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[Article]

Laboratory Dose Confirmation of Copper Sulfate for Treating Fungus on Channel Catfish Eggs

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Abstract.—Two dose confirmation studies are required by the U.S. Food and Drug Administration to verify the effectiveness of a candidate before approval as a new animal drug is awarded; the two studies provide independent substantiation of the results. This laboratory study was designed to compare an untreated control and a 10-mg/L copper sulfate ($CuSO_4$) treatment to control fungus (*Saprolegnia* spp.) on eggs of channel catfish *Ictalurus punctatus* at 23.5°C in a flow-through system. The eggs were treated once daily until the embryos reached the eyed stage (five treatments). When hatching was complete for all viable eggs (day 10), the fry were counted to determine the percent survival in each treatment. Infestation by a fungus identified morphologically and by polymerase chain reaction as *Saprolegnia* spp. was severe in the control fish, resulting in 8% survival. The mean percent survival of fry hatched from the $CuSO_4$-treated eggs was significantly higher (mean, 55%; range, 27–71%).

On fish farms during the spawning season, channel catfish *Ictalurus punctatus* broodstock are moved to ponds and provided with containers for spawning. Containers are routinely inspected and egg masses are brought into a hatchery to be observed and inventoried, making hatchery management a key component of catfish culture (Tucker and Robinson 1990; Avery and Steeby 2004). Incubation of channel catfish eggs can range from 5 to 10 d and is dependent on water temperature and age of the eggs brought into the hatchery (Tucker and Robinson 1990; Avery and Steeby 2004). The 2003 Catfish Health and Production Practices survey (USDA 2003) reported that 8.3% of channel catfish eggs brought into hatcheries were lost to fungal infections (saprolegniasis). Unfertilized eggs contribute to egg mortality because these eggs are easily colonized by fungus. The only compounds approved by the U.S. Food and Drug Administration (FDA) for fungus control on eggs are formalin (37–40% formaldehyde) and 35% hydrogen peroxide.

Common genera of the class Oomycetes (water molds) that have been implicated in saprolegniasis in channel catfish are *Saprolegnia* and *Achlya* (Tucker and Robinson 1990; Hawke and Khoo 2004). *Aphanomyces* spp. and *Saprolegnia* spp. have been identified in recent studies with catfish eggs (authors' unpublished data); however, *Achlya* spp. were not found. These data suggest that species causing saprolegniasis on channel catfish eggs are more diverse than previously considered and may be defined by locality and other environmental factors.

Copper sulfate ($CuSO_4$) is used as a waterborne therapeutant for ichthyophthiriasis and winter fungus in commercial and recreational fish ponds (MacMillan 1985; Tucker and Robinson 1990; Bly et al. 1992; Noga 1996) and as an approved algicide and molluscicide by the U.S. Environmental Protection Agency (EPA). It is not approved by the FDA for therapeutic use in aquaculture. However, regulatory action on $CuSO_4$ has been deferred pending the outcome of ongoing research at the Harry K. Dupree–Stuttgart National Aquaculture Research Center (SNARC). The effectiveness of $CuSO_4$ in controlling ichthyophthiriasis in channel catfish has been demonstrated (Straus 1993, 2008a; Schlenk et al. 1998), as has its effectiveness in controlling saprolegniasis on channel catfish eggs (Straus et al. 2009). Copper sulfate is usually administered daily during the egg incubation period (USDA 2003; Steeby and Avery 2005) until the eggs become "eyed" (i.e., eye pigment is first seen in the embryos). Treatment is stopped at the eyed stage because there may be embryos within the same rearing unit that are hatching and the dose could be toxic to the fry (Straus 2008b). The purpose of this study is to confirm the effectiveness of a single daily dose of $CuSO_4$ to treat for fungus, specifically *Saprolegnia* spp., on channel catfish eggs in a flow-

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through system; this dose confirmation study is required by the FDA to verify that $CuSO_4$ is effective at the optimum dose determined by Straus et al. (2009).

Methods

Four aluminum hatching troughs were used in this study. They were similar to those typically used in the catfish industry except for being divided into individual compartments, each of which contained a plastic mesh basket to hold eggs until hatching. Each compartment held 35 L of water and was supplied with a separate water inlet and a stand-pipe outlet covered with a deep-water release. As in a typical hatchery, the flow rate was set at 1.25 L/min and adjusted daily to allow for a 28-min water exchange. Each compartment was fitted with a rotating plastic paddle fastened to a powder-coated steel shaft that was driven by an electric motor for water circulation and aeration. Troughs were powder-coated light blue to prevent corrosion caused by the $CuSO_4$ and to aid in blinding the experiment for quality assurance and statistical purposes. Powder coating consists of a pigment encapsulated in resin; no solvents are involved.

Groundwater was filtered through a 75-μm canister filter and flowed through the compartments continuously. The study was maintained under 12 h light : 12 h dark conditions with fluorescent lighting. Total alkalinity and total hardness (as $CaCO_3$) were measured at the beginning and end of the study by titration methods (APHA, American Water Works Association, and Water Pollution Control Federation 2005) and were 211.5 ± 2.1 mg/L (mean ± SD) and 95.0 ± 0.5 mg/L, respectively. The pH (Orion Research 720A Meter, Thermo Electron Corporation, Beverly, Massachusetts) was measured at the beginning and end of the study and averaged 7.7 ± 0.1. Dissolved oxygen and water temperature were monitored daily in each experimental unit (YSI Model 95; YSI Environmental, Yellow Springs, Ohio), registering 70 ± 2.6% saturation and 23.5 ± 0.2°C, respectively.

Four egg masses less than 24 h old were collected from channel catfish broodstock spawned at SNARC, moved to the laboratory, and prepared for distribution into the trough compartments. The egg mass was gently divided by hand into similar portions (100.1 ± 5.1 g); each portion was weighed and placed into a mesh basket of an individual compartment for a 1-h acclimation period. The adhesive matrix of each egg mass portion remained intact. To count the eggs, representative samples (17.3 ± 1.7 g) from each spawn were weighed and the adhesive matrix was dissolved with 1.5% sodium sulfite (Sigma Chemical Co., St. Louis, Missouri). These counts ($n = 3$) provided an estimate of the number of eggs in each larger portion from the same spawn.

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) used in this experiment was Triangle Brand Copper Sulfate (Phelps Dodge Refining Corporation, El Paso, Texas). A 35-g/L $CuSO_4$ stock solution was prepared with deionized (DI) water before treatment. The optimum $CuSO_4$ concentration of 10 mg/L was determined in a previous range-finding study (Straus et al. 2009). Eggs were exposed to a control treatment (no $CuSO_4$ added) or a 10-mg/L $CuSO_4$ treatment. A different spawn was used in each of the four troughs ($n = 4$) in the present study because anecdotal evidence suggests that there can be considerable differences in hatching success among individual spawns. There were two replicates of each treatment in each trough (two control treatment compartments and two $CuSO_4$-treated compartments).

To blind the experiment, the compartments within each trough were sequentially numbered and treatments were randomly assigned; the 20-mL $CuSO_4$ treatment was a 1:1 mixture of stock solution and DI water in an amber vial; control treatments were dosed with 20 mL of DI water. Following the 1-h acclimation period, treatments were applied to each egg mass by pouring the contents of the respective vial into the water opposite the egg basket; only the study director was aware of the treatment concentrations.

The experiment was set up and eggs were initially and subsequently treated in the morning. Treatments were administered at 24-h intervals until the embryos developed eyes. Treatments were discontinued in a trough when any embryo within that trough developed eyes.

Eggs were naturally infected with *Saprolegnia* spp. A daily record of fungal growth in each compartment was maintained. An ordinal fungal infection scale was used to indicate the total measured surface area (cm^2) covered by fungus: 0 = no fungus present, 0.5 = fungal coverage of less than 1 cm diameter, 1 = from 1 to less than 2 cm diameter, 2 = from 2 to less than 3 cm diameter, and so on.

Fungal infection was verified by visual examination of the eggs for characteristic signs of fungus. A small sample was removed by forceps to be microscopically examined by a fish pathologist for the typical mycelial and hyphal development. Fungus samples from the control treatments of each spawn were cultured on yeast-extract glucose agar. This was made with 500 mg yeast extract, 10 g glucose, 500 mg K_2HPO_4, and 250 mg $MgSO_4 \cdot 7H_2O$ dissolved in 500 mL DI to which 7.5 g agar was added. The mixture was autoclaved for 30 min and cooled to 50°C, then 500 μL of 68-mg/mL (in DI) streptomycin sulfate and 1,000 μL of 34-mg/mL (in ethanol) chloramphenicol were added. Cultures

were morphologically identified to genus and verified by polymerase chain reaction (PCR) amplification and sequencing of the rRNA gene internal transcribed spacer (ITS) regions.

The end of the experiment was determined to be 3 d after hatching began; this was sufficient time for all viable eggs to hatch. Healthy yolk sac fry sank or swam to the bottom of the trough after hatching and congregated in the corners of the trough. Fry that were designated as prematurely hatched (PMH) typically hatched earlier than the beginning of normal hatching, appeared deformed, and showed a lack of normal mobility; these fry were a waxy yellow color instead of the golden brown color associated with healthy fry and did not congregate in the corners of the trough. Healthy fry were siphoned from each compartment and preserved in 70% ethanol to be counted later. Prematurely hatched fry were siphoned into a separate container and live PMH fry were counted immediately. Success of the treatment was determined by the percentage of fry that hatched from the eggs; living fry were counted individually to establish percent survival.

Water samples for copper (Cu) analyses were taken at midwater depth adjacent to the egg basket 30 s after treatments were applied. Samples were filtered through a 0.22-μm-screen nylon filter (Weltje et al. 2003; Straus et al. 2009), acidified to 1% (by volume) with nitric acid and stored for later analysis (within 1 month). Samples were analyzed for Cu concentration with an Optima DV2000 Perkin-Elmer inductively coupled plasma optical emission spectrometer (ICP-OES; Perkin-Elmer, Waltham, Massachusetts) according to standard methods (APHA, American Water Works Association, and Water Pollution Control Federation 2005). The measured dissolved Cu concentration divided by the calculated total Cu concentration of the treatment equals the percent of Cu recovered; this was used to verify the treatment rate. A groundwater sample was analyzed for a suite of chemical parameters by the Water Quality Laboratory at the Arkansas Water Resources Center (University of Arkansas, Fayetteville).

PCR analysis.—The ITS region of the nuclear rRNA repeat was amplified with primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) according to White et al. (1990). The PCR master mix was prepared according to the manufacturer's protocol (Takara ExTaq, Chemicon International, Temecula, California). Tubes were cycled in a DNA Engine Peltier thermal cycler (Bio-Rad Life Science Research, Hercules, California) as follows: 98°C for 2 min; 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 1 min; 72°C for 7 min, and 4°C hold. The PCR products were evaluated by gel electrophoresis. Samples were cleaned with the PCR Product PreSequencing Kit (USB Corporation, Cleveland, Ohio) according to the manufacturer's protocol. Cleaned PCR products were sent to the University of Chicago, Cancer Research Center, DNA Sequencing Facility (Chicago, Illinois) for analysis. Edited sequences were compared with those in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST).

Statistical analysis.—The study was conducted in a randomized design with four troughs; an individual spawn was used in each trough and there were two replicates of each treatment in each trough (i.e., two control treatment compartments and two $CuSO_4$-treated compartments). The fry survival rate was analyzed by the Proc GLIMMIX procedure (version 9.1.3; SAS Institute, Cary, North Carolina) for differences between the control and $CuSO_4$ treatments. The model "treatment" used the fixed effect and "trough" was the random effect within the model statement. A significant difference was indicated at $\alpha < 0.05$.

Results and Discussion

Eye pigment was first seen in the embryos during afternoon observations on day 5 and all treatments were stopped. Hatching was complete by day 10. A small amount of fungus was first detected on most of the control treatment eggs by day 2 and on some of the $CuSO_4$-treated eggs by day 3.

Based on morphometric ITS sequence characterization, fungus samples from all troughs were identified as *Saprolegnia* spp. Two sequences were deposited in GenBank (accession numbers FJ236032 and FJ236033). Multiple sequence identification supports the introductory comment that species causing saprolegniasis are diverse and possibly defined by various unidentified factors. Species-level determination could not be made morphologically due to lack of oogonial development. However, new research by Hulvey et al. (2007) suggests that species identifications based on morphological characteristics are difficult and uncertain; they concluded that species cannot be circumscribed solely on the basis of sexual features and taxonomic criteria must be comprehensively re-evaluated.

Fungal growth was severe in the control treatments resulting in 8% survival, and the mean survival of hatched fry in the $CuSO_4$-treated eggs was significantly higher (55%; Table 1). Very little fungus was present in treatments receiving $CuSO_4$ (2.1 cm^2) except in one

TABLE 1.—Copper sulfate dose, fry survival rate (mean ± SD; $n = 4$) and P-value, percent of survival that was considered prematurely hatched (PMH) fry, and mean highest fungal area in the laboratory dose confirmation study of the effectiveness of copper sulfate in controlling fungus on channel catfish eggs.

Dose (mg/L)	% Survival	P-value[a]	% PMH	Fungal area (cm^2)
0	8.2 ± 13.9		0.5 ± 0.7	9.3 ± 3.3
10	54.6 ± 14.8	0.0003	1.2 ± 0.6	2.1 ± 2.2

[a] Differences were considered significant at $\alpha < 0.05$.

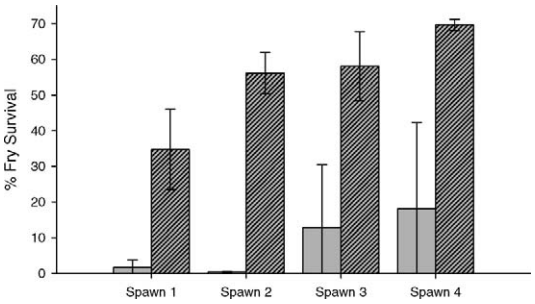

FIGURE 1.—Fry survival rates (mean ± SD, $n = 4$) for control (solid bars) and $CuSO_4$-treated eggs (cross-hatched bars) of the four replicate spawns in the laboratory dose confirmation study of the effectiveness of $CuSO_4$ in controlling fungus on channel catfish eggs.

replicate that had large areas of unfertilized eggs that act as a source of nutrition for fungal growth; these eggs were easily identifiable since pigmentation does not develop as in normal eggs. In this replicate, fungal growth was low and remained in check while being treated with $CuSO_4$, but increased dramatically on day 8 through the end of the experiment. Additionally, one of the control replicates from two different spawns (spawns 3 and 4 in Figure 1) had higher survival than in other controls in this study or in previous studies in this laboratory.

The mean egg survival in the catfish industry has been estimated to be approximately 60% (Wolters 2001) when, along with other best management practices, formalin and iodine are used as needed to control fungal infections. In the present study, fry survival in the control treatments ranged from 0.2% to 35% and in the $CuSO_4$ treatments ranged from 27% to 71% among egg masses (Figure 1). These results underscore the importance of using several spawns to replicate the treatments. Differences in hatching success among individual egg masses can be attributed to various factors, including (1) the fungal susceptibility of an individual spawn, (2) the amount of unfertilized eggs in an individual spawn, and (3) other nonfungal pathogens affecting an individual spawn (i.e., bacteria, ciliates, etc.). This variability is evident by the large SD values in fry survival. Further research should address reasons for this variability.

The average survival in the $CuSO_4$ treatment included 1% of the eggs that were considered to be PMH fry. There was no apparent correlation of PMH fry to treatment. The lower percentage of PMH fry in the control is attributed to higher fungal growth, hence lower survival. Preliminary observations at SNARC suggest that a portion of PMH fry may recover and appear normal. Future studies should investigate the survival of PMH fry for longer durations.

The Cu concentration of the control treatment water was 0.00 mg/L, which is typical of SNARC groundwater. The calculated concentration of the $CuSO_4$ treatment was 2.54 mg/L Cu and the measured concentration was 2.21 ± 0.24 mg/L Cu; the mean dissolved Cu concentration of the treatment was 86.9% of the calculated concentration. Straus et al. (2009) demonstrated the elimination rate of various concentrations of $CuSO_4$, and discussed the reasoning behind choosing a 30-s sampling time after the treatment has been applied. Data to obtain the dissolved Cu concentration from the 10-mg/L $CuSO_4$ profile demonstrates that approximately 7% of the total Cu (unfiltered sample) is bound and removed by filtering (Straus et al., 2009). This is similar to results of the present study.

The results of the water analyses for chemical characteristics are presented in Table 2. A detailed account of the water chemistry is necessary for studies involving metal toxicity or efficacy, though much of this information is missing from earlier references. Computer-modeling research requires such data to determine metal toxicity, speciation, and binding capacities. As an example, the biotic ligand model has recently been incorporated by the EPA in their water quality criteria documents (Di Toro et al. 2001; USEPA 2003a, 2003b).

Extension personnel recommend $CuSO_4$ for fungus control in catfish hatcheries because of its effectiveness, history of safe use, and low cost. This laboratory dose confirmation experiment is an important part of the effectiveness portion of the new animal drug approval process, but a separate dose confirmation experiment in a commercial hatchery will be necessary to provide independent substantiation. The potential approval of $CuSO_4$ for fungus control will provide hatchery managers and extension personnel additional legal options when treating egg fungus. Future studies required for FDA approval will research the safety of $CuSO_4$ to catfish eggs and the environment.

TABLE 2.—Water characteristics determined by the Water Quality Laboratory at the Arkansas Water Resources Center for the laboratory dose confirmation study of the effectiveness of copper sulfate in controlling fungus on channel catfish eggs; BDL = below detection limit.

Characteristic	Concentration[a]
Aggressive index[b]	12.36
Bicarbonate[c]	219.4
Calcium	25.07
Carbonate[c]	BDL
Chloride	61.28
Conductivity[d]	591
Copper	BDL
Fluoride	0.07
Iron	0.01
Magnesium	6.26
Manganese	BDL
Nitrate-nitrogen	1.00
Nonpurgeable organic carbon[e]	1.51
Phosphate	0.15
Potassium	6.98
Sodium	BDL
Sulfate	0.02
Total dissolved solids	378.24
Dissolved organic carbon[e]	1.66
Total suspended solids	1.80
Zinc	BDL

[a] mg/L unless noted otherwise.
[b] A general indicator of the tendency for corrosion to occur; no units for concentration.
[c] As $CaCO_3$.
[d] μmhos/cm.
[e] As carbon; nonpurgeable organic carbon was determined on an unfiltered sample, dissolved organic carbon on a 0.45-μM-filtered sample.

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