USE OF LOW VACUUM ELECTRON MICROSCOPY TO QUICKLY ESTIMATE BACTERIAL POPULATIONS ON INCUBATING SALMONID EGGS

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ABSTRACT

Low vacuum scanning electron microscopy (SEM) was used to quickly estimate microbial populations attached to the external egg membrane of land-locked fall chinook salmon (*Oncorhynchus tshawytscha*) eyed eggs. The eggs required no preservation or treatment prior to placement inside the SEM in low vacuum mode, and could be viewed for approximately 10-15 min before severe desiccation occurred. Bacterial numbers were estimated for 7 d post-eyed stage eggs reared in vertical-flow tray incubators and treated with either 1,667 mg/L formalin for 15 min daily in the AM or 1,667 mg/L formalin for 15 min twice daily (AM and PM). Estimated bacterial levels were twice as high on the eggs, and percent hatch was significantly lower, in the AM-treated trays compared to the eggs in trays receiving treatments in both the AM and PM. As a result of estimated bacterial loads, three trays in the AM group were shifted to both AM and PM treatments at eight days post-eyed stage. Survival to hatch in these was significantly greater compared to trays that were not switched. This study documents the feasibility of using low vacuum SEM to quickly estimate bacterial populations on eyed eggs and subsequently customize anti-microbial chemical treatments during hatchery rearing.

Keywords

Chinook salmon, *Oncorhynchus tshawytscha*, electron microscope, bacteria, eyed eggs, formalin

INTRODUCTION

Enumeration of microbial populations associated with salmonid egg incubation has been performed using culture media and plating techniques (Trust

1972; Barker et al. 1989, 1991; Barnes et al. 1999, 2000a). More recently, Stephenson et al. (2003) used high vacuum scanning electron microscopy (SEM) to count individual bacteria on landlocked fall chinook salmon (*Oncorhynchus tshawytscha*) eyed eggs. The results from these techniques are valid. However, all of them are labor intensive and have a potential lag time of several days between the collection of samples and reporting of the results.

Because bacteria may play some role in egg mortality (Sauter et al. 1987; Barker et al. 1989, 1991; Stephenson et al. 2003), estimating the size of bacterial populations during egg incubation could provide the basis for a prescribed anti-microbial chemical treatment regime. Such treatments are usually conducted at the same concentration and duration throughout incubation, despite fluctuations in microbial levels (Trust 1972; Barnes et al. 1997, 2000b, 2001).

The objective of this study was to evaluate the use of low vacuum SEM to quickly estimate the number of bacteria attached to the external membrane of incubating landlocked fall chinook salmon eggs, so that anti-microbial treatments could be quickly adjusted in a production setting.

METHODS

A JEOL 5600LV SEM (JEOL USA, Inc., Peabody, Massachusetts) was used for this study. It was operated in low vacuum mode at a magnification of 3000x, acceleration voltage of 20 kV, and a pressure of 30 to 33 Pa. Eggs were not preserved or treated in any way prior to viewing under low vacuum conditions.

The landlocked fall chinook salmon eggs examined under the microscope were spawned on 10/15/2002 and incubated under the conditions described by Barnes et al. (1999). After autopicking to remove any dead eggs on 11/11/2002, nine Heath vertically-stacked incubator trays (Flex-a-lite Consolidated, Tacoma, Washington) were loaded at 6,422 (1500 ml) eyed eggs per tray. Six trays received formalin (37% formaldehyde, 6 to 14% methanol; Parasite-S, Western Chemical Inc., Ferndale, Washington) treatments at a concentration of 1,667 mg/L for 15 min daily at 08:00. Three trays received the same formalin treatment at 08:00, but also received an additional, identical formalin treatment at 15:30. Three of the trays in the once-daily treatment stack were switched on 11/20/2002 (eight days post-eyed stage) to twice-daily formalin treatments until complete hatch on 12/2/2002.

Chemical treatments were started on 11/12/2002 and discontinued on 12/2/2002, at which time egg mortalities were recorded from all nine trays.

All formalin treatments were administered using a Masterflex model 7524-00 microprocessor peristaltic pump (Cole-Parmer Instrument Company, Chicago, Illinois). Because of the well-established benefits of post-eyed stage formalin treatments on Lake Oahe chinook salmon eggs (Barnes et al. 1997; 2003), and because the primary thrust of this experiment was to evaluate the utility of viewing live eggs in a low vacuum SEM, no true control (i.e. trays that received no chemical treatments) was included in this experiment.

Ten to twelve eggs were removed from the bottom tray in each treatment group on 11/19/2002 and transported in plastic bags containing incubation

water 18 km (20 min) from McNenny State Fish Hatchery to the electron microscope at Black Hills State University, Spearfish, South Dakota. The procedure employed during the electron microscopy session was as follows, with the approximate time for each step in parenthesis:

1. The SEM was focused under high vacuum conditions on a lead ball roughly the same size as the salmon eggs to be viewed, and subsequently the microscope was switched to low vacuum mode (5 min).
2. A single salmon egg was placed on a delicate task wiper, blotted to remove excess water, and put into the microscope chamber (2 min).
3. The egg was viewed and its image was captured. The number of bacteria attached to the external egg membrane was estimated from two randomly selected sites (15 min).
4. Steps 2 and 3 were repeated until three eggs from each treatment had been viewed. Bacterial estimates from the eggs were averaged and converted to number of bacteria/mm2.

Data were analyzed using analysis of variance with the SPSS (9.0) statistical analysis program (SPSS 1999). Pairwise mean comparisons were performed using Fisher's Protected Least Significance Difference, with significance predetermined at $P < 0.05$ (Ott 1984). All embryo survival percentage data were arcsine transformed prior to analysis to stabilize the variances (Ott 1984).

RESULTS AND DISCUSSION

The external egg membranes of the salmon eggs were clearly visible for approximately 15 min until egg desiccation occurred. No fungal growth was observed on any of the eggs, but bacteria could be readily observed. There were an estimated 1,500 to 3,000 bacteria/mm^2 from the eggs receiving once daily formalin treatments and between 0 and 1,000 bacteria/mm^2 on the eggs treated with formalin twice daily.

Survival to hatch was significantly different between the trays of eyed-eggs receiving once-daily formalin treatments through hatch, compared to those trays receiving twice-daily formalin treatments and those trays that were switched from once-daily to twice-daily treatments midway during incubation (Table 1). However, the increase in survival with increasing formalin treatment may not be biologically significant, because the eggs receiving once-daily formalin experienced only 0.75% greater mortality than eggs in the other two groups.

Table 1. Mean (± SE) percent survival to hatch for eyed landlocked fall chinook salmon eggs incubated in vertically-stacked incubation trays subjected to one of three formalin treatment regimes. Means followed by different letters are significantly different at P ≤ 0.05 (n = 3).

Treatment	Hatch
Daily 15 min, 1,667 mg/L formalin	94.79 + 0.10z
Daily 15 min, 1,667 mg/L formalin for first 9 d followed by twice for the remaining 13 d	95.65 + 0.24y
Twice daily 15 min, 1,667 mg/L formalin	95.54 + 0.19y

Our results show that using a low vacuum SEM is a novel technique that can be used to very quickly estimate microbial populations on salmonid eggs. It is also possible that using a multiple specimen holder for the SEM could reduce the time required for estimation even further. Although this would save loading time, it would also increase the risk of specimen dehydration.

We recognize that scanning electron microscopes with low vacuum capabilities might not be in close proximity to many hatcheries incubating salmonid eggs. However, in those situations where it is available, it is possible that chemical usage could quickly be adjusted in relation to estimated microbial population size. Treatments could be increased as microbial populations increase and decreased as populations decrease. Although in our experiment chemical treatments were increased in response to elevated microbial populations, it is highly probable that chemical treatments could also be adjusted downward, particularly during the lower bacterial numbers associated with initial egg incubation (Barnes et al. 1999, 2000a). Given the public pressure to decrease chemical outflows in hatchery effluents (Winton 2001), methods to decrease hatchery chemical discharges or even more quantitatively justify the use of chemicals during hatchery rearing are extremely important. Costs associated with chemical use during egg incubation might also decrease with increased monitoring of microbial populations attached to incubating eggs.

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