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Title: Evaluation of Three Embryo Disinfectants on Hatching Success in Four Freshwater Ornamental Fish Species

Running Head: Effects of Disinfectants on Ornamental Fish Embryos

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[A]Abstract

Maintaining optimum conditions for embryo incubation is a critical consideration in finfish aquaculture. To this end, prophylactic disinfectant protocols have been widely used in the culture practices of many finfish species. Empirical evaluations of safety and effectiveness for disinfectants in ornamental fish species, however, are uncommon. In this study, we evaluated hatching success in embryos of four ornamental fishes (Redtail Sharkminnow *Epalzeorhynchos bicolor,* Rainbow Sharkminnow *Epalzeorhynchos frenatum*, Featherfin Squeaker *Synodontis eupterus,* and Upside-Down Catfish *Synodontis nigriventris*) after immersion in three common disinfectant solutions: 1,500 mg/L formalin [15 min], 100 mg/L iodine [15 min], 1,000 mg/L hydrogen peroxide [15 min], and hatching water [15 min]. All disinfectants were administered according to either the manufacturer's recommendation or protocols found in published literature for other species. Of the concentrations used, hydrogen peroxide and formalin can be safely used as a prophylactic treatment for the majority of species evaluated with no detrimental effects. In addition, in Redtail Sharkminnows, embryos treated with formalin exhibited numerically higher hatch success with less variation among replicates than the control treatment. For all species, immersion in 100 mg/L iodine [15 min] resulted in significantly lower hatching success when compared with all other treatments.

[A]Introduction

The aquatic environment is home to numerous pathogens that can negatively impact the production capability of aquaculture operations. The embryonic stage is one of the most vulnerable periods of an aquatic organism's life. At this stage the organism is sessile and susceptible to fungal and bacterial infections due to its immature immune system (Liu et al. 2014). During the embryonic stage, gas and metabolite exchange occurs across the chorion, which may be vulnerable to colonization by pathogens. Fungi or bacteria, which can rapidly

proliferate in the culture environment, may attach to the surface of developing embryos, decreasing permeability, and potentially smothering the growing larvae. Dead embryos can quickly diminish water quality, negatively impacting conspecifics, which can manifest in further mortalities of incubating embryos or newly hatched larvae. In an attempt to mitigate mortality and reduce the abundance of potentially pathogenic microbes associated with embryos and the incubation environment, commercial aquaculture hatcheries commonly employ a variety of strategies to increase survival and hatching of embryos which can range from the use of chemical disinfectants to implementation of targeted biosecurity practices. Furthermore, prophylactic disinfection of embryos is a common biosecurity practice among hatcheries (Stuart et al. 2010) and is critical to prevent the introduction and spread of pathogens in an aquaculture setting.

Bacteria occur ubiquitously in the aquatic environment and can be pathogenic or mutualistic in the development of a fish (Bergh 2000). The majority of pathogenic bacteria that may negatively affect teleosts can be broadly categorized in four groups: ulcer forming or systemic gram-negative bacteria, external gram-negative bacteria, systemic gram-positive bacteria and slow-growing acid fast bacteria (Roberts et al. 2009). The gram negative bacteria in the genus *Flavobacterium* are of high concern for adversely impacting fish production and have been reviewed extensively (Hansen et al. 1992; Pavlov and Moksness 1993; Bergh 2000; Sudova et al. 2007; Wagner et al. 2008; De Swaef et al. 2016).Virtually any surface can be colonized by bacteria in an aquatic environment, including the mucosal lining of skin and surface of fish embryos (Oppenheimer 1955; Hansen and Olafsen 1989). Most disease causing bacteria are secondary opportunistic pathogens that take advantage of a compromised immune system as a result of a stressed host, however, some may be obligate or primary pathogens (Roberts et al. 2009). Suboptimal environmental conditions are a common stressor and can lead to suppression of a fish's immune system or create a habitat in which pathogens may flourish (Roberts et al. 2009). Bacteria in the genus *Flavobacterium* (formerly *Flexibacter*) may also be able to penetrate the egg chorion and infect the forming embryo inside (Bergh 2000). Egg ulceration caused by *Flavobacterium* spp. bacteria has been demonstrated to result in premature hatching in common Wolffish, *Anarhichas lupus* (Pavlov and Moksness 1993). Additionally, asphyxiation of the developing embryo from bacterial overgrowth is also a concern in a hatchery setting (Hansen et al. 1992; De Swaef et al. 2016).

Pathogenic Oomycetes, colloquially referred to as fungi and fungal spores, are common in the hatchery environment and can be present in both fresh or saltwater and inhabit a wide range of temperatures (Monticini 2010). Fungi heavily impact aquaculture with the most influential species occurring in the family *Saprolegniaceae* (Noga 1993). Fungi most often feed saprophytically, consuming organics and dead substrates (Monticini 2010). On fish embryos, mortality is believed to be caused by the breaching of the chorionic membrane from hyphal growth, causing osmoregulatory difficulties for the embryo (Liu et al. 2014). Fungal colonization usually begins in non-viable or unfertilized eggs and spreads to the developing healthy embryos (Monticini 2010). In trout hatcheries, saprolegniasis of embryos has been reported to impair production by 20-40% (Forneris et al. 2003).

There is a wide assortment of disinfectant chemicals and associated protocols used to treat teleost embryos. Suitability varies with the target pathogen, as well as the tolerance of the embryo to therapeutic levels of the disinfectant, which in many cases can be species specific (Sudova et al. 2007; Chambel et al. 2014; De Swaef et al. 2016). Efficacy of the chosen chemical can vary greatly and may be dependent on species, contact time, stage of embryo development, application method, and disinfectant concentration. Among the more common disinfectant chemicals used in prophylactic treatment of fish embryos are hydrogen peroxide, formalin, and iodine. This is partially due to their availability, effectiveness in treating a range of pathogens, and status as FDA approved or low regulatory status.

The objective of this study was to identify if prophylactic treatment of embryos using formalin, hydrogen peroxide, and iodine would improve hatching success of the Upside-Down Catfish *Synodontis nigriventris*, Featherfin Squeaker *Synodontis eupterus*, Redtail Sharkminnow *Epalzeorhynchos bicolor,* and Rainbow Sharkminnow *Epalzeorhynchos frenatum* when administered via an immersion bath. While studies evaluating susceptibility of the four study species to known bacterial and fungal pathogens are generally scarce, both *Epalzeorhynchos* species are susceptible to systemic infection by the gram-positive bacteria *Streptococcus iniae*, although cases were limited to juvenile and adult fish (Russo et al. 2006; Russo and Yanong 2009). Common pathogens such as *Streptococcus* spp., *Flavobacterium* spp., *Aeromonas* spp. and *Saprolegnia* spp. can infect all four species (R. Yanong, personal communication) and therapeutic doses of formalin, hydrogen peroxide, or iodine have been indicated to have both bactericidal and fungicidal properties (Murray et al. 1995, Yanong and Erlacher-Reid, 2012). As

there is a dearth of published data on efficacy of embryo disinfection methods for ornamental species, results from this experiment will aid in identification of effective hatching protocols for four economically significant ornamental fish species.

[A]Methods

[B]Experimental Design

Broodstock for all species were acquired from commercial tropical fish farms in Florida 24 to 48 hours prior to spawning. Individual fish were held in plastic 18.9 L buckets with holes drilled for water exchange, which were situated in 1030 L rectangular concrete tanks. Tanks were supplied with 380 L/h degassed well water. Broodstock were returned to the farms where they originated following spawning. For each species (*S. nigriventris*, *S. eupterus*, *E. bicolor E. frenatum*) a separate experiment was conducted to evaluate the effect of three embryo disinfectant protocols on hatching success. Eggs used in this study were obtained from hormone induced spawning trials using gonadotropin releasing hormone analogs (GnRHa's) and procedures outlined in Sipos et al. (2019). *E. frenatum* and *E. bicolor* were spawned using similar methods. Prior to injection with spawning aids, each fish was anesthetized in a tricaine methane-sulfonate bath (Tricaine-S, Syndel, Ferndale, WA, USA) at a concentration of 150 mg/L, which was buffered with 300 mg/L sodium bicarbonate. Males and females of both *Epalzeorhynchos* species received a 10 % priming dose and a 90 % resolving dose of the GnRHa drug preparation staggered 6 hours apart at 0.5 µL/g of the fish's bodyweight. *S. eupterus* and *S. nigriventris* were also spawned using analogous protocols. Both *Synodontis* catfish received a single dose of the GnRHa drug preparation at 0.5 µL/g of the fish's bodyweight. Spawning aids in all species were administered as an intramuscular injection at the base of the dorsal fin. Ovulation occurred 3-10 hours post resolving injection for the *Epalzeorhynchos* species while ovulation was observed at 16-24 hours post injection for both *Synodontis* species. At time of ovulation, females were stripped of oocytes and fertilized with milt from a single male. Each spawn was then placed into a 1 L floating screen bottom (400 µm screen) container and incubated within a recirculating system until subsamples were to be collected. Embryo samples used for subsequent experiments resulted from spawns collected from at least two individual females and were combined approximately 1.5 hours post fertilization and pooled prior to use. Embryos at this time ranged in development from late cleavage to the early morula stage.

Three experimental disinfectant solutions and one control group containing system water were evaluated for each species. Disinfectant solutions and concentrations evaluated were 1,500 mg/L formalin (Parasite-S [37 % formaldehyde], Syndel, Ferndale, WA, USA), 100 mg/L iodine (Ovadine [10 % povidone-iodine buffered solution,] Syndel, Ferndale, WA, USA), and 1,000 mg/L hydrogen peroxide (3% hydrogen peroxide, Equate, Bentonville AR). Concentrations of each disinfectant were chosen based on aquaculture drug guidelines provided by the American Fisheries Society Fish Culture Section (Bowker et al. 2016) as well as commercial recommendations of the approved product supplier. Disinfection was implemented as a one-time bath, and the duration of embryo contact time for the disinfectant was standardized among treatments (15 minutes) according to FDA guidelines for dosing of the approved drugs (hydrogen peroxide and formalin) for finfish embryos and previous studies with Lake Trout *Salvelinus namaycush* (Rach et al. 2005) and Rainbow Trout *Oncorhynchus mykiss* (Amend 1974; Schreier et al. 1996). Solutions were prepared by mixing a predetermined quantity of disinfectant into respective containers containing 1 L of hatching water to achieve the treatment concentrations previously stated. New disinfectant solutions were made the day of each trial for each of the four species examined. Each treatment contained 10 replicates, with each replicate comprised of 50 embryos that were stocked into 150 mL screen (40 µm) bottom cups. Embryos were transferred from a homogenized pool to replicate cups using 3 mL transfer pipettes. Screen bottomed containers which included embryos, were nested within an additional 150 mL sample container filled with 80 mL of the respective disinfectant solutions or hatching water (control) and allowed to remain static for 15 minutes. Following disinfectant immersion, screened sample containers were transferred to a recirculating system where the containers were incubated until hatching. The recirculating system was 1,120 L and was comprised of six 110 L aquaria, a gravity fed header tank, 175 L sump, bubble wash bead filter and foam float to suspend hatching cups within aquaria. Hatch success was evaluated within 24 hours post fertilization for the *Epalzeorhynchos* species and 36 hours for *Synodontis* species due to natural differences in incubation time. Prior to enumeration, sample cups were placed into a 300 mg/L tricaine methanesulfonate solution (MS-222, Western Chemical Inc., Ferndale, WA) to euthanize larvae. Larvae were then counted using transmitted light for increased contrast. Larvae that appeared to be successfully hatched but deceased prior to euthanasia were considered as hatched.

[B]Water Quality

Water quality parameters for broodstock holding and hatching systems were tested prior to and during spawning trials. Dissolved oxygen (DO) and temperature were measured using a YSI ProComm II meter (YSI Inc., Yellow Springs, OH). Total ammonia nitrogen (TAN), nitrite-nitrogen, pH, hardness ($CaCO_3$), and alkalinity ($CaCO_3$) were measured using standard colorimetric assays according to the manufacturer's protocols (Hach Company, Loveland, CO).

[B]Data Analysis

A single experimental embryo disinfection trial was completed for every species. Each trial had a total of 10 replicates per experimental treatment (N=4) with the exception of *E. bicolor* having 9 replicates for the hydrogen peroxide, formalin, and control treatments due to damaged specimen cups. Hatch success for each replicate was calculated by dividing total larvae present in treatment cup by the initial amount of stocked embryos per cup (50). A Shapiro-Wilk test was used to assess normality, and a Levene's test was conducted to evaluate homogeneity of variances among treatments. Distributions of hatch success data for all species violated assumptions of normality and homoscedasticity. Non-normal hatch success data was analyzed using a one-way Kruskal-Wallis test followed by a Wilcoxon signed rank test with a Holm correction for post hoc pairwise comparisons among treatments. Coefficient of variation (CV) values (CV = [Standard deviation / mean] x 100) were generated for hatching data for each treatment to examine degree of variance. A P-value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using JMP Pro v13.0.0 (SAS Institute, Cary, NC). Percent values are rounded to the nearest whole number and are represented as mean ± standard deviation (SD).

[A]Results

[B]Hatch Success

In each species hatch success was significantly affected by embryo disinfectant ($P <$ 0.001). In *E. frenatum* the highest mean hatch success was observed in the control (24 ± 9%) and the hydrogen peroxide treated embryos (22 ± 9%) , which were statistically similar ($P = 0.382$, Table 1). Formalin treated embryos yielded a hatch success (10 ± 9%) approximately half that of the control and hydrogen peroxide treatments while the iodine treatment resulted in 100% mortality (Table 1). For *E. bicolor* both formalin and hydrogen peroxide treated embryos exhibited hatching success comparable to that of the control treatment ($P = 0.742$ and 0.252, respectively) although formalin performed significantly better than hydrogen peroxide ($P <$

0.001, Table 1). Iodine exposure once again resulted in complete mortality for replicates evaluated with this species.

Hydrogen peroxide and control treatment groups produced similar hatching results (40 ± 9% and 36 ± 6%) for *S. nigriventris* embryos ($P = 0.566$); with hydrogen peroxide performing significantly better than both formalin and iodine exposure protocols (Table 1). *S. eupterus* had the highest hatch rates amid the formalin (36 ± 14%) and control treatments (40 ± 12%) ($P = 0.569$, Table 1). The hydrogen peroxide (26 ± 8%) and iodine (1 ± 1%) treatments resulted in embryo hatching success significantly less than what was recorded for the control group during this experiment (Table 2). Levene's test for each species indicated variance was unequal among mean hatch success values obtained from each treatment ($P \leq 0.015$). No apparent trends in the coefficients of variation were observed among disinfectants, however calculated values elucidated treatments within species that performed with greater consistency (Table 2).

[B]Water Quality

Mean (± SD) water quality parameters recorded for the flow through spawning systems for all experiments were: TAN 0.0 ± 0.0 mg/L, nitrite-nitrogen 0.0 ± 0.0 mg/L, pH 8.0 ± 0.0, hardness 440.33 ± 32.37 mg/L $CaCO_3$, alkalinity 192.38 ± 8.55 mg/L $CaCO_3$, temperature 25.23 ± 0.51 °C and DO 7.90 ± 0.62 mg/L. Water quality parameters recorded for the recirculating hatching system for each experiment are depicted in Table 3.

[A]Discussion

When prophylactically treating embryos, dosage of disinfectant, exposure duration, and species can greatly affect the hatch success. Among the four species investigated, the variability in hatch success following immersion in each chemical tested underscores the need to identify the most appropriate drug and application protocol for successful disinfection of embryos. Our studies indicate that 1,000 mg/L hydrogen peroxide applied to embryos in a static immersion for 15 minutes does not detrimentally effect hatch success when compared to the control treatment ($P \geq 0.252$) in *E. frenatum*, *E. bicolor,* and *S. nigriventris*. Results also show that while concentrations of 1,500 mg/L formalin did not confer any increase in hatching success, this disinfectant may be applied for the designated 15-minute duration to prophylactically treat embryos without adverse effects on hatch rate ($P \geq 0.053$) in *E. bicolor*, *S. eupterus,* and *S. nigriventris*.

Given the broad diversity among fishes in aquaculture, it is reasonable to expect the effects of chemotherapeutants on developing embryos to be species specific. Although iodine produced poor hatch success for the four ornamental species tested in this trial, previous literature has reported increased hatching success at higher concentrations and/or longer application times using this chemical (Amend 1974; Khodabandeh and Abtahi 2006; Chambel et al. 2014). Conversely, in Black Sea Turbot *Scophthalmus maximus* embryos treated with concentrations of 100-3,000 mg/L iodine for 10 minutes, all had lower hatching success and higher rates of deformity than untreated embryos (Aydın 2011). A study using the ornamental species Black Tetra *Gymnocorymbus ternetzi*, Zebra Danio *Danio rerio,* and Freshwater Angelfish *Pterophyllum scalare,* treated embryos with 1, 5, and 15 mg/L iodine for a duration of 24 hours and observed greater or equal performance in hatch success compared to the control group (Chambel et al. 2014). These results suggest an increased exposure time with a substantially reduced concentration may be a more viable approach for embryo disinfection using iodophors; especially in sensitive ornamental species. Moreover, in the same study marginally higher concentrations of iodine (25 mg/L) resulted in 0% survival exhibiting a low safety margin for the drug (Chambel et al. 2014).

In a study evaluating the efficacy of povidone-iodine (PVPI) as an embryo disinfectant in laboratory reared zebrafish *Danio rerio*, unbuffered PVPI administered for 5 minutes at a concentration of 50 mg/L resulted in significantly higher embryo mortality than the control at 6 hours post fertilization (Chang et al. 2016). In the same study, mortality at 24 hours post fertilization was significantly higher than the control for embryos treated with concentrations of 25 mg/L and 50 mg/L of unbuffered PVPI, as well as 50 mg/L buffered PVPI, indicating toxicity at these concentrations (Chang et al. 2016). Although our experimental concentration of 100 mg/L iodine and contact time of 15 minutes mirrored protocols used in state hatcheries for salmonid embryos (Wagner et al. 2008) and successful toxicity studies done on Rainbow Trout embryos (Amend 1974), the observed embryo mortality in all our tested species supports the contention that the development of overarching guidelines for embryo disinfection may be inappropriate and emphasizes the need to formulate species specific protocols (Chambel et al. 2014; De Swaef et al. 2016).

Embryos in this trial were incubated in a recirculating aquaculture system of low stocking density, with low dissolved organics, and high filtration rates. In general, these conditions are not

conducive to rapid growth of bacteria and fungi as environmental requirements may be limiting. In contrast, in a production setting, producers often stock systems with higher quantities of embryos and larvae and may not monitor or adjust water quality parameters as often as in a laboratory experimental setting. Higher organic loads which result from higher stocking densities can also influence the activity and thus the toxicity of administered disinfectants. In a study focused on salmonid embryo disinfection, a volume ratio of 1:1, embryo : 100 mg/L iodine solution was used, at the end of one hour the amount of iodine left in solution was not able to be measured (Chapman and Rogers 1992). A more pronounced decrease in iodine concentration was also observed when embryos were not rinsed and contained both milt and ovarian fluid (Chapman and Rogers 1992). Results from this study highlight an important consideration when administering chemicals to systems with substantial organic loads. The ratios of embryos to iodine solution used in our experiment were possibly much higher than even the highest treatment (1:4) tested by Chapman and Rogers (1992). The greater quantity of active iodine in relation to embryo volume in the current experiment could have had deleterious effects on embryos, translating to poor hatch rate regardless of duration of treatment (Table 1).

Water quality parameters may further affect the efficacy or toxicity of the chemotherapeutant in the aquatic environment. Iodine becomes more toxic to embryos below pH of 6 and becomes a less effective disinfectant in environments with a pH of 8 or above (Torgersen and Håstein 1995). In this study, hatching in all experimental trials occurred in pH ranges of 7.5-8.0 (Table 3). Hydrogen peroxide may become more toxic at higher temperature and will decrease the pH of the water being treated (De Swaef et al. 2016). This investigation took place in a temperature-controlled laboratory and water used for the experiments contained sufficient alkalinity to buffer acute fluctuations in pH (Table 3).

The rate at which fish embryos develop is highly variable and toxicity of the disinfectant can fluctuate depending on developmental stage. In all species used in this study, hatch occurred within 36 hours post fertilization while salmonids can take up to 7 weeks to hatch (Hinshaw 1990). A large body of literature exists which focuses on embryo disinfection of salmonids as a result of their prolonged duration as a vulnerable embryo. A study examining the effect of iodine disinfection of Orange-spotted Grouper *Epinephelus coioides* determined that embryos survived the iodine treatment better at the eyed stage compared to the cleavage stage (Tendencia 2001). A 30% increase in hatch rate was observed when non-eyed Rainbow Trout embryos were treated

with 566 mg/L of hydrogen peroxide for 15 minutes every other day (FDA 2007). Research with non-eyed Common Carp *Cyprinus carpio* embryos treated with sodium chloride, formalin, and iodine, until they became eyed, demonstrated an increased hatch rate for all treatments when compared to the control (Khodabandeh and Abtahi 2006). Embryos selected for the current experiment ranged in development from late cleavage to the early morula stage. Future investigations which delayed disinfection of embryos until later stages of maturity would further aid in optimization of species-specific procedures. As relative fecundity limited replicates to 50 embryos for this study, future studies which examine disinfection protocols on larger commercial scales are warranted to ensure results from this trial are scalable. Additionally, evaluation of survival and development of larval fish beyond the hatching stage could shed light on the persistent effects of disinfection protocols in these species.

The uses of chemical disinfectants in aquaculture are widespread and have a range of applications. In a production setting the majority of chemotherapeutant use is prophylactic and aimed towards disease management and mortality mitigation; however, disinfectants may also be used to reduce the horizontal transmission of pathogens between conspecifics and facilities by sanitizing egg surfaces (Torgersen and Håstein 1995). Embryo disinfection should also be implemented as a critical biosecurity step during the transfer of embryos between aquaculture facilities, in addition to disinfection of fomites related to said transfer (Noga 2010). Disinfection procedures are also used to create axenic or gnotobiotic larvae for model organisms in research facilities (Chang et al. 2015; De Swaef et al. 2016). To best prescribe a disinfectant and concentration it is essential to know desired results of drug application as axenic levels of disinfection may compromise hatch success.

Further consideration should be given when selecting a disinfectant with regard to safety to humans. Although iodine appeared toxic to our study species embryos at the given concentration and duration of treatment (Table 1), handling of the chemical by the applicator is comparatively safe. Iodine has been reported to be used as a germicide in human drinking water at concentrations as high as 20 mg/L with no ill effects (Punyani et al. 2006). Safety while using hydrogen peroxide varies with concentration. When using 35 % hydrogen peroxide interactions with the user may be caustic and proper skin and eye protection should be used (Rach et al. 2005). However, the concentration of 1,000 mg/L hydrogen peroxide used in this study could be considered quite safe. Peroxide is often given preference based on environmental safety, as the

byproducts of hydrogen peroxide reacting are water and oxygen (Yanong 2011). Formaldehyde (formalin), although an FDA approved treatment for finfish eggs is also carcinogenic (Roberts et al. 2009). While working with formaldehyde, the Occupational Safety and Health Administration (OSHA) set permissible exposure limits of airborne formalin at 8 hours of 0.75 ppm and 15 minutes for concentrations of 2 ppm (OSHA 2011). Storage of formalin should be cool, however if temperatures drop to below 40º F, paraformaldehyde is formed; furthermore, contact with the white precipitate is extremely toxic and can kill fish (Francis-Floyd and Pouder 1996).

Based on the results obtained from this experiment, it is recommended to use a dose of 1,500 mg/L formalin administered as an immersion bath for 15 minutes to prophylactically treat *E. bicolor* embryos. Using this method, the highest hatch success of 78 ± 5 % was achieved in this species and the lowest variability in performance was also observed. Although the hatch success between the control and formalin treated fish was similar ($P = 0.742$) the coefficients of variation differed greatly with a CV of 46.8 in the control group and a CV of 6.9 in the formalin treated embryos (Table 2). Drugs which have lower CV values may be chosen by the producer to yield a more consistent response from the chosen prophylaxis. Although 1,000 mg/L hydrogen peroxide exposure did not outperform the control treatment in *E. frenatum* and *S. nigriventris*, use of this drug may be beneficial in aquatic environments with reduced water quality or known pathogens when incubating embryos of these species. The 1,500 mg/L formalin exposure treatment is suggested to treat *S. eupterus* embryos as it did not detrimentally effect hatching and may be advantageous in a commercial production setting. The dose of 100 mg/L iodine for a 15-minute immersion is not recommended to treat embryos of the four ornamental species used in this study. Further species-specific investigations which focus on disinfectant concentration and contact time are urged to develop empirically based recommendations that are both safe for the developing embryo and may prophylactically address pathogenic microbes which can adversely impact commercial hatching protocols.

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[B]Tables

Table 1. Mean hatch success percentages ± standard deviations for embryos from four species of ornamental fish exposed to four disinfectant chemicals. Disinfectant concentrations and exposure times were as follows: 1,500 mg/L formalin [15 min], 100 mg/L iodine [15 min], 1,000 mg/L hydrogen peroxide (H_2O_2) [15 min], and hatching water (control) [15 min]. Different letters within columns denote statistically significant differences among treatment groups.

Treatment	*E. frenatum*	*E. bicolor*	*S. eupterus*	*S. nigriventris*
Control	24 ± 9 z	62 ± 29 zy	40 ± 12 z	36 ± 6 z
Iodine	0 ± 0 x	0 ± 0 x	1 ± 1 x	0 ± 0 y
H_2O_2	22 ± 9 z	47 ± 17 y	26 ± 8 y	40 ± 9 z
Formalin	10 ± 9 y	78 ± 5 z	36 ± 14 zy	30 ± 7 z

Table 2. Levene's test *P* values and coefficient of variation values among treatments for each species. The – symbol signifies 0 hatch success.

	E. frenatum	*E. bicolor*	*S. eupterus*	*S. nigriventris*
P	0.010	<0.001	0.015	0.003
Control	36.2	46.8	30.4	16.1
Iodine	-	-	161.0	-
H_2O_2	39.7	35.9	32.1	23.6
Formalin	88.4	6.9	40.3	21.7

Table 3. Experimental water quality for recirculating hatching systems. TAN (total ammonia nitrogen), nitrite-nitrogen, hardness ($CaCO_3$), alkalinity ($CaCO_3$) and DO (dissolved oxygen) are measured as mg/L, while temp (temperature) data was recorded in Celsius (°C).