 3.6
		1:2	100.0 ± 0.0	32.3 ± 2.3
	0.75% rock	1:1	100.0 ± 0.0	28.0 ± 0.0
	salt	1:2	100.0 ± 0.0	25.7 ± 0.6

for unextended, pooling across treatments). Duration of motility was not significantly affected by the type of diluent activator solution ($P = 0.88$, F = 0.12, d.f. = 2) or the milt dilution ratio ($P = 0.22$, F = 1.6, d.f. = 2). There were no significant interaction terms in the general linear model ($P > 0.06$).

Test 2: Effects of Extender With Low Sperm:Egg Ratios

The mean percent survival to hatch ranged from 83.8 to 88.7% among extended milt treatments and from 81.5 to 86.5% among un-extended controls (Fig. 1). There was no significant effect of extender use ($P = 0.56$, F = 0.3, d.f. = 1) or sperm:egg ratio ($P = 0.22$, F = 1.6, d.f. = 1) on the percent hatch. Similarly, the percentage of deformities was not significantly affected by extender use ($P = 0.98$, F < 0.01, d.f. = 1) or sperm:egg ratio ($P = 0.11$, F = 2.9, d.f. = 1).

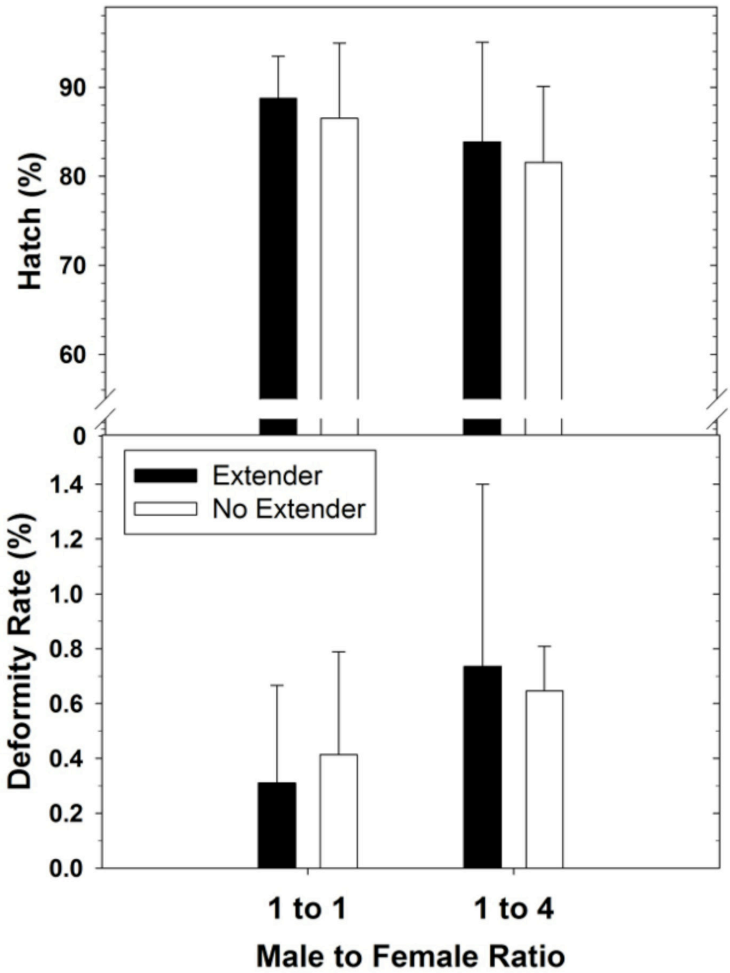

Figure 1. Comparison of the percent hatch (top panel) and percent deformities (bottom panel; mean ± SD, N = 5) among eggs fertilized with extended milt or non-extended milt, using male:female ratios of 1:1 (control) or 1:4.

Test 3: Effect of Extender on Cutthroat Trout

There was no significant difference in survival to the eyed egg stage between eggs fertilized with extended sperm (84.0 ± 9.5%) and controls fertilized by adding milt directly to the bowl of eggs (74.7 ± 30.0%). However, the high variance in the control group was notable, largely due to one replicate which had only 40% survival to the eyed stage.

Discussion

Our results from Test 1 indicated that non-extended sperm can be activated when mixed with an equal volume of water. In that test, we observed that male milt quantity varied from about 0.3 to 20 mL per fish. So, especially at lower milt volumes, it would not take much contamination with water to activate at least pockets of sperm, which could inhibit fertilization. Blood (Ingermann et al. 2010), urine (Poupard et al. 1998) and ovarian fluid (Rucker et al. 1960, Billard 1983, Ingermann et al. 2010) may also prematurely activate sperm prior to thorough mixing.

Fortunately, extender solutions have been developed to store sperm in an un-activated state, extending the storage life of sperm (Billard 1983, McNiven et al. 1993, Henderson and Dewar 1959). Extenders typically try to match the osmolality and pH of seminal fluid (Petit et al. 1973, Ingermann et al. 2002). For example, the best extenders for striped bass (*Morone saxatilis*) (Jenkins-Keeran and Woods 2002) and sea lamprey (*Petromyzon marinus*) sperm (Ciereszko et al. 2002) were those that matched the osmolality of the seminal fluid. Sperm extenders were developed for preserving sperm for extended periods of time, i.e., several weeks. What has received little attention, however, is the use of sperm extenders during routine spawning to prevent premature activation. Proper handling procedures can minimize water dripping into the milt, but blood and urine in the milt are harder to mitigate with handling per se.

In our studies, we evaluated the use of sperm extenders during stripping or for diluting small volumes of sperm to promote better coverage over eggs and fertilization. The extender used in this study required at least a 1:2 dilution if activated with water and a 1:3 dilution to get consistent 100% activation with rock salt diluent. Graybill (1968) also observed a ≥1:2 dilution was needed for activation of extended coho salmon (*Oncorhynchus kisutch*) sperm using fresh water; The extender solution had a K^+ concentration of 2.98 g/L or 83 mM, so activation occurred at about 41.5 mM when using water as an activator (did not measure K^+ concentration in water) and 27.7 mM K^+ (1:3 dilution) using rock salt diluents (K^+ concentration of salt diluents unknown). This concentration is much higher than the threshold for activation reported by Ingermann et al. (2010). Baynes et al. (1981) observed that KCl concentrations as low as 1 mM inhibited activation of rainbow trout sperm in the absence of NaCl, but when present (150 mM), at least 13 mM KCl was required to inhibit activation. So, our findings corroborate previous studies that have demonstrated that variables other than K+ concentration alone are involved with sperm activation, such as changes in osmotic pressure (Stoss 1983, Orfão et al. 2011). For example, Bates et al. (1996) noted for channel catfish (*Ictalurus punctatus*) sperm that a drop in osmolality from physiological levels of 273 mosmol/kg to 132 mosmol/kg (about a two-fold dilution) led to complete activation. Changes in transmembrane potential have been shown to activate rainbow trout sperm (Blaber and Hallett 1988); the electrical potential was three times greater when K+ concentrations dropped, than for the same drop in Na+ ion concentration. Sperm from cyprinid species also has been shown to activate after decreasing osmolality by half (Morisawa et al. 1983b).

In this study, sperm motility duration did not differ between hatchery well water and either of the 0.75% salt diluents. However, sperm of Atlantic salmon (*Salmo salar*) studied by Ellis and Jones (1939)

and of salmonids in studies reviewed by Scott and Baynes (1980) remained motile longer if activated by dilute salt solutions rather than freshwater. Ginsburg (1972) reviewed several studies and found this relationship as well, but there were other studies he reviewed in which this motility difference was not observed. The discrepancy among studies may be related to the salt concentration used, which may have a profound effect on motility duration, which decreases as salinity exceeds a narrow optimum concentration (Ginsburg 1972; Billard 1978).

The goal of our second test was to determine whether the dilution of sperm in extender helped improve egg fertilization in a situation where milt volume and presumably sperm number were limited compared to the number of eggs fertilized. A few studies (e.g., Billard et al. 1974, Scott and Baynes 1980) have described how the dilution of sperm using diluent can influence motility. What has received little attention, however, is the uniform distribution of sperm, which has been identified as a factor that can influence egg fertilization (Snook 2005). Rainbow trout sperm motility has been shown to decrease precipitously after 15 sec (Stoss 1983) and in principle the time required to thoroughly mix sperm with eggs after activation may be sufficient to prevent some eggs from coming in contact with motile sperm. This issue is likely most prevalent when the volume of milt used is low relative to the number of eggs fertilized. In our test, the pre-dilution of sperm in extender did not significantly improve fertilization rates. Although not statistically significant, hatch rates were approximately 2% higher when the extender was used. Oplinger and Wagner (2015) evaluated the use of sperm extenders containing antibiotics and found a 0.75% greater hatch rate among eggs fertilized using extended sperm compared to controls where the sperm was not extended. Thus there is some evidence that extender use leads to slight increases in fertilization, albeit more evaluation is required. Slight increases in viability could translate into significant increases in fish numbers in large production hatcheries or could be beneficial in situations where species conservation is of interest and there is a need to produce as many fish as possible.

The use of extender for cutthroat trout sperm indicated no negative effect of extender use on survival to the eyed stage. The variance observed in the controls indicated that while some batches have high egg survival when milt is stripped directly onto the eggs, others do not. So, although extender use did not significantly improve fertilization, egg survival was more consistent in eggs fertilized with extended sperm. A similar reduction in variance was observed in the small-scale trials performed by Oplinger and Wagner (2015). There are many factors that can affect fertilization percentages such as nutrition, stress, genetics, overripe eggs and age of female (Coward et al. 2002). Sperm quality has also been shown to vary temporally, typically declining later in the spawning season (Büyükhatipoglu and Holtz 1984, Hajirezaee et al. 2010, Johnson et al. 2013). For the cutthroat trout in this study, the gametes were collected during the middle of the spawning season. While these factors may also be influencing fertilization success at the wild traps that were the impetus for this study, factors relating to premature activation of sperm may be mitigated with the use of extender. In addition to standard hatchery practices minimizing water dripping into milt, extender use in routine hatchery spawning scenarios could also lead to less variance and incremental improvements in fertilization. We recommend further testing of extender use on a production scale. Also a controlled quantitative study to assess the effect of contaminants (e.g., blood, water, ovarian fluid) added to extender solutions is recommended.

The literature on extenders is extensive for evaluations of sperm storage methods, storage duration and motility after storage, but our current research is the first application of extender use reported in the literature that we are aware of for mitigating

premature sperm activation during normal spawning operations. Extender use did not compromise sperm motility and is recommended for use for preventing premature sperm activation. Sufficient dilution to achieve sperm activation, e.g. ≥ 1:3, is easily achieved if small amounts of extender solutions are used.

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