Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide

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Abstract

Egg survival, sperm motility and physiological stress responses (plasma cortisol, glucose and chloride) of rainbow trout (*Oncorhynchus mykiss*) broodstock were compared among three anesthetics: tricaine methanesulfonate (MS-222), clove oil in the form of AQUI-S® (a proprietary mix of 50% isoeugenol and other ingredients) and carbon dioxide gas. Concentrations of 60 mg/l tricaine, 20 mg/l isoeugenol (40 mg/l AQUI-S) and 220–275 mg/l carbon dioxide were based on preliminary tests and chosen to standardize induction time among anesthetics. Plasma glucose, chloride and cortisol concentrations indicated that none of the anesthetics used after crowding and netting completely eliminated the stress response. The return to prestress cortisol levels differed among the three anesthetics. Fish anesthetized with AQUI-S had significantly lower cortisol concentrations at 1 or 7 h postimmersion than the other anesthetics and controls, but were elevated at 24 h. Plasma cortisol in tricaine- and CO_2-treated fish returned to prestress levels within 7 and 24 h, respectively, whereas cortisol levels in control fish remained elevated at 24 h. Sperm motility and duration of motility were assessed for a practical range of concentrations: tricaine, 15–100 mg/l; AQUI-S, 10–100 mg/l; CO_2, 50–173 mg/l. The percentage of motile sperm was unaffected by anesthetic treatment, averages ranging from 68% to 87%. However, duration of motility decreased as anesthetic concentration increased, averages ranging from 55 to 36 s for tricaine and from 56 to 37 s for AQUI-S. Duration of sperm motility was low (31–43 s) for all levels of CO_2 tested. Fish recovery time was significantly longer for fish anesthetized by AQUI-S (370 s) than the either CO_2 or tricaine (192 and 199 s, respectively). Gender had no effect on recovery time. Egg survival to the eyed stage and to hatch was not significantly different among anesthetic treatments and controls. No

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delayed mortality was observed for any of the fish handled and bled for the test. Results indicated that tricaine, AQUI-S and CO_2 were all suitable for broodfish anesthesia, but the longer recovery time and lower cost for AQUI-S may make it more useful than the alternatives. None of the anesthetics wholly suppressed the stress responses during a typical spawning process, but did help reduce the duration of the stress responses and eased handling without compromising egg viability. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Anesthetics are widely used in the aquaculture industry to aid in handling fish and to minimize stress. Stress can have a negative impact on captive fish, with effects including reduced immunocompetence, increased susceptibility to disease, reduced egg quality and spermatocrit and reduced growth (Pickering, 1981; Campbell et al., 1992; Iwama et al., 1997). Physiological changes in fish in response to stressful stimuli (stressors) are termed stress responses (Iwama et al., 1997). Plasma glucose, cortisol and chloride are a few of the physiological variables that respond to stressors, serving as indicators of stress (Pickering, 1981).

In the past, a number of different anesthetics have been used or evaluated for aquacultural applications such as quinaldine, benzocaine, 2-phenoxyethanol, etomidate, metomidate and tricaine methanesulfonate (Muench, 1958; Schoettger and Julin, 1967; Gilderhus et al., 1973; Dawson and Gilderhus, 1979; Limsuwan et al., 1983; Gilderhus and Marking, 1987). Carbon dioxide in its various forms has also been evaluated (Fish, 1943; Booke et al., 1978; Post, 1979; Gelwicks et al., 1998). Currently, only carbon dioxide and tricaine methanesulfonate (tricaine or MS-222) are approved for use as fish anesthetics by the U.S. Food and Drug Administration (FDA). Tricaine is expensive and requires a 21-day withdrawal period before fish can be consumed (Schnick et al., 1986). Canada currently requires a 5-day withdrawal period for tricaine if water temperatures are above 10 °C (Cho and Heath, 2000).

Clove oil (70–90% eugenol) has recently been discovered as an effective fish anesthetic, although it has been used by humans for centuries in Indonesia and elsewhere as a topical anesthetic (Soto and Burhanuddin, 1995; Peake, 1998). The active ingredient, eugenol, is a derivative of clove oil, a viscous liquid extracted from the leaves, buds and stem of the *Eugenia caryophyllata* tree (Isaacs, 1983). The isomer isoeugenol is approved in the U.S. as a food additive and eugenol is approved as a direct food substance generally recognized as safe. The commercial product, AQUI-S®, contains 50% isoeugenol and is currently in the process of evaluation for approval as a fish anesthetic by the FDA. It is approved for such use, with zero-withdrawal time required (i.e., fish can be consumed immediately after use of anesthetic), in New Zealand, Australia, Chile and the Faroe Islands (personal communication, Robert Campbell, AQUI-S New Zealand). Eugenol is an attractive alternative to tricaine because of shorter induction times at similar concentrations and lower cost (Keene et al., 1998). The longer recovery time for fish anesthetized with eugenol (Keene et al., 1998)

may also be a useful trait for situations such as marking, surgery or spawning, where it is desirable to work with the fish for a longer period after they are removed from the anesthetic (Prince and Powell, 2000). It has been used successfully in other countries for harvest of farmed salmon (personal communication, Robert Campbell, AQUI-S New Zealand).

Tricaine is widely used to anesthetize broodstock (Piper et al., 1982), with little apparent effect on egg viability. Despite numerous toxicological studies with anesthetics (Marking, 1967; Dawson and Marking, 1973; Siwicki, 1984; Gelwicks et al., 1998), research on mature fish and the effects of anesthesia on gamete viability is limited. Exceptions include the work of Allison (1961), who demonstrated that tricaine affected the motility of trout sperm at concentrations as low as 19 mg/l. In addition, Hovda and Linley (2000) noted that egg viability was unaffected by use of hypothermia (-1.5 to -3.0 °C) for anesthesia of Pacific salmon (*Oncorhynchus* spp.). Redman et al. (1998) noted that electroanesthesia did not affect egg viability, but significantly reduced broodstock survival. The effect of clove oil on egg viability of broodstock, or directly on sperm viability is not known.

The physiological stress responses of juvenile chinook salmon (*O. tshawytscha*) and juvenile rainbow trout (*O. mykiss*) to clove oil or AQUI-S anesthesia have been studied recently (Cho and Heath, 2000; Davidson et al., 2000), but broodstock have not been tested. Similarly, other studies examining the stress response of salmonids to anesthetics have focused on juvenile fish (Strange and Schreck, 1978; Barton and Peter, 1982; Laidley and Leatherland, 1988) or only one anesthetic (Smit et al., 1979). Other than Cho and Heath (2000), who compared the physiological responses to tricaine and clove oil, a comparison of physiological stress responses to AQUI-S with that for other available anesthetics has not been conducted. This study was conducted to compare the effects of three anesthetics—AQUI-S, tricaine and carbon dioxide gas—on the stress responses of rainbow trout broodstock as well as the viability of fertilized eggs from anesthetized parents. Duration of activity and percent motility of sperm in anesthetic was also examined with the objective of comparing anesthetics and their effects.

2. Methods

Preliminary tests were conducted with both fingerling and adult fish to determine anesthetic concentrations that would result in loss of equilibrium within 2–3 min. This standardized the induction time so that the stress response could be compared appropriately. For broodstock testing, the concentrations of the anesthetics were 40 mg/l AQUI-S and 60 mg/l tricaine, and for carbon dioxide ranged from 220 to 270 mg/l. Because of its high viscosity, AQUI-S stock solution was warmed in a breast pocket before diluting 1.2 ml in 100 ml of warm deionized water. This was added to 30 l of hatchery well water at 8–9 °C in a polyethylene container. Tricaine stock solution (100 mg/ml; Argent Chemical, Redmond, WA) was made a few days prior to the experiment, kept chilled in a cooler and the appropriate quantity added directly to another container. Carbon dioxide was delivered from a bottled gas cylinder via an airstone until the target concentration was reached. Concentrations of carbon dioxide were measured (± 5 mg/l) by colorimetric titration using a commercial test kit (Hach Chemical, Loveland, CO). The pH of the anesthetic solutions

was measured with a digital meter calibrated on the day of the test. The CO_2 treatment pH dropped to 5.04, the tricaine solution was 7.3, the AQUI-S solution was 7.8 and the hatchery-water control was 7.8. Total hardness of the hatchery spring water was 90 mg/l as $CaCO_3$.

2.1. Sperm motility

One day prior to the egg viability test, milt from five males was collected and pooled as follows. The males were anesthetized in 60 mg/l tricaine, dipped in clean hatchery water, wiped ventrally with paper towels and the expelled milt pooled in a plastic bowl. The pooled milt was transferred into a glass bottle which was placed in an ice-water bath until fertilization a few minutes later. For the sperm tests (duration of motility and percent motility), 10 µl of milt was placed onto a prefocused microscope slide. A second 10-µl volume of 0.7% NaCl was placed next to the milt so they were not touching. This was followed by a third 10-µl volume of the test anesthetic. Target anesthetic concentrations after mixing on the slide were: tricaine, 15, 25, 30, 50 and 100 mg/l; isoeugenol, 5, 10, 25, 50 mg/l (10, 20, 50, 100 mg/l AQUI-S) and CO_2, 50, 103 and 173 mg/l.

To activate the sperm a cover slip was placed onto the microscope slide which served to mix the three solutions. This method is similar to that of Terner (1986) who recommended a 2:1 ratio of saline to milt. We adjusted this by totaling the saline and anesthetic into a volume which was twice that of the milt volume. At the moment the cover slip was placed, a timer was started and duration and percent motility were noted. The end point for duration of motility was defined as when all forward movement of sperm had ceased even if vibratory motion continued. Percent sperm motility was counted as the percentage of motile sperm during the first several seconds after activation. Controls were also run using 10 µl of milt activated with 20 µl of 0.7% NaCl. Three slides were viewed for each anesthetic test concentration and controls.

2.2. Effect of anesthetics on egg viability

Rainbow trout of the Ten Sleep strain were checked for maturity 2 days before the experiment (1 December 1999) and 120 females with extrudible eggs were transferred to 800-l circular tanks. Males were similarly maintained in separate tanks. Individual fish weight ranged from 0.54 to 1.22 kg. Both males and females were dip-netted individually from the tanks into the anesthetic solutions and induction time recorded. Induction time was defined as the elapsed time from entry to total loss of equilibrium (Stage 4 anesthesia; Keene et al., 1998). Upon loss of equilibrium, the fish remained in anesthetic solution an additional minute before attempting to handle them. The eggs from five 3-year-old females were pooled on cloth netting to drain away the ovarian fluid. Milt from 4-year-old males was collected in two separate pans, one for males that were anesthetized and one for those that were not (control). The egg pool was divided roughly in half, one portion fertilized by two males that had been anesthetized, the other by milt from two controls. After the females were spawned, the recovery time in fresh hatchery spring water was recorded. Recovery time was defined as the elapsed time required for the fish to regain full equilibrium. The process described above was

repeated four times for each anesthetic treatment and for controls which were spawned without the use of anesthesia.

Two minutes after fertilization in a salt diluent (0.7% NaCl), the eggs were rinsed and transferred to incubation trays, randomizing location of treatments and replicates. After the eggs had reached the eyed-egg stage (eye-up), they were mechanically shocked by pouring to cull infertile eggs and dead eggs were counted the following day. After hatching, the remaining infertile and dead eggs were enumerated and any deformed fry culled and counted as well. Egg mortality at eye-up and hatch were expressed as a percentage of total eggs at the start of incubation. The percentage of abnormal fry was expressed as a percentage of hatched fry.

2.3. Stress test

A stress test was conducted on 17 May 2000 with 3.5-year-old females of the same strain of rainbow trout. Treatment–time combinations (e.g., AQUI-S 1 h, tricaine 7 h, etc.) were assigned randomly to each of 13 individual compartments (3×10 m) within two serial outdoor raceways. Twenty-two fish were added to each compartment and acclimated for 2 weeks before blood collection. Twenty fish sampled from each compartment comprised the replication for the study. The serial raceways each received 42.5 l/s hatchery spring water at 8–9 °C with no recirculation. Fish were held off feed for 2 days prior to the experiment. Fish for each sample ($N=20$) were concentrated by a crowding screen and netted into 30 l of an anesthetic solution. Fish for the prestress sample were put into 60 mg/l tricaine and their blood collected at 0830 h. The concentrations of the three anesthetics were: tricaine, 60 mg/l; isoeugenol, 20 mg/l (40 mg/l AQUI-S) and CO_2, 270 to 275 mg/l. A fresh solution of anesthetic was mixed up for each of the three sampling times: 1, 7 and 24 h. Sampling times were limited by space and fish numbers. Effort was made to net the fish out of the raceway as soon as the fish were crowded. For the test, all 20 fish from a given compartment were put into either of two 30-l solutions of anesthetic (same anesthetic in each) as quickly as possible. Induction time was noted when about half of the fish in the container lost equilibrium. At 1 min after loss of equilibrium, a crew of four quickly handled each fish as if attempting to extrude the gametes (grabbing the caudal peduncle with one hand and massaging the abdominal cavity with the other), mimicking the typical spawning process at the hatchery. The fish were returned to the raceway compartment to recover. Control fish were put into 30 l of hatchery well water and subdued by handling for the spawning process without the aid of anesthesia. At 1, 7 and 24 h after this handling procedure, the fish were crowded once more, netted and anesthetized in 60 mg/l tricaine. Blood was collected by heparinized (1% ammonium heparin) syringe from the caudal vasculature. Blood was kept on a ice-water bath until it was centrifuged for 5 min at 13,750 rpm using a fixed horizontal rotor (Beckman Microfuge, Palo Alto, CA). The plasma was transferred to another 1.5-ml microcentrifuge tube and kept on ice until it was frozen at -45 °C.

The plasma was later analyzed for chloride concentration by amperometric–coulometric titration (Haake-Buchler chloridometer, Saddle Brook, NJ). Plasma cortisol concentrations were determined from 20-μl samples by enzyme immunoassay after extraction with ethyl ether (Barry et al., 1993). Eight cortisol standards from 0 to 375 ng/ml were

used to form the standard curve. Plasma glucose concentrations were determined by an enzymatic (hexokinase) method (Sigma Diagnostics, procedure 16-UV, Saint Louis, MO). The assay was conducted in 96-well plates and read at 340 nm with an ELISA plate reader (Titertek Multiskan MCC/340 MK II, Flow Laboratories, McLean, VA).

2.4. Statistical analysis

An α-level of 0.05 was used for all analyses which were performed by SPSS software, version 7.0 (SPSS, 1993). Preliminary assessment of the normal distribution assumption was made with the Kolmogorov–Smirnov test and examination of box plots, stem and leaf plots and histograms. Two-way analysis of variance (ANOVA) was used to compare glucose, chloride and cortisol concentrations among treatments (AQUI-S, tricaine, CO_2 and control) and sampling periods (0, 1, 7 and 24 h). Time and treatment were considered fixed factors in the full factorial model. The Least Significant Difference test was used for mean comparison if one- or two-way ANOVA tests were significant. One-way ANOVA was used to compare induction and recovery time among the four treatments. Induction time and recovery time differences between males and females were analyzed using a separate *t*-test for each anesthetic. Egg eye-up and hatching data were each compared among the seven combinations of female anesthetic–male anesthetic (AQUI-S×AQUI-S, AQUI-S×control, tricaine×tricaine, tricaine×control, CO_2×CO_2, CO_2×control, control×control) using the Kruskal–Wallis test. Log_{10}-transformed abnormal fry data were similarly compared among the seven groups using one-way ANOVA.

3. Results

3.1. Sperm motility

The duration of motility was significantly influenced by the presence of the three anesthetics at all concentrations used. Compared to the controls, which had an average duration of 87 ± 6 s, all anesthetic treatments had a significant reduction in duration (mean of 43 ± 10 s). For the tricaine and AQUI-S treatments, the lower doses had longer durations. Within the tricaine treatments, duration of sperm movement in the 15 and 25 mg/l doses was significantly longer than at higher doses of 50 and 100 mg/l (Table 1). In the AQUI-S treatments, sperm in 10 mg/l were motile significantly longer than in either 50 or 100 mg/l. The durations for the CO_2 concentrations ranged from 31 to 43 s and were not significantly different from each other. The percent of sperm motility was not influenced by the type of anesthetic or concentration. The average ($\pm$S.D.) motility for the controls was $83\pm6\%$ and $80\pm7\%$ averaged across all treatments.

3.2. Effect of anesthetics on egg viability

Induction times averaged 130–138 s among the three anesthetics and were not significantly different. However, mean recovery time was significantly longer for AQUI-S (370 s) than either CO_2 or tricaine (192 or 199 s, respectively; Table 2).

Table 1
Average ($N=3$) duration of sperm motility and percentage of sperm that were motile (sperm motility) after exposure to various concentrations of tricaine methanesulfonate, AQUI-S and carbon dioxide

Anesthetic	Treatment concentration (mg/l)	Duration sperm motility (s)	Sperm motility (%)
Control	–	87±6 a	83±6
Tricaine	15	55±11 bc	73±6
	25	63±5 b	85±5
	30	42±6 cd	82±10
	50	35±4 d	70±0*
	100	36±6 d	82±3
AQUI-S	10	56±6 bc	72±3
	20	45±1 cd	83±12
	50	37±7 d	68±8
	100	37±1 d	83±12
CO_2	50	39±3 d	87±6
	103	43±4 cd	87±8
	173	31±5 d	83±8

Mean (±S.D.) values with a different letter are significantly different ($P\leq0.05$).
* $N=2$.

Recovery and induction time were independent of gender. Male induction time averaged 111, 116 and 135 s for AQUI-S, tricaine and CO_2, respectively, whereas female induction time averaged 138, 140 and 139 s. Male recovery time averaged 334, 283 and 220 s for AQUI-S, tricaine and CO_2, respectively, whereas female recovery time averaged 386, 165 and 181 s.

Survival of eggs from anesthetized parents to the eyed egg stage and to hatching did not differ from controls (Table 3), regardless of whether one or both parents had been anesthetized. There were substantial differences in variability (e.g., S.D., Table 3) among replicates in some groups, especially the carbon dioxide treatment. This may indicate an interaction between egg quality from individual females and type of anesthetic, but further research is needed to pursue this hypothesis.

3.3. Stress test

Among the three anesthetics, induction times for fish used for blood collection ranged from 76 to 140 s and did not significantly differ ($P=0.102$). Cortisol concentrations varied

Table 2
Mean ($N=28$) and range of induction time and recovery time for rainbow trout broodstock anesthetized by 40 mg/l AQUI-S, 60 mg/l tricaine methanesulfonate or 220–270 mg/l carbon dioxide gas

	Induction time (s)		Recovery time (s)	
	Mean	Range	Mean	Range
AQUI-S	130 z	80–210	370 z	153–755
Tricaine	133 z	48–225	199 y	87–657
CO_2	138 z	98–219	192 y	71–523

Means that share a common letter are not significantly different ($P>0.05$).

Table 3
Percent survival (±S.D., $N=4$) to the eyed egg stage (eye-up) and hatching, as well as the percentage of abnormal fry, of eggs from rainbow trout parents anesthetized with one of three anesthetics or not anesthetized (control)

Anesthetic for female	Anesthetic for male	Eye-up (%)	Hatch (%)	Abnormal (%)
AQUI-S	AQUI-S	98.8±1.6	96.0±3.2	0.27±0.08
AQUI-S	Control	99.3±0.4	96.1±1.6	0.21±0.07
Tricaine	Tricaine	97.0±2.5	91.8±4.6	0.50±0.41
Tricaine	Control	94.3±9.5	89.1±8.5	0.23±0.11
CO_2	CO_2	88.4±17.8	84.3±17.4	0.22±0.18
CO_2	Control	93.1±10.8	90.1±10.5	0.23±0.09
Control	Control	98.8±0.7	97.0±0.7	0.09±0.06

significantly over time and among anesthetic treatments and the interaction term of the general linear model was also significant. Subsequent one-way ANOVA tests for each time period indicated significant differences among anesthetic treatments. At 1 h, the cortisol concentrations of fish anesthetized with AQUI-S were significantly lower than the other three treatments ($P=0.006$; Fig. 1); fish anesthetized by tricaine or CO_2 had significantly elevated cortisol concentrations above prestress levels ($P<0.001$) that were not signifi-
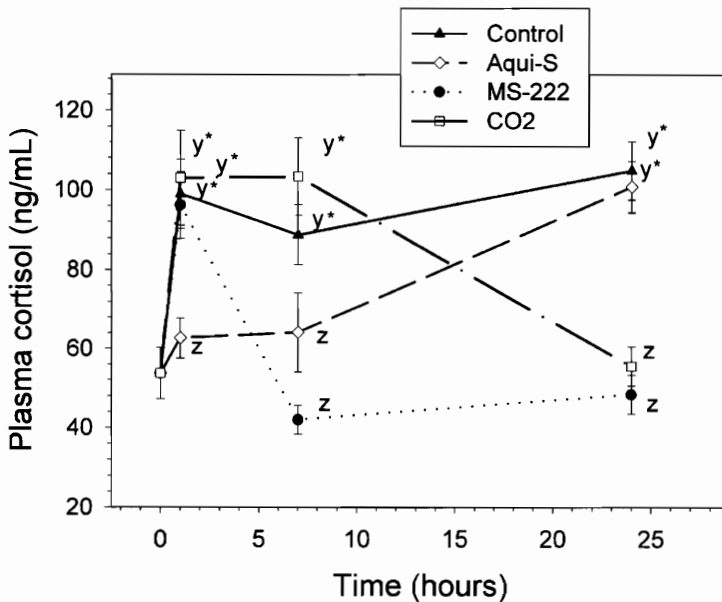

Fig. 1. Plasma cortisol concentrations of female rainbow trout broodstock anesthetized by AQUI-S, tricaine, CO_2, or not anesthetized, and sampled at three time periods. Means (±S.D.; $N=20$) within a sampling time that are not significantly different share a common letter or no letters. Means with asterisk were significantly different from the prestress value.

Fig. 2. Plasma glucose concentrations of female rainbow trout broodstock anesthetized by AQUI-S, tricaine, CO_2, or not anesthetized, and sampled at three time periods. Means ($\pm$S.E.; $N=20$) within a sampling time that are not significantly different share a common letter or no letters. Means with asterisk were significantly different from the prestress value.
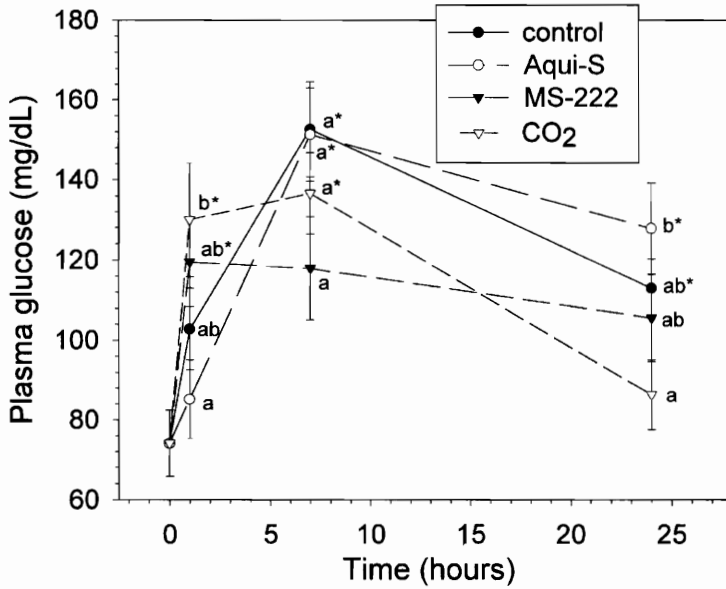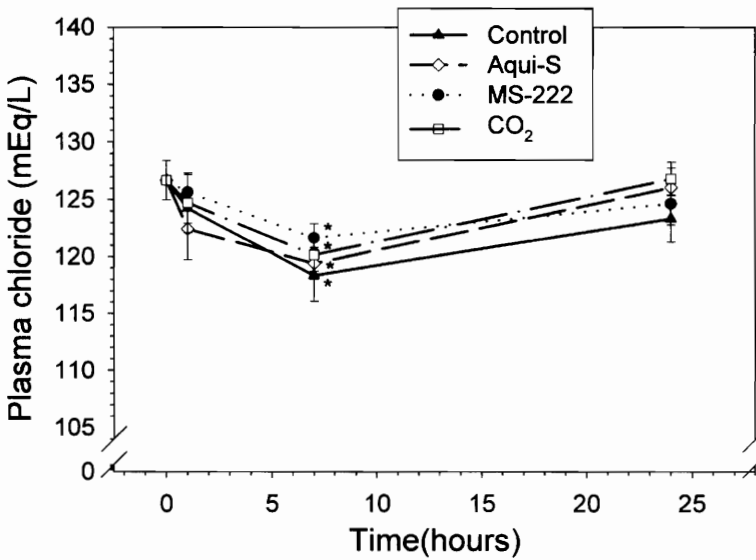

Fig. 3. Plasma chloride concentrations ($\pm$S.E.; $N=18-20$) of female rainbow trout broodstock anesthetized by AQUI-S, tricaine, CO_2, or not anesthetized, and sampled at four time periods. Means with asterisk were significantly different from the prestress value.

cantly different from handled controls. Use of tricaine, however, resulted in a quicker return (within 7 h) of cortisol to prestress levels. Fish anesthetized with CO_2 returned to prestress levels within 24 h, whereas control and AQUI-S groups still had elevated cortisol concentrations 24 h after sham spawning.

Plasma glucose concentrations increased in response to the handling stress, peaking in the 7-h sample for all treatments but the tricaine group (Fig. 2). At 1 h, the glucose concentrations for the AQUI-S treatment were significantly lower than those for the CO_2 treatment ($P=0.035$), but did not differ from controls. At 7 and 24 h, glucose levels for all three anesthetic treatments did not differ significantly from controls. At 24 h, similar to the cortisol results, glucose levels for fish anesthetized by AQUI-S were still significantly elevated above the prestress level ($P<0.001$) and above the concentrations noted for CO_2-treated fish ($P=0.029$).

Chloride concentrations compared among anesthetic treatments for each time period indicated no significant differences for any period. Within a given treatment however, there were some significant changes over time for all treatments: chloride levels were depressed at 7 h relative to prestress concentrations (Fig. 3). Samples from fish with large external lesions resulting from abrasions had depressed chloride concentrations, so these six samples were deleted from the analysis. Interestingly, these same samples did not appear as outliers for the cortisol or glucose data.

4. Discussion

The use of anesthetics for broodfish greatly facilitated the handling of both sexes during the spawning process. No delayed mortality was observed in the weeks following the tests for any of the fish handled and bled. This study was the first to evaluate egg viability from salmonid broodstock exposed to chemical anesthetics. Anesthetic use did not adversely affect egg survival, nor did it improve survival over that of controls. Nonchemical anesthesia with either hypothermia (Hovda and Linley, 2000) or electricity (Maxfield et al., 1971) similarly had no effect on egg viability.

Isoeugenol proved to be an effective anesthetic for broodstock handling and a dose of 20 mg/l (35–40 mg/l AQUI-S) was optimal in this study. Higher doses (>80 mg/l AQUI-S) induced anesthesia much faster in preliminary tests, but elicited a violent head shaking response from fish upon placement in the anesthetic bath. Keene et al. (1998) recommended doses of 40–60 mg/l eugenol for juvenile rainbow trout and Peake (1998) similarly recommended 60 mg/l for nonsalmonid fishes. Waterstrat (1999) recommended 100 mg/l clove oil for use with channel catfish (Ictalurus punctatus). The longer recovery time for fish anesthetized with AQUI-S is in agreement with other studies with juvenile fish (Keene et al., 1998; Peake, 1998).

The stress indicators generally showed that the three anesthetics did not prevent the physiological stress responses to broodfish during spawning, but did speed up recovery. The stress response is likely due to the initial crowding and netting stressors that occurred before the fish entered the anesthetic. In control fish, handling during spawning was an additional stressor. Barton et al. (1986) found that cumulative stressors in chinook salmon caused a cumulative increase in the physiological stress responses, including plasma

cortisol. This cumulative increase can significantly affect survival (Sigismondi and Weber, 1988). In this study, instead of a cumulative increase in stress indicator levels, the stress response was prolonged in handled controls. It is possible that anesthesia prior to handling (e.g., prior to crowding and dip-netting in this study) might reduce the stress response, however Barton and Peter (1982) observed little benefit to fingerling rainbow trout anesthetized with tricaine prior to transport.

Other work with anesthetics have indicated that control of the corticosteriod stress response is dependent upon the type of anesthetic and the concentration used. Anesthetics themselves may also be stressors. For example, Barton and Peter (1982) found that 15-min exposure to 50 mg/l tricaine, as well as to 0.5 ml/l phenoxyethanol, induced a stress response in juvenile rainbow trout. In striped bass (*Morone saxatilis*), Davis et al. (1982) also observed an increase in corticosteroids in response to 25 mg/l tricaine, 2.5 mg/l quinaldine, or 0.1 mg/l etomidate. However, tricaine at 50 mg/l effectively controlled the stress response of chinook salmon yearlings, but prolonged exposure (180 min) to a lower sedating dose (25 mg/l) elevated cortisol (Strange and Schreck, 1978). A prolonged low dose of AQUI-S (17 mg/l for 30 min) was similarly shown to induce a stress response in rainbow trout (Davidson et al., 2000). The opposite effect was observed by Thomas and Robertson (1991) who noted that plasma cortisol in red drum (*Sciaenops ocellatus*) significantly increased in response to 80 mg/l tricaine or 20 mg/l quinaldine sulfate, but cortisol increased minimally in response to 10 mg/l tricaine. However, the anesthetics were used in circular tanks which were slowly refilled after anesthesia, diluting the concentration as the tank refilled. An immobilizing dose of metomidate (7 mg/l) failed to increase plasma cortisol or glucose (Thomas and Robertson, 1991). Electroanesthesia, either as pulsed or continuous DC, proved to be more stressful than handling for juvenile bull trout (*Salvelinus confluentus*; Barton and Dwyer, 1997). These results suggest different physiological responses to the anesthetics.

The results of the stress test also support this hypothesis, since cortisol and glucose patterns were different for each anesthetic. AQUI-S depressed both glucose and cortisol responses, except for an inexplicable increase in cortisol in the 24-h sample. Davidson et al. (2000) similarly noted that cortisol concentrations in rainbow trout exposed to 17 mg/l AQUI-S for 30 min returned to normal within 4 h, but a second peak in the stress response (glucose and cortisol) occurred at 16–24 h. The cause for this secondary response is unknown, but Davidson et al. (2000) suggested that AQUI-S may have a persistent irritant effect on sensitive tissues. Davidson et al. (2000) also noted a rise in plasma protein associated with the secondary rise in plasma cortisol. Elevated plasma protein has also been associated with tricaine anesthesia of rainbow trout, independent of plasma cortisol levels (Laidley and Leatherland, 1988).

Although Hovda and Linley (2000) previously reported on the effects of CO_2 anesthesia on viability (98.9% hatch), this study was the first to assess the stress responses to anesthesia using CO_2 gas. Fish anesthetized by CO_2 had an initial rise in plasma cortisol similar to that of controls, but returned to prestress levels sooner. The hyperglycemia and hypochloremia in the CO_2 treatment was similar to that for tricaine and to other stress studies (Wedemeyer, 1972). The lower pH in the CO_2 treatment (5.0) apparently had no additive effect on the stress responses. Similar pH levels have induced significant increases in plasma glucose or cortisol in some studies (Goss and Wood,

1988; Whitehead and Brown, 1989), but not others (Brown and Whitehead, 1995). Exposure to low pH in this study was limited to a few minutes, whereas exposure in the pH stress studies cited above was for several days. The induction and recovery times noted for fish in CO_2 in this study were similar to those reported by Gilderhus and Marking (1987) and by Gelwicks et al. (1998) who generated CO_2 by mixing $NaHCO_3$ and H_2SO_4.

While anesthesia had no impact on egg survival if males were anesthetized, direct contact between anesthetic and sperm did affect the duration the sperm were migrating. Allison (1961) similarly found a reduction of brook trout (Salvelinus fontinalis) sperm motility to less than 10 s at tricaine concentrations ranging from 19 to 75 mg/l. Billard (1981) found no reductions in rainbow trout sperm motility when working with tricaine concentrations of 4–150 mg/l, but he was working with a buffered diluent which was added to the anesthetic and he suggested that the decrease in motility could be attributed to low pH caused by the addition of anesthetics, rather than the anesthetics per se. Reductions in sperm motility have been found with northern pike (Esox lucius) at low pH (<7.0) and chain pickerel (Esox niger) at low (<6.0) and high (>7.0) pH (Duplinsky 1982). In our work the pH was low for the CO_2 treatments, 5.0, but was stable for the AQUI-S, 7.8, and tricaine, 7.3, treatments compared to the pH value of the hatchery water which was 7.8. Therefore, in this study, reductions in motility as a result of low pH might be explainable within the CO_2 treatments, but not for AQUI-S or tricaine. In a hatchery setting where an anesthetic was being used on broodstock, the general practice is to dip the anesthetized fish into a container of clean water prior to spawning to wash off residual anesthetic that might interfere with fertilization. In addition, sperm is generally expressed into a dry container in which great care is taken to avoid introduction of dripping water. These practices would likely negate any of the reductions in motility duration found during this study, as demonstrated by the egg survival data. Electroanesthesia (Jennings and Looney, 1998) may be more useful for situations in which rapid induction and rapid recovery are necessary, but has been observed to induce rapid increases in corticosteroids itself (Barton and Dwyer, 1997). The longer recovery time for fish anesthetized with AQUI-S could be helpful for situations such as marking, spawning and surgery. Assuming AQUI-S is approved for use with food fish, the lack of a withdrawal time for releasing anesthetized fish would be useful for field applications, e.g., releasing fish after field handling or for harvesting farmed fish. The lower cost, deeper anesthesia and lack of cumbersome compressed gas cylinders would also make it an attractive alternative to tricaine and CO_2.

Acknowledgements

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