

COMMUNICATION

## Efficacy of 35% PEROX-AID (Hydrogen Peroxide) in Reducing an Infestation of *Gyrodactylus salmonis* in Freshwater-Reared Rainbow Trout

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### Abstract

*Gyrodactylus salmonis* is a monogenean ectoparasite that can infest a variety of captive-reared salmonid fishes. The physical damage inflicted during severe infestations can cause osmoregulatory disturbances and potentially render individuals more vulnerable to secondary pathogens. If not treated, *G. salmonis* infestations can reduce growth and survival in affected fish populations. Many chemical compounds have been used to treat *Gyrodactylus* infestations; however, little information has been published about the use of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for *Gyrodactylus* control. Consequently, we conducted a trial to evaluate the efficacy of H<sub>2</sub>O<sub>2</sub> in reducing a natural infestation of *G. salmonis* in freshwater-reared, adult rainbow trout *Oncorhynchus mykiss*. Triplicate tanks of adult rainbow trout (20 fish per tank; length = 45.6 ± 5.8 cm and weight = 1.3 ± 0.4 kg, mean ± SD) were exposed to a static bath of H<sub>2</sub>O<sub>2</sub> at a target dosage of 50 mg/L or hatchery water (sham treatment) for 30 min/d on two alternate days. Treatment efficacy was assessed at 2 and 7 d posttreatment via light microscopy examination of skin scrapes (one per fish) taken from 10 fish per tank on each day. At 2 d posttreatment, the mean abundance of *G. salmonis* in the H<sub>2</sub>O<sub>2</sub>-treated group (0.1 ± 0.3 *G. salmonis* individuals per skin scrape) was significantly different from that observed in the sham-treated group (34.4 ± 43.2 individuals per skin scrape). Also, at 7 d posttreatment, the mean abundance of *G. salmonis* in the H<sub>2</sub>O<sub>2</sub>-treated group (0.1 ± 0.3 individuals per skin scrape) was significantly different from that observed in the sham-treated group (38.5 ± 77.4 individuals per skin scrape). The percent reduction in mean abundance (treated group compared with control group) was greater than 99% at both 2 and 7 d posttreatment. In conclusion, the H<sub>2</sub>O<sub>2</sub> treatment regimen that we used significantly reduced a natural infestation of *G. salmonis* in freshwater-reared, adult rainbow trout.

*Gyrodactylus* are monogeneans that have a direct life cycle (i.e., require no intermediate host) and are ectoparasitic on fish (Buchmann and Bresciani 2006). The genus comprises over 400 recognized species worldwide; however, the actual number of

species could be as high as 20,000 (Harris et al. 2004). Approximately 60% of the recognized species are specific to one fish host, while others can infect multiple fish species (Bakke et al. 2002, 2007). *Gyrodactylus salmonis* is of particular concern in North American fish culture because it has been recovered from seven salmonid species spanning three salmonid genera (Cone et al. 1983; Harris et al. 2004). *Gyrodactylus* have short life spans (days to weeks) and low fecundity (averaging one to five offspring per individual). However, infestations can increase rapidly in populations of intensively cultured salmonids because the organisms can complete their entire life cycle on a single fish, are protogynous hermaphrodites (within each organism, the female reproductive system matures before the male reproductive system), are highly progenetic (reproduce as larvae and juveniles), and are viviparous (bear live young; Bakke et al. 2007). Moreover, when young are released from parents, they are fully formed and already contain developing embryos in utero (Beverly-Burton 1994). In captive-reared fish populations, brief fish-to-fish contact is all that is required to transmit *Gyrodactylus* among fish (Bakke et al. 2007). *Gyrodactylus* can also be transferred among tanks and raceways via dip nets and other hatchery equipment if such equipment is not properly disinfected. Infestations of *G. salmonis* resulting in disease occur most frequently during winter and early spring when water temperatures are at or below 8°C (Beverly-Burton 1994), although infestations are also possible in fall as water temperatures decline in temperate climates (Bakke et al. 2007). Like other members of the genus, *G. salmonis* attaches to a host via an opisthaptor (posterior hold-fast device; Bakke et al. 2007). The physical damage inflicted during severe infestations can cause osmoregulatory disturbances and potentially render individuals more vulnerable to secondary pathogens (Cone and

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Odense 1984; Cusack and Cone 1986a, 1986b; Cusack et al. 1988). The primary behavioral sign of *Gyrodactylus* infestation is flashing, as if the fish are trying to rub away the irritation (Beverley-Burton 1994). External gross disease signs vary with *Gyrodactylus* and host fish species and with environmental conditions (Wells and Cone 1990) but can include erosion of fins, pale, discolored flanks, thickened cuticle, obvious secretions of mucus, and emaciation (Beverley-Burton 1994). Other authors have noted epidermal thinning and decreased mucus production (Cusack and Cone 1986b; Wells and Cone 1990). *Gyrodactylus*-induced mortality has been reported (Cusack and Cone 1986b); however, mortality is highly variable among individuals, and infestation thresholds above which death is inevitable may be impossible to determine (Bakke et al. 2007). Nonetheless, severe infestations can reduce growth and survival in affected fish populations. Such adverse effects can often be minimized by improving environmental rearing conditions for fish or by administering chemotherapeutic treatments (Thoney and Hargis 1991).

Many chemical compounds and treatment regimens have been used to treat gyrodactylid and other monogenean infestations (e.g., Schmahl 1991; Thoney and Hargis 1991; Buchmann and Bresciani 2006; Schelkle et al. 2009). In the United States, only formalin (37% formaldehyde) is approved for such use by the U.S. Food and Drug Administration (FDA). However, there are human and environmental safety issues associated with the use of formalin, and many fish culturists are looking for safer alternatives. One potential alternative to formalin is hydrogen peroxide ( $H_2O_2$ ), a relatively safe compound that is used as a bleaching agent in the textile industry, as an antimicrobial agent in cheese production, for the treatment of drinking water (Marking et al. 1994), and in human health care as a first aid antiseptic and oral debriding agent. Hydrogen peroxide has also been used to treat a variety of external fungal, bacterial, and parasite infections on fish and fish eggs (e.g., Marking et al. 1994; Mitchell and Collins 1997; Speare and Arsenault 1997; Lumsden et al. 1998; Howe et al. 1999; Rach et al. 2000a, 2000b, 2003; Montgomery-Brock et al. 2001, 2004; Buchmann and Kristensson 2003; Sitjà-Bobadilla et al. 2006; Russo et al. 2007; Bravo et al. 2010). Fish species sensitivity to  $H_2O_2$  varies (Rach et al. 1997; Gaikowski et al. 1999), although tolerance to this chemical can be increased by low-level preexposure (Tort et al. 1998). Hydrogen peroxide has relatively little environmental impact because it breaks down into water and oxygen (Rach et al. 1997; Treasurer and Grant 1997) and is relatively safe for users because no harmful fumes are released during application (Rach et al. 1997). In January 2007, FDA approved 35% PEROX-AID (35% active  $H_2O_2$ ; manufactured by Eka Chemicals, Marietta, Georgia, and distributed in the USA by Western Chemical, Ferndale, Washington) for a variety of therapeutic uses in aquaculture. Although 35% PEROX-AID is the only  $H_2O_2$  product currently approved for use in U.S. aquaculture, it is not approved for use to control or reduce infestations of fish ectoparasites. To generate data needed to expand the current

$H_2O_2$  label claim to allow such use in the United States, data are needed to demonstrate treatment efficacy against specific ectoparasites. Consequently, we conducted a trial to evaluate the effectiveness of  $H_2O_2$  administered at a target dosage of 50 mg/L for 30 min/d in a static bath on two alternate days to reduce a natural infestation of *G. salmonis* in freshwater-reared, adult rainbow trout *Oncorhynchus mykiss*.

## METHODS

*Experimental design and data collection.*—The trial was conducted in October 2010 at the U.S. Fish and Wildlife Service (USFWS) Ennis National Fish Hatchery (NFH), Ennis, Montana. The null hypothesis was that (1) mean abundance of *G. salmonis* in test tanks treated with  $H_2O_2$  is equal ( $P \geq 0.05$ ) to that in test tanks not treated with  $H_2O_2$  or that (2) the percent reduction in mean abundance of *G. salmonis* ( $H_2O_2$ -treated group compared with sham-treated group) is less than 90%. Both parts of the null hypothesis had to be rejected to demonstrate treatment efficacy.

The reference fish population comprised 160 adult rainbow trout (length =  $45.6 \pm 5.8$  cm and weight =  $1.3 \pm 0.4$  kg, mean  $\pm$  SD). Completely randomized designs (Petersen 1985) were used to allocate fish from the reference population to each of six test tanks (20 fish per tank) and assign treatments to test tanks. The fiberglass, circular, test tanks (1.8 m diameter) had a water depth of 0.56 m and were plumbed with first-pass hatchery water (125 L/min). An additional 30 fish from a reference population were impartially collected via dipnetting, and one skin scrape was taken from each of these fish to estimate mean abundance and prevalence of *G. salmonis*. The skin scrape was taken from the right side of each fish with the sharp edge of a scalpel blade (width of blade,  $\sim 1$  cm) beginning at the anterior attachment point of the dorsal fin and proceeding posteriorly for approximately 5 cm. The skin scrape was transferred to a clean glass slide, diluted with a drop of hatchery water, cover-slipped, and examined at  $40\times$  magnification with an Olympus BH-2 compound microscope (Olympus America, Center Valley, Pennsylvania). All *G. salmonis* individuals on each slide were counted, and the total number counted on all slides divided by the number of fish examined (for each treatment group) is herein referred to as mean abundance. Prevalence was determined by dividing the number of fish with a *G. salmonis* abundance of one or more individuals by the number of fish examined. Of the 30 fish collected to estimate the baseline *G. salmonis* infestation level, 20 were then randomly selected, euthanized in a solution of tricaine methanesulfonate (TRICAINES, Western Chemical, Ferndale, Washington), and necropsied to characterize baseline fish health. Necropsied fish were visually evaluated for gross appearance of major external and internal organs and tissues and did not show any clinical signs indicating bacterial or fungal infections or secondary ectoparasites. Finally, 3 of the 30 fish collected to estimate the baseline *G. salmonis*

infestation level were randomly selected and used for the collection of *Gyrodactylus* specimens for the identification of the parasite to species, and prepared as follows. A skin scrape was taken from the left side of each fish and transferred to a petri dish containing a small amount of saline solution. *Gyrodactylus* specimens were collected from the petri dish with a disposable pipette and transferred to a glass vial containing a solution of alcohol and formic acid for preservation. The preserved specimens were later identified as *G. salmonis* by qualified staff at the USFWS La Crosse Fish Health Center (FHC), Onalaska, Wisconsin, according to methods described in Cone et al. (1983).

The trial consisted of a 3-d acclimation period, a 3-d treatment period, and a 7-d posttreatment period. On days 1 and 3 of the treatment period, H<sub>2</sub>O<sub>2</sub> was administered to treated tanks as a static bath at a target concentration of 50 mg/L for 30 min/d, while a sham (hatchery water) treatment was administered to control tanks. Water samples were collected from each test tank approximately 15 min into each 30-min treatment for H<sub>2</sub>O<sub>2</sub> dose verification. Samples were uniquely coded, and on each treatment day, two samples (one H<sub>2</sub>O<sub>2</sub> treated and one sham treated) were randomly selected and analyzed for H<sub>2</sub>O<sub>2</sub>. The titrimetric method described by Jeffery et al. (1989:372–373) was used to verify H<sub>2</sub>O<sub>2</sub> concentrations in the water samples.

Mortality, general fish behavior, and fish-feeding behavior were monitored daily. Feed administered to test fish was Silver Cup extruded, brood, sinking, 6-mm pellets (Silver Cup Fish Feed, Murray, Utah). Fish were fed daily (including treatment days) via belt feeders suspended above each tank at a rate of 0.5% of their estimated initial mean body weight. Feeding (appetite) behavior was evaluated and scored by observing feeding fish and characterizing behavior as “aggressive = 2,” “semi-aggressive = 1,” or “nonaggressive = 0.” Aggressive appetite behavior meant that fish appeared to be actively feeding and that nearly all feed offered was consumed. Semiaggressive appetite behavior meant that some fish appeared to be feeding actively and that some fish appeared to not be feeding. Nonaggressive appetite behavior meant that the fish appeared to not be feeding. Water temperature and dissolved oxygen concentration were measured daily with a YSI temperature and dissolved oxygen model 95 meter (Yellow Springs Instruments, Yellow Springs, Ohio). On treatment day 1 and posttreatment day 7, source water hardness and alkalinity (reported as CaCO<sub>3</sub>) were measured with a Hach titrator and reagents (Hach, Loveland, Colorado), and pH was measured with a YSI EcoSense pH and temperature pen.

At 2 and 7 d posttreatment, the *G. salmonis* infestation level was assessed by examination via light microscopy of skin scrapes (one per fish) taken from 10 fish impartially collected from each tank on each day. Skin scrapes were taken and prepared, and *G. salmonis* counts were made as previously described. Dorsal fins were hole-punched to identify fish sampled at 2 d posttreatment, and these fish were returned to their respective tanks to maintain fish-loading densities. Fish sampled on posttreatment day 2 were not sampled on posttreatment day 7.

**Statistical analysis.**—At 2 and 7 d posttreatment, mean abundance of *G. salmonis* was compared between treatment groups with a mixed-model, nested analysis of variance (ANOVA; SYSTAT 2007) at significance level of  $P < 0.05$ . In this analysis, test tank was the experimental unit, fish was the observational unit, treatment was the fixed factor, and test tank nested within treatment was the random factor. To compensate for *G. salmonis* counts of zero in some fish, the count for each fish was increased by one and log<sub>e</sub>-transformed before analysis. The least-squares means from the ANOVA were back-transformed ( $e^{\text{treatment group mean}}$ ) to geometric means, which were used to calculate percent reduction in mean abundance (H<sub>2</sub>O<sub>2</sub>-treated group compared with sham-treated group) as follows:

$$\text{Percent reduction in mean abundance} = 100 - \left[ 100 \times \frac{(\text{geometric mean}_{\text{H}_2\text{O}_2} - 1)}{(\text{geometric mean}_{\text{sham}} - 1)} \right]$$

## RESULTS

Before the trial started, estimated mean abundance and prevalence of *G. salmonis* in the reference population were 31.2 ± 38.8 individuals per skin scrape (i.e., per fish) and 100%, respectively. At 2 d posttreatment, mean abundance of *G. salmonis* in the H<sub>2</sub>O<sub>2</sub>-treated group (0.1 ± 0.3 individuals per skin scrape) was significantly ( $P < 0.001$ ) different from that observed in the sham-treated group (34.4 ± 43.2 individuals per skin scrape; Table 1). Also, at 7 d posttreatment mean abundance of *G. salmonis* in the H<sub>2</sub>O<sub>2</sub>-treated group (0.1 ± 0.3 individuals per skin scrape) was significantly ( $P < 0.001$ ) different from that observed in the sham-treated group (38.5 ± 77.4 individuals per skin scrape) (Table 2). Percent reduction in mean abundance was greater than 99% at both 2 and 7 d posttreatment. Mean prevalence in H<sub>2</sub>O<sub>2</sub>-treated tanks decreased to 13% (range, 10–20%) at 2 d posttreatment and 7% (range, 0–10%) at 7 d posttreatment. Conversely, mean prevalence of *G. salmonis* in sham-treated tanks was 93% (range, 90–100%) at 2 d posttreatment and 100% at 7 d posttreatment.

No fish died during the acclimation, treatment, or posttreatment periods. General fish behavior was characterized as normal in both H<sub>2</sub>O<sub>2</sub>-treated and sham-treated tanks. During the treatment period, fish appetite behavior was described as semi-aggressive to aggressive, and the mean appetite behavior score ranged from 1.3 to 1.7 in H<sub>2</sub>O<sub>2</sub>-treated tanks and was 1.3 in sham-treated tanks. During the posttreatment period, fish appetite behavior was described as aggressive, and the mean behavior score in H<sub>2</sub>O<sub>2</sub>-treated and sham-treated tanks was 1.9.

All water quality variables measured during the trial were considered suitable for rearing healthy rainbow trout. Mean water temperature was 12.0°C (range, 12.0–12.1°C), and mean dissolved oxygen concentration was 7.2 mg/L (range, 6.8–7.7 mg/L). Source water hardness, alkalinity, and pH were 254 ± 17.0 mg/L (mean ± SD), 146 ± 5.7 mg/L, and 8.0 ± 0.05,

TABLE 1. Tank and overall abundance, determined as the mean number of individuals per skin scrape (SD), of *Gyrodactylus salmons*; prevalence of infestation of rainbow trout; and percent reduction in the mean abundance of *G. salmons* in H<sub>2</sub>O<sub>2</sub>-treated tanks compared with sham-treated tanks on posttreatment day 2. Mean abundance values with different lowercase letters are significantly ( $P < 0.05$ ) different from each other.

Abundance and prevalence	H <sub>2</sub> O <sub>2</sub> -treated tanks			Sham-treated tanks		
	1	2	3	1	2	3
Tank mean abundance	0.1 (0.3)	0.1 (0.3)	0.2 (0.4)	35.3 (40.8)	29.2 (32.7)	38.8 (56.8)
Overall mean abundance		0.1 (0.3) z			34.4 (43.2) y	
Percent reduction in mean abundance				99.4		
Prevalence (%)	10	10	20	90	100	90
Mean prevalence (%)		13			93	

respectively. The average concentration of H<sub>2</sub>O<sub>2</sub> administered to the two H<sub>2</sub>O<sub>2</sub>-treated tanks that were sampled was 57.9 mg/L, or 116% of the 50-mg/L target dose [i.e., (58.5 + 57.4) / 2]. Hydrogen peroxide was not detected in the sham-treated tanks.

## DISCUSSION

In our trial, H<sub>2</sub>O<sub>2</sub> administered at a target dosage of 50 mg/L as a static bath for 30 min/d on two alternate days was effective in reducing a natural infestation of *G. salmons* in a population of freshwater-reared, adult rainbow trout. Mean parasite abundance in treated tanks was significantly different from that in control tanks, and prevalence was substantially reduced. Conversely, prevalence and mean parasite abundance in control tanks at the end of the trial were similar to that in the reference population before the start of the trial. In addition, the treatment regimen used appeared safe because there was no mortality and fish ate consistently throughout the trial. The minor differences observed between posttreatment days 2 and 7 were attributed to fish-to-fish variability as opposed to posttreatment therapeutic effects. Unlike antibiotics, chemotherapeutants such as H<sub>2</sub>O<sub>2</sub> provide virtually no prolonged therapeutic effect after treatment has been terminated and the chemical has been flushed from the rearing system. If fish are reared in a facility in which *G. salmons* is present in the water supply, it is likely the fish will become reinfested.

Results from this study have been submitted to FDA to support expanding the current use of H<sub>2</sub>O<sub>2</sub> for this indication. If approved, fish culturists in the United States would be able to administer H<sub>2</sub>O<sub>2</sub> at treatment regimens ranging from 50 mg/L for 30 min/d on two alternate or consecutive days to 100 mg/L for 60 min/d on three alternate or consecutive days. The approved treatment regimen range is based on data to demonstrate efficacy at the lowest dose, duration, and frequency and safety to fish at the highest dose, duration, and frequency. Data to demonstrate that the highest dosage is safe to salmonids was previously generated and accepted by FDA (NADA 141-255). This range is noteworthy because the treatment regimen used in our trial significantly reduced the abundance of *G. salmons* but did not eliminate them. Ultimately, it will be the responsibility of the fish culturist working in cooperation with their fish health professional to determine the treatment regimen within the approved range that will best suit their needs.

There is limited evidence in peer-reviewed literature that supports the effectiveness of H<sub>2</sub>O<sub>2</sub> to reduce other *Gyrodactylus* infestations in freshwater-reared salmonids. Rach et al. (2000b) showed that 170, 280, and 560 mg/L H<sub>2</sub>O<sub>2</sub> administered for 30 min/d on three alternate days were effective in controlling *Gyrodactylus* infestations in rainbow trout. We are also aware of unpublished data indicating that other H<sub>2</sub>O<sub>2</sub> treatment regimens (50 mg/L for 60 min/d, 100 mg/L for 30 min/d, and 150 mg/L for 15 min/d, all administered on three alternate days) were

TABLE 2. Tank and overall abundance, determined as the mean number of individuals per skin scrape (SD), of *Gyrodactylus salmons*; prevalence of infestation of rainbow trout; and percent reduction in the mean abundance of *G. salmons* in H<sub>2</sub>O<sub>2</sub>-treated tanks compared with sham-treated tanks on posttreatment day 7. Mean abundance values with different lowercase letters are significantly ( $P < 0.05$ ) different from each other.

Abundance and prevalence	H <sub>2</sub> O <sub>2</sub> -treated tanks			Sham-treated tanks		
	1	2	3	1	2	3
Tank mean abundance	0.0 (0.0)	0.1 (0.3)	0.1 (0.3)	25.7 (35.8)	39.4 (43.9)	50.4 (125.6)
Overall mean abundance		0.1 (0.3) z			38.5 (77.4) y	
Percent reduction in mean abundance				99.7		
Prevalence (%)	0	10	10	100	100	100
Mean prevalence (%)		7			100	

effective in reducing *G. salmonis* infestations (mean abundance and prevalence) in coaster brook trout *Salvelinus fontinalis* and lake trout *S. namaycush* (Mark Gaikowski, U.S. Geological Survey, personal communication). Although results from our trial showed that H<sub>2</sub>O<sub>2</sub> administered at 50 mg/L for 30 min/d on two alternate days was effective, users should be aware that extremely low doses might be ineffective. For example, Russo et al. (2007) found that treating green swordtail *Xiphophorus hellerii* with 3 or 6 mg/L H<sub>2</sub>O<sub>2</sub> for 24 h or 11 or 16 mg/L H<sub>2</sub>O<sub>2</sub> for 1 h was ineffective at controlling *Gyrodactylus* infestations.

Gilmore et al. (2010) listed the three gyrodactylids—*G. salmonis*, *G. colemanensis*, and *G. nerkae*—that commonly infest freshwater salmonids in North America. Based on several factors, such as similar body morphology and life history, we speculate that H<sub>2</sub>O<sub>2</sub> would reduce infestations of *G. colemanensis* and *G. nerkae* at least as effectively as it reduced *G. salmonis* in our trial. However, demonstrating the efficacy of H<sub>2</sub>O<sub>2</sub> against *G. colemanensis* and *G. nerkae* might not be cost effective because *G. salmonis* appears—by far—to be the most frequently occurring of the three species. In an article describing the taxonomy of *Gyrodactylus* parasitizing certain salmonid fishes of North America, Cone et al. (1983) reported that *G. salmonis* was the most commonly found species in a survey of solicited *Gyrodactylus* samples. In addition, *Gyrodactylus* samples submitted to the USFWS La Crosse FHC from 25 different hatcheries (mostly located in the Pacific Northwest region of the USA) were all *G. salmonis* (Eric Leis, USFWS La Crosse FHC, personal communication). Based on this information, approval of a therapeutant specific for reducing infestations of *G. salmonis* would probably be sufficient to address most of the issues fish culturists might have on rearing freshwater salmonids infested with *Gyrodactylus*. Based on our results, H<sub>2</sub>O<sub>2</sub> administered at a target dosage of 50 mg/L as a static bath for 30 min/d on two alternate days is suitable for this purpose.

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