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ARTICLE

Comparative Value of Fish Meal Alternatives as Protein Sources in Feeds for Hybrid Striped Bass

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Abstract

Numerous alternative proteins have been assessed and reported as adequate for fish meal (FM) sparing; however, few studies have directly compared the value of alternative proteins in side-by-side comparisons. Previous research also suggests that changes in dietary protein source may affect fillet quality, but comprehensive data on this subject are lacking. We assessed the production performance and consumer acceptance of hybrid Striped Bass (White Bass *Morone chrysops* × Striped Bass *M. saxatilis*; mean weight ± SE = 43.4 ± 0.2 g) reared on a control diet containing menhaden FM (30% FM) as the primary protein source or on experimental feeds containing approximately 10% menhaden FM and one of the following protein sources: soybean meal (10% FM–46% SBM), poultry byproduct meal (10% FM–21% PBM), grain distillers' dried yeast (10% FM–36% GDDY), corn gluten meal (10% FM–21% CGM), or distillers' dried grains with solubles (10% FM–33% DDGS). Weight gain (1,055–1,323%), specific growth rate (SGR; 1.29–1.40% of body weight [BW]/d), and feed intake (2.10–2.28% BW/d) were equivalent among groups, except that fish receiving the 10% FM–36% GDDY feed exhibited reduced performance (weight gain = 929%; SGR = 1.23% BW/d; feed intake = 1.95% BW/d) that appeared to be related to feed palatability. Feed conversion ratio was relatively consistent among the dietary treatments, with only the highest (1.39; for 10% FM–33% DDGS) and lowest (1.16; for 10% FM–21% PBM) values being significantly different. Dietary protein source had no effect on fillet color or consumer acceptance; consumers were unable to differentiate between control and experimental portions in 64% of comparisons. Each of the alternatives used in the present study appeared, to various degrees, to be suitable alternatives to FM. The results of this and other trials suggest that the FM content in feeds for hybrid Striped Bass can be significantly reduced beyond the current standard levels without negative effects on production performance or product acceptance.

Aquaculture currently consumes a majority of the fish meal (FM) produced in the world (Tacon and Metian 2008; FAO 2012), with FM used as a primary protein source in aquafeeds. As the aquaculture industry continues to grow and becomes increasingly reliant on industrially compounded aquafeeds, demand for FM will also increase. Given that FM is a largely finite resource, FM-rich feed formulations will become increasingly unsustainable in both economic and environmental terms. Alternative sources of protein are typically terrestrial in nature and are either plant based (e.g., crude soy, corn, and wheat derivatives; Trushenski et al. 2006; Gatlin et al. 2007) or animal based (e.g., poultry byproduct meal [PBM] and blood meal; Bureau 2006). More recently, these "traditional alternatives" have been joined by an increasing number of novel protein sources, such as refined protein concentrates and isolates (Blaufuss and Trushenski 2012) and waste streams from other industrial processes, such as biofuel coproducts (Gause and Trushenski 2011a, 2011b) and rendered seafood processing waste (Bechtel and Johnson 2004; Hardy et al. 2005).

Fish meal sparing has been evaluated in many taxa representing a wide array of niches, including coldwater, coolwater, and warmwater fish; marine and freshwater fish;

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and carnivorous and omnivorous fish. These studies have evaluated numerous alternatives, including the traditional and novel protein sources mentioned above (e.g., Lewis and Kohler 2008; Thompson et al. 2008, 2012; Salze et al. 2010; Gause and Trushenski 2011a, 2011b; Schaeffer et al. 2011; Trushenski and Kohler 2011; Blaufuss and Trushenski 2012; Colburn et al. 2012; Slawski et al. 2012). Most often, researchers have evaluated the sparing of graded levels of FM by using one alternative or an alternative protein blend. For example, grain distillers' dried yeast (GDDY; Gause and Trushenski 2011a, 2011b), PBM (Webster et al. 2000; Pine et al. 2008; Rawles et al. 2009; Trushenski and Kohler 2011), soybean meal (SBM; Gallagher 1994; Webster et al. 1999; Laporte and Trushenski 2012), corn gluten meal (CGM; Lewis and Kohler 2008), and other alternatives have all been assessed in feeds for hybrid Striped Bass (White Bass *Morone chrysops* × Striped Bass *M. saxatilis*) in order to determine how much FM can be replaced. Based on production performance criteria, these researchers reported that dietary FM could be reduced to 0–20% of the diet without impacting production performance. Although these studies were beneficial for determining the substitution value of individual alternatives to FM, there remains the need for assessing comparative value in direct side-by-side comparisons. For example, in separate studies, a greater level of FM sparing was possible with turkey meal (FM reduced to 0% without production performance effects; Muzinic et al. 2006) than with SBM (FM reduced to 5% without production performance effects; Laporte and Trushenski 2012); was FM sparing greater with turkey meal because it is a superior ingredient, or was the difference simply an artifact of the particular trial and the diets, livestock, and rearing conditions involved? Some studies have used a comparative approach with multiple alternative sources (Webster et al. 1999, 2000), but few have compared multiple FM alternatives with a constant amount of FM in the diet.

Fish meal sparing may affect production performance, but it may also influence fillet quality and consumer acceptance of farm-raised seafood. For example, the fillets of Rainbow Trout *Oncorhynchus mykiss* that were fed a plant-protein-based feed in a long-term feeding trial were rated as having higher hardness, less sweetness, and a trend to lower juiciness than fillets from fish that were fed a FM-based diet (de Francesco et al. 2004). Similarly, D'Souza et al. (2006) reported significant differences in fillet taste between Rainbow Trout that were given diets containing increasing levels of SBM at the expense of FM. However, others have reported that FM sparing has little effect on fillet taste profile or acceptance (Skonberg et al. 1998; de Francesco et al. 2007). Rasmussen (2001) suggested that while feed composition may have a strong effect on fillet composition and yield, sensory qualities (e.g., flavor, odor, and texture) are only slightly affected. Fillet color, however, may be altered drastically by the presence of FM alternatives, potentially resulting in undesirable appearance and reduced acceptance by consumers (Skonberg et al. 1998; D'Souza et al. 2006; Li et al. 2007; Joseph et al. 2009). Although the reported effects of FM alternatives on sensory qualities and color of fillets vary, ensuring that consumer acceptance is not diminished by the use of reduced-FM feeds is critical.

Accordingly, we assessed production performance and fillet quality of hybrid Striped Bass that were reared on diets containing menhaden FM, SBM, CGM, PBM, distillers' dried grains with solubles (DDGS), or GDDY as the principal dietary protein source.

METHODS

Preparation and analyses of diets.—The control formulation used in the present work was formulated to be broadly representative of commercial feeds used in hybrid Striped Bass production and to be consistent with the FM-based formulations commonly used in hybrid Striped Bass nutrition research at the Fisheries and Illinois Aquaculture Center (Gause and Trushenski 2011a; Laporte and Trushenski 2012). Thus, the control formulation contained approximately 40% protein, 15% lipid, and 30% menhaden FM (30% FM diet; Table 1). Experimental feeds were formulated to include approximately 10% menhaden FM along with one of the five principal alternative source of protein (Table 1): 10% FM–46% SBM, 10% FM–21% PBM, 10% FM–36% GDDY, 10% FM–21% CGM, and 10% FM–33% DDGS. Attempts were made to keep the formulations as similar as possible except for the test ingredients. However, adjustments to other ingredients, including soy protein concentrate, soy protein isolate, wheat bran, and the supplemental oils, were necessary to maintain crude protein and lipid levels and to meet the known nutrient and energy requirements of hybrid Striped Bass (NRC 2011). Researchers at the Fisheries and Illinois Aquaculture Center have evaluated most of these ingredients in previous trials with hybrid Striped Bass (Lewis and Kohler 2008; Gause and Trushenski 2011a, 2011b; Trushenski et al. 2011; Laporte and Trushenski 2012). To facilitate comparisons with these studies, the same grades of alternative proteins and FM used in the previous work were used in preparing feeds for the present trial (Table 2). All feeds were prepared as extruded, 5–6-mm pellets by Wenger, Inc. (Sabetha, Kansas). Briefly, the extruded feeds were prepared using a single-screw extruder, according to the following conditions: the conditioner steam level was 19.4–20.7 kg/h, the conditioner water level was 42.4–55.1 kg/h, the feed screw speed was 60–72 revolutions per minute (rpm), the extruder shaft speed was 327–424 rpm, and the barrel temperature increased from 50°C to 110–120°C through barrel to die. Using a pilot-scale twin-screw extruder, feed production was performed in two replications for each diet at conditioner steam levels of 0.11–0.16 kg/min, extruder water of 0.11–0.19 kg/min, and screw speeds of 230–300 rpm. Feeds were top-dressed with the appropriate lipid blends, dried, packaged, shipped to the Fisheries and Illinois Aquaculture Center, and stored under refrigeration for approximately 2 months prior to initiation of the study. During use, working stores of feed were kept at ambient temperature in plastic tubs; as the feed was used, the tubs were refilled from the bulk stores kept under refrigeration throughout the trial.

TABLE 1. Formulation and proximate composition (mean ± SE) of feeds given to hybrid Striped Bass (FM = menhaden fish meal; GDDY = grain distillers' dried yeast; CGM = corn gluten meal; DDGS = distillers' dried grains with solubles; PBM = poultry byproduct meal; SBM = soybean meal).

Component	30% FM	10% FM–36% GDDY	10% FM–21% CGM	10% FM–33% DDGS	10% FM–21% PBM	10% FM–46% SBM
Ingredient (g/kg)						
FM	309	103	103	103	103	104
GDDY	0	364	0	0	0	0
CGM (60% protein)	0	0	214	0	0	0
DDGS	0	0	0	325	0	0
PBM (pet-food grade)	0	0	0	0	214	0
SBM (47.5%)	298	304	305	304	304	461
Soy protein concentrate	6	10	10	72	10	52
Soy protein isolate	6	7	10	53	10	46
Wheat bran	239	69	202	0	220	178
Menhaden fish oil	48	49	62	48	47	65
Canola oil	49	49	48	49	46	48
Methionine	0	0	0	1	1	2
Mineral premix	1	1	1	1	1	1
Dicalcium phosphate	15	15	16	15	15	16
Sodium phosphate	15	15	16	15	15	16
Vitamin premix	1	1	1	1	1	1
Stay-C (35%)	1	1	1	1	1	1
Choline chloride	6	6	6	6	6	6
Carboxymethyl cellulose	4	4	4	4	4	4
Proximate composition (g/kg)						
Dry matter	958 ± 1	947 ± 1	940 ± 1	952 ± 1	933 ± 1	934 ± 1
Protein	397 ± 2	432 ± 2	373 ± 2	418 ± 2	428 ± 2	402 ± 2
Lipid	139 ± 5	165 ± 5	155 ± 5	162 ± 5	150 ± 5	148 ± 5
Ash	120 ± 5	82 ± 5	87 ± 5	77 ± 5	99 ± 5	78 ± 5

Feeds were analyzed in triplicate to confirm proximate composition (Table 1). Moisture content was determined gravimetrically after freeze drying (Freezone 6; Labconco Corporation, Kansas City, Missouri); freeze-dried samples were pulverized and used for the determination of ash, protein, and lipid content. Ash was determined after incineration in a muffle furnace for 4 h at 650°C. Lipid content was determined gravimetrically by following a modified Folch extraction method (Folch et al. 1957). Protein content was determined by using a protein analyzer (FP-528; Leco Corporation, St. Joseph, Michigan). Although all feeds were formulated to be isoproteic (40%) and isolipidic (15%)—that is, to contain equal amounts of crude protein and lipid—some deviations were observed. These differences were attributed to differences in expected and actual ingredient composition (e.g., dry matter content being higher or lower than anticipated) and errors in the feed manufacturing process (e.g., slight inaccuracies in ingredient measurement or incomplete mixing). The consequences of these inaccuracies are considered in the Discussion.

Experimental design and feeding trial.—The feeding trial was conducted in an indoor recirculation system comprising thirty 170-L tanks, mechanical filtration (sand filter; Pentair Pool Products, Sanford, North Carolina), biological filtration (submerged media biofilter bed), and a supplemental aeration system with a 24-h photoperiod (i.e., 24 h of light). Each diet was randomly assigned to five replicate tanks, each stocked with six juvenile hybrid Striped Bass (mean weight ± SE = 43.4 ± 0.2 g) purchased from Keo Fish Farm (Keo, Arkansas). Fish were fed the assigned feeds once daily to apparent satiation for 190 d until they reached a marketable or near-marketable size (≥0.45 kg [1 lb]). Temperature and dissolved oxygen were monitored daily throughout the study by using a YSI oxygen meter (Model 55; YSI, Yellow Springs, Ohio), whereas alkalinity, total ammonia-nitrogen (N), nitrite-N, and nitrate-N were measured periodically (semi-monthly, with samples collected after feeding) using standard water quality testing equipment and reagents (Hach Co., Loveland, Colorado). Throughout the trial, water quality characteristics were maintained within ranges that are considered suitable for hybrid Striped Bass culture (Kohler 2000): temperature was 24.4°C (range = 18.3–29.5°C), dissolved oxygen concentration was 8.0 mg/L (range = 6.9–9.9 mg/L), alkalinity was 228 mg/L (range = 100–400 mg/L),

TABLE 2. Proximate composition and amino acid profile of test ingredients used in feeds for hybrid Striped Bass (FM = menhaden fish meal; GDDY = grain distillers' dried yeast; CGM = corn gluten meal; DDGS = distillers' dried grains with solubles; PBM = poultry byproduct meal; SBM = soybean meal; NR = value not reported).

Component	FM	GDDY	CGM	DDGS	PBM	SBM
Proximate composition (%, as fed)						
Dry matter	92.0	94.4	92.0	89.2	93.0	88.9
Protein	61.0	44.0	63.0	30.4	63.0	46.7
Lipid	8.0	6.9	2.8	7.8	12.5	1.2
Ash	19.1	2.6	7.6	4.3	15.0	5.8
Fiber	0.9	3.1	1.8	6.7	2.5	3.8
Amino acid profile (%, as fed)						
Arginine	3.7	2.0	2.0	1.1	4.2	3.4
Histidine	1.4	1.0	1.3	0.7	1.3	1.2
Isoleucine	2.9	2.1	2.6	1.1	2.3	2.2
Leucine	4.5	5.2	10.2	2.5	4.2	3.0
Lysine	4.7	2.3	1.2	0.9	3.6	3.0
Methionine	1.7	1.0	1.6	0.6	1.1	0.7
Cystine	0.6	0.6	1.2	0.4	0.8	0.7
Phenylalanine	2.4	2.4	1.4	1.3	2.3	2.4
Tyrosine	1.9	2.0	3.2	0.8	1.7	1.7
Threonine	2.5	1.9	2.1	1.0	2.3	1.8
Tryptophan	0.6	0.4	0.3	0.2	0.4	0.7
Valine	3.2	2.5	3.0	1.5	3.0	2.3
Alanine	NR	3.3	5.6	NR	4.2	2.1
Aspartic acid	NR	3.7	4.0	NR	5.0	5.6
Glutamic acid	NR	6.7	13.6	NR	7.8	8.8
Glycine	4.2	1.8	1.7	0.6	5.8	2.1
Proline	NR	2.6	6.0	NR	4.5	2.5
Serine	2.2	2.2	3.2	1.8	2.5	2.5

total ammonia-N was 0.23 mg/L (range = 0.03–0.39 mg/L), nitrite-N was 0.04 mg/L (range = 0.01–0.11 mg/L), and nitrate-N was 7.34 mg/L (range = 1.55–12.00 mg/L). Variation in water temperature was the result of variation in ambient temperature: although the system is located indoors, the building has limited insulation and no air conditioning, and thus the system is subject to seasonal shifts in temperature. Variation in other water quality characteristics primarily reflects maturation of the biofilters during the course of the trial and some variability in the frequency of backflushing and buffer (sodium bicarbonate) addition. Although the ranges are sizeable, it is important to note that these values were observed over the course of a 190-d trial and do not reflect major daily or weekly shifts in water quality. All fish husbandry practices, including the euthanasia and sampling procedures described below, were conducted according to the standards of the Institutional Animal Care and Use Committee, Southern Illinois University, under Animal Care and Use Protocol Number 11-028.

Sample collection and analyses.—After the end of the feeding trial, all fish were harvested, counted, and group-weighed by tank to determine average individual production performance. Fish were then euthanized by immersion in salted, ice–water slurries (individual slurry baths for each tank). Five fish were randomly selected from each tank, individually weighed, and dissected to collect boneless, skinless J-cut fillets (no belly flap), livers, and intraperitoneal fat masses for the determination of organosomatic indices. Fillets were pooled by tank, packaged in plastic bags, and stored frozen (−70°C) for subsequent color and consumer panel evaluations. Production performance metrics were calculated using the following equations:

$$\text{Weight gain (\%)} = 100 \times \frac{(\text{average individual final weight} - \text{average individual initial weight})}{\text{average individual initial weight}},$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{average individual dry matter feed intake}}{\text{average individual weight gain}},$$

$$\text{Specific growth rate (SGR; \% body weight/d)} = 100 \times \frac{\log_e(\text{average individual final weight}) - \log_e(\text{average individual initial weight})}{\text{days of feeding}},$$

$$\text{Feed intake (\% body weight/d)} = 100 \times \frac{\text{average individual dry matter feed intake}}{(\text{average individual initial weight} \times \text{average individual final weight})^{0.5}/\text{days of feeding}},$$

$$\text{Hepatosomatic index (HSI)} = 100 \times \frac{\text{liver weight}}{\text{fish weight}}, \quad \text{and}$$

$$\text{Liposomatic index (LSI)} = 100 \times \frac{\text{intraperitoneal fat weight}}{\text{fish weight}}.$$

Fillet color and taste test evaluations.—Skinless fillets were stored at −70°C for 8 months prior to the color evaluation and taste test. Fillets were thawed for approximately 24 h under refrigeration (4°C), followed by approximately 3 h in cold water under ambient conditions (~25°C). Thawed fillets were rinsed with cool water and were scanned with a Hunterlab MiniScan EZ colorimeter (Hunter Associates Laboratory, Inc., Reston, Virginia) to quantify color in terms of lightness (L*; 100 = white, 0 = black), red–green (a*; green: a* < 0; red: a* > 0), and yellow–blue (b*; blue: b* < 0; yellow: b* > 0) parameters. Treatment mean L*, a*, and b* values were used to calculate color distance (ΔE) values as

$$\Delta E = \sqrt{(L^*_{\text{control}} - L^*_{\text{experimental}})^2 + (a^*_{\text{control}} - a^*_{\text{experimental}})^2 + (b^*_{\text{control}} - b^*_{\text{experimental}})^2},$$

with the assumption that ΔE values of 2.3 or greater correspond to a "just noticeable difference" (Mahy et al. 1994). Afterwards, the fillets were portioned to yield ~5- × 4- × 2-cm (length × weight × thickness) pieces of shank fillet for use in the taste test. Fillet portions were rinsed with cool water again, packaged in plastic bags, and stored under refrigeration (4°C) for approximately 16 h prior to final preparation and presentation to participants in the taste test.

Calls for volunteers were electronically distributed to various administrative units at Southern Illinois University Carbondale (e.g., the Zoology Department and the Department of Animal Science, Food, and Nutrition), yielding 15 individuals willing

to participate in the taste test as untrained consumer panelists. Although demographic information was not formally assessed, the panel included roughly equal numbers of men ($n = 7$) and women ($n = 8$) and a relatively broad age range (individuals in their 20s, 30s, 40s, and 50s). Before starting the taste test, participants were given verbal and written instructions regarding the purpose of the test and the general concept of a triangle test and were then asked to sign the required consent forms. Participants were then provided with bottled water and specific instructions (i.e., waiting a few minutes between tests, cleansing their palates with water between samples, how to fill in the questionnaires, etc.) and were presented with the fish portions for their first triangle test. All taste test methods, including the detailed procedures described below, were conducted according to the standards of the Human Subjects Committee, Southern Illinois University, under Protocol Number 12179.

While the taste test participants were being briefed, the fillet portions were cooked and plated in an adjacent room. Fillet portions were evenly spaced in a single layer on disposable aluminum baking sheets (dietary treatments on separate trays) and sprayed lightly with a salt solution (62 g [2.2 oz] of sea salt dissolved in 946 mL [2 cups] of tap water). The portions were then baked in a standard oven at 177°C (350°F) until they reached an internal temperature of 63°C (145°F; ~10 min). Cooked portions were transferred to numerically labeled, disposable plates and were assembled into three-portion groups for the triangle tests. Each triangle test was designed to compare the fillets of fish fed an experimental feed with the fillets of fish fed the control (30% FM) feed. Thus, the 30% FM treatment was randomly assigned to one portion or two portions within each triangle test, and an experimental treatment was assigned to the other portion(s).

For each triangle test, participants were asked to taste each portion (retasting was allowed) and then fill out a short questionnaire that asked (1) which of the portions was different, (2) how easy it was to tell the difference between the portions, (3) whether the different portion was more or less appealing to them and why, and (4) which of the three offered portions they preferred overall (Figure 1). If participants were not sure which of the portions was different, they were allowed to guess. Each participant completed five triangle tests.

Statistical analyses.—Production performance metrics and fillet color were analyzed by one-way ANOVA (GLIMMIX procedure in the Statistical Analysis System [SAS] version 9.2; SAS Institute, Cary, North Carolina) to determine whether significant differences existed among dietary treatment groups. Dunnett's pairwise comparison tests were applied to facilitate comparison between the experimental groups and the control group. Tukey's honestly significant difference pairwise comparison tests were also applied to allow for comparisons between the experimental groups. For these analyses, tanks were used as the experimental units ($N = 5$).

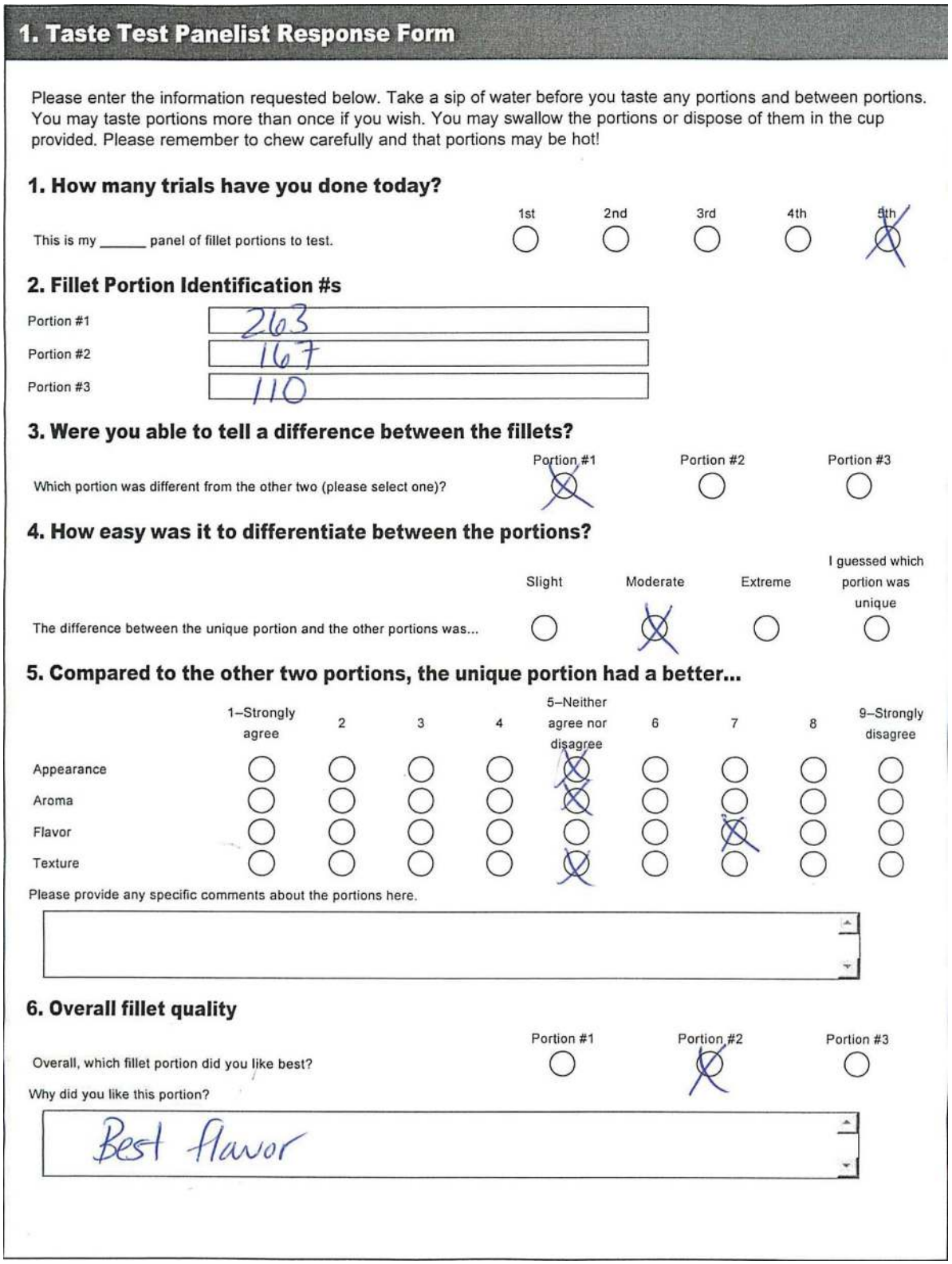
Based on the volume of 30% FM fillet portions that were available for the taste test, each of the experimental treatments was compared with the 30% FM control in 14 (DDGS and PBM) or 15 (CGM, GDDY, and SBM) individual triangle tests, which served as the experimental units for these analyses ($N =$ 14 or 15). Questionnaires were first scored as to whether the unique portion in a given triangle test was correctly identified and whether the participant's overall preference was for a 30% FM control portion or an experimental portion. These data were first analyzed by using a chi-square analysis for equal proportions to test whether there were significant differences between the frequencies of correct versus incorrect portion identifications. Subsequently, the same data were subjected to a secondary chi-square analysis to determine whether there were significant differences in the frequency of unique portion identifications among the dietary treatments (FREQ procedure in SAS). The results of these tests indicated that there were no differences between the treatments in terms of scoring, and the frequency of incorrect identifications (64% of the triangle tests) was significantly greater than the frequency of correct identifications (36%). Overall fillet preference responses were analyzed further by using the same statistical approach. In this case, the secondary chi-square analysis revealed a significant treatment effect on portion preferences; thus, post hoc Fisher's exact tests were used for pairwise comparisons of treatment groups. Remaining questionnaire responses (i.e., ease of unique portion identification, whether the unique portion was more or less appealing, etc.) were dependent on the participants' correct identification of the unique fillet portion. Given the significant number of incorrect identifications, these data were not analyzed.

All analyses were conducted using SAS version 9.2, and differences or effects were considered significant at P-values less than 0.05.

RESULTS

Hybrid Striped Bass production performance was significantly affected by dietary protein source (Table 3). Final weight (mean ± SE = 452 ± 17 g), weight gain (929 ± 44%), SGR (1.23 ± 0.02% body weight/d), and feed intake (1.95 ± 0.09% body weight/d) were all significantly reduced among fish that received the 10% FM–36% GDDY feed in comparison with fish that were given the 30% FM control feed (final weight = 581 ± 17 g; weight gain = 1,244 ± 44%; SGR = 1.37 ± 0.02% BW/d; feed intake = 2.28 ± 0.08% BW/d). All other experimental treatments were equivalent to the control, except for the 10% FM–33% DDGS group, which exhibited a significantly reduced final weight (502 ± 17 g) but was otherwise statistically similar to the control. The FCR (range = 1.16–1.29), HSI (range = 1.49–1.78), and LSI (range = 2.96–3.42) varied among treatments, but none of the experimental groups differed from the 30% FM control, and the ranges of values observed were relatively small. Survival (range = 93–100%) and dress-out (range = 24–26%) did not vary among treatments.

During harvest, treatment-blinded researchers made anecdotal observations regarding the outward appearance of the fish.



1. Taste Test Panelist Response Form

Please enter the information requested below. Take a sip of water before you taste any portions and between portions. You may taste portions more than once if you wish. You may swallow the portions or dispose of them in the cup provided. Please remember to chew carefully and that portions may be hot!

1. How many trials have you done today?

This is my _____ panel of fillet portions to test. 1st ○ 2nd ○ 3rd ○ 4th ○ 5th ⊗

2. Fillet Portion Identification #s

Portion #1: 263
Portion #2: 167
Portion #3: 110

3. Were you able to tell a difference between the fillets?

Which portion was different from the other two (please select one)? Portion #1 ⊗ Portion #2 ○ Portion #3 ○

4. How easy was it to differentiate between the portions?

	Slight	Moderate	Extreme	I guessed which portion was unique
The difference between the unique portion and the other portions was...	○	⊗	○	○

5. Compared to the other two portions, the unique portion had a better...

	1–Strongly agree	2	3	4	5–Neither agree nor disagree	6	7	8	9–Strongly disagree
Appearance	○	○	○	○	⊗	○	○	○	○
Aroma	○	○	○	○	⊗	○	○	○	○
Flavor	○	○	○	○	○	○	⊗	○	○
Texture	○	○	○	○	⊗	○	○	○	○

Please provide any specific comments about the portions here.

6. Overall fillet quality

Overall, which fillet portion did you like best? Portion #1 ○ Portion #2 ⊗ Portion #3 ○

Why did you like this portion?

Best flavor

FIGURE 1. Example of a completed questionnaire filled out by a taste test participant in comparing the fillets of hybrid Striped Bass fed an experimental diet (10% FM plus an alternative protein source) with the fillets of fish fed the control feed (30% FM). [Figure available in color online.]

TABLE 3. Production performance and fillet characteristics of hybrid Striped Bass reared on feeds containing a typical level of menhaden fish meal (30% FM) or a reduced level (10% FM) coupled with various alternative protein sources (GDDY = grain distillers' dried yeast; CGM = corn gluten meal; DDGS = distillers' dried grains with solubles; PBM = poultry byproduct meal; SBM = soybean meal). Values are presented as least-squares means ± pooled SEs; *P*-values associated with one-way ANOVA tests are provided. Results of Tukey's honestly significant difference pairwise comparison tests are indicated by lowercase letters (within a given row, means with a letter in common are not significantly different; $P > 0.05$). Results of Dunnett's pairwise comparison tests are indicated by asterisks (means with asterisks are significantly different from the mean for the 30% FM control group; $P < 0.05$).

Variable	30% FM	10% FM–36% GDDY	10% FM–21% CGM	10% FM–33% DDGS	10% FM–21% PBM	10% FM–46% SBM	*P*
Initial individual weight (g)	43 ± 1	44 ± 1	43 ± 1	44 ± 1	44 ± 1	43 ± 1	0.350
Final individual weight (g)	581 ± 17 zy	452 ± 17* w	516 ± 17 yxw	502 ± 17* xw	622 ± 17 z	545 ± 17 yx	<0.001
Weight gain (%)	1,244 ± 44 zy	929 ± 44* x	1,095 ± 44 yx	1,055 ± 44* yx	1,323 ± 44 z	1,182 ± 44 zy	<0.001
Specific growth rate (% body weight/d)	1.37 ± 0.02 zy	1.23 ± 0.02* x	1.30 ± 0.02 yx	1.29 ± 0.02* yx	1.40 ± 0.02 z	1.34 ± 0.02 zy	<0.001
Feed intake (% body weight/d, dry matter)	2.28 ± 0.09 z	1.95 ± 0.09* y	2.10 ± 0.09 zy	2.28 ± 0.09 z	2.14 ± 0.09 zy	2.21 ± 0.09 zy	0.012
Feed conversion ratio (dry matter)	1.28 ± 0.06 zy	1.29 ± 0.06 zy	1.27 ± 0.06 zy	1.39 ± 0.06 z	1.16 ± 0.06 y	1.27 ± 0.06 zy	0.018
Survival (%)	100 ± 2	93 ± 2	100 ± 2	100 ± 2	100 ± 2	97 ± 2	0.178
Dress-out (%)	25 ± 1	25 ± 1	24 ± 1	24 ± 1	26 ± 1	25 ± 1	0.380
Hepatosomatic index	1.62 ± 0.05 zy	1.49 ± 0.06 y	1.64 ± 0.06 zy	1.78 ± 0.06 z	1.78 ± 0.06 z	1.73 ± 0.03 zy	0.014
Liposomatic index	3.15 ± 0.20 zy	3.43 ± 0.21 zy	3.95 ± 0.20* z	4.38 ± 0.20* z	3.42 ± 0.20 zy	2.96 ± 0.20 y	<0.001
Fillet color							
Lightness parameter L* (100 = white, 0 = black)	56.9 ± 1.3	56.8 ± 1.3	56.7 ± 1.3	57.3 ± 1.3	54.9 ± 1.3	56.2 ± 1.3	0.514
Red–green parameter a* (green: a* < 0; red: a* > 0)	1.8 ± 0.5	1.4 ± 0.5	2.3 ± 0.5	1.6 ± 0.5	1.8 ± 0.5	1.4 ± 0.5	0.488
Yellow–blue parameter b* (blue: b* < 0; yellow: b* > 0)	10.5 ± 0.8	11.6 ± 0.8	12.3 ± 0.8	10.7 ± 0.8	11.3 ± 0.8	11.7 ± 0.8	0.320
Color distance (ΔE)	0.0	1.2	1.9	0.5	2.2	1.4	—

All five tanks of fish that were fed the 10% FM–21% CGM feed and one tank that received the 10% FM–36% GDDY feed were noted as having distinctly yellow skin pigmentation relative to fish in the other dietary treatments, which were described as having bluish-silver pigmentation. However, fillet color analysis indicated no significant difference among the dietary treatments in terms of L* (mean ± SE = 56.4 ± 0.3), a* (1.7 ± 0.1), or b* (11.3 ± 0.2), and all ΔE values were less than the threshold of 2.3 (Table 3).

As indicated above, the overall frequency of incorrect portion identifications (64% of triangle tests) was significantly greater than the frequency of correct identifications (36%), but there was no difference in identification accuracy among the dietary treatments (Table 4). Overall, there was no difference between the frequency of 30% FM control portions being preferred (44% of triangle tests) and the frequency of experimental portions being preferred (56%); however, treatment groups varied significantly in terms of fillet portion preference. Specifically, the 30% FM control portion was more frequently preferred in taste tests involving 10% FM–36% GDDY portions (73% of triangle tests) and was less frequently preferred in tests involving the 10% FM–21% PBM portions (14%), with the other treatments yielding intermediate preference frequencies.

DISCUSSION

The results of this study are broadly similar to previous reports of hybrid Striped Bass reared on reduced-FM feeds (Table 5) and suggest that PBM, CGM, DDGS, and SBM can be used to spare a significant amount of menhaden FM without affecting growth. The 10% FM–21% PBM feed did yield significantly higher weight gain than the 10% FM–21% CGM and 10% FM–33% DDGS feeds, but performance was only marginally higher than that associated with the 10% FM–46% SBM and 30% FM feeds. Previous research on sparing and replacement of FM with CGM (Lewis and Kohler 2008), PBM (Webster et al. 2000; Pine et al. 2008; Rawles et al. 2009; Trushenski

TABLE 4. Results of triangle taste tests comparing fillets of hybrid Striped Bass reared on feeds containing various alternative protein sources (experimental portions; GDDY = grain distillers' dried yeast; CGM = corn gluten meal; DDGS = distillers' dried grains with solubles; PBM = poultry byproduct meal; SBM = soybean meal) with the fillets of fish that received feed containing a typical level of menhaden fish meal (FM, 30%; control portions). Values are expressed as a percentage of the total number of triangle tests. Values with a letter in common are not significantly different ($P > 0.05$). *P*-values associated with chi-square tests for equal proportions (overall percentages of correct versus incorrect identifications or preferences for control versus experimental portions) are also provided.

Variable	10% FM–36% GDDY	10% FM–21% CGM	10% FM–33% DDGS	10% FM–21% PBM	10% FM–46% SBM	Overall percentage	*P*
Percentage of triangle tests in which the unique fillet portion was correctly identified	40	27	21	50	40		0.514
Percentage of correct identifications						36 y	
Percentage of incorrect identifications						64 z	0.014
Percentage of triangle tests in which a 30% FM control portion was preferred over an experimental portion	73 z	53 zy	29 yx	14 x	33 zyx		0.028
Percentage with preference for control portion						44	
Percentage with preference for experimental portion						56	0.332

and Kohler 2011), DDGS (Webster et al. 1999), and SBM (Gallagher 1994; Webster et al. 1999; Laporte and Trushenski 2012) also demonstrated that each of these feedstuffs is a suitable FM alternative in feeds for hybrid Striped Bass. Research with other alternatives (e.g., blood meal, meat-and-bone meal, and soy protein isolate) similarly demonstrated that some level of FM may be spared in hybrid Striped Bass feeds. For these and similar ingredients in hybrid Striped Bass feeds, the reported apparent digestibility coefficients for protein ($ADC_{protein}$) vary but tend to be lower than those reported for menhaden FM; $ADC_{protein}$ values (presented from lowest to highest) were 54% for brewers' yeast, 55–78% for PBM, 65% for DDGS, 79% for CGM, 80–86% for SBM, and 88–95% for menhaden FM (Sullivan and Reigh 1995; Gaylord et al. 2004; Thompson et al. 2008; Barrows et al. 2011). Nonetheless, SBM, CGM, PBM, and DDGS are relatively well utilized by hybrid Striped Bass and can be used to spare dietary FM in feeds for these fish without significantly compromising growth performance. Our data, in combination with the production performance results summarized from the available literature, also suggest that hybrid Striped Bass are quite amenable to FM sparing and accept a wide range of alternative proteins, with limited effects on growth and growth efficiency. Collectively, the results outlined in Table 5 indicate that in most cases, dietary FM content may be significantly reduced in hybrid Striped Bass feeds relative to what are considered typical inclusion rates at this time.

With the exception of the 10% FM–36% GDDY feed, deviations from intended dietary protein and lipid content were generally reflected in the observed production performance. Although the 10% FM–33% DDGS feed used in this study had a higher protein and lipid content than the control feed, the lower $ADC_{protein}$ for DDGS may explain why weight gain for that treatment was lower than the weight gain associated with the control feed. Despite having the lowest protein content, the 10% FM–21% CGM feed resulted in weight gain similar to that achieved with the control, 10% FM–33% DDGS, and 10% FM–46% SBM feeds, perhaps due to the comparatively high $ADC_{protein}$ for CGM. The high $ADC_{protein}$ of these feedstuffs when used in feeds with properly balanced amino acid profiles that meet known requirements may allow for additional FM sparing (i.e., FM levels lower than those used in the current study) or even complete replacement of FM. Laporte and Trushenski (2012) demonstrated that FM could be reduced to 5% in a SBM-based feed for hybrid Striped Bass, thus halving the amount of FM used in the current study. Pine et al. (2008) and Rawles et al. (2009) achieved complete replacement of FM with PBM in diets for hybrid Striped Bass. The current results show that FM can be spared by CGM at greater levels than previously demonstrated (Lewis and Kohler 2008). When combined with other alternative protein sources, DDGS could be used in a FM-free feed (Webster et al. 1999). These past studies as well as the current study demonstrate that even with some variation in protein and lipid content (i.e., as was observed here), if digestible protein and amino acid profiles are adequate, then there are numerous alternatives that can be used to spare significant levels of FM in diets for hybrid Striped Bass. The apparent limiting factor when sparing FM is the poor palatability of feeds, as was observed for the 10% FM–36% GDDY feed administered to hybrid Striped Bass in the current experiment.

Despite adequate digestible protein, a proper amino acid profile, and being overformulated for hybrid Striped Bass (i.e., higher in protein and lipid density than the other feeds used), the 10% FM–36% GDDY feed was associated with significant reductions in fish final weight and weight gain relative to the other feeds. The FCR for the 10% FM–36% GDDY feed in the current study was similar to that of the control treatment; therefore, palatability may be the causal factor associated with reduced performance in this treatment group. These observations are in contrast to previous results (Gause and Trushenski 2011a) demonstrating that in a GDDY-based feed, FM could be reduced to 7.5% without impairing growth of hybrid Striped Bass; in fact, slight improvements in weight gain and feed intake were reported relative to fish that were given a 30% FM control diet. The primary difference between the previous trial and the current trial is the method of feed manufacture. In the previous trial (Gause and Trushenski 2011a), the feeds were press-pelleted, whereas the current feeds were extruded. It is possible that the extrusion process caused some change in the 10% FM–36% GDDY formulation, rendering it less palatable to hybrid Striped Bass. It has been suggested that a reduced intake of extruded feeds is a function of longer gastric retention times (Hilton et al. 1981; Booth et al. 2002), resulting in a reduction in weight gain but an improvement in FCR. However, if this had been the case, we would expect the 10% FM–36% GDDY feed to be the best-performing feed given that it was the most nutrient dense and presumably had adequate digestible protein and a balanced amino acid profile. Further, any effects of extrusion on gastric retention time would presumably extend to the other formulations as well. As all of the feeds used in this study were manufactured at the same time by using the same methods and sources of bulk ingredients, it would seem that some organoleptic property of GDDY is responsible for the reduced intake of the extruded feed. The addition of a palatant could potentially increase intake (Papatryphon and Soares 2000); however, Gause and Trushenski (2011b) reported that the addition of soluble canola protein concentrate (as a palatant) to a GDDY-based feed failed to elicit an increase in feed consumption.

Dietary protein source did not have an apparent effect on fillet quality or consumer preference, as panelists were unable to correctly differentiate between the fillets of fish that received the control feed and the experimental feeds in the majority of cases. For those tests in which the unique portion was correctly identified, there was no significant effect of treatment, indicating that none of the diets yielded fillets that were more or less distinct from the fillets of fish fed the 30% FM feed (Table 3). Although the experimental treatments varied in terms of whether their fillets were preferred over the 30% FM control fillets, the apparent preference for fillets from the 10% FM–21% PBM, 10% FM–33% DDGS, or 10% FM–46% SBM treatment may not be particularly meaningful, as tasters were largely unable to identify the unique portions during the taste test. The apparent preference for 30% FM fillets over 10% FM–36% GDDY fillets may be similarly arbitrary. In other words, since panelists were unable to correctly differentiate between dietary treatments, it seems unlikely that their overall preferences were based on treatment-related differences between portions. It is important to note that our panelists were not trained prior to participating in the taste test. Sensory analysis of seafood may involve rigorously trained panelists to objectively describe product attributes or may involve untrained panelists providing subjective feedback (Hyldig 2010). Although discriminative tests like triangle tests are generally considered objective, they do not always involve trained panelists. Panelists with special training to perceive and articulate differences in seafood products might have been able to discriminate between fillets representing the feeds evaluated in this experiment. However, in a similar study involving culinary students, chef instructors, and other culinary professionals with sensitive, trained palates (Smith et al., in press), these panelists were generally unable to differentiate between hybrid Striped Bass raised on grow-out diets (similar to those used in the present work) containing FM/fish oil or SBM/soybean oil as primary ingredients. Regardless of whether trained panelists are able to differentiate between hybrid Striped Bass reared on different diets, it seems unlikely that the average consumer would be able to detect differences among fish that have been fed the diets assessed in the present work.

The lack of a detectable difference in taste among the treatments suggests that replacing FM with the tested alternatives is unlikely to affect the taste of farmed hybrid Striped Bass. However, some FM alternatives (e.g., CGM) contain significant levels of the yellow carotenoid pigments lutein and zeaxanthin (Park et al. 1997), which may alter fillet color and product acceptability. Changes in fillet color have been reported in previous FM sparing studies with Rainbow Trout (Peterson et al. 1966; Skonberg et al. 1998; D'Souza et al. 2006) and in a study of Channel Catfish *Ictalurus punctatus* that were fed diets with higher levels of carotenoids (Li et al. 2007). Specifically, as FM was spared with plant-based alternatives, increasing whiteness (L*) and decreasing redness (a*) in Rainbow Trout fillets were observed (Skonberg et al. 1998; D'Souza 2006). Channel Catfish that were fed diets supplemented with various carotenoids exhibited a significant increase in the yellowness (b*) of the fillets (Li et al. 2007). In either case, deviations from what is considered a "normal" fillet color can negatively affect consumer perception and product acceptability (Skonberg et al. 1998). None of the diets we evaluated had a significant effect on fillet color, although we observed a numeric increase in skinless fillet yellowness among fish that received the 10% FM–21% CGM diet and the skin of these fish was noticeably more yellow in color. Brook Trout *Salvelinus fontinalis* that were given diets containing carotenoid-rich paprika extracts exhibited similar changes in skin pigmentation (Peterson et al. 1966). Trushenski et al. (2010) reported that the use of corn oil-based feeds (containing carotenoids) in lieu of fish oil-based feeds did not predictably alter the color of hybrid

TABLE 5. Summary of research on fish meal (FM) replacement in feeds for hybrid Striped Bass. All studies involved sunshine bass (female White Bass × male Striped Bass) except Gallagher (1994) and Gallagher and LaDouceur (1995), which involved palmetto bass (female Striped Bass × male White Bass). Weight gain, feed conversion ratio (FCR), and feed intake of fish that were fed reduced-FM experimental feeds are reported qualitatively in relation to those of fish that were given FM-based control feeds (NR = not reported).

Protein, lipid content (%); control	Alternative protein	FM content (%)	Weight gain (%)	FCR	Feed intake	Reference
44, 10; 16% FM	Meat-and-bone meal	15.01	↑	=	=	Bharadwaj et al. 2002
		12.96	↑	↓	=	
		10.92	↑	=	=	
		8.88	↑	↓	↑	
		6.84	↑	=	=	
		4.79	↑	=	=	
		2.75	↑	=	=	
39, 12; 10% FM	Soy protein concentrate	6.70	=	↓	↓	Blaufuss and Trushenski 2012
		3.30	=	↓	↓	
		0.00	↓	=	↓	
40, 12; 10% FM	Soy protein isolate	7.50	=	=	=	Blaufuss and Trushenski 2012
		5.00	=	=	=	
		2.50	=	=	=	
		0.00	=	=	=	
41, 14; 24% FM	Corn gluten meal	20.00	=	=	=	Lewis and Kohler 2008
		16.00	↓	=	=	
		12.00	↓	=	=	
		8.00	↓	↑	=	
		4.00	↓	↑	↓	
		0.00	↓	↑	↓	
		10.00	=	=	=	
		0.00	=	=	=	
40, 10; 30% FM	Turkey meal	20.00	=	=	=	Muzinic et al. 2006
		10.00	=	=	=	
		0.00	=	=	=	
37, 11; 30% FM	Poultry byproduct meal	20.00	=	=	=	Pine et al. 2008
		10.00	=	=	=	
		0.00	=	=	=	
45, 12; 25% FM	Poultry byproduct meal	16.20	=	=	=	Rawles et al. 2009
		7.50	=	=	=	
		0.00	=	=	=	
40, 6; 30% FM	Meat-and-bone meal	0.00	↑	=	=	Webster et al. 2000
40, 6; 30% FM	Meat-and-bone meal, hempseed meal	0.00	=	=	=	Webster et al. 2000
40, 6; 30% FM	Poultry byproduct meal	0.00	=	↑	=	Webster et al. 2000
40, 6; 30% FM	Meat-and-bone meal, canola meal	0.00	=	↑	=	Webster et al. 2000
40, 15; 30% FM	Grain distillers' dried yeast	22.50	=	=	=	Gause and Trushenski 2011
		15.00	↑	=	=	
		7.50	=	=	=	
		0.00	↓	↑	↓	
41, 14; 20% FM	Poultry byproduct meal	10.00	=	=	=	Trushenski and Kohler 2011
		0.00	↓	=	=	

TABLE 5. Continued.

Protein, lipid content (%); control	Alternative protein	FM content (%)	Weight gain (%)	FCR	Feed intake	Reference
40, 14; 30% FM	Soybean meal	20.00	=	=	=	Laporte and Trushenski 2012
		15.00	=	=	=	
		10.00	=	=	=	
		5.00	=	=	=	
		0.00	↓	↑	=	
38, 10; 30% FM	Soybean meal	15.00	=	=	NR	Webster et al. 1999
		7.50	↓	↑		
35, 10; 47% FM	Soybean meal	35.70	=	=	NR	Gallagher 1994
		23.00	=	=		
		16.50	=	=		
35, 10; 47% FM	Blood meal	42.00	=	=	NR	Gallagher and LaDouceur 1995
		35.50	=	=		
		24.50	↓	↑		
35, 10; 47% FM	Enzyme-hydrolyzed poultry product	35.20	=	=	NR	Gallagher and LaDouceur 1995
		23.50	=	=		
		11.70	=	=		
35, 10; 47% FM	Low-ash poultry product	35.20	=	=	NR	Gallagher and LaDouceur 1995
		23.50	=	=		
		11.70	=	=		

Striped Bass and did not affect the yellowness of skinless fillets at all. Joseph et al. (2009) also reported somewhat conflicting effects of dietary composition on hybrid Striped Bass fillet color; full replacement of FM with PBM yielded skinless fillets that were lighter in color but also more red and yellow. These results do not offer a consistent pattern of dietary influence on hybrid Striped Bass fillet color, but they do suggest that the effects, if present, are likely to be relatively minor and restricted to the skin. Moreover, when numeric or statistical differences in L*, a*, and b* values are recorded, it is important to interpret these in the context of what is readily perceptible to the human eye; numeric differences aside, our ΔE values suggest that the skinless fillet colors we observed did not reach this threshold. Further research may be necessary to confirm this and to determine whether there is a threshold for carotenoid concentration in feeds that would result in an undesirable color change in the fillets. Given that the market for seafood may be largely driven by price (Wessells et al. 1994; Tveteråas et al. 2012) and the effects of FM sparing on hybrid Striped Bass fillet quality and acceptance were inconsequential, the sparing of costly FM with the alternative proteins we assessed would seem beneficial.

With the exception of GDDY, each of the alternatives used in the present study appeared to be suitable replacements for FM. Grain distillers' dried yeast may still prove a viable alternative if (1) it is fed at a lower percentage of the diet and used together with other alternatives or (2) a palatant is added to increase acceptance. Although replacement of FM with PBM resulted in marginally improved production, CGM, DDGS, and SBM also resulted in production performance similar to that obtained from the control diet, suggesting that these alternatives may also prove suitable. The availability of a range of sustainable, lower-cost FM substitutes will continue to allow the aquaculture industry to explore options for lowering feed costs and increasing sustainability. Furthermore, the results of this study and other trials (Table 5) suggest that the FM content in feeds for hybrid Striped Bass may be significantly reduced without negative effects on production performance. However, life stage and rearing conditions and their influences on nutritional demands should be taken into consideration when applying these results to feeds and feeding strategies for hybrid Striped Bass.

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Controlling Mortality Caused by External Columnaris in Largemouth Bass and Bluegill with Chloramine-T or Hydrogen Peroxide

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ARTICLE

Controlling Mortality Caused by External Columnaris in Largemouth Bass and Bluegill with Chloramine-T or Hydrogen Peroxide

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Abstract

Columnaris (causative agent, *Flavobacterium columnare*) is a widespread fish disease of concern among fish culturists in the USA. If left untreated, an entire population of fish may become infected, and morbidity and mortality may reach high levels. In virtually all instances, columnaris outbreaks require intervention to prevent significant losses. A number of sanitizing agents, most notably chloramine-T (CLT) and hydrogen peroxide (HP), have been used to control mortality associated with a variety of bacterial pathogens causing external infections. However, the majority of trials conducted to demonstrate the effectiveness of these chemicals, thereby gaining U.S. Food and Drug Administration approval for their use in treating fish infected with columnaris, have been conducted on salmonids. Accordingly, we conducted seven experiments to evaluate the effectiveness of CLT or HP to control mortality associated with external columnaris in Florida Largemouth Bass *Micropterus salmoides floridanus* and Bluegill *Lepomis macrochirus*. Treatment with CLT or HP significantly reduced cumulative mortality in five of the seven experiments. Cumulative mortality was strongly correlated to pretreatment mortality in treated and control tanks in the five Largemouth Bass experiments, suggesting that intervention at later stages of columnaris progression may result in less favorable outcomes. Odds ratios calculated for individual experiments indicated varying degrees of success in controlling mortality; however, meta-analysis of all experiments indicated treatment with either CLT or HP significantly increased probability of survival, regardless of fish species or test article. These results demonstrate that both chemicals can be effective in controlling mortality associated with external columnaris in Largemouth Bass and Bluegills and that timely treatment of fish will likely result in lower overall mortality.

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Columnaris (causative agent, *Flavobacterium columnare*; formerly *Flexibacter columnaris*) is an acute-to-chronic external or systemic bacterial disease affecting freshwater-reared finfish worldwide (Bullock et al. 1986). Infections begin when *F. columnare* invades epithelial tissues, most commonly affecting the gills and buccal, opercular, dorsal, and caudal surfaces (Post 1987). Lesions form as the infection progresses and are often observed at the base of the dorsal fin or on the caudal fin, lending columnaris its colloquial names, "saddleback" and "fin rot." If left untreated, external lesions may penetrate blood vessels or the body cavity, leading to systemic infections.

Columnaris can be presumptively diagnosed based on clinical signs, including the presence of the aforementioned lesions and long, slender, possibly filamentous, rod-shaped, Gram-negative bacteria (Post 1987) exhibiting the characteristic "haystack" formation and "flexing" behavior of *F. columnare* (Noga 2000). In virtually all instances, columnaris outbreaks require intervention to prevent significant losses. Shedding of bacteria from the epithelial surfaces of infected fish may create a self-perpetuating, population-wide infection (Post 1987). If left untreated, mortalities in overcrowded or unsanitary conditions may reach 70% or higher among young and most susceptible fishes (Post 1987). During outbreaks, fish culturists can often minimize mortality by improving environmental rearing conditions, administering chemotherapeutic bath treatments, or both.

A number of external sanitizing agents, including chloramine-T (CLT) and hydrogen peroxide (HP), have been used to control mortality caused by *Flavobacterium* in a variety of freshwater finfish, including *F. branchiophila* (a causative agent of bacterial gill disease [BGD]) and *F. columnare*. Chloramine-T ($C_7H_7ClNNaO_2S{\cdot}3H_2O$) is a biocide used worldwide as a disinfectant and antiseptic. Although CLT has been used for years under authorization of publically held Investigational New Animal Drug (INAD) exemptions and has been shown to effectively control mortality associated with BGD in freshwater-reared salmonids (From 1980; Speare and Ferguson 1989; Bullock et al. 1991; Thorburn and Moccia 1993; Ostland et al. 1995; Bowker and Erdahl 1998; Bowker et al. 2008) and columnaris in a variety of freshwater finfish, it is not yet approved by the U.S. Food and Drug Administration (FDA) for use on fish.

Hydrogen peroxide (H_2O_2) is used as a bleaching agent in the textile industry, as an antimicrobial agent in cheese production and treatment of drinking water (Marking et al. 1994), and in human healthcare as a first aid antiseptic and oral debriding agent. It has also been used to treat a variety of external fungal, bacterial, and parasite infections and infestations 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; Buchmann and Kristensson 2003; Montgomery-Brock et al. 2001, 2004; Sitjà-Bobadilla et al. 2006; Russo et al. 2007; Bravo et al. 2010; Bowker et al. 2012a). The only product approved by the FDA for controlling mortality in fishes associated with *Flavobacterium* is 35% PEROX-AID (active ingredient is 35% HP; Eka Chemical, Marietta, Georgia). Specifically, it can be used to control mortality associated with BGD in freshwater-reared salmonids and external columnaris in coolwater finfish and channel catfish *Ictalurus punctatus* (USFDA 2012), although efficacy has been observed in controlling *Flavobacterium*-related mortality in other fishes (USFWS AADAP 2011).

Although field trials suggest that CLT and HP are effective in controlling *Flavobacterium*-related mortality in a variety of fish (Bonnie Johnson, USFWS, personal communication), relatively few rigorous, experimental trials have been conducted, and therefore the approved uses of these compounds are restricted to the claims previously described for HP. Furthermore, few studies have directly compared the suitability of CLT and HP in controlling external columnaris disease-related mortality. Therefore, we conducted a series of experiments in which Florida Largemouth Bass *Micropterus salmoides floridanus* and Bluegills *Lepomis macrochirus* diagnosed with external columnaris were treated with CLT or HP according to different treatment regimens.

METHODS

Test Facility, Test Fish, and Test Articles

Experiments were conducted over a 15-month period from July 2007 to September 2008 at the Florida Bass Conservation Center's Richloam Fish Hatchery, Webster, Florida. Test fish were Bluegills or Florida Largemouth Bass fingerlings collected from onsite reference populations presumptively diagnosed with external columnaris. Test articles were Chloramine-T (100% CLT; H&S Chemical, Covington, Kentucky) and 35% PEROX-AID. Fiberglass, rectangular test tanks (1.8 $\times$ 0.45 m, water depth = 0.45 m, rearing volume $\approx$ 382 L) supplied with degassed, aerated well water (same water source as in reference population tanks) were used for all experiments.

Experimental Design and Procedures

Completely randomized designs (Peterson 1985) were used to allocate fish from reference populations to test tanks and assign treatments to tanks. Each experiment comprised a 1-d pretreatment acclimation period, a treatment period of variable length depending on the drug and treatment regimen, and a 14-d posttreatment period. Static treatments were administered by turning off water to tanks, adding test article or sham water treatments, and mixing tank contents. Water flow was returned to the original rate after 60 min.

Before experiments started, 10 moribund fish were collected from the reference population and evaluated for fish health. The external and internal tissues of all fish examined appeared normal, with the exception of skin lesions characteristic of external columnaris, which were found on all fish. Presumptive diagnoses of columnaris were made via microscopic examination of wet-mount skin-scrapes prepared for each fish, which revealed motile bacteria morphologically similar to

F. columnare in all samples. Pretreatment mortality (i.e., mortality on the day before fish were allocated to test tanks) ranged from 0.1% to 4.7% of the reference population. Except where noted below, mean length of test fish was calculated by collecting and individually measuring 20 reference-population fish, whereas mean weight of test fish was based on sample-counting (Piper et al. 1982) three groups (50 fish/group) of reference population fish.

Experiment 1.—Florida Largemouth Bass were treated with 20 mg/L CLT for 60 min on three consecutive days. The reference population (pretreatment mortality = 0.20%) comprised approximately 21,630 bass fingerlings (mean weight = 4.7 g, mean length = 7.0 cm) held in a single concrete raceway supplied with water to achieve 1.0 exchanges/h. Twelve test tanks (six control tanks and six treated tanks) were stocked with fish from the reference population by weight to achieve target densities 314 fish/tank (3.9 g fish/L). However, at the end of the experiment, when all live fish remaining were hand-counted and mortalities were accounted for, we determined that initial stocking numbers had actually ranged from 292 to 316 fish/tank (3.7–4.0 g fish/L). Water flow to test tanks was 23.7 L/min; thus, water turnover rate was 3.8 exchanges/h. During the experiment, fish were fed a diet formulated for Florida Largemouth Bass at Richloam Fish Hatchery (based on typical trout/salmon feed) and prepared by Nelson and Sons, Inc. (Tooele, Utah) at a daily rate of 5% of estimated body weight delivered every 5 h by Louden-style pneumatic feeders. On day 12 of the posttreatment period, a total of eight moribund fish collected from control tanks were examined for external columnaris. There were no moribund fish in the treated tanks; hence, no treated fish were collected and examined.

Experiment 2.—Florida Largemouth Bass were treated with CLT at 20 mg/L of water for 60 min on three consecutive days. The reference population (pretreatment mortality = 0.08%) comprised approximately 98,800 bass fingerlings (13.6 g, 10.8 cm) held in a single concrete raceway supplied with water to achieve 1.0 exchanges/h. Eight test tanks (four control tanks and four treated tanks) were stocked with fish from the reference population at 800 fish/tank (36.5 g fish/L). Water flow to test tanks was 23.7 L/min, and the water turnover rate was 3.8 exchanges/h. Fish were fed as in experiment 1.

Experiment 3.—Bluegills were treated with CLT at 20 mg/L of water for 60 min on three alternate days. The reference population (pretreatment mortality = 4.40%) comprised approximately 2,400 Bluegill fingerlings (15.8 g, 8.8 cm) held in a single fiberglass rectangular tank supplied with water to achieve 0.9 exchanges/h. Eight test tanks (four control tanks and four treated tanks) were stocked with fish from the reference population at 200 fish/tank (7.6 g fish/L). Water inflow to test tanks was 37.9 L/min, and the water turnover rate was 6.0 exchanges/h. Fish were fed the same diet used in experiment 1, except the daily ration was 3% of estimated body weight. Three moribund fish were collected from each test tank on days 3–5 of the treatment period, and one to three moribund fish were collected from each test tank during the first six posttreatment days; all of these fish were examined for external columnaris.

Experiment 4.—Florida Largemouth Bass were treated with CLT at 20 mg/L of water for 60 min on three alternate days. The reference population (pretreatment mortality = 0.76%) comprised approximately 20,000 bass fingerlings (17.7 g, 12.5 cm) held in a single fiberglass tank supplied with water to achieve 1.1 exchanges/h. Eight test tanks (four control tanks and four treated tanks) were stocked with fish from the reference population at 275 fish/tank (12.2 g fish/L). Water flow to test tanks was 18.9 L/min, and the water turnover rate was 3.0 exchanges/h. Fish were fed in the same manner as described for experiment 1. Three moribund fish were collected from each test tank on days 2–5 of the treatment period, and three fish (most of which were moribund) were sampled from each test tank on posttreatment days 3–5; all of these fish were examined for external columnaris.

Experiment 5.—Florida Largemouth Bass were treated with HP at 150 mg/L of water for 60 min on three consecutive days. The reference population, test tank loading densities, and general experimental conditions were as described for experiment 2 because these two experiments were conducted concurrently.

Experiment 6.—Florida Largemouth Bass were treated with HP at 50 mg/L of water for 60 min on three alternate days. The reference population, test tanks loading densities, diet and feeding rate, and general experimental conditions were as described for experiment 4 because these two experiments were conducted concurrently.

Experiment 7.—Bluegills were treated with HP at 50 mg/L of water for 60 min on three alternate days. The reference population (pretreatment mortality = 3.65%) comprised approximately 1,700 Bluegill fingerlings (16.5 g, 10.8 cm) held in a single fiberglass tank supplied with water to achieve 0.7 exchanges/h. Twelve test tanks (six control tanks and six treated tanks) were stocked with fish from the reference population at 100 fish/tank (4.6 g fish/L). Water flow to test tanks was 11.4 L/min, and the water turnover rate was 1.8 exchanges/h. Twice daily, Bluegills were hand fed the same diet used in experiment 1 at a daily rate of 2% of estimated body weight. Three moribund fish were collected from each test tank on days 2–5 of the treatment period, and three fish were sampled from each test tank on posttreatment days 4–7; all of these fish were examined for external columnaris. Fish sampled from treated tanks appeared healthy, whereas eight of the fish sampled from controls tanks appeared healthy and the remaining 10 fish were moribund.

Data Collection

Mortalities were recorded daily. Fish collected and examined for external columnaris were examined grossly to assess external and internal organs: skin and gills were examined for presence of lesions and tissue necrosis, and wet mounts of skin scrapes and gill filaments were examined microscopically for the presence of motile bacteria presumptively identified as *F. columnare*.

TABLE 1. Mean (ranges in parentheses) water quality metrics and analytically verified concentrations of drugs, as measured during experiments conducted to evaluate the effectiveness of chloramine-T or hydrogen peroxide to control mortality associated with columnaris in Largemouth Bass and Bluegills.

Experiment	Water temperature (°C)	Dissolved oxygen (mg/L)	Alkalinity (mg/L)	Hardness (mg/L)	pH	Verified concentration (mg/L)	Deviation from target dose (%)
1	25.2 (24.1–26.4)	15.8 (10.7–17.7)	245	373	8.1	19.5 (16.7–21.6)	2.5
2	24.8 (24.1–25.4)	15.2 (10.2–19.2)	264	381	7.9	20.5 (18.5–22.9)	2.5
3	22.2 (19.0–24.8)	11.9 (8.5–16.3)	317	337	7.6	22.1 (21.3–23.6)	10.5
4	24.8 (22.5–27.2)	13.3 (8.8–17.6)	320	345	7.6	20.5 (20.0–21.1)	2.5
5	24.8 (24.1–25.4)	15.2 (10.2–19.2)	364	382	7.9	153.8 (136.0–165.8)	2.5
6	24.8 (22.5–27.2)	13.9 (8.8–17.7)	320	350	7.6	50.3 (46.8–53.1)	0.6
7	24.9 (23.8–26.6)	15.2 (10.6–18.1)	345	370	7.6	48.6 (46.3–51.4)	2.8

General fish behavior was assessed daily and characterized as normal or abnormal for each tank, and any abnormal behaviors (e.g., lethargy, hyperactivity, flashing, piping at the surface) were described. Feeding behavior was also assessed daily and characterized as "2 = aggressive" (fish appeared to be actively feeding and that nearly all feed offered was consumed), "1 = semiaggressive" (some fish appeared to be feeding actively but some fish did not appear to be feeding), or "0 = nonaggressive" (none of the fish appeared to be feeding).

Water temperature and dissolved oxygen (DO) concentration were measured at least once daily during each experiment, whereas water hardness, alkalinity, and pH were measured at the beginning and end of each experiment from water samples collected from one randomly selected test tank. Water temperature and DO concentration were measured with an OxyGuard Handy Polaris Portable DO Meter (OxyGuard International A/S, Birkerød, Denmark). Water hardness and alkalinity were measured with a Hach titrator and reagents (Hach Co., Loveland, Colorado), and pH was measured with a Hach sensION1 portable pH meter. All water quality parameters measured were within ranges considered adequate for rearing Largemouth Bass and Bluegill (Table 1).

Water samples were collected from each test tank during each treatment period and analyzed to verify CLT or HP dose. The titrimetric method described by Jeffery et al. (1989) was used to determine HP concentrations. A Hach DR/890 Colorimeter was used to measure free and total chlorine, and these values were used to calculate CLT concentrations as described by Dawson et al. (2003).

Data Analysis

Mortality data analysis was done using the PROC GLIMMIX (logit link; Wolfinger and O'Connell 1993) function of the Statistical Analysis System software (version 9.2, SAS Institute, Cary, North Carolina), comparing treated fish to control fish on each day of the treatment and posttreatment periods.

Individual experiment odds ratios and associated asymmetric 95% confidence intervals (CIs) were calculated (PROC GLIMMIX); 95% CIs > 1 indicated that treatment significantly improved the probability of survival. An overall odds ratio and associated asymmetric 95% CI were calculated using the meta-analysis function of MedCalc software (version 12.3.0.0, MedCalc Software, Mariakerke, Belgium). The meta-analysis test for heterogeneity revealed significant variation among the experiments; thus, the random effects model (DerSimonian and Laird 1986) was used to generate a more conservative estimate of the overall odds ratio.

For analysis of individual experiments, tank was considered the experimental unit ($N = 4$ or 6, depending on the experiment); for the meta-analysis, experiments were considered the experimental unit. All hypotheses were tested at an $\alpha = 0.05$ (two-tailed tests).

Microsoft Excel software (Microsoft Corporation 2010), SYSTAT 12 software (SYSTAT 2007), and SigmaPlot 12 software (SYSTAT 2011) were used to generate summary statistics (e.g., mean, standard deviation, range) for water temperature, dissolved oxygen concentration, other water chemistry measurements, and mean daily mortality. Linear regressions (SYSTAT 2011) were used to determine if there was a relation between pretreatment mortality of Florida Largemouth Bass in the reference population and the cumulative mortality among (1) treated fish or (2) control fish. For each regression, pretreatment mortality was the independent variable, and cumulative mortality was the dependent variable.

RESULTS

A significant difference was detected in mortality between treated and control groups at some point during each experiment. However, at the end of the posttreatment periods, cumulative mortality of treated fish was significantly less than that of control fish in five of the seven experiments (experiments 1, 3, 4, 6, and 7). Odds ratios calculated for individual experiments indicated significantly greater probabilities of survival among treated fish compared with control fish in the same five experiments for which significant differences in cumulative mortality were observed (range = 1.25–3.03). However, the overall odds ratio resulting from the meta-analysis was 1.83 (95% CI = 1.43–2.35), indicating a significant beneficial effect of treatment, regardless of fish species or test article. Cumulative mortality was strongly and positively related to pretreatment mortality in treated ($r^2 = 0.98$) and control ($r^2 = 0.96$) tanks in the five Florida Largemouth Bass experiments. In both treated

and control groups, higher mortality rates in the reference population before the start of the experiments were associated with higher cumulative mortality rates.

Dose verification levels were within ±20% of the target dose in each experiment (Table 1), and no CLT or HP was detected in samples collected from control tanks. Mean water temperature for each experiment ranged from 22.2°C to 25.2°C, and individual measurements ranged from 19.0°C to 27.2°C (Table 1). Mean DO concentrations for each experiment ranged from 11.9 to 15.8 mg/L, and individual measurements ranged from 8.5 to 19.2 mg/L. Except where noted below, fish behavior was considered normal in all test tanks during each experiment, and fish fed at least semiaggressively on the first day of the experiment and aggressively thereafter.

Experiment 1

At the end of this experiment, mean cumulative mortality of Florida Largemouth Bass treated with CLT was 26.8% (SD = 5.4, range = 20.5–34.0%), which was significantly ($P = 0.0100$) less than the mean of 35.3% for control fish (SD = 3.4, range = 32.8–41.9%). A significant difference between groups was evident after experiment day 2 (Figure 1). The odds ratio for this experiment was 1.49 (95% CI, 1.12–1.97). Active *F. columnare* were observed on all skin scrapes taken from fish sampled from control tanks during the posttreatment period.

Experiment 2

At the end of this experiment, mean cumulative mortality of Florida Largemouth Bass treated with CLT was 17.9% (SD = 6.6, range = 13.0–27.6%), which was not significantly ($P = 0.4842$) different from the control fish mean of 21.9% (SD = 8.9, range = 15.8–33.9%), However, significant differences were detected on experiment days 3–12 (Figure 2). The odds ratio for this experiment was 1.29 (95% CI, 0.56–2.94). General fish behavior was characterized as abnormal (hyperactive) in seven of eight tanks during the treatment period but normal in all tanks during the posttreatment period.

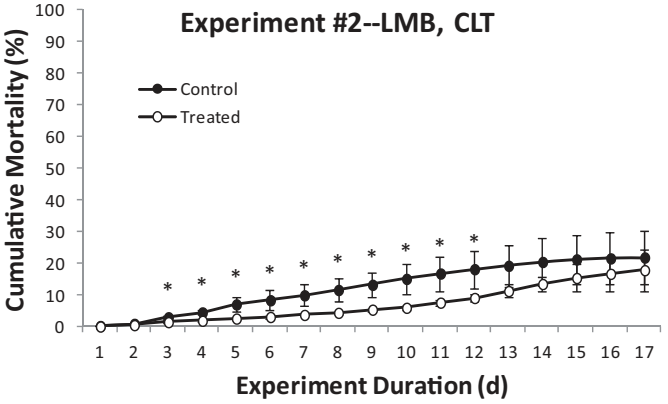

FIGURE 2. Mortality of Florida Largemouth Bass (LMB) observed in experiment 2. Treated fish were exposed to chloramine-T (CLT) at 20 mg/L of water for 60 min/d on three consecutive days, whereas control fish received sham treatments (four replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was not significantly ($P = 0.4842$) different from that of control fish.

Experiment 3

At the end of this experiment, mean cumulative mortality of Bluegills treated with CLT was 12.9% (SD = 2.3, range = 9.8–15.5%), which was significantly ($P = 0.0304$) less than the control fish mean of 26.9% (SD = 10.3, range = 18.7–41.5%). In addition, a significant difference in mortality was detected between treated and control groups from day 10 through the end of the experiment (Figure 3). The odds ratio for this

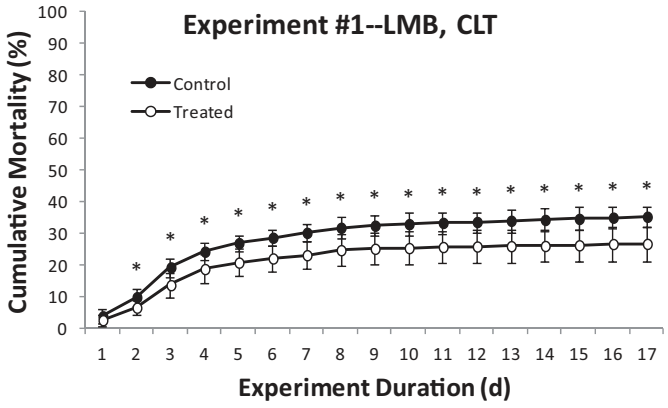

FIGURE 1. Mortality of Florida Largemouth Bass (LMB) observed in experiment 1. Treated fish were exposed to chloramine-T (CLT) at 20 mg/L of water for 60 min/d on 3 consecutive days, whereas control fish received sham treatments (six replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was significantly ($P = 0.0100$) less than that of control fish.

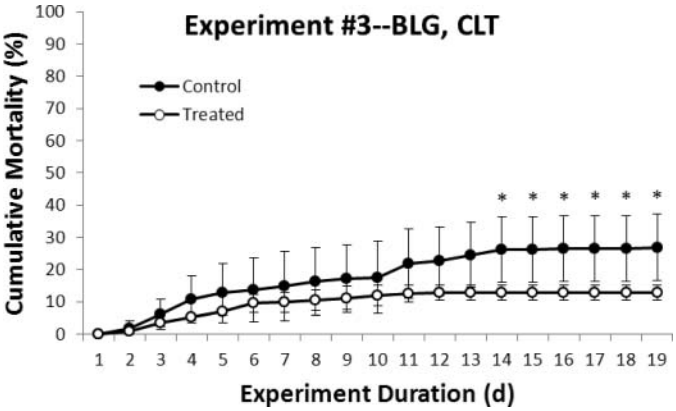

FIGURE 3. Mortality of Bluegills (BLG) observed in experiment 3. Treated fish were exposed to chloramine-T (CLT) at 20 mg/L of water for 60 min/d on three alternate days, whereas control fish received sham treatments (four replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was significantly ($P = 0.0304$) less than that of control fish.

experiment was 2.50 (95% CI, 1.13–5.54). Feeding behavior was characterized as nonaggressive during the treatment period and from nonaggressive to aggressive during the posttreatment period. Bacteria presumptively identified as *F. columnare* were observed on skin scrapes from 7 fish sampled from treated tanks and 11 fish sampled from control tanks during the treatment period. Bacteria presumptively identified as *F. columnare* were observed on skin scrapes from all fish sampled from control tanks but not on the samples collected from treated tanks during the posttreatment period.

Experiment 4

At the end of this experiment, mean cumulative mortality of Florida Largemouth Bass treated with CLT was 45.5% (SD = 6.3, range = 41.0–54.9%), which was significantly (P = 0.0034) less than the control fish mean of 62.6% (SD = 2.9, range = 59.5–66.2%). In addition, a significant difference in mortality was detected between treated and control groups for the last 10 d of the experiment (Figure 4). The odds ratio for this experiment was 1.97 (95% CI, 1.38–2.80). Bacteria presumptively identified as *F. columnare* were observed on skin scrapes from most (20 of 24) fish sampled during the treatment period, and from all 12 fish sampled from control tanks and from 1 fish sampled from treated tanks during the posttreatment period.

Experiment 5

At the end of this experiment, mean cumulative mortality of Florida Largemouth Bass treated with HP was 17.6% (SD = 4.9, range = 12.1–22.8%), which was not significantly (P = 0.3779) different from the control fish mean of 21.1% (SD = 5.47, range = 13.3–22.8%). However, a significant difference in mortality was detected between treated and control groups on experiment days 9–13 (Figure 5). The odds ratio for this experiment was 1.25 (95% CI, 0.70–2.23).

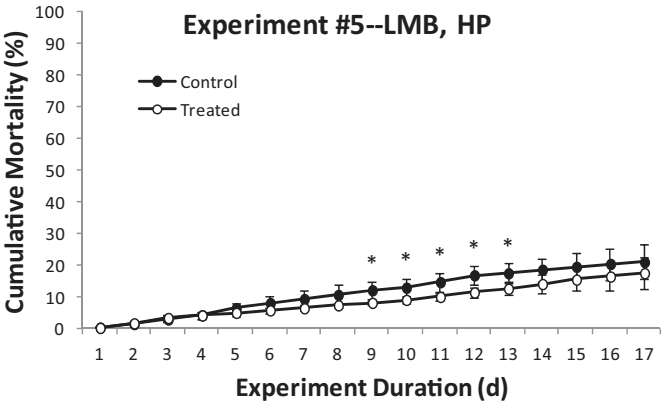

FIGURE 5. Mortality of Florida Largemouth Bass (LMB) observed in experiment 5. Treated fish were exposed to chloramine-T (CLT) at 150 mg/L of water for 60 min/d on three consecutive days, whereas control fish received sham treatments (four replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was not significantly (P = 0.3779) different from that of control fish.

Experiment 6

At the end of this experiment, cumulative mortality of Florida Largemouth Bass treated with HP was 49.0% (SD = 9.3, range = 36.8–57.4%), which was significantly (P = 0.0085) less than the control fish mean of 74.5% (SD = 8.3, range = 67.6–85.2%). In addition, a significant difference in mortality was detected between treated and control groups on day 3 through the end of the experiment (Figure 6). The odds ratio for this experiment was 3.03 (95% CI, 1.50–6.14). Virtually all fish sampled during the treatment period were presumptively

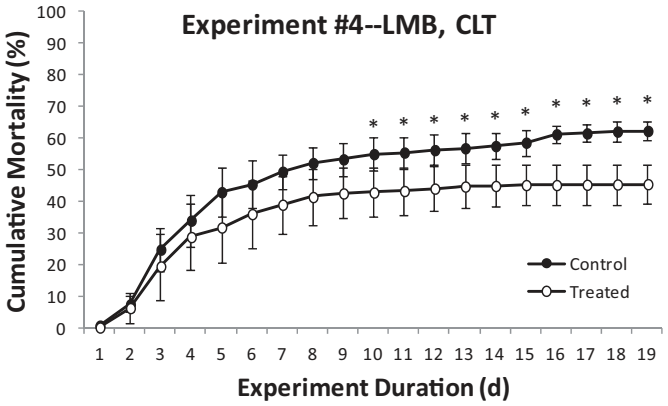

FIGURE 4. Mortality of Florida Largemouth Bass (LMB) observed in experiment 4. Treated fish were exposed to chloramine-T (CLT) at 20 mg/L of water for 60 min/d on three alternate days, whereas control fish received sham treatments (four replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was significantly (P = 0.0034) less than that of control fish.

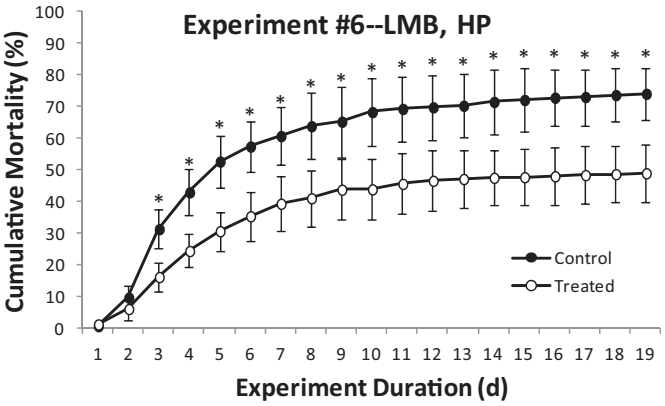

FIGURE 6. Mortality of Florida Largemouth Bass (LMB) observed in experiment 6. Treated fish were exposed to hydrogen peroxide (HP) at 50 mg/L of water for 60 min/d on three alternate days, whereas control fish received sham treatments (four replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was significantly (P = 0.0085) less than that of control fish.

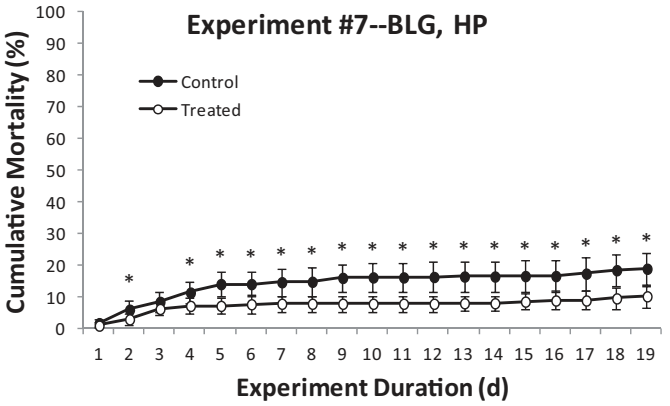

FIGURE 7. Mortality of Bluegills observed in experiment 7. Treated fish were exposed to hydrogen peroxide (HP) at 50 mg/L of water for 60 min/d on three alternate days, whereas control fish received sham treatments (six replicate tanks/treatment). Asterisks indicate days on which a significant difference ($P < 0.05$) was detected between treated and control groups; at the end of the trial, cumulative mortality of treated fish was significantly ($P = 0.0051$) less than that of control fish.

diagnosed with columnaris, and bacteria presumptively identified as *F. columnare* were observed on skin scrapes from 1 fish sampled from treated tanks and all 12 fish sampled from control tanks during the posttreatment period.

Experiment 7

At the end of this experiment, cumulative mortality of Bluegills treated with HP was 10.3% (SD = 3.4, range = 5.8–15.3%), which was significantly ($P = 0.0051$) less than the control fish mean of 19.0% (SD = 5.2, range = 13.4–24.6%). In addition, a significant difference in mortality was detected between treated and control groups on experiment day 2 and day 4 through the end of the experiment (Figure 7). The odds ratio for this experiment was 2.21 (95% CI, 1.37–3.59). Nearly all (34 of 36) fish sampled during the treatment period were presumptively diagnosed with external columnaris. During the posttreatment period, none of the fish sampled from the treated tanks exhibited clinical signs of columnaris; however, at least one fish sampled from each control tank was presumptively diagnosed with the disease.

DISCUSSION

Our results demonstrate that CLT and HP can be effective in controlling mortality associated with external columnaris disease in Florida Largemouth Bass and Bluegills. Treatments successfully reduced mortality associated with columnaris in five of seven experiments (experiments 1, 3, 4, 6, and 7). Although a significant reduction in mortality was detected at some point in the other two experiments (experiments 2 and 5, which were conducted concurrently), mortality did not vary significantly on the last 4–5 d of either experiment. However, the treatment protocols we employed are not completely consistent with the likely disease management approach used in a hatchery setting. The constraints of effectiveness-testing experiments (as generally accepted by FDA) limit treatment to a single treatment cycle; however, if reinfection was suspected, most fisheries professionals would have confirmed this suspicion and considered retreating the affected population. We speculate some degree of reinfection occurred in experiments 2 and 5 and contributed to the loss of statistical significance during the course of the experiments. If our experimental designs had allowed for treatments to be reapplied, it seems likely that the increases in mortality observed in experiments 2 and 5 could have been curbed, perhaps resulting in statistically significant differences between treated and control groups. Comparing our results to previous research is challenging because there is relatively little peer-reviewed information about the effectiveness of CLT or HP in controlling mortality associated with *Flavobacterium* infections in finfish other than salmonids (Speare and Arsenault 1997; Bowker and Erdahl 1998; Rach et al. 2000a). Nonetheless, our results are broadly consistent with other reports of using CLT and HP in the treatment of columnaris. For example, Rach et al. (2003) showed that survival of fingerling Walleyes *Sander vitreus* infected with columnaris was significantly improved when treated with HP at 50 mg/L of water for 60 min/d on three alternate days compared with untreated fish; however, exposure to higher concentrations of HP (75 or 100 mg/L) resulted in reduced survival. Similarly, Altinok (2004) reported higher survival in *F. columnare*-infected Goldfish *Carassius auratus* following a single 60-min treatment with CLT at 5, 10, 15, or 20 mg/L of water compared with untreated fish, but increasing the dose to 25 mg/L was less beneficial. Survival of fingerling Channel Catfish *Ictalurus punctatus* with columnaris was significantly improved after being treated with HP at 50, 75, or 100 mg/L of water for 60 min/d on three alternate days, but these fish did not exhibit sensitivity to the higher doses (Rach et al. 2003). Results from these studies suggest varying sensitivity to CLT and HP and a "Goldilocks paradigm" (i.e., CLT and HP doses that are too low or too high may yield less desirable results than one that is just right for the species or lifestage; Gaikowski et al. 1999; Russo et al. 2007; Gaikowski et al. 2008; Bowker et al. 2011).

Excluding circumstances when treatment concentrations exceed a fish's tolerance threshold, available data suggest that both CLT and HP are effective in controlling or reducing mortality associated with columnaris. Results of the side-by-side experiments evaluating CLT and HP underscore this point. Experiments 2 (CLT) and 5 (HP) were conducted concurrently with fish from the same reference population of Florida Largemouth Bass, and the mortality patterns (Figure 8) and odds ratios observed in these experiments were nearly identical. Experiments 3 (CLT) and 7 (HP) were not conducted concurrently, but were conducted with Bluegills from reference populations with similar pretreatment mortality; mortality and odds ratios (Figure 9) reported for these experiments were also similar. Chloramine-T and HP are characterized as microbicides with broad-spectrum antiseptic properties (McDonnell and Russell 1999); thus, it is perhaps not surprising they yield similar results when used to

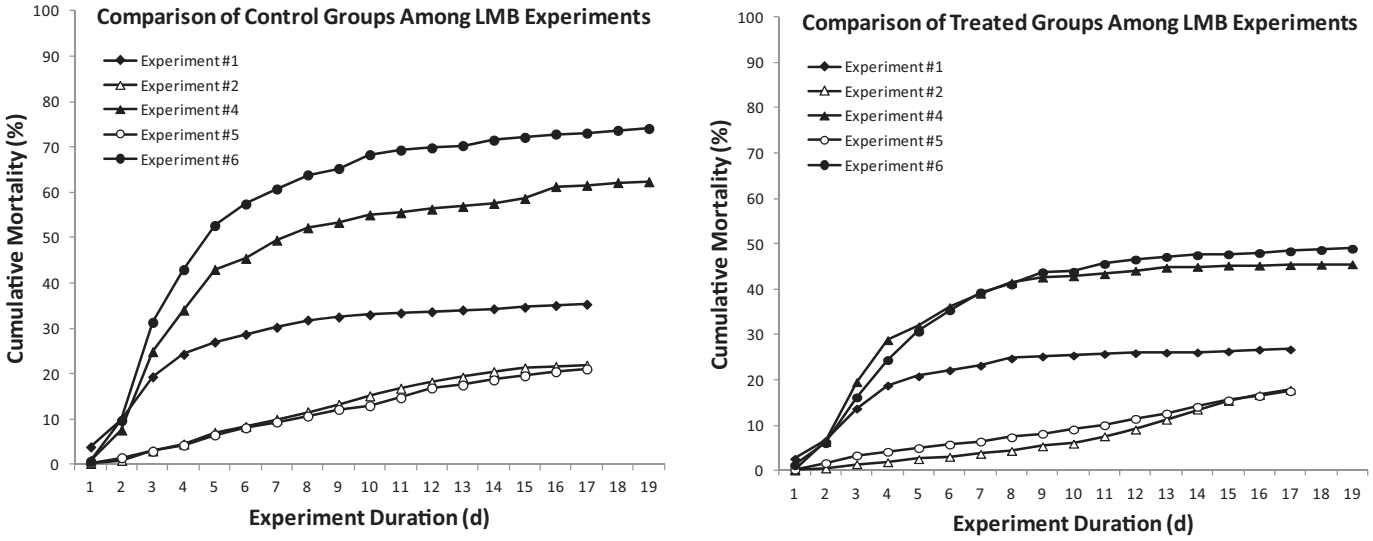

FIGURE 8. Mortality curves for treated and untreated groups of Florida Largemouth Bass (LMB) in experiments 1, 2, 4, 5, and 6.

treat external infections such as columnaris. Both compounds act via nonselective, oxidative processes that damage peptide linkages, proteins, and other cellular constituents and disrupt essential cellular processes (Maris 1995). Microorganisms, such as bacteria, are especially susceptible to these oxidative processes, which kill the organism quickly, often by disrupting their cellular membrane (Maris 1995). Because of the nonspecificity of this mode of action, microorganisms do not develop resistance to CLT or HP as can occur with antibiotics with specific cellular targets (Masten and Haneke 2002). However, chemicals such as CLT and HP provide virtually no prolonged therapeutic effect after treatment has been terminated and the chemical has been flushed from the rearing system

Our side-by-side comparison experiments indicate that CLT and HP are equally effective in controlling or reducing mortality associated with columnaris in Florida Largemouth Bass and Bluegills, which is also supported by the results of the meta-analysis. However, the significance and magnitude of mortality reduction varied considerably among our experiments. In experiments 1, 2, 5, and 7 the difference in mortality between treated and control groups was less than 10%, whereas in experiment 6, mortality was reduced by more than 25%. Many factors can affect the outcome of experiments conducted to evaluate the effectiveness of a drug to control mortality caused by an infectious fish pathogen. First, the effectiveness of a waterborne treatment is determined by mode of action and may be

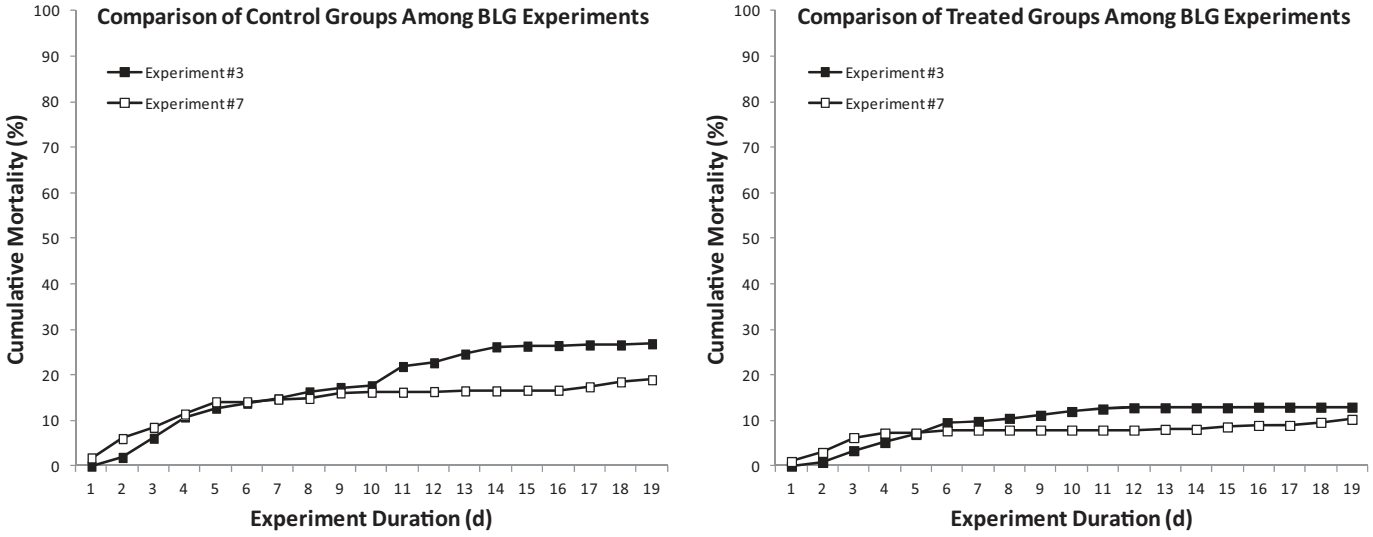

FIGURE 9. Mortality curves for treated and untreated groups of Bluegills (BLG) in experiments 3 and 7.

influenced by water chemistry and other environmental variables. For example, Thomas-Jinu and Goodwin (2004) observed copper sulfate, pyrazinedium dibromide (diquat), potassium permanganate, CLT, and HP, to vary in their ability to control mortality of channel catfish infected with different strains of *F. columnare*; pyrazinedium bromide was extremely effective, copper sulfate was not, and the various oxidizers yielded intermediate results. These authors also emphasized that the effectiveness of oxidizers could be strongly influenced by the oxidizable organic load in the system, which could neutralize a significant amount of the antiseptic, effectively reducing the treatment dose. The effectiveness of a drug, particularly an oral antibiotic, may also be affected by water temperature, especially if the temperature is on the lower or upper end of the fish's optimal thermal range. Second, as results from our Largemouth Bass experiments show, the extent of a disease's progression before intervention (i.e., pretreatment mortality) has a substantial influence on cumulative mortality in both treated and control groups during an experiment: the lower the mortality at the start of the study, the lower the cumulative mortality in the treated and control tanks and the smaller the difference between the two. Clearly, water quality, pretreatment mortality, and disease progression should be taken into consideration when assessing the relative merits of waterborne treatment options.

Microbicidal treatment should not be considered the principal means of disease management. Although relatively inexpensive, such treatments add to overall production costs. Assuming a 3,780 L (1,000 gal) culture tank and current pricing for the test articles used in this study, treating with CLT (3 treatments, 20 mg/L) would cost US$4.53, and treating with HP (3 treatments, 50 mg/L) would cost $9.03. Rather, a comprehensive disease management plan should be based primarily on procedures to minimize the likelihood of a disease outbreak, with treatment protocols as a secondary approach should these prevention measures prove insufficient. Such strategies may include using groundwater sources to minimize introduction of bacteria and other disease vectors, disinfecting incoming surface water with ultraviolet light or ozone, avoiding or minimizing the effects of chronic (e.g., poor water quality, improper fish densities) and acute stressors (e.g, handling, transport), and administering effective vaccines (Bowker et al. 2012b). Improving culture conditions by increasing water flow is another strategy to consider; however, in our studies, increased water exchange rates in test tanks ($\geq$2.5 times that of reference population tanks) was insufficient to control mortality without some other intervention. Regardless of the specific strategies used, any disease management plan should include routine inspection of fish for signs of distressed or diseased fish and protocols to ensure corrective steps are taken at the first sign of disease. In the case of columnaris, if treatment is recommended, CLT and HP will probably yield similar results; however, it is also likely that either microbicide will be more effective in controlling mortality if administered during the early stages of disease progression. Furthermore, we speculate that other nonspecific oxidizing microbicides similar to CLT and HP would yield similar results in this context.

In conclusion, our results indicate that either CLT administered at 20 mg/L or HP at 50 mg/L for 60 min/d on three alternate or consecutive days can be effective in controlling mortality associated with external columnaris disease in Florida Largemouth Bass and Bluegills. Future research should focus on evaluating the efficacy of these two drugs for the control of external columnaris-induced mortality in other warmwater finfishes.

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Growth of Juvenile Scup Fed Two Commercial Diets

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COMMUNICATION

Growth of Juvenile Scup Fed Two Commercial Diets

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Abstract

Juvenile Scup *Stenotomus chrysops* 0.35–0.95 g were fed two different commercial diets for 9 weeks to determine growth rates. Diets were hand-distributed to four replicate groups of 45 fish, four times a day. Survival among all treatments was high (90.0–92.5%), and both diets supported good growth. After five weeks, Scup fed diet 1 were significantly greater in length and weight than fish fed diet 2. These differences remained significant throughout the rest of the study. Specific growth rate (SGR) values were 5.8%/d for fish fed diet 1 and 5.5%/d for diet 2, while feed conversion ratios (FCR) were 1.22 for diet 1 and 1.25 for diet 2. Relative growth rate (RGR) values were diet 1 = 3,689% and diet 2 = 3,077%, and daily weight gain (DWG) values were diet 1 = 0.38 g/d and diet 2 = 0.31 g/d. Scup fed diet 1 had significantly greater final live weight, total length, weight gain, SGR, RGR, and DWG measurements than did fish fed diet 2. Juvenile Scup exhibited high growth rates and low feed conversion ratios when fed the two commercial diets, identifying them as a strong candidate species for commercial aquaculture.

Scup *Stenotomus chrysops* is a temperate marine species belonging to the family Sparidae. They are an important food fish that inhabit coastal waters of the USA from Cape Cod, Massachusetts, to Cape Hatteras, North Carolina, although occasionally a few are caught north of Cape Cod (Bigelow and Schroeder 1953). Scup are managed by the Atlantic States Marine Fisheries Commission (ASMFC) and the Mid-Atlantic Fishery Management Council (MAFMC) under Amendment 8 to the Summer Flounder, Scup, and Black Sea Bass Fishery Management Plan (FMP).

Scup are commercially important, annual landings from 2003 to 2008 averaging 4,200 metric tons (Terceiro 2009) valued at $6,000,000 (NOAA 2013). This species is also highly prized by anglers, recreational catches averaging 1,913 metric tons during the same period (Terceiro 2009). Scup are highly valued by commercial and recreational fishermen for its taste.

Successful and economical culture of marine finfish requires an understanding of nutritional requirements to optimize growth, survival, and economic viability of an aquaculture venture.

The protein quality of Scup is superior to several other Southern New England marine fish species (Jhaveri et al. 1984), making it a nutritious and desirable food product. Scup is a low-fat source of protein, low in sodium, and high in niacin, phosphorus, vitamins B6, B12, and selenium (NOAA 2013).

Successful commercial aquaculture of other sparids has been occurring elsewhere, such as the Gilthead Bream *Sparus aurata* in the Mediterranean Sea and the Red Seabream *Pagrus major* in the Asia–Pacific region (Basurco et al. 2011). Sparids are now intensively reared in ponds, tanks, raceways, and cages, most of the current production coming from cages. In 2006, the Mediterranean production of Gilthead Bream was 107,620 metric tons, Greece being the leading producer; in the Asia–Pacific region 75,754 metric tons of Red Seabream were produced, Japan being the main producer (Basurco et al. 2011). Recently in the Mediterranean region, culture of other sparid species has been tested to assess commercial aquaculture potential of Dentex *Dentex dentex*, Red Porgy *Pagrus pagrus*, Twoband Bream *Diplodus vulgaris*, and some sparid hybrids. The successful rearing of other sparid species is evidence of the potential for Scup in commercial culture. Rapid growth rates exhibited by Scup under culture conditions suggest that they are a promising species for aquaculture, in either land-based systems or in cage culture (Perry et al. 2009). Scup are hardy, readily accept commercial diets, tolerate handling, and exhibit high survival in holding tanks.

A feeding study involving Scup was conducted at the National Marine Fisheries Service (NMFS) laboratory in Milford, Connecticut, during the summer of 2008 to evaluate the

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aquaculture potential of Scup and to measure growth of juvenile Scup fed two commercial diets containing different levels of protein and lipid. These data complement results from a previous study (Perry et al. 2009) that compared the growth of juvenile Scup fed three commercial diets. The commercial diet that provided the greatest growth in that experiment was used in our study, along with a new commercial diet, to provide further information relating to the growth of juvenile Scup under culture conditions.

METHODS

Fish collection and experimental design.—During mid-July 2008, approximately 600 age-0 Scup were collected in the Atlantic Ocean off Milford, Connecticut, with an otter trawl containing a fine-mesh (1.61 cm square) liner in the net. Fish were transported to the Milford Laboratory in coolers with aeration. At the laboratory, fish were weighed, measured, and sorted by size. We indiscriminately selected 45 fish (0.35–0.95 g) for stocking into 250-μm-mesh baskets (61 × 25.4 × 20.3 cm), and one basket was placed into eight separate 76-L glass aquaria.

In this experiment we compared two diets with four replicate aquaria per treatment. Aquaria were placed into one large water bath black tank (5.9 × 1.2 × 0.5 m). Adjacent aquaria received different feed types. Aeration was provided to each aquarium using air stones. Light intensity was 1,000 lx at the water surface and photoperiod was held constant at 9 h light: 15 h dark for the duration of the experiment. Seawater at ambient temperature was delivered through a 5-μm sand filter to each aquarium from one seawater manifold line. Flow rate was maintained at 1.5 L/min per replicate during the study. Measurements of dissolved oxygen (DO) concentration, percent oxygen saturation, temperature, and salinity were taken daily in each aquarium (YSI model 85, Yellow Springs Instruments, Inc., Yellow Springs, Ohio).

Growth, both total length (TL; mm) and wet weight (g) of all fish was measured weekly. All mesh baskets, aquaria, and the large black tank were cleaned at that time.

Commercial diets.—Scup were hand-fed four times daily one of the two commercial diets (separate manufacturers) at an initial ration of 12% body weight daily. Manufacturers' analysis of the feeds was 55.5–58.5% protein and 13.4–14.5% lipid for diet 1, and 59% protein and 16% lipid for diet 2. All pellet sizes of the diets were commercially produced. Nutritional composition of each diet is listed in Table 1. The feeding regime for both diets fed to the juvenile Scup was increased over time: week 1 = 200–400-μm pellets, week 2 = 400–600-μm pellets, weeks 3 and 4 = 500–900-μm pellets, weeks 5 and 6 = 0.8–1.0-mm pellets, and weeks 7, 8, and 9 = 1.2–1.4-mm pellets. When pellet size was increased, feed ration was gradually decreased to a final amount of 5% body weight daily. The experiment was terminated after 9 weeks.

Calculations and statistical analysis.—Specific growth rate (SGR), relative growth rate (RGR), and daily weight gain (DWG) were calculated using the following formulas (where, wt_1 = mean weight (g) at the initial time point, wt_2 = mean weight (g) at the final time point, and ET = elapsed time (d) between measures of weight).

$$\text{SGR} = [\log_e(wt_2) - \log_e(wt_1)]/ET \times 100,$$
$$\text{RGR} = (wt_2 - wt_1)/(wt_1) \times 100,$$
$$\text{DWG} = (wt_2 - wt_1)/ET.$$

Feed conversion ratio (FCR) was calculated as the weight of feed consumed/(final total biomass − intitial total biomass).

All data were analyzed for statistical differences among treatments with feed type as the independent variable and instantaneous growth rate, as weight (G_W) or length (G_L) as the dependent variable. Analysis of variance (ANOVA) was used for comparisons of weight gain, SGR, RGR and DWG. Data expressed as percentages were normalized using arcsine transformation before statistical analyses. When our final data resulted in an unbalanced design, due to low mortality in some treatments, we chose to avoid the uncertainty involved in estimating degrees of freedom for an F-test (Kliegl et al. 2007; Quené and van den Bergh 2004; Pinheiro and Bates 2000). We used a likelihood ratio test and highest posterior density intervals (HPD intervals) to compare the final lengths and weights of the Scup. Statistical analyses were performed using the open source statistical software R (R Development Core Team 2009) and the lme4 package to fit linear mixed models (Bates et al. 2010).

TABLE 1. Ingredient content and feed composition of diets (dry weight basis).

Variable	Diet 1	Diet 2
Feed size (μm)	200–1,400	200–1,200
Protein (%)	55.5–58.5	59
Lipid (%)	13.4–14.5	16
Ash (%)	13.1–13.7	13
Moisture (%)	6.3–7.8	7
Fiber, maximum (%)	3	1.9

RESULTS AND DISCUSSION

Linear mixed model results indicated differences in weight and length of Scup fed each diet. Scup fed diet 1 weighed significantly more than those fed diet 2 after 35 d (HPD Interval $P < 0.05$). These differences continued to be statistically significant for the remainder of the study (HPD interval $P < 0.05$, Figure 1). Scup fed diet 1 grew from an initial mean length of 36.8 mm (SE, 0.2) to a final length of 109.6 mm (SE, 0.6), an increase of 72.8 mm. Fish fed diet 2 increased in mean length from 36.7 mm (SE, 0.2) to 104.7 mm (SE, 0.7), a gain of 68.0 mm. Juvenile Scup fed diet 1 began with an mean initial weight of 0.64 g (SE, 0.01) and grew to 24.27 g (SE, 0.4), a gain of 23.63 g (Figure 1).

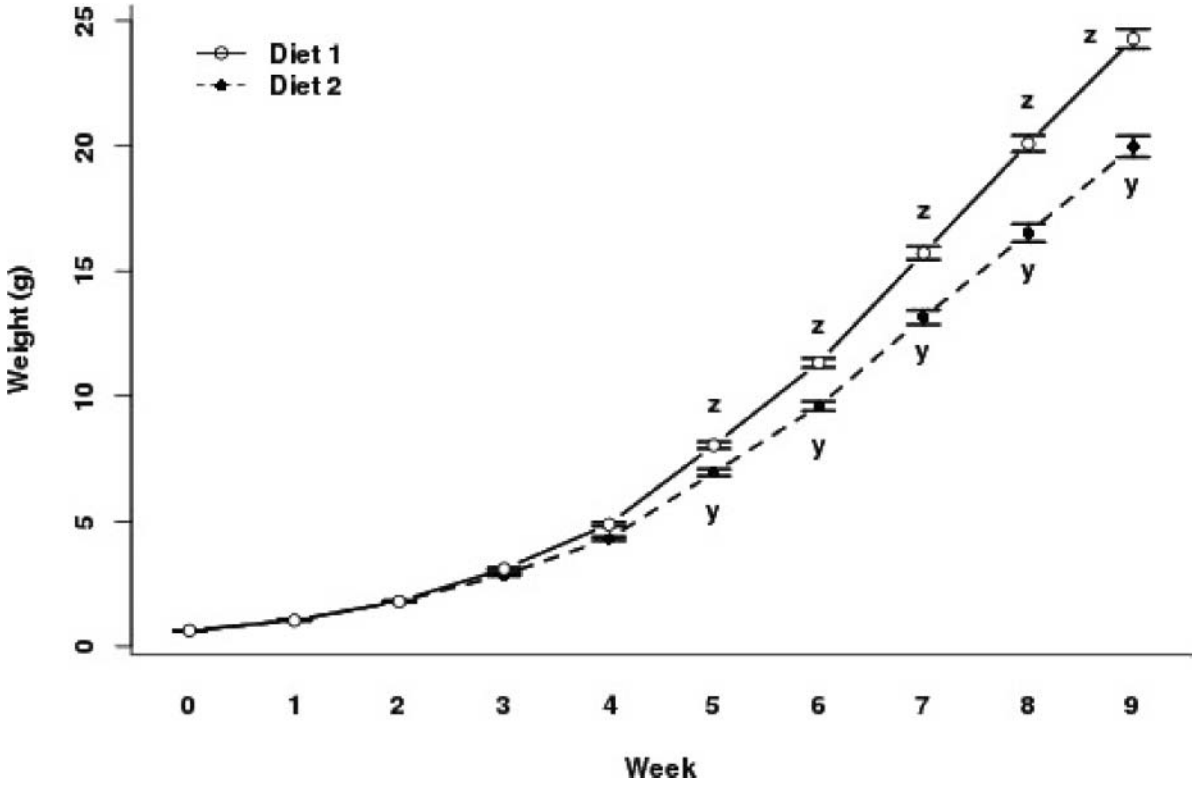

FIGURE 1. Mean weight (and SE) versus time for juvenile Scup fed two commercial diets. Error bars are SE of four replicate groups per treatment. Different letters for a given week indicate statistically significant differences ($P < 0.05$) between diets.

Fish fed diet 2 increased in mean weight from 0.63 g (SE, 0.01) to 19.97 g (SE, 0.4), a gain of 19.34 g. Minor differences in diet formulations may have an impact on growth rates of fish. The ingredient composition of diet 1 may have met the particular nutritional requirements of Scup better than the composition of diet 2. Feed manufacturers derive their protein and lipid from differing sources and the ingredient formulation of diet 1 may have allowed nutrients to be more readily metabolized and absorbed by the Scup. Also, the fiber content was slightly higher in diet 1, which may have had an effect on the metabolism of the fish (Table 1). Commercial diets have not been developed to specifically address the nutritional requirements of juvenile or adult Scup. Diet 1 is formulated for use with all marine fish, and diet 2 has been used to culture seabream. Our study tested commercially available feeds since growers presently rely on these products for marine fish culture operations. Ideally, nutritional studies comparing laboratory formulated diets with a range of protein and lipid levels may provide more information on the precise diet compositions that result in optimal growth.

Diet 1 contained lower levels of protein and lipid than diet 2 (Table 1). Our previous feeding study found that juvenile Scup grew significantly larger on the feed that contained the highest levels of protein and lipid (Perry et al. 2009). Although the dietary protein content (55.5–58.5%) of diet 1 was higher, the lipid content (13.4–14.5%) was lower than those values used in growth studies of other juvenile sparids. Dietary protein and lipid relations have been reported for Dentex (45–57% protein, 17–21% lipid; Skalli et al. 2004; Espinós et al. 2003; Ait Ali et al. 2008), and Red Sea Bream (52% protein, 15% lipid; Takeuchi et al. 1991). High levels of protein or lipid in fish diets may not necessarily result in the highest growth. Successful culture of fish utilizing diets with lower amounts of protein and lipid is advantageous since these are the most expensive components of an aquafeed and are often supplied from limited resources, such as fish meal and oil. Our results indicate that the high growth rates of Scup fed the diet containing lower protein and lipid levels make this species an economically attractive candidate for aquaculture.

Weight gain, SGR, RGR and DWG were significantly higher ($P < 0.001$) for Scup fed diet 1 (Table 2). The FCR (1.22) for fish fed diet 1 was not significantly different from fish fed diet 2 (Table 2). Specific growth rates of Scup in this study were higher than those measured in other juvenile sparids, irrespective of the feed used (Table 2). Feed conversion ratios based on diet 1 and 2 were similar to those measured in other juvenile sparids of similar size. Growth rates we observed were similar or greater than those reported for sparid juveniles given similar diets over a comparable period: Dentex (SGR = 3.12%/d and FCR = 0.90 [Skalli et al. 2004], SGR = 4.0%/d and FCR = 1.8 [Espinós et al. 2003], SGR = 2.4%/d and FCR = 1.3 [Ait Ali et al.

TABLE 2. Effect of two commercial diets on growth of juvenile Scup. Values in the same row with different letters are significantly different ($P < 0.001$).

	Mean (SE)	
Feed	Diet 1	Diet 2
Initial weight (g)	0.64 (0.01)	0.63 (0.01)
Weight gain (g)	23.63 (0.17) z	19.34 (0.27) y
Relative growth rate (%)	3,689 (53.3) z	3,077 (44.9) y
Specific growth rate (%/d)	5.8 (0.02) z	5.5 (0.02) y
Daily weight gain (g/d)	0.38 (0.003) z	0.31 (0.004) y
Feed conversion ratio	1.22 (0.03)	1.25 (0.01)

2008]) and Red Seabream (RGR = 218–463%; Takeuchi et al. 1991).

Water quality parameters remained acceptable throughout the course of the experiment for all treatments. Dissolved oxygen concentration ranged from 4.7 to 7.2 mg/L (SE, 0.4) and oxygen saturation levels ranged from 62.8% to 89.5% during the study. Seawater temperatures ranged from 19.4°C to 24.9°C with a median of 23.0°C. Among aquaria, salinity ranged from 23.1‰ to 26.0‰, with a median of 25.5‰. Survival averaged 91.7% for all treatments over the study period and was not affected by diet.

Juvenile Scup grew well when fed either diet (Table 2). Suitable and sufficient dietary protein and lipid levels in commercial feeds for young marine finfish are necessary for normal growth and development. During our feeding experiment, juvenile Scup readily accepted two different commercial feeds and had high survival rates under culture conditions. Scup exhibited rapid growth rates and low feed conversion ratios on the commercial diet containing lower levels of protein and lipid. These results provide additional information regarding growth of scup under culture conditions and further indicate the potential value of this species for commercial aquaculture.

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pH Dynamics of Tricaine Methanesulfonate (MS-222) in Fresh and Artificial Seawater

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COMMUNICATION

pH Dynamics of Tricaine Methanesulfonate (MS-222) in Fresh and Artificial Seawater

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Abstract

Tricaine methanesulfonate (MS-222) solutions are used routinely for clinical and experimental anesthesia of a variety of aquatic organisms. It is commonly accepted that MS-222 alters pH when added to freshwater, and buffering freshwater anesthetic solutions is considered to be the standard of care. However, buffering saltwater solutions is considered by many to be unnecessary; some individuals question whether any water with high natural mineral hardness needs to be buffered when MS-222 is added. To assess the need for buffering, we studied the dynamics of pH change when adding clinically relevant amounts of MS-222 to artificial seawater and to freshwater of varying hardness. We found that artificial seawater does not possess adequate buffering capacity to prevent a physiologically significant drop in pH when MS-222 is added, and hardness does not accurately predict the reaction of water pH to the addition of MS-222 in clinically relevant concentrations. Our data for salt water suggest that MS-222 solutions should be buffered to saturation with sodium bicarbonate and in freshwater should be titrated to the particular water source to satisfy the optimal pH of the species being anesthetized.

Tricaine methanesulfonate (MS-222) is one of the most commonly used anesthetic agents in aquaculture and the aquarium industries and by veterinarians. It is the only anesthetic labeled for use in finfish intended for food in the USA and is used for a wide variety of species, including marine and freshwater teleosts and elasmobranchs, as well as for amphibians and aquatic invertebrates. Conventionally, the powder form is either added directly to a volume of the existing holding water, or a concentrated stock solution is prepared in advance. Dose ranges vary widely, from less than 50 mg/L of water for sedation of finfish to over 10 g/L for euthanasia of amphibians (Carpenter 2013).

As the salt of a strong acid (methanesulfonic acid), MS-222 dissociates when added to water, eliciting a drop in pH that is immediately dependent on the buffering capacity of the water used as the diluent (Smit et al. 1977). When deionized water, with its near neutral pH and nonexistent buffering capacity, is used as the diluent, the addition of only 25 mg/L MS-222 is documented to lower the pH of the solution from about 7.0 to 4.0 (Smit et al. 1978). In response to this effect, MS-222 is routinely buffered by the addition of sodium bicarbonate ($NaHCO_3$), although use of sodium hydroxide has also been described (Ohr 1976; Smit and Hattingh 1979). The current literature suggests two common buffering protocols: (1) the addition of equal amounts of MS-222 and sodium bicarbonate by dry weight, and (2) the saturation of the aqueous component with bicarbonate following the addition of MS-222 (Sladky 2001; Harms 2003). The belief that natural or artificial seawater has sufficient inherent buffering capacity has led to a common practice by some practitioners of not buffering MS-222 solutions for use with marine organisms (Ohr 1976; Noga 1996).

The potential impact of an MS-222 caused drop in pH of water should be of concern for producers and scientists because it can impact survival and health, even when anesthesia duration is relatively short. Significant skin and corneal ulceration has been demonstrated in freshwater fish exposed to unbuffered MS-222 at normal anesthetic doses for 25–35 min (Davis et al. 2008). Gill lamellar epithelium is damaged by exposure to an acute pH drop from 6.8 to 5.6 (Daye et al. 1976). The damage may impact survival of some exposed individuals, and impacts on experimental results can include demonstrable histologic changes, disruption of blood acid–base balance, and changes in hematologic and serum values. Common Carp *Cyprinus carpio*, tilapia (species in the genera *Oreochromis*, *Sarotherodon,* and *Tilapia*), and Rainbow Trout *Oncorhynchus mykiss* anesthetized with bicarbonate buffered MS-222 have much more stable acid–base blood

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metrics, fewer hemodilution effects, and lower blood glucose and lactate values than fish exposed to unbuffered MS-222 (Smit and Hattingh 1979). Rainbow Trout subjected to unbuffered MS-222 anesthesia experience a significant decrease in renal ascorbic acid concentration, an indirect measure of ACTH production and the activation of the hypothalamus-pituitary axis (Wedemeyer 1970).

Our studies examine the influence of water hardness and two currently recommended buffering practices on the pH dynamics of clinically relevant concentrations of aqueous MS-222 solutions, including artificial seawater.

METHODS

Experiments were performed using 250 mL of water maintained in 400-mL borosilicate glass beakers as measured via gradations on the beakers. Each beaker was rinsed thoroughly with tap water followed by a deionized water rinse prior to use in the trial. Samples were maintained at room temperature (20–22°C) on the bench top throughout the trial. Beakers were open to the air for the duration of the trial. Evaporation was considered to be nominal for the short duration of these trials.

A bench top pH meter (Fisher Scientific Accumet Model 25) was used to measure pH to the nearest hundredth of a pH point. Prior to each trial, the instrument was calibrated using standard buffers of pH 4.01, pH 7.00 and pH 10.01 (Thermo Fisher Scientific, Beverly, Massachusetts). Quantities of MS-222 (Finquel, Argent Chemical Laboratories) and sodium bicarbonate (Fisher Chemicals) were weighed (μg) in plastic weigh boats via a microbalance (Sartorium CP 225D). Artificial seawater was prepared using Instant Ocean salt solution according to the manufacturer's directions to a specific gravity of 1.026, as measured with a salinity refractometer (VISTA, Model A366ATC). Water hardness for the artificial seawater was calculated from the concentration of Ca^{++} ions expected to be present in the solution when mixed as described. Hardness for the freshwater samples was measured using a commercially available test kit (LaMotte Hardness Liquid Version; Code 4482-LI-01) according to package instructions.

Measurements showed excessive variability in the initial pH readings of water from the same source prior to the addition of MS-222 or other chemicals. Each sample was subsequently aerated for 30 min with room air administered via standard aquarium air pump and air stone, then allowed to rest uncovered on the bench top for a minimum of 12 h before beginning the experiment. This procedure allowed the water to obtain a stable equilibrium with atmospheric CO_2 and was successful in eliminating the initial variability, which we felt was sufficiently large to affect the interpretation of our experiments.

For each water sample, pH was measured immediately prior to the addition of MS-222 and, when applicable, sodium bicarbonate. Plastic weigh boats containing the chemicals were emptied into the beaker, then rinsed gently in the diluent water to ensure complete dissolution of the dry matter. Samples rested uncovered on the bench top between pH readings, and each was gently mixed with a stir bar for 3–4 s prior to readings.

In experiment 1, MS-222 was added to samples of four different waters: deionized water, two tap waters (A and B) from geographically distinct municipal water sources, and artificial seawater. The pH value was measured 5 min following the addition of MS-222 at five different doses. Each trial at a specific dose was performed in triplicate.

Experiment 2 was performed using five different doses of MS-222, each applied to deionized water, the locally available tap water source (tap water B), and artificial seawater. Samples were buffered at a 1:1 ratio with sodium bicarbonate. The sodium bicarbonate and MS-222 were added simultaneously, then gently stirred for 60 s with a magnetic stir bar. The pH was measured at 5 min for three replicates of each experimental combination.

In experiment 3, trials were performed with samples from the same three water sources used in experiment 2; the highest and lowest doses of MS-222 from that study were used to investigate the impact of buffering with sodium bicarbonate past the point of water saturation. Sufficient sodium bicarbonate to exceed the saturation point of the diluent was added immediately after the dissolution of MS-222. All trials were performed in triplicate.

To investigate the dynamics of pH over time, single trials were performed with MS-222 doses of 100 and 1,000 mg/L in all four source waters used in buffering experiment 1. These trials were performed using the same technique as experiment 1, and no buffer was added. Only one extended trial was performed with each water source, and pH values were measured and presented at expanding time intervals out to 1,440 min following the addition of MS-222.

In experiments 1–3, each trial was performed in triplicate to provide evidence of repeatability. This is common in basic chemical investigations, however, the assumption of normal distribution of data cannot be made with three replicates, nor is it appropriate to use parametric statistical analysis commonly employed in biological and agricultural studies. For this reason we used the median, the nonparametric analog of the more commonly reported mean, as the primary summary statistic, and we included the range of the data to facilitate reader assessment of data variability.

RESULTS

All water sources tested demonstrated the expected pH decrease almost immediately following addition of MS-222 (Table 1). The effect was dose-dependent and resulted in a notable pH drop (>0.5 points) in all water sources within 5 min, even at the lowest MS-222 dose of 100 mg/L of water. There was no apparent relationship between the total hardness of the water sample and the magnitude of the pH drop; the smallest pH drop for MS-222 at 1,000 mg/L was seen in the tap water sample with the lowest hardness value.

TABLE 1. Median pH values for three replicates of water samples of varying hardness taken 5 min after addition of various doses of MS-222.

MS-222 without buffer	Median (range) pH at MS-222 dose					Hardness as $CaCO_3$ (mg/L)
	0 mg/L	100 mg/L	250 mg/L	500 mg/L	1,000 mg/L	
Deionized water	6.34[a] (5.84–7.94)	3.99 (3.95–4.24)	3.48 (3.41–3.54)	3.24 (3.24–3.34)	3.14 (3.09–3.17)	0
Tap water A	7.05 (7.00–7.93)	6.51 (6.39–7.02)	[b]	[b]	3.38 (3.06–3.41)	50
Tap water B	8.18 (7.31–8.77)	7.28 (6.93–7.46)	7.23 (6.93–7.27)	6.53 (6.45–6.69)	5.59 (5.22–5.74)	20
Artificial seawater	7.92 (7.55–8.31)	6.91 (6.79–6.98)	5.97 (4.59–6.27)	4.60 (4.59–5.6)	4.18 (3.76–4.93)	1,000

[a]Due to the lack of electrically conductive ions in pure deionized water, values obtained by this (or any glass electrode) pH meter are inaccurate (Youmans 1972).
[b]Values are not available because trials were not performed at all doses with this diluent.

Addition of an equal mass of sodium bicarbonate with MS-222 in three different water sources resulted in a reduced pH drop compared with solutions not buffered (Table 2). The pH of the buffer-treated water remained much closer to the original value and exhibited less dose dependency, especially in the case of deionized water. The difference in pH between buffered and unbuffered samples at the lowest MS-222 concentration was lower for artificial seawater than for hard tap water. However, this relationship did not hold at higher doses of MS-222, where buffering of the tap water samples maintained the pH at a higher value than the artificial seawater.

In all water samples, buffering to the point of saturation with sodium bicarbonate resulted in a higher pH at 5 min than buffering at 1:1, and in the case of both deionized water and tap water, the pH at 5 min after addition of drug was even higher than the starting pH (Table 3). Buffering of the artificial seawater with sodium bicarbonate to saturation did not prevent the pH drop entirely, though it did stay much closer to the starting value than the 1:1 MS-222 to sodium bicarbonate buffering protocol.

All of the water sources tested except deionized water showed a recovery of pH over time following introduction of MS-222 without supplemental buffering. At an MS-222 dose of 100 mg/L, near complete recovery was seen within 6 h in most water samples (Table 4). At the higher MS-222 dose of 1,000 mg/L (a commonly used euthanasia dose for finfish), pH recovery was notably less complete for all water sources even after 24 h (Table 5).

DISCUSSION

The addition of MS-222 to water with minimal innate buffering capacity (deionized) resulted in a large drop in pH. Contrary to recommendations in current texts on fish medicine suggesting supplemental buffering is not required in water with a hardness ($CaCO_3$) value of >50 mg/L (Noga 1996), we found additions of MS-222 resulted in a drop in pH that may impact survival of organisms in water samples with $CaCO_3$ values at or greater than 50 mg/L. The magnitude of the pH drop in all samples except deionized water was nearly equivalent at the lowest dose of MS-222, and the pH value measured at 5 min seemed dependent on the starting pH. As the dose of MS-222 was increased, the pH changes seen in the different water samples were noted to be inconsistent, probably as the variable quantity of buffering ions present in the water sample were proportionally reacted by the dissociated hydrogen ions from methanesulfonic acid. At the highest dose of MS-222 (1,000 mg/L) we tested, the highest pH was retained in the water with the lowest hardness (tap water B), indicating some factor other than total hardness is impacting pH dynamics in MS-222 solutions.

TABLE 2. Median pH values for three replicates of water samples 5 min after addition of various doses of MS-222 with and without the addition of sodium bicarbonate at equal weight to MS-222.

MS-222 with or without buffer	Median (range) pH at MS-222 dose				
	0 mg/L	100 mg/L	250 mg/L	500 mg/L	1,000 mg/L
Deionized water					
Unbuffered	6.34[a] (5.84–7.94)	3.99 (3.95–4.24)	3.48 (3.41–3.54)	3.24 (3.24–3.34)	3.14 (3.09–3.17)
With $NaHCO_3$		6.57 (6.53–6.62)	6.44 (6.42–6.65)	6.48 (6.47–6.61)	6.53 (6.52–6.55)
Tap water (B)					
Unbuffered	8.18 (7.31–8.77)	7.28 (6.93–7.46)	7.23 (6.93–7.27)	6.53 (6.45–6.69)	5.59 (5.22–5.74)
With $NaHCO_3$		7.65 (7.00–7.65)	7.32 (7.18–7.37)	7.02 (6.96–7.02)	6.84 (6.76–6.85)
Artificial seawater					
Unbuffered	7.92 (7.55–8.31)	6.91 (6.79–6.98)	5.97 (4.59–6.27)	4.60 (4.59–5.60)	4.18 (3.76–4.93)
With $NaHCO_3$		6.98 (6.97–6.98)	6.68 (6.67–6.70)	6.52 (6.48–6.53)	6.33 (6.32–6.36)

[a]Due to the lack of electrically conductive ions in pure deionized water, values obtained by this (or any glass electrode) pH meter are inaccurate (Youmans 1972).

TABLE 3. Median and range (in parentheses) of pH for three replicates of several water samples 5 min after addition of MS-222, using two different buffering protocols with sodium bicarbonate.

MS-222 with or without buffer	Median (range) pH at MS-222 dose		
	0 mg/L	100 mg/L	1,000 mg/L
Deionized water			
Unbuffered	6.34[a] (5.84–7.94)	3.99 (3.95–4.24)	3.14 (3.09–3.17)
Buffered 1:1 with $NaHCO_3$		6.57 (6.53–6.62)	6.53 (6.52–6.55)
Buffered to saturation		8.13 (8.12–8.29)	7.97 (7.96–8.18)
Tap water (B)			
Unbuffered	8.18 (7.31–8.77)	7.28 (6.93–7.46)	5.59 (5.22–5.74)
Buffered 1:1 with $NaHCO_3$		7.65 (7.00–7.65)	6.84 (6.76–6.85)
Buffered to saturation		8.30 (8.24–8.36)	8.13 (8.13–8.16)
Artificial seawater			
Unbuffered	7.92 (7.55–8.31)	6.91 (6.79–6.98)	4.18 (3.76–4.93)
Buffered 1:1 with $NaHCO_3$		6.98 (6.97–6.98)	6.33 (6.32–6.36)
Buffered to saturation		7.75 (7.75–7.77)	7.60 (7.59–7.72)

[a]Due to the lack of electrically conductive ions in pure deionized water, values obtained by this (or any glass electrode) pH meter are inaccurate (Youmans 1972).

The equal parts of sodium bicarbonate buffering protocol did reduce the pH drop in freshwaters when MS-222 concentrations were lower than 500 mg/L. In deionized water, the addition of sodium bicarbonate in equal amounts by weight with MS-222 stabilized the water pH around 6.5, regardless of the dose of MS-222. In the tap water and artificial seawater, a dose-dependent pattern of pH change was obtained, suggesting a more complex interaction between the buffering bicarbonate ions and buffering ions of the water sample itself. Even at the lowest concentrations of MS-222 we tested with this buffering protocol, we saw a decrease in pH that could potentially cause metabolic issues in some species.

The second common buffering protocol, adding sodium bicarbonate to saturation, completely prevented the pH drop in deionized and tap water. As an amphoteric compound that can act as either an acid or base, bicarbonate added to water in large quantities is expected to generate hydroxide ions and result in an alkaline solution. In both deionized and tap water, even at the higher dose of MS-222, the pH measured 5 min after the addition of the chemicals was >8.0, as the quantity of hydroxide ions produced overwhelmed the hydrogen ions from the dissociation of MS-222. While less studied than the effects of acidic pH on aquatic organisms, severe alkaline changes in pH of freshwater are also likely to have negative physiologic effects, particularly on those species that are sensitive to high pH environments (e.g., discus *Symphysodon* spp.; Noga 1996). Producers should take care to ensure that freshwater anesthetic solutions are not being overbuffered. The ideal quantity of buffer for a given source of freshwater will depend on its innate buffering capacity. It should be possible for aquaria or aquaculture facilities using a stable water source to determine this with a few simple trials to measure the pH of their water after the addition of the desired dose of MS-222 with varying quantities of buffer.

Results indicate the artificial seawater we tested does not have adequate buffering capacity to counteract the pH drop from addition of MS-222 in commonly used anesthetic concentrations. Even MS-222 at 100 mg/L buffered with an equal amount of sodium bicarbonate decreased pH by nearly 1 point, which would be expected to affect the acid–base balance of marine fishes. When bicarbonate buffer was added to the point of saturation, pH values at 5 min post MS-222 addition were notably higher than obtained without the buffer, but still lower than the pH of the initial water sample. When working with marine organisms acclimated to an optimal pH >8.0, physiologic

TABLE 4. Change in pH over time of various water samples after the addition of 100 mg/L MS-222.

Water source	pH value by time (min)								
	0	1	3	5	10	60	360	1,020	1,440
Deionized water	8.96	4.92	4.12	4.24	4.20	4.15	4.24	4.10	4.01
Tap water A	7.05	6.63	6.50	6.51	6.44	6.65	6.88	6.67	7.13
Tap water B	7.81	7.68	7.49	7.46	7.23	7.41	7.91	8.20	8.46
Artificial seawater	8.11	7.88	7.76	7.76	7.67	7.81	7.92	8.05	8.20

TABLE 5. Change in pH over time of various water samples after the addition of 1000 mg/L MS-222.

Time (min)	0	1	3	5	10	60	360	1,020	1,440
Deionized water	6.45	3.17	3.17	3.16	3.15	3.14	3.16	3.00	3.02
Tap water A	7.00	3.37	3.39	3.38	3.36	3.38	3.40	3.22	3.27
Tap water B	7.42	5.85	5.59	5.59	5.63	5.86	6.22	7.02	6.95
Artificial seawater	8.24	5.46	5.21	5.21	5.18	5.46	5.57	6.51	7.15

consequences of the pH drop would be expected for most species, and practitioners would be wise to buffer anesthetic solutions with sodium bicarbonate to the point of saturation. We used artificial seawater in our trials to help reduce variability in water composition; however, variability in actual elemental composition of artificial salt solutions and natural seawater could alter the ability of seawater to buffer MS-222 additions.

Few investigations have been made into the temporal aspect of buffering MS-222 solutions. At lower concentrations typically used for teleost anesthesia our trials show that many source waters have the ability to stabilize towards a physiologically safe pH within 6 h. This reaction is not sustained at higher concentrations of MS-222. This effect is expected to be affected by environmental conditions such as temperature and atmospheric carbon dioxide concentrations and may not be consistent across situations. In many clinical situations, it will frequently be more practical to simply buffer anesthetic solutions than to prepare anesthetic solutions far enough in advance to take advantage of this reaction. However, there may occasionally be situations where the addition of sodium bicarbonate is undesired or impossible, and the innate buffering capacity of a water sample over time would be useful. The temporal dynamics of pH in MS-222 solutions could also have potential importance for sedation and transport situations where animals are contained in a low-dose anesthetic baths for extended periods of time.

Our studies have also demonstrated that pH trends when MS-222 is added to water are not solely dependent on total hardness of the water. Although we did not investigate alkalinity and specific mineral content of the water, they should be expected to impact how a given sample of water reacts to the addition of MS-222. Though total hardness and alkalinity are generally considered to move in parallel due to the primary contribution of limestone ($CaCO_3$) in many sources of groundwater, this is not always the case. Our experiments suggest that producers using MS-222 in salt water should consider buffering MS-222 stock solutions to saturation with sodium bicarbonate to prevent adverse effects on fishes from rapid pH changes in water. Our results for freshwater solutions suggest that buffering protocols should be titrated to a particular water source so that the proportion of buffer to MS-222 results in a pH approaching the desired value for the species being anesthetized.

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Identification of Gender in Yellow Perch by External Morphology: Validation in Four Geographic Strains and Effects of Estradiol

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ARTICLE

Identification of Gender in Yellow Perch by External Morphology: Validation in Four Geographic Strains and Effects of Estradiol

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Abstract

External morphological criteria that enable the rapid determination of gender have been developed for Yellow Perch *Perca flavescens*. Criteria are based upon (1) shape of the urogenital papilla (UGP), (2) size of the UGP relative to the anal (AN) opening, and (3) coloration of the UGP. In females, the UGP appeared (1) rounded at the anterior margin, (2) pointed at the posterior margin and had a V or U shape (crescent shaped in mature animals), (3) generally lacked reddish coloration, and (4) narrower relative to the AN. In males, the UGP (1) appeared circular or oval around the entire margin, (2) typically displayed reddish coloration, and (3) was generally wider relative to the AN. To verify accuracy of these criteria, gender was verified internally in perch of various sizes, sex, and maturity from four domesticated geographic strains ($n = 1{,}389$). For all perch tested, accuracy was 97.3% for both sexes, 98.8% for females, and 95.9% for males. To experimentally verify accuracy of these criteria, juvenile Yellow Perch ($n = 913$) were treated with dietary 17β-estradiol (E2; 15 mg/kg diet) or a control diet. Accuracy was 97.7% for control females and 95.1% for control males, which diminished to 63.9% for the E2-treated females and 57.6% for E2-treated males. We developed a gender identification algorithm that will enable sorting of sexes for many uses including (1) collection of broodstock by new aquaculture producers, (2) management of broodstocks for existing producers, (3) improved selection criteria for genetic selection programs, (4) studies on gender-specific differences in Yellow Perch physiology, and (5) the unharmed release of fish in field and aquaculture settings. Uncoupling of external UGP morphology from actual gender in E2-treated perch can enable producers and biologists to detect exposure to estrogenic compounds in areas where endocrine disruption is suspected.

The ability to determine gender in finfish has been essential to the successful development of fisheries management, basic research, and aquaculture. However, reliable external identification methods have not been developed for many important finfish species. While gender can be identified by the release of sperm or eggs from sexually mature fish, such criteria cannot be used outside of the spawning season and is not applicable for juveniles, sexually immature adults, or sexually monomorphic species. Therefore, the need for reliable determination of gender and reproductive stage has driven the development of diagnostic

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methods involving the use of catheterization or biopsies (Ross 1984), biochemical methods (Le Bail and Breton 1981; Craik and Harvey 1984; Pottinger et al. 2005), molecular markers (Devlin et al. 1994; Griffiths et al. 2000; Nagler et al. 2004), and imaging techniques (e.g., ultrasonography, endoscopy, and laparoscopy; Moccia et al. 1984; Ortenburger et al. 1996; Novelo and Tiersch 2012) for a number of finfish species. While such methods are generally precise, there are drawbacks such as the need for laboratory infrastructure that is not available to all researchers or aquaculture producers and deployment of complicated equipment and procedures in a field or aquaculture setting. Such methods are time consuming, require substantial expertise, and can involve stress and injury to the fish. Consequently, methods that enable rapid, accurate identification of gender in juvenile and adult finfish, without equipment or complicated procedures, are highly desirable.

The development of criteria for external gender identification using external morphology (e.g., body shape, genital papilla), body coloration, or both has only been described for very few stenohaline freshwater species in North America. These would include Fathead Minnow *Pimephales promelas* (Ankley et al. 2001), Bluegill *Lepomis macrochirus* (McComish 1968), Largemouth Bass *Micropterus salmoides* (Parker 1971), Northern Pike *Esox lucius* (Casselman 1974), and Muskellunge *E. masquinongy* (Lebeau and Pageau 1989). While the methods described for Northern Pike and Muskellunge appear to be the only ones that are sufficiently robust to enable gender identification in immature fish, comparatively less is known for percid fishes.

Yellow Perch *Perca flavescens* are an ecologically and economically important percid with a wide geographic distribution in North America. One particular aspect of Yellow Perch physiology is that females grow larger and faster than males and this growth can be estrogen-dependent (Malison et al. 1985, 1988; Goetz et al. 2009). Given there are differences in growth rate and in the functional relationships between body weight and length (and maturation) among geographic strains of Yellow Perch (Brown et al. 2002; Wang et al. 2009; Rosauer et al. 2011), one could hypothesize that differences occur in the external morphology of the urogenital papilla (UGP) between strains, juveniles, and adults.

Based on this knowledge, we developed criteria for the external identification of gender in Yellow Perch using fish from four geographic strains (Choptank River, Maryland; Perquimans River, North Carolina; Sassafras River, Maryland; Lake Winnebago, Wisconsin) that vary in size and maturity. We also experimentally tested the validity of these criteria using estrogen-treated juvenile perch (Malison et al. 1985; Goetz et al. 2009). The present study describes external morphology of the UGP of male and female Yellow Perch, criteria for determining gender using UGP morphology, a decision algorithm to enable systematic identification of gender in these geographic perch strains, and observational and experimental data on the accuracy of these criteria.

METHODS

General conditions for animal husbandry.—Yellow Perch were maintained and euthanized in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin– Milwaukee. All perch were obtained from captive broodstocks maintained at the School of Freshwater Sciences (Milwaukee, Wisconsin). These broodstocks were originally derived from gametes taken from wild Yellow Perch from several geographic locations, including the Choptank River (Chesapeake Bay, Maryland), Perquimans River (Albemarle Sound, North Carolina), Sassafras River (Chesapeake Bay, Maryland), and Lake Winnebago (Fond du Lac, Wisconsin) (Grzybowski et al. 2010; Rosauer et al. 2011). Spawning of adults, production of feed-trained fingerlings, and general husbandry for the Choptank, Perquimans, and Winnebago strains have been previously described (Rosauer et al. 2011). Gonadal somatic index (GSI) in many of the perch used for this work was generally minimal (<5%) as several groups (with exception as described below) had not undergone the environmental cycling required for sexual maturation (Hokanson 1977). Gonadal somatic index was determined from random samples for specific strains only (as reported below or in the Results section). The GSI was calculated as follows (Morrison et al. 1985):

$$\text{GSI} = \text{gonad weight in grams} \times (\text{body weight in grams} - \text{gonad weight in grams})^{-1} \times 100.$$

Specific rearing conditions for Choptank, Perquimans, Winnebago, and Sassafras strains.—Yellow Perch from the Perquimans and Choptank strains were F_3 progeny derived from parents (F_2 broodstock) spawned in March and April 2011, respectively. Data on sex identification for the Choptank and Perquimans perch were obtained from an on-going growth performance trial that is part of a broodstock genetic improvement program. Conditions for the performance trial were similar to those previously described (Rosauer et al. 2011), but perch (~48 per strain) were sampled at 6 and 9 months of age and maintained on a high protein–high energy diet (pellet size, 3.0-mm; Bio Vita Fry, Bio-Oregon, Longview, Washington); GSI was not determined for these perch.

To experimentally validate UGP morphological criteria, additional F_3 progeny were treated with dietary 17β-estradiol (E2). Approximately 70 Yellow Perch from the Choptank (3.0 ± 0.2 g, mean ± standard error of the mean) strain and 70 perch from the Perquimans (6.4 ± 0.2 g) strain were separately stocked into eight, circular, 120-L fiberglass tanks per strain ($n = 913$ total fish). Tanks were blocked by strain (Choptank or Perquimans) and treatment (control and estrogen), resulting in four replicate tanks consisting of Choptank control, Choptank E2, Perquimans control, and Perquimans E2. Tanks were supplied with flow-through water at 20 ± 1.5°C and photoperiod was maintained at 16 h light : 8 h dark. Yellow Perch

were acclimated for a period of 3 weeks. Following acclimation, the perch were weighed and measured and fed a diet containing 15 mg E2 (Sigma-Aldrich, St. Louis, Missouri) per kilogram of diet (pellet size, 2.0 mm; Bio Vita Fry, Bio-Oregon,) or a control diet treated with vehicle (ethanol) alone. Diets were prepared as previously described (Goetz et al. 2009) and perch were fed by hand to apparent satiation twice daily. At the end of the 15-week period, all perch in each tank were anesthetized with tricaine methanesulfonate (MS-222; 100 mg/L), weighed and measured, and euthanized by decapitation. Gender was determined externally for all perch at this time (criteria described below) followed by internal confirmation of gender by examination of the gonad (fish were 11 months old at this time). Gonads were removed from random samples and preserved in zinc-buffered formalin (10%) fixative (Z-fix, Anatech, Battle Creek, Michigan) for subsequent histological analyses. To determine GSI, fixed gonads were blotted dry on paper towel and then weighed to the nearest 0.001 g using a calibrated analytical balance (Voyager Pro, Ohaus, Parsippany, New Jersey). Gonad weights were corrected to account for weight loss (Anderson and Neumann 1996).

Two additional groups of F_3 Choptank Yellow Perch, not used for the growth performance trial, were also tested for gender identification. The first group represented perch that were held under similar conditions (water source, temperature, and photoperiod) as previously reported (Rosauer et al. 2011) and were 12 months old at the time of sampling. The second group of perch were separately maintained in a 2,000-L fiberglass tank and fed a maintenance ration (2% of biomass per day) using a commercially available feed (3.0 mm; BioVita Fry, Bio-Oregon). Photoperiod was maintained at 14 h light: 10 h dark and temperature was allowed to fluctuate over the seasons, but never exceeded 22°C in summer or went below 10°C in the winter. This second group was 13 months old at the time of sampling. At the time of sampling for sex identification, gonads were weighed from a subsample of these excess Choptank male and female perch for GSI determination; GSI was 1.57 ± 0.76% for females and 3.77 ± 0.82% for males.

Yellow Perch that comprise the Sassafras strain (24 months old) were obtained as feed-trained fingerlings in April 2010 and were held in a circular 2,400-L fiberglass tank. Photoperiod was maintained at 14 h light: 10 h dark and temperature was allowed to fluctuate over the seasons, but never exceeded 22°C in summer or went below 10°C in the winter. Once the perch reached a size of 40 g body weight, they were maintained on a maintenance ration of 2% biomass/d (3.0 mm; Bio Vita Fry). At the time of sampling, many of the male perch were spermiating. Consequently, a subsample of gonads from each sex was weighed to determine GSI; GSI was highly variable in females (8.6 ± 5.6%) compared with males (2.54 ± 0.2%).

Winnebago strain Yellow Perch were F_2 progeny resulting from parental crosses of genetically selected broodstock (F_1) spawned in May 2009. Perch were reared, maintained, fed, and environmentally cycled as previously described (Rosauer et al. 2011). Perch were sampled for gender identification at 39 months of age. While these perch were confirmed to have spawned in May 2011 and May 2012, individual spawning events were not recorded for May 2012. Because these perch had been cycled for spawning, gonads were randomly sampled for weight to determine GSI; female GSI was 4.0 ± 1.5% and male GSI was 0.2 ± 0.01%.

Morphological criteria for external gender identification.—The morphological criteria used to externally determine gender in Yellow Perch from these various geographic strains were (1) shape of the UGP, (2) coloration of the UGP, and (3) size of the UGP relative to that of the anus (AN). After determination of sex had been made, the perch were euthanized as previously described and confirmation of sex was accomplished by internal examination of the gonads according to initial criteria described by Parker (1942) and expanded criteria reported by Malison et al. (1986). Since the work of Parker (1942) demonstrated that genital and urinary systems share a single duct, we have called this area where the urogenital opening occurs, the UGP.

Image capture and processing.—Images of the UGP and gonads were taken using a Canon Power Shot SX40HS camera equipped with a 35 × , 24–840-mm zoom lens (f-stop, 2.7–5.8). Images were imported into Adobe Photoshop CS6 (version 13.0, 64 bit, San Jose, California) for processing, zooming, and cropping. To ensure the best possible representation of coloration and appearance of the AN and UGP regions, images were processed using the clone stamp tool to reduce reflected light (see Figure 1) and white- and color-balancing (see Figure 2) to compensate for variable lighting conditions (Adobe Photoshop CS6). Adobe Illustrator (16.0.0) was used for organization of the images.

Statistical analyses for datasets of untreated and estrogen-treated fish.—A completely randomized design (CRD) was used to examine and compare external and internal sex identification of four strains of untreated Yellow Perch for three size categories (see Table 1). The strains examined were Choptank, Perquimans, Sassafras, and Winnebago. The size categories (determined from overall size distributions) were defined in terms of TL and were: 80–170 mm, 171–210 mm, and 211–290 mm. Since perch were used from various populations, specific size categories are absent for certain geographic strains.

To answer questions regarding the efficacy of using external gender identification for untreated Yellow Perch, two analyses were performed. The first test was a binomial proportions test to determine whether external gender identification is effective in predicting the true gender of the perch, as determined by internal gender confirmation, over all strains, size categories, and true gender of the perch. The dependent, or *Y*-variable, was binary (1 = correct external gender identification and 0 = incorrect). The binomial proportions analysis compared the frequency count of the number of correctly identified external gender identifications to the number of incorrect external gender identifications.

The second test was a logistic analysis for determining whether strain, size category, or true gender of the perch influences the success of external gender identification. The same binary dependent variable was used as described above. The independent classification variables—Internal Gender

Confirmation, Strain, and Size Category—were used. If a significant ($P < 0.05$) Wald chi-square test was obtained, differences of least squares means on maximum likelihood estimates of the effects was used as a pairwise multiple comparison procedure. Odds ratios were obtained and used to determine the probability of correct external sex identification compared with the probability of incorrect identification for each comparison.

A CRD was used to examine and compare external and internal gender identification of two strains of estrogen-treated Yellow Perch for two treatments (see Table 2). The strains examined were Choptank and Perquimans, and the treatments applied were identified as Control and E2. As described above, a binomial proportions test was used to determine whether external gender identification is effective in predicting the true gender of the perch. Significant differences were declared at $P \leq 0.05$ from Wald chi-square tests. Odds ratios were obtained and used to determine the probability of correct external gender identification compared with the probability of incorrect identification for each significant effect comparison. A first logistic analysis used a three-factor full model with interactions of the categorical variables: Strain (Choptank and Perquimans), True Gender of the perch (based on internal examination, female or male), and Treatment (control or E2), to determine which factors influence the success of external gender identification in Yellow Perch. A second logistic analysis further examined odds ratios for significant main effects from the first analysis.

Analysis of the GSI of the Choptank and Perquimans Yellow Perch in the dietary E2 study were analyzed separately by two-way ANOVA using transformed data, with treatment (control or E2) and sex (female or male) as independent variables (main effects). Following significant ANOVA differences ($P < 0.05$), pairwise comparisons were performed using the Tukey–Kramer method.

In all cases, the stated P-value ($P < 0.05$) for individual comparisons is a general cut-off value (Steel and Torrie 1980). All values are reported as the mean, range, raw counts, or percentages. Analyses were performed using PC SAS software (version 9.2, 2002–2008; SAS Institute, Inc., Cary, North Carolina).

RESULTS

Appearance of the UGP in Untreated Female and Male Yellow Perch

The UGP is located just posterior to the AN opening (Figure 1). In females (Choptank, Perquimans, and Sassafras strains) of all size categories (80–170 mm, 171–210 mm, and 211–290 mm TL), the margin of UGP typically occurs in a "U" or "V" shape that is oriented towards the anal fin, and the width of the UGP is equivalent to, or narrower than, that of the AN opening (Figure 1B and 1D). Color of the UGP in these smaller Yellow Perch is either pale (Figure 1D) or light brown (Figure 1B). In the females sampled from the Winnebago strain (211–290 mm TL), the margin of the UGP had either a U shape or crescent shape (Figure 1F) and appeared dark brown, but was always equivalent (or less) in width to that of the AN margin. In addition to the crescent-shaped appearance of the UGP in the Winnebago strain, another unique observation for this strain was the presence of AN swelling in females (Figure 1F), which was not observed in similarly sized perch from the Choptank, Perquimans, or Sassafras strains (Figure 1A–D; in each panel, an inset figure shows the gonads of the corresponding perch).

In male Yellow Perch of all strains and sizes, the margin of the UGP was typically round or oval in shape, wider than that of the AN opening, and coloration ranged from pale brown to red (Figure 1A, C, E). Interestingly, we also observed AN swelling (Figure 1E) in males of the Winnebago strain (211–290 mm TL), which was not observed in similarly sized males from the Choptank, Perquimans or Sassafras strains.

Appearance of the UGP and Gonads of Yellow Perch Treated with E2

The UGP (and gonads) of control males and females (Figure 2A, B) of both strains (Choptank and Perquimans) appeared as described for untreated Yellow Perch shown in Figure 1A–D. By contrast, external features of the UGP in E2-treated males and females (Choptank and Perquimans strains) were generally similar in appearance (Figure 2C, D). Specifically, the UGP of E2-treated males (Figure 2C) had less developed coloration (similar to that of control females, Figure 1B), was U shaped and the width appeared equal to that of the AN. The margin of the UGP in E2-treated females appeared as a U or V shape, but the width of the UGP margin often appeared to be greater than that of the AN opening (Figure 2D). In each figure panel, an inset figure shows the gonads of the corresponding control and E2-treated males and females (Figure 1A–D). While confirming gender internally, gross examination of the gonads revealed that the testes and ovaries of E2-treated perch were smaller in size (Figure 2E, F). Specifically, the testes of E2-treated males (Figure 2C, E) were translucent, less developed, and difficult to detect. While histology was not performed on the gonads from these fish, the stage of development could be approximated macroscopically. The maturity stage of E2-treated male Yellow Perch most closely resembled the "resting stage" as described by Turner (1919) or "sterile" as described by Malison et al. (1986). The ovaries of E2-treated females (Figure 2D, F) were also less developed, but often displayed a hemorrhagic red coloration and the posterior was filled with a clear fluid. The maturity stages of control and E2-treated female perch are best described as being in stage III (developing early) and stage IID (maturing virgin), respectively (Treasurer and Holliday 1981).

Analyses of External Gender Identification Criteria for all Strains and Size Categories in Untreated Yellow Perch

Data on the accuracy of external gender identification in untreated male and female Yellow Perch were obtained from a total of 1,389 perch across all strains and sizes (Table 1). When evaluating data summarized in Table 1 (untreated, $n =$ 1,389), there was a highly significant difference between the

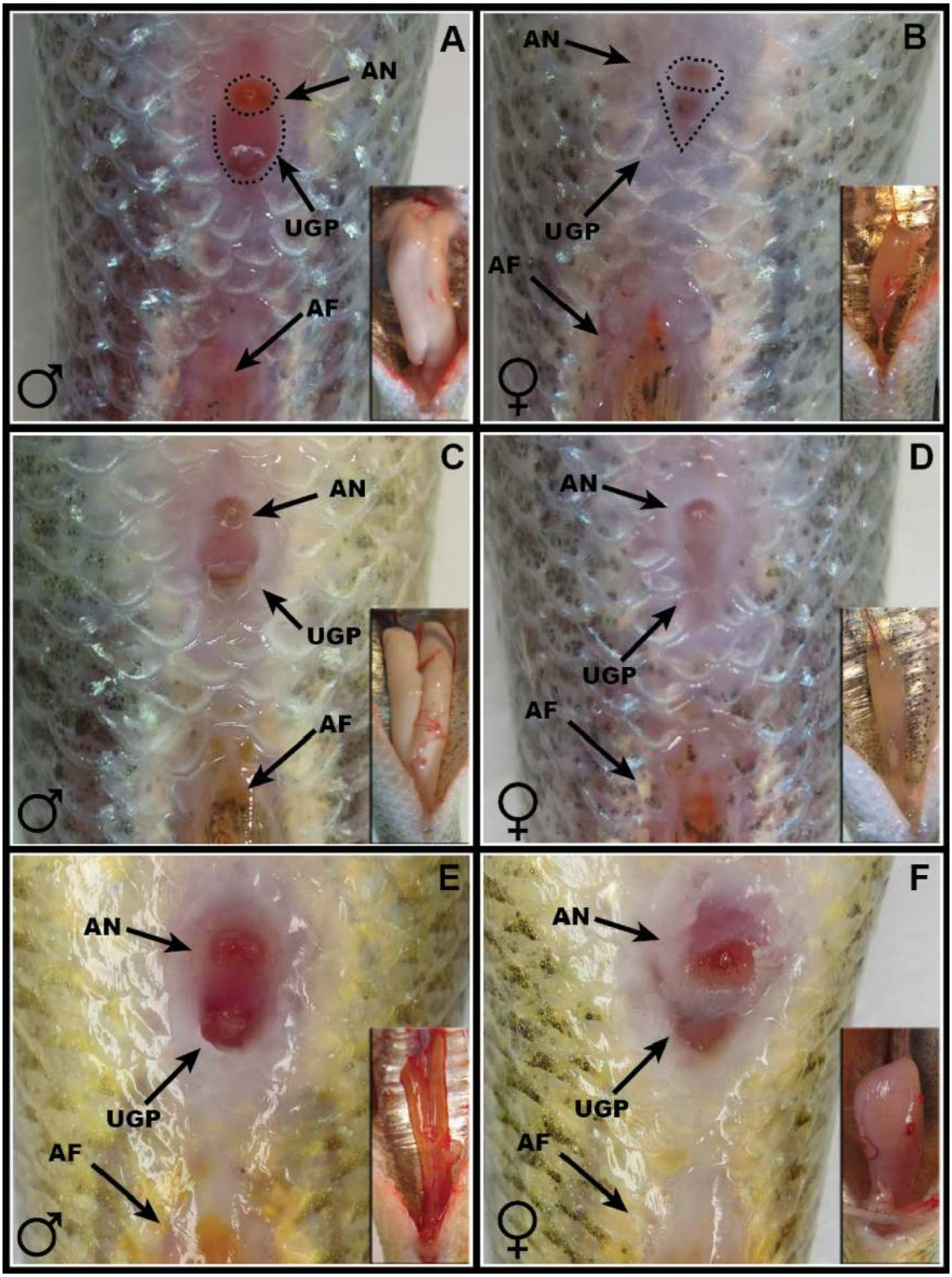

FIGURE 1. External morphology of the urogenital papilla in untreated male and female Yellow Perch from three distinct size-classes. An example of sex confirmation is included in each panel as an inlay. Representative examples were all externally identified correctly. (**A**) Male (♂) Sassafras strain Yellow Perch in size category 1 (80–170 mm). (**B**) Female (♀) Sassafras strain Yellow Perch in size category 1 (80–170 mm). (**C**) Male Sassafras strain Yellow Perch in size category 2 (171–210 mm). (**D**) Female Sassafras strain Yellow Perch in size category 2 (171–210 mm). (**E**) Male Winnebago strain Yellow Perch in size category 3 (211–290 mm). (**F**) Female Winnebago strain Yellow Perch in size category 3 (211–290 mm). Morphological landmarks are indicated in bold block letters as follows: AN = anal opening, UGP = urogenital papilla, AF = anal fin. Dashed lines indicate general shape of the UGP in males (panel A) and females (panel B). Of noticeable contrast between each sex is the rounded UGP in males, the V- or U-shaped UGP in females, darker reddish coloration of the UGP in males versus females, and the UGP generally wider than the AN in males and generally narrower than the AN in females.

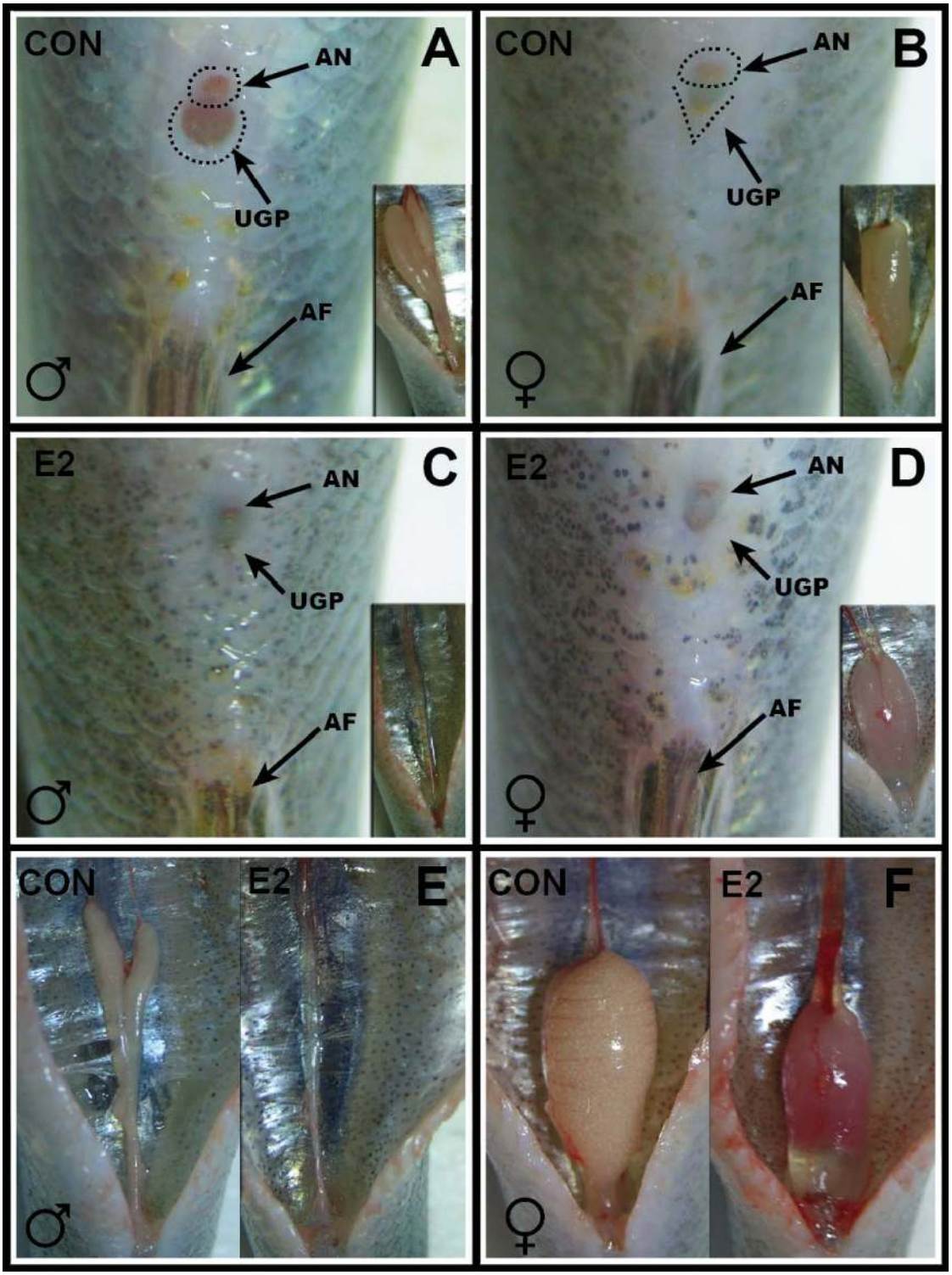

FIGURE 2. External morphology of the urogenital papilla of control and E2-treated male (♂) and female (♀) Yellow Perch. An example of sex confirmation is included in each panel (A–D) as an inlay. Representative examples were all externally identified correctly except the E2-treated male (Choptank and Perquimans strains) in panel C. (**A**) Control male Yellow Perch. (**B**) Control female Yellow Perch. (**C**) E2-treated male Yellow Perch. (**D**) E2-treated female Yellow Perch. (**E**) Internal reproductive organ comparison of control and E2-treated male Yellow Perch. Poor testicular development in the E2-treated males indicates a treatment-induced effect. (**F**) Internal reproductive organ comparison of control and E2-treated female Yellow Perch. It was typical to see hemorrhagic coloration, fluid at the posterior end, and lack of fully formed eggs in the ovaries of E2-treated females. Morphological landmarks are highlighted in bold block letters as follows: AN = anal opening, UGP = urogenital papilla, AF = anal fin. Dashed lines indicate general shape of the UGP in males (panel A) and females (panel B). Of noticeable contrast between each sex is the rounded UGP in males, the V- or U-shaped UGP in females, darker reddish coloration of the UGP in males, and the UGP generally wider than the AN in males and generally narrower than the AN in females. However, E2-treated males externally demonstrated morphological characteristics that more closely resembled those of control females than control males.

TABLE 1. Yellow Perch external sex determination by strain and size. Groups of Yellow Perch from four geographically distinct strains ($n = 1{,}389$) and three distinct size categories were externally scored for sex determination using external morphological criteria. TL range = range of total length in millimeters; mean TL of the group is in parentheses. BW range = range of body weight in grams; mean BW of the group is in parentheses. n = total number of perch in group, (n correct) = total number of perch in group identified correctly, % = percentage [(number of perch identified correctly/total number of perch in identified group) × 100] of perch in group identified correctly, ND = no data.

		Male				Female				Both sexes			
Strain	Size category	TL range (mean)	BW range (mean)	n (n correct)	%	TL range (mean)	BW range (mean)	n (n correct)	%	TL range (mean)	BW range (mean)	n (n correct)	%
Choptank	80–170 mm	105–170 (143.8)	15–74 (40.2)	102 (100)	98.0	81–169 (138.7)	12–93 (39.0)	67 (67)	100.0	81–170 (141.8)	12–93 (39.7)	169 (167)	98.8
	171–210 mm	171–209 (183.1)	56–145 (82.9)	94 (90)	95.7	171–210 (193.0)	63–158 (97.9)	107 (105)	98.1	171–210 (188.4)	56–158 (90.9)	201 (195)	97.0
	211–290 mm	211–285 (234.1)	110–234 (186.0)	181 (179)	98.9	212–266 (240.6)	117–351 (210.3)	59 (59)	100.0	211–285 (235.7)	110–351 (192.0)	240 (238)	99.2
Perquimans	80–170 mm	75–170 (160.7)	40–71 (56.4)	33 (31)	93.9	77–170 (145.2)	57–91 (67.3)	11 (10)	90.9	75–170 (156.8)	40–91 (59.1)	44 (41)	93.2
	171–210 mm	171–210 (186.3)	63–193 (90.3)	140 (130)	92.9	173–210 (192.3)	67–171 (98.3)	103 (100)	97.1	171–210 (188.7)	63–193 (93.4)	247 (230)	93.1
	211–290 mm	212–252 (227.5)	145–263 (196.4)	13 (8)	61.5	211–256 (225.7)	123–320 (190.0)	23 (23)	100.0	211–256 (226.3)	123–320 (192.3)	36 (31)	86.1
Sassafras	80–170 mm	120–170 (156.6)	17–68 (48.7)	77 (76)	98.7	144–170 (158.7)	36–73 (53.0)	44 (44)	100.0	120–170 (157.4)	17–73 (50.3)	121 (120)	99.2
	171–210 mm	171–203 (180.5)	61–105 (76.9)	45 (44)	97.8	172–208 (189.6)	66–132 (97.2)	88 (86)	97.7	171–208 (186.5)	61–132 (90.3)	133 (130)	97.7
	211–290 mm	ND	ND	0 (0)	ND	211–213 (212.0)	136–142 (139.0)	2 (2)	100.0	211–213 (212.0)	136–142 (139.0)	2 (2)	100.0
Winnebago	80–170 mm	ND	ND	0 (0)	ND	ND	ND	0 (0)	ND	ND	ND	0 (0)	ND
	171–210 mm	195–210 (205.1)	81–128 (112.6)	13 (13)	100.0	ND	ND	0 (0)	ND	195–210 (205.1)	81–128 (112.6)	13 (13)	100.0
	211–290 mm	211–237 (219.6)	102–218 (142.2)	41 (38)	92.7	212–270 (244.5)	92–342 (196.9)	146 (146)	100.0	211–270 (239.0)	92–342 (184.9)	187 (184)	98.4
Total		75–285 (197.5)	15–263 (104.4)	739 (709)	95.9	77–270 (192.4)	12–351 (124.1)	650 (642)	98.8	75–285 (195.0)	12–351 (113.5)	1,389 (1,351)	97.3

number of correct external gender identifications and the number of incorrect identifications (97.3% and 2.7%, respectively; binomial proportion test: $Z = 35.23$, $P < 0.0001$), indicating that the technique of external gender identification is very effective in predicting the true gender of Yellow Perch over both sexes, all four strains, and three size categories tested. Despite the single group (Perquimans strain, size category 3) of perch that exhibited lower accuracy in the identification of males only (61.5%), the accuracy of our criteria for determining true gender, as applied to all strains and sizes, was 97.3% for both genders combined (Table 1).

The results of the main effects logistic model showed significant differences in the success of external gender identification between males and females ($P = 0.0028$) for all strains and size categories as well as differences between strains ($P = 0.004$) for both sexes of Yellow Perch and all size categories. No effect of size category on the success of external gender identification was found. Other logistic models showed no significant Strain × Size Category or Strain × True Gender interaction effects. Main effect logistic models were further examined for Strain and True Gender of fish independent of one another. Significant differences for Strain in the success of external gender identification were found for both sexes and all size categories of perch (Wald chi-square = 19.16, df = 3, $P = 0.0003$). The Perquimans strain had statistically lower chances of external gender identification being successful than did the other three strains, which were not different from one another (Table 1). Specifically, odds ratio estimates show that the Choptank strain was 4.2 times as likely as Perquimans strain for external gender identification to be successful (98.3% versus 90.8% across both sexes and all sizes; $P = 0.0003$). Sassafras strain was 4.386 times as likely as Perquimans to be successfully identified externally (98.9% versus 90.8% for both sexes and all sizes; $P = 0.0075$). Winnebago was 4.566 times as likely as Perquimans for external gender identification to be successful (99.2% versus 90.8% for both sexes and all sizes; $P = 0.0149$).

Significant differences for True Gender in the success of external gender identification were found for all strains and size categories of Yellow Perch (Wald chi-square = 9.26, df = 1, $P = 0.0023$), and females were 3.4 times (odds ratio estimate) as likely as males to be successfully identified by external examination ($P = 0.0023$), which corresponds to the reduced frequency of successful identifications (for all strains and size categories) in males (95.9%) versus females (98.8%) (Table 1).

TABLE 2. Yellow Perch external sex determination after treatment with E2, grouped by strain (total $n = 913$). Groups of Yellow Perch were fed either control feed (sprayed with 100% ethanol as the carrier and allowed to evaporate completely) or E2-treated feed (15 mg E2/kg diet, dissolved in the carrier then sprayed on feed and allowed to evaporate completely) for 15 weeks and examined to determine gender using external morphological criteria. TL range = range of total length in millimeters; mean TL of the group is in parentheses. BW range = range of body weight in grams; mean BW of the group is in parentheses. n = total number of perch in group, (n correct) = total number of perch in group identified correctly, % = percentage [(number of perch identified correctly/total number of perch in identified group) $\times$ 100] of perch in group identified correctly.

		Choptank				Perquimans				Both strains			
Treatment	Sex	TL range (mean)	BW range (mean)	n (n correct)	%	TL range (mean)	BW range (mean)	n (n correct)	%	TL range (mean)	BW range (mean)	n (n correct)	%
Control	Male	146–200 (174.1)	38–108 (69.5)	131 (125)	95.4	147–210 (180.6)	40–144 (79.3)	156 (148)	94.9	146–210 (174.1)	38–144 (69.5)	287 (273)	95.1
	Female	151–220 (190.1)	49–140 (93.4)	127 (125)	98.4	164–221 (191.4)	57–146 (95.5)	116 (113)	97.4	151–221 (190.1)	49–146 (93.4)	243 (238)	97.9
	Total	146–220 (182.0)	38–140 (81.3)	258 (250)	96.9	147–221 (185.2)	40–146 (86.2)	272 (261)	96.0	146–221 (182.0)	38–146 (81.3)	530 (511)	96.4
E2	Male	139–197 (171.2)	32–92 (59.7)	97 (58)	59.8	145–199 (177.6)	45–96 (70.2)	106 (59)	55.7	139–199 (171.2)	32–96 (59.7)	203 (117)	57.6
	Female	150–215 (184.6)	37–124 (76.6)	101 (63)	62.4	165–217 (192.3)	52–129 (90.9)	79 (52)	65.8	150–217 (184.6)	37–129 (76.6)	180 (115)	63.9
	Total	139–215 (178.1)	32–124 (68.3)	198 (121)	61.1	145–217 (183.9)	45–129 (79.1)	185 (111)	60.0	139–217 (178.1)	32–129 (68.3)	383 (232)	60.6
Grand total		139–220 (180.3)	32–140 (75.7)	456 (371)	81.4	145–221 (184.7)	40–146 (83.3)	457 (372)	81.4	139–221 (180.3)	32–146 (75.7)	913 (743)	81.4

Analysis of External Gender Identification Criteria for Yellow Perch Treated with E2

Data on the accuracy of external gender identification in treated male and female Yellow Perch were obtained from a total of 913 perch for both strains (Choptank and Perquimans) and treatments (control and E2) (Table 2). The results of the three-factor logistic model showed significant main effect differences in the success of external gender identification between males and females (Wald chi-square = 4.12, df = 1, $P = 0.0424$), as well as main effect differences between control and E2-treated perch (Wald chi-square = 106.57, df = 1, $P < 0.0001$). No effect of strain on external gender identification was found, nor were there any significant interactions (Table 2). Odds ratios showed that control-treated Choptank females were 37.7 times more likely to be successfully identified to gender (98.4% versus 62.4%) compared with E2-treated Choptank females (Table 2). Control Perquimans females were 19.6 times more likely to have gender successfully identified (97.4% versus 65.8%) than were E2-treated Perquimans females. Control-treated Choptank males were 14 times more likely to be successfully identified to gender (95.4% versus 59.8%) than were E2-treated Choptank males (Table 2). Control Perquimans males were 14.7 times more likely to have their gender successfully identified (94.9% versus 55.7%) than were E2-treated Perquimans males (Table 2).

A single factor logistic model with Treatment (control and E2) as the main effect was used to determine the odds ratio probabilities for both strains and gender of Yellow Perch. A significant treatment effect was obtained that matched the three-factor logistic model (Wald chi-square = 125.06, df = 1, $P < 0.0001$), and the odds ratio estimate showed that control-treated fish were 17.5 times more likely to be correctly identified to gender (96.4% versus 60.6%) than were the E2-treated fish (Table 2). A single factor logistic model with Gender (male and female) as the main effect was used to determine whether there was a significant gender effect for both of the strains and treatments tested. No significance was obtained. The odds ratio showed that females are 1.3 times more likely to have gender correctly identified than were males, but there was no significant P-value associated with this odds ratio suggesting that there were no real differences in the accuracies for correct gender identification between males (79.6%) and females (83.5%) for the two strains and treatments tested. The finding of this odds ratio pairwise test from the single factor logistic model for true gender contrasts with the overall three-factor logistic model for the control- and E2-treated perch, which shows a significant effect (Wald chi-square = 4.12, df = 1, $P = 0.0424$) of true gender on accuracy of correct gender identification. Furthermore, this finding contrasts with the original main-effects logistic test showing a significant effect (Wald chi-square = 9.26, df = 1, $P = 0.0023$) of gender on accuracy of correct gender identification for the untreated perch (all strains and sizes) shown in Table 1. These differences are probably attributed to (1) the smaller sample size of the data set used for control and E2-treated fish, and (2) the strong (overall) effect that E2 treatment had on reducing accuracy of gender identification in male and female Yellow Perch (Table 2).

The GSIs for the Choptank strain fish were significantly affected by treatment ($F_{1, 156} = 56.99$, $P < 0.001$) (control or E2) and sex ($F_{1, 156} = 159.06$, $P < 0.001$) (male or female) (two-way ANOVA). The GSI values for control Choptank perch were 1.0 $\pm$ 0.05% ($n = 40$) for females and 0.5 $\pm$ 0.06% ($n = 40$) for males. The GSI values for E2-treated Choptank fish were 0.75 $\pm$ 0.03% ($n = 41$) for females and 0.1 $\pm$ 0.03% ($n = 39$) for males, and these values were significantly

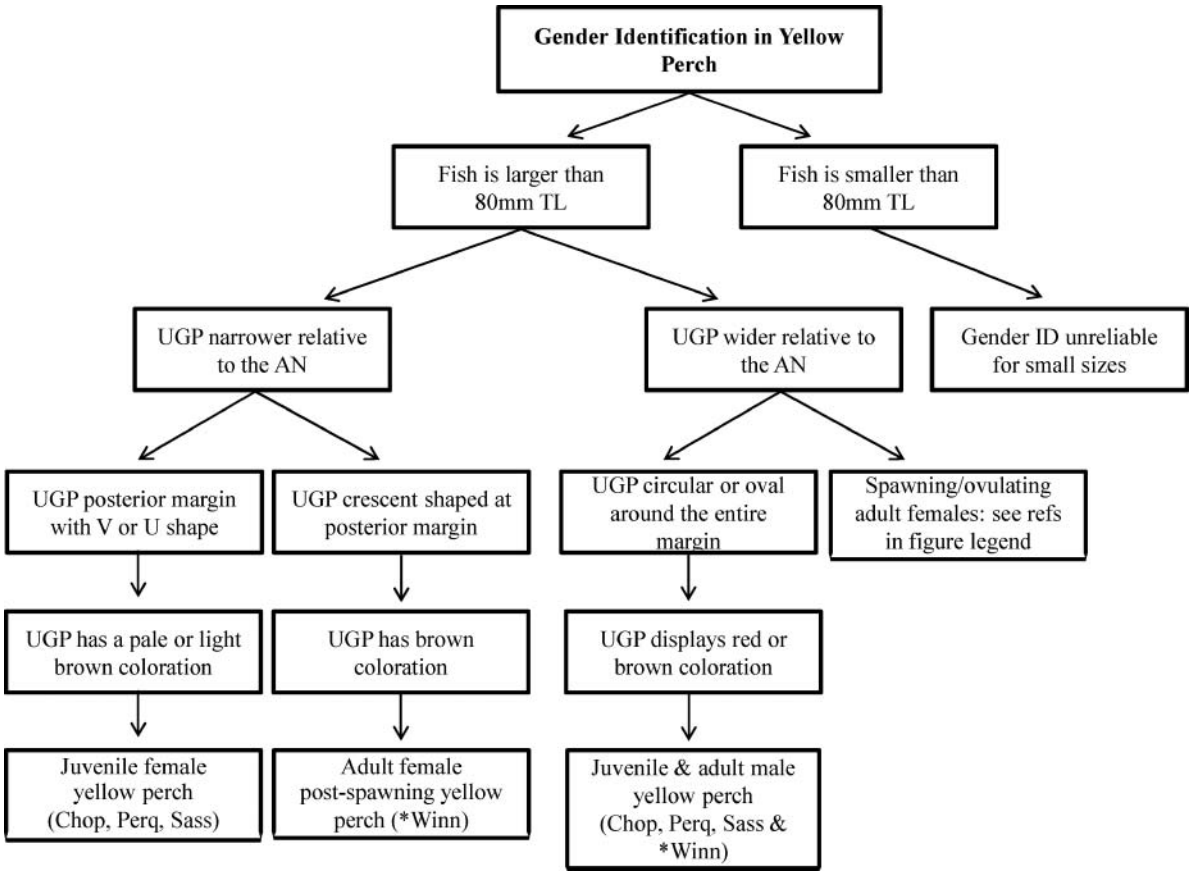

FIGURE 3. Gender identification algorithm for four domesticated geographic strains of Yellow Perch. Abbreviations: AN = anus, UGP = urogenital papilla, Chop = Choptank strain, Perq = Perquimans strain, Sass = Sassafras strain, Winn = Winnebago strain (*Winn indicates males and females had AN swelling that was not present in other strains). See Parker (1942), Hinshaw (2006), and Garling et al. (2007) for images and description of the UGP in spawning and ovulating females.

different from their respective control groups (Tukey–Kramer pairwise comparison: $P < 0.05$). The GSIs for the Perquimans strain fish were significantly affected by treatment ($F_{1,150} = 46.77$, $P < 0.001$) (control or E2) and sex ($F_{1,150} = 365.35$, $P < 0.001$) (male or female) (two-way ANOVA), and GSI values for control Perquimans perch were $0.85 \pm 0.05\%$ ($n = 34$) for females and $0.19 \pm 0.04\%$ ($n = 40$) for males. The GSI values for E2-treated Perquimans fish were $0.58 \pm 0.02\%$ for females and $0.03 \pm 0.002\%$ ($n = 43$) for males, and these values were significantly different from their respective control groups (Tukey–Kramer pairwise comparison: $P < 0.05$).

Yellow Perch Gender Identification Algorithm

We have developed a decision algorithm for identifying gender of Yellow Perch that uses four primary criteria: (1) total length of the perch, (2) width of the UGP relative to the AN, (3) shape of the UGP, and (4) color of the UGP (Figure 3). This algorithm will assist groups working with Yellow Perch to externally identify gender in their perch populations.

DISCUSSION

A rapid method for external identification of gender in Yellow Perch using differences in morphology and appearance of the external UGP has been developed. This method is highly accurate and can be used to sex juvenile and adult Yellow Perch of four domesticated geographic strains. In the female Yellow Perch we examined, the margin of the UGP typically appeared as a U or V shape that was oriented towards the anal fin, was narrower than the margin of the AN, and displayed either a pale or light brown coloration. In male Yellow Perch, the margin of the UGP was typically round or oval in shape and was wider than that of the AN opening, and coloration ranged from pale brown to a darker red. Using these criteria, differences in the appearance of the external morphology of the female and male Yellow Perch UGP can be detected in fish as small as ~85 mm TL and 15 g body weight. Across all strains and sizes of perch tested, these criteria successfully identified the true gender of male and female Yellow Perch with an accuracy of 97%. Using logistic regression analyses, we can confirm that differences in the accuracy of external gender identification do exist between

geographic strains and sex of the perch, and lower accuracy occurs in the Perquimans strain than in others and in males versus females.

Recently, Malison et al. (2011) reported external morphological criteria that enabled them to identify the gender of a single geographic midwestern strain (from Lakes Mendota and Cherokee in Dane County, Wisconsin; Malison and Held 1992) of Yellow Perch within a size range of 80–280 mm TL (weights were not reported). While our criteria are quantitatively more accurate and can be valid in four geographic strains, the differing criteria do converge but with some exceptions. Specifically, Figures 1 and 2 in Malison et al. (2011) show the female UGP as being crescent shaped and occurring with AN swelling. We only observed females with a crescent-shaped UGP in the Winnebago strain, which was not the typical phenotype observed for females from the other geographic strains (Choptank, Perquimans, or Sassafras) of similar size that were examined in this study. Additionally, we observed AN swelling in both males and females of the Winnebago Strain. By contrast, Malison et al. (2011) reported AN swelling in females only. Consequently, we propose that the crescent-shaped UGP and AN swelling (males and females) are either characteristic of Midwestern strains of Yellow Perch or may be characteristic of sexually mature perch that are in postspawning condition, which was noted for perch of the Winnebago strain in our study. Our findings underscore the differences in UGP morphology between geographic strains and sexual maturity or reproductive history in Yellow Perch. Despite some remaining questions regarding the appearance of the UGP in sexually mature female Yellow Perch across various geographic strains, the appearance of the UGP in spawning females (not shown) is quite distinctive and has been previously documented (Parker 1942; Hinshaw 2006; Garling et al. 2007).

Given all the morphological characteristics identified as necessary to successfully determine gender in Yellow Perch, we developed a decision algorithm (Figure 3). Using the principal features of UGP width relative to AN, UGP color, and UGP shape, we propose a logical framework to enable identification of gender in juvenile and adult male and female Yellow Perch. Given that our criteria have only been developed and validated using four geographic strains of Yellow Perch that were greater than 85 mm TL, we provide the caveat that these identifying characters will need to be tested in perch of varying maturity and from other geographic strains.

To experimentally validate our observational criteria for external identification of gender in Yellow Perch, we treated juvenile Yellow Perch with dietary E2 or a control diet. In both geographic strains treated and examined (Choptank and Perquimans), we found that accuracy of external gender identification was significantly reduced in E2-treated males (57.6%) and females (63.9%) when compared with untreated males (95.1%) and females (97.9%). For E2-treated males, we observed that the margin of the UGP tended to have a U or V shape (indicative of females), lacked distinctive coloration, and appeared equivalent in size to the AN margin, which resulted in the low (57.6%) accuracy for identifying males. A difference in appearance of the UGP in E2-treated and control male Yellow Perch suggests that dietary E2 treatment feminizes the male secondary sexual traits that are characteristic for the UGP of this gender. Interestingly, the region of the UGP in E2-treated females routinely appeared wider than the AN margin and lacked coloration, which caused convergence in gender-specific UGP criteria. Accordingly, convergence of these criteria resulted in a lowered accuracy (63.9%) for identifying the true gender (via external identification) of E2-treated females. Given the difficulties with correctly identifying true gender in E2-treated Yellow Perch and the reduced GSI in these fish, it is likely that E2 treatment delays (via negative feedback) development of primary and secondary sexual characteristics in female Yellow Perch.

It is well known that steroids and steroid antagonists and agonists can affect the reproductive development (primary and secondary sexual characteristics) of gonochoristic finfishes (Sumpter 1997). In this vein, disruption by E2 treatment of the secondary sexual characteristics that enable external identification of gender in Yellow Perch via UGP morphology are consistent with findings of estrogen-induced feminization in other North American species, including Fathead Minnow, Threespine Stickleback *Gasterosteus aculeatus*, and Rainbow Darter *Etheostoma caeruleum* (Hahlbeck et al. 2004; Parrott and Blunt 2005; Elias et al. 2007). Furthermore, the reduced GSI of E2-treated males and females (compared with controls) is consistent with reported results of E2-dependent delayed gonadal development in other finfish species (Scholz and Klüver 2009) and with previous work in Yellow Perch (Malison et al. 1986). While these findings are new for Yellow Perch, the effects of dietary E2 on male and female UGP morphology serves as independent experimental validation of the accuracy of our criteria for using external UGP morphology to identify gender in Yellow Perch. Additionally, the apparent uncoupling of external UGP morphology from actual gender in E2-treated Yellow Perch could indicate exposure to estrogenic substances in settings where endocrine-disrupting chemicals can have an influence.

Access to a robust and cost-effective method for identifying and sorting sexes can have foreseeable benefits to Yellow Perch aquaculture producers and researchers. For aquaculture, the ability to easily recognize gender can enable new producers to collect (without harm to the animal) founder broodstocks from wild populations and enable established producers to manage broodstocks before and during breeding. With respect to aquaculture research, our group is developing genetically improved Yellow Perch broodstocks that exhibit faster growth. In the absence of precise methods for gender identification, it has been necessary to use relaxed selection criteria that require the retention of the upper 40% of performers to allow for a sufficient number of smaller-sized males to develop the next generation of select perch broodstocks (Grzybowski et al. 2010; Rosauer et al. 2011). Now that a more precise method exists for the identification of gender in Yellow Perch, it is possible to implement a bimodal selection regime that focuses on the upper

10–15% of female and male performers, which can further accelerate genetic gain for growth in these domesticated perch strains. For researchers, the ability to easily identify gender in Yellow Perch can enable much-needed, multitank, replicate experiments aimed at studying gender-specific differences in behavior, nutrition, immunology, physiology, and toxicology in this species.

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Temperature Modulation of Growth and Physiology of Juvenile Guadalupe Bass

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NOTE

Temperature Modulation of Growth and Physiology of Juvenile Guadalupe Bass

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Abstract

Fingerling Guadalupe Bass *Micropterus treculii* were cultured in 18, 21, 24, 27, or 30°C water for 6 weeks. Temperature affected the growth of the Guadalupe Bass when it was measured by increases in TL but not when measured by increase in mass. The optimal temperature for growth of Guadalupe Bass was 27–28°C. Condition factor increased linearly with temperature, indicating that the fish were plumper at warmer temperatures. The mean hematocrit was 37 and not significantly affected by temperature, although a trend of increasing hematocrit with increasing temperature was apparent. The liver index varied significantly with temperature, and the maximum index was at 24°C. The results of this study provide basic thermal information and some base line hematological values for hatchery production and management of Guadalupe Bass.

The Guadalupe Bass *Micropterus treculii* is a stream-dwelling black bass native to the Edwards Plateau region of southcentral Texas. It is a species of special concern in Texas due to limited natural distribution and genetic introgression with introduced black bass, particularly the Smallmouth Bass *Micropterus dolomieu* (Whitmore 1983; Warren et al. 2000; Littrell et al. 2007; Bonner and Bean 2008). The stocking of hatchery-produced Guadalupe Bass fingerlings is a part of the recovery effort for this species. Beyond some information on spawning and hatching conditions (Carmichael and Williamson 1986) and toxicity of nitrogenous wastes (Tomasso and Carmichael 1986), little is known of the environmental requirements of Guadalupe Bass. To develop a broader base of environmental information for hatchery managers, this study determined the effects of temperature on growth and body condition of Guadalupe Bass. We also observed the effect of rearing temperature on hematocrit, liver index, and body condition, which are indicators of general fish health (Barton et al. 2002).

METHODS

Approximately 400 Guadalupe Bass fingerlings (59–108 mm TL) were obtained from the A.E. Wood Fish Hatchery (Texas Parks and Wildlife Department) in May 2011. The fish were trained to consume prepared feed by generally following Carmichael and Williamson (1986). Briefly, fish were held in indoor fiberglass tanks receiving constantly flowing spring water (22°C). Initially, fish were offered a mixture of dried, crushed krill and silverside and a prepared pellet (BioDiet Grower©; Bio-Oregon, Longview, Washington: 43% protein, 14% oil, 1% fiber). During training, feed was offered at least 10 times/d, and the proportion of prepared pellet was increased as fish began to feed consistently. During training, approximately 5% of the fish died. Fish were transferred to experimental systems after they had been readily taking prepared pellets for at least 2 weeks.

Thirty individuals were stocked into each of six 300-L water recirculating systems (Living Streams, Frigid Units, Toledo, Ohio), and then water temperatures were adjusted at a rate of approximately ±0.5°C per day (starting water temperature was approximately 22°C) until temperatures in Living Streams were nominal 18, 21, 24 (two Living Streams), 27, or 30°C with a thermostat resolution of ±1.0°C. After all Living Streams reached their nominal temperatures, fish in each were anesthetized with tricaine methanesulfonate at 2 g/L of water, weighed individually, and returned to the Living Stream from which they were collected. Averaged fish weight was 4.7 g (SD, 1.47). Mean fish weight varied significantly (analysis of variance [ANOVA]; $P < 0.001$) among Living Streams; means ranged from 3.9 to 5.3 g, being higher in the warmer tanks, which was due to thermally controlled differential growth during the acclimation period. After weighing, fish were exposed to experimental temperatures for 6 weeks and were fed to satiation once per day. During the

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6-week study, 6% of the fish died: seven fish in the 18°C treatment and three fish in the 21°C treatment.

Temperature and dissolved oxygen were monitored daily using a YSI 85-10 m (YSI, Inc., Yellow Springs, Ohio). The mean temperature for each Living Stream was the same as the nominal temperature with a maximum coefficient of variation of 2.1%. Mean oxygen concentrations ranged from 87% of saturation in the highest temperature treatment to 96% of saturation in the lowest temperature treatment, the maximum coefficient of variation being 3.8%. Total ammonia nitrogen (TAN) was measured by direct Nesslerization (APHA 1989) three times during the experimental period and fell below detectable limits (0.01 mg/L) each time. The pH was measured using an Accumet 15 pH meter (Fisher Scientific, Pittsburgh, Pennsylvania) three times during the experimental period and ranged from 8.5 to 8.6. Artificial sea salt was added to the culture water to maintain adequate ions and buffering capacity. It was measured daily using the YSI 85-10 and was 1.2 (±0.2) during the course of the study.

After a 6-week exposure to experimental temperatures, fish were anesthetized as described previously, weighed and measured (TL), and liver and blood samples were collected. For both length and mass, growth was calculated as percent gain. Blood was collected into a heparinized capillary tube from the hemal arch after severing the caudal peduncle. Capillary tubes were immediately centrifuged and hematocrit determined. After fish were weighed, livers were removed and weighed separately. Liver index was expressed as a percent of body mass represented by the liver. Fulton's condition factor (K) was calculated according to Anderson and Neumann (1996).

Regression analysis was applied to all data sets, and significance was set at $\alpha = 0.05$. Based on previous experience defining thermal–growth relationships in fishes (e.g., Atwood et al. 2003; Sullivan and Tomasso 2010), we fitted a second-degree polynomial to the growth data. Mean responses of individuals in each Living Stream were entered into analyses, resulting in an N of six in each analysis.

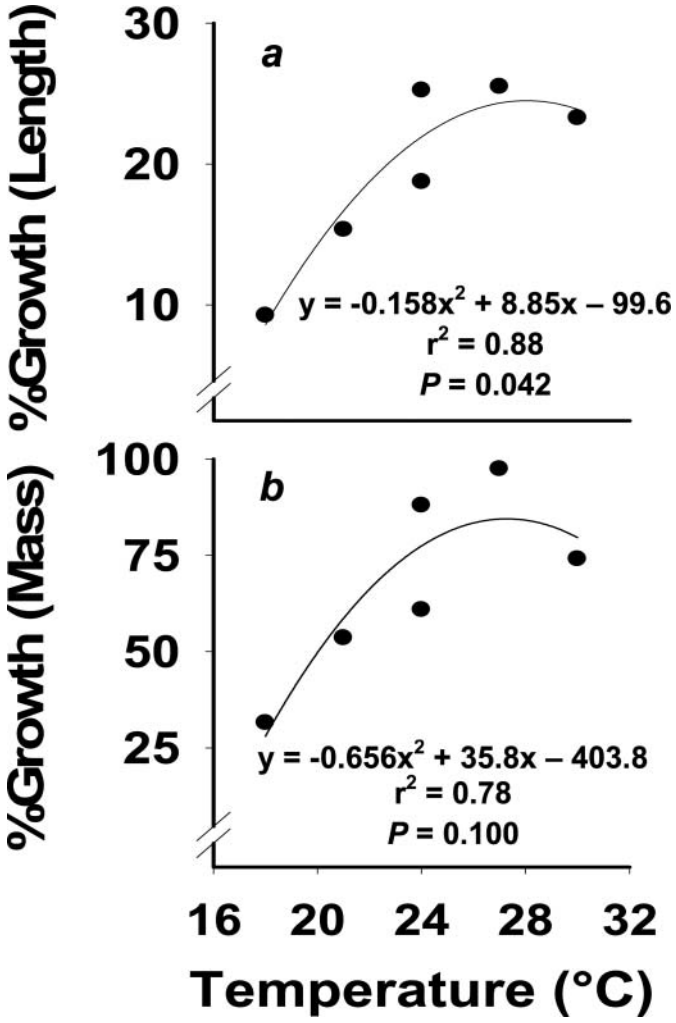

FIGURE 1. The effect of temperature on growth **(a)** TL and **(b)** wet weight of Guadalupe Bass after 6 weeks of exposure to five discrete experimental temperatures.

RESULTS AND DISCUSSION

Temperature affected the growth of the Guadalupe Bass as measured by increases in TL (Figure 1a). However, a significant treatment effect was not observed when growth was measured by mass (Figure 1b). The polynomials used to describe the temperature and growth relationship described 88% of the variability in the length observations and 78% of the mass observations. The optimal temperature for growth of Guadalupe Bass was estimated to be 28°C for length and 27°C for mass. Decreases in growth rates were observed at 30°C, indicating that the bass were in a thermal environment that was beyond their maximum metabolic scope (Neill and Bryan 1991). Estimated optimal growth temperature for Guadalupe Bass was similar to that reported for Largemouth Bass *Micropterus salmoides* (26–30°C; Jobling 1981) but differed from that of Smallmouth Bass (22°C; Whitledge et al. 2006). The low N (6) may be responsible for the failure to observe a significant treatment effect for mass and creates some uncertainty with regard to our estimate of optimal temperature.

Condition factor increased linearly with temperature (Figure 2), indicating that the fish were plumper at warmer temperatures. This is similar to results of Largemouth Bass where significantly greater condition factors were observed at 26°C and 32°C versus 20°C (Tidwell et al. 2003). To try and understand why K was different across treatments, we plotted mass versus length for all fish at the end of the study. The relationship was continuous ($\text{mass} = 0.000009 \cdot \text{length}^{3.004}$) and consistent ($r^2 = 0.95$), indicating that warmer (faster-growing) fish and cooler (slower-growing) fish will have the same K at a given mass. Hence, the differences we detected in K may be attributed to normal allometric growth rather than a thermal effect.

The mean hematocrit was 37 and not significantly affected by temperature, although a trend of increasing hematocrit with

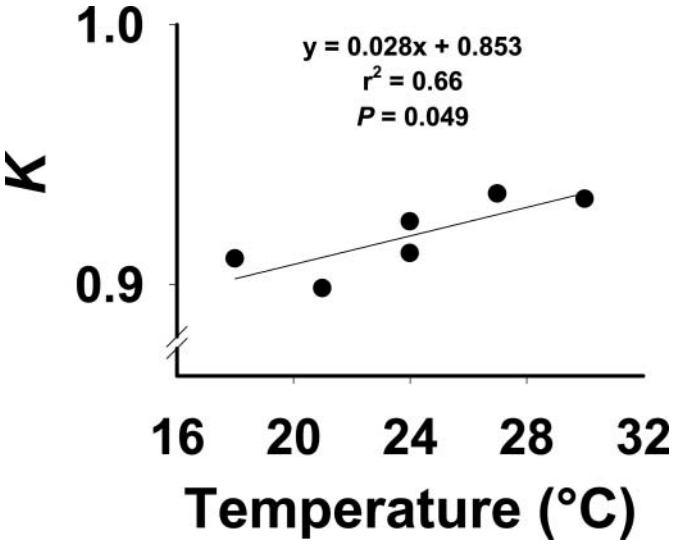

FIGURE 2. The effect of temperature on body condition (K) of Guadalupe Bass after 6 weeks of exposure to five discrete experimental temperatures.

increasing temperature was apparent. As temperature increases, oxygen demand by fish will increase while the solubility of oxygen in water and plasma decreases. Any increase in hematocrit under these conditions is probably directed toward increasing the ability of the animal to deliver oxygen to the tissues (reviewed in Nikinmaa 2006). However, diet and activity may also play a role in variability in hematocrit (Denton and Yousef 1975).

The liver index varied significantly with temperature with a maximum index at 24°C (Figure 3b). During periods of surplus energy intake, fish store energy in muscle and liver leading to changes in liver size relative to fish body size (Busacker et al. 1990). As stored energy is used, liver size decreases. In this study, relative liver size increased in fish reared at lower temperatures, peaked, and declined in fish reared at the higher temperatures. Relative liver size peaked at temperatures 3–4°C below the temperature for maximum growth. Perhaps the earlier decline in relative liver size represents a period when energy stored as liver fat and glycogen supplemented daily rations to support thermally controlled increases of metabolic rate and growth. The temperature at which fish growth decreases represents the point where metabolic scope is decreasing due to the rapid increase of standard metabolism (Neill and Bryan 1991). Similar peaks in relative liver size have been reported for Largemouth Bass (Heidinger and Crawford 1977; Brown and Murphy 2004).

Current recovery efforts are focused on the South Llano River (Texas) Guadalupe Bass population, which is 3% hybridized with Smallmouth Bass (Bean et al. 2013). Recovery efforts include capturing of broodstock from the South Llano River and subsequent back-stocking of hatchery-produced fingerlings. The results of this study provide basic thermal information for use during hatchery production of Guadalupe Bass and management of populations in the field.

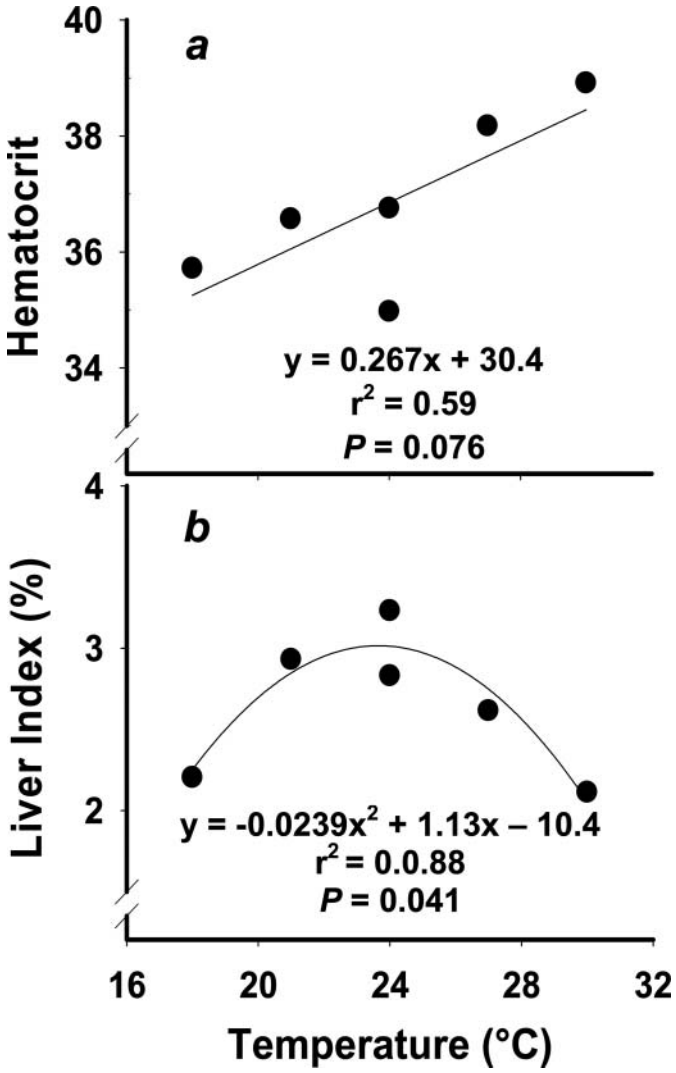

FIGURE 3. The effect of temperature on **(a)** hematocrit and **(b)** liver index in Guadalupe Bass after 6 weeks of exposure to five discrete experimental temperatures.

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Use of Aluminum Sulfate to Reduce pH and Increase Survival in Fingerling Striped Bass Production Ponds Fertilized with Nitrogen and Phosphorus

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ARTICLE

Use of Aluminum Sulfate to Reduce pH and Increase Survival in Fingerling Striped Bass Production Ponds Fertilized with Nitrogen and Phosphorus

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Abstract

Previous studies revealed inorganic nitrogen (300 μg/L) and phosphorus (30 μg/L) additions thrice weekly is effective in controlling the density of *Prymnesium parvum*, a flagellated haptophytic golden alga with ichthyotoxicity in fish culture ponds. However, increased pH resulting from this treatment is also toxic to fry and fingerlings of Striped Bass *Morone saxatilis*. We evaluated the efficacy of aluminum sulfate (alum) in lowering pH in ponds fertilized for *P. parvum* control. The effects of alum on dissolved N and P concentrations, as well as the efficacy of this fertilization treatment for *P. parvum* control, were also examined. Striped Bass fingerlings (27.93 mm total length) were raised for 42 d in 10 plastic-lined ponds (0.1 ha) fertilized for *P. parvum* control. Five ponds received alum applications at a rate of 1 mg/L for each 1 mg/L phenolphthalein alkalinity when afternoon pH was ≥9.0. The remaining five ponds served as controls and received no alum. Among alum ponds, morning pH was reduced by an average of 1.2 units and afternoon pH by an average of 0.8 units compared with controls. However, the alum treatment did not maintain afternoon pH below 9.0. Alum reduced concentrations of total P, soluble reactive P, total Kjeldahl N, nitrite + nitrate N, and total N but not ammonium N. The fertilization regimen was not as aggressive in controlling *P. parvum* in alum ponds as it was in control ponds, which may be attributed to N and P removal by alum. Striped Bass production was poor in all ponds, probably because of high pH. Future studies should investigate higher alum treatment rates (e.g., phenolphthalein alkalinity : alum = 1:1.5 or 1:2) along with modification of the timing or frequency of fertilization.

The flagellated haptophytic golden alga, *Prymnesium parvum*, causes large losses of fish in brackish aquaculture ponds when cell densities and ichthyotoxin concentrations are not controlled (Tal and Shelubsky 1952; Shilo Shelubsky and Shilo 1953; Guo et al. 1996). In 2001 *P. parvum* was first identified as the cause of fish mortalities at the Texas Parks and Wildlife

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Department (TPWD) Dundee Fish Hatchery (DFH) and in 2002 at Possum Kingdom Fish Hatchery. Since 2001, TPWD has investigated methods for controlling *P. parvum* cell densities and attendant ichthyotoxicity (Dorzab and Barkoh 2005; Smith 2005a, b; Barkoh et al. 2003, 2004, 2008, 2011; Kurten et al. 2007, 2010, 2011). Current control strategies include the use of ammonium sulfate, $(NH_4)_2SO_4$, or a copper-based algaecide (Cutrine-Plus; Applied Biochemists, Alpharetta, Georgia) to reduce cell densities, and potassium permanganate ($KMnO_4$) has been used to mitigate ichthyotoxicity (Barkoh and Fries 2005; Barkoh et al. 2010). These strategies have been relatively successful at DFH and Possum Kingdom Fish Hatch (Kurten et al. 2010); however, there are considerable risks associated with using these approaches in pond culture of sensitive fish such as fry and fingerlings of Striped Bass *Morone saxatilis*.

Several factors contribute to making ammonium sulfate or Cutrine-Plus use in fingerling production ponds tedious or risky. Effective use of ammonium sulfate to lyse *P. parvum* cells requires knowledge of current cell densities, pond water conductivity, pH, temperature, and total ammonia concentration to determine if treatment is warranted and to calculate the appropriate un-ionized ammonia (NH_3) application rate (Shilo Shelubsky and Shilo 1953; Barkoh et al. 2003, 2004, 2010). The ability to anticipate future temperature and pH dynamics in ponds is also important because sensitive fish species can be vulnerable to elevated NH_3 (Bergerhouse 1994; Oppenborn and Goudie 1993; Ashe et al. 1996; Harcke and Daniels 1999; Barkoh et al. 2004) associated with increased temperature and pH (Emerson et al. 1975). The range of NH_3 concentrations that effectively lyses *P. parvum* cells without also causing significant fish mortality is narrow (0.14–0.25 mg/L; Barkoh et al. 2004, 2010) and can change rapidly when weather changes pond pH and water temperatures. In early spring, cool water temperatures and low pH can make target NH_3 concentrations that lyse *P. parvum* cells ($\geq$0.14 mg/L; Barkoh et al. 2003, 2010) difficult to achieve. Cultine-Plus use in phase-1 (35–40 mm TL) Striped Bass fingerling production ponds is inappropriate because it also kills phytoplankton and can be toxic to zooplankton and aquatic insects (McKnight et al. 1983; Welch et al. 1990; Guo et al. 1996; Irwin et al. 1997), thereby disrupting the pond food web that supports fingerling production. In addition, using ammonium sulfate or Cultine-Plus is labor-intensive and costly.

We have investigated inorganic fertilization of aquaculture ponds as an alternative to current *P. parvum* control treatments since 2001 (Barkoh and Fries 2005). We have not determined whether the mechanism of *P. parvum* control is through reduction in allelopathy or through competitive exclusion by other algae, or a combination of both, but we have found that inorganic fertilization (N at 300 μg/L plus P at 30 μg/L of water) is a viable method for eliminating *P. parvum* from ponds (Kurten et al. 2007). However, elevated pH and NH_3 were two issues that emerged with the development of this fertilization treatment. We have resolved the high NH_3 problem by using nitrate (NO_3) instead of ammonium (NH_4) as the source of inorganic nitrogen (N; Kurten et al. 2010), but we were unable to resolve the high pH issue with cottonseed meal treatments (Kurten et al. 2011). As a result, alternatives needed investigation.

Alum (aluminum sulfate; $Al_2(SO_4)_3 \cdot 14H_2O$) is acidic in water and can reduce total alkalinity and pH by neutralizing carbonate and bicarbonate compounds, with greater efficiency in reducing pH when applied to water with low initial total alkalinity (Boyd 1979a, 1990; Wilkinson 2002). Alum treatments of 15–25 mg/L of water have been reported to lower pH by 0.4–1.5 units in 48 h (Boyd 1979a). Mandal and Boyd (1980) used alum to reduce pH by 0.3 units in 8 and by 0.8 units in16 d, whereas Masuda and Boyd (1994) applied alum at 20 mg/L to lower pH by 0.8. These results suggest alum may be useful for controlling pH in Striped Bass production ponds where the inorganic fertilization treatment is applied. However, a major concern with using alum in fertilized ponds, such as in this study, is that alum precipitates phosphorus as insoluble aluminum phosphate (Boyd 1979a; Masuda and Boyd 1994; Wilkinson 2002), making it unavailable for phytoplankton growth. Poor phytoplankton growth can result in inadequate zooplankton to support successful Striped Bass fingerling production (Anderson 1994a; Westers 2001). We investigated the efficacy of alum in maintaining pH at <9 in ponds subjected to our inorganic fertilization regimen for *P. parvum* control (Kurten et al. 2007, 2010). We also examined the effect of alum on P and N and on the effectiveness of the inorganic fertilization regimen in controlling *P. parvum*.

METHODS

Pond filling, fertilization, and alum treatment.—This study was conducted in 10 plastic-lined ponds (0.1 ha) at the DFH, Archer County, Texas, from 7 April through 31 May 2008 during phase 1 (35–40 mm) Striped Bass fingerling production. Ponds were filled 2 weeks (day −14) before fry stocking (day 0) with water from Lake Diversion, the water source to the hatchery. The water quality characteristics (in mg/L) were as follows: total suspended solids = 19, total dissolved solids = 2,923, Cl = 1,031, SO_4 = 654, Ca = 173, Mg = 51, Na = 550, K = 14, and alkalinity = 99. Ponds were randomly assigned to control and treatment groups of five replicates each. Control ponds received no alum treatments, whereas treatment ponds received alum when afternoon pH was 9.0 or higher. Alum treatment concentrations were estimated from phenolphthalein alkalinities, which were determined on approximately 300-mL samples of pond water via the sulfuric acid titration procedure in Standard Methods (2320 B, APHA 1998; Boyd 1979b). Water samples were collected from ponds in white, opaque, 1-L polyethylene bottles from depths of 25–30 cm. Titrations were completed within 25 min after sample collection. Alum treatments were applied at a 1:1 ratio (i.e., for every 1 mg/L phenolphthalein alkalinity, alum at 1 mg/L was applied to the pond; Boyd 1990). Alum was applied by broadcasting onto the pond water surface on the windward side of the pond and allowing wind action to disperse it across the pond.

All ponds were subjected to the same inorganic and organic fertilization regimens. Inorganic fertilization consisted of three applications per week of N at 300 μg/L plus P at 30 μg/L beginning on day −14 and ending on day 39 (day 0 was day of fish stocking), 3 d before harvest of fingerlings. Potassium nitrate (KNO_3; N = 13.5%, P = 0%, K = 46.2%) was the sources of inorganic N, and phosphoric acid (H_3PO_4; N = 0%, P = 54%, K = 0%) was the sources of inorganic P. Organic fertilization followed a standard TPWD regimen of three applications of cottonseed meal during the production season: 170.3 kg/ha during pond filling (day −10), 56.8 kg/ha on day 1, and 56.8 kg/ha on day 8.

Water quality.—Water temperature, pH, and dissolved oxygen (DO) concentrations were measured twice daily (0700 and 1500 hours) with a YSI 650 MDS handheld meter fitted with a YSI 600 XL multiprobe sonde (Yellow Springs Instruments, Yellow Springs, Ohio). Water samples were taken twice a week for nutrient analysis from 25 to 30 cm below the pond water surface using opaque white (1 L; for N) and brown (250 mL; for P) polyethylene bottles. Water samples were packed on ice and shipped overnight to the TPWD Environmental Contaminants Laboratory (San Marcos, Texas) for analysis. Total P (TP), orthophosphate-P (PO_4-P), total Kjeldahl N (TKN), nitrite plus nitrate-N (NO_{2+3}-N), and ammonium-N (NH_4-N) were sampled each Tuesday and Friday after pond filling. These analyses were performed using USEPA-approved methods (USEPA 1983) or standard methods (APHA 1998). Methods and detection limits were as follows: TP (USEPA 365.4; P = 22 μg/L), PO_4-P (USEPA 365.3; P = 1.7 μg/L), TKN (USEPA 351.2; N = 83 μg/L), NO_{2+3}-N (USEPA 353.2; N = 7 μg/L), and NH_3-N (USEPA 350.1; N = 19 μg/L). Inorganic N was estimated as the sum of NO_{2+3}-N and NH_3-N, organic N by subtracting NH_3-N from TKN, and organic P by subtraction PO_4-P from TP (USEPA 1983; APHA 1998).

Cell density and toxicity.—Cell counts of *Prymnesium parvum* and ichthyotoxicity were monitored twice weekly using standard TPWD fish hatchery procedures (Southard and Fries 2005). Cell densities were estimated by examining 10-μL fresh unfixed water samples from ponds with a hemacytometer at 400 × magnification. Ichthyotoxicity bioassays were performed using Fathead Minnow *Pimephales promelas* fry as test animals at 28°C. Individual bioassays used water collected from each pond, and four test animals each were exposed to 100 mL undiluted water, 100 mL undiluted water plus 2 mL cofactor, or water diluted by 1/5 with *P. parvum*-free water plus 2 mL cofactor. Four fish were also placed in undiluted *P. parvum*-free water for control. The cofactor solution consisted of 0.003 M 3,3′-iminobispropylamine and 0.02 M tris buffer (pH, 9.0), which functioned to increase the toxicity of *P. parvum* ichthyotoxin, allowing the otherwise sublethal levels to be detectable. Mortalities of test animals were determined after 2 h, and toxicity in terms of ichthyotoxicity units (ITU), was determined as follows: mortality in undiluted water indicated a high level of toxicity (25 ITU); mortality in 1/5 water dilution plus cofactor indicated moderate toxicity (5 ITU), mortality in undiluted water plus cofactor indicated low toxicity (1 ITU), and zero mortality in all bioassays meant no toxicity (0 ITU).

Fish production.—At day 14, ponds were stocked at approximately 500,000 fry/ha (500,010–504,720 fry/ha) with 4-d-old Striped Bass fry (mean total length, 6 mm) hatched at DFH. Fry were counted into 57-L vats (1 vat per pond) with a Jensorter fry counter (model FC2; Jensorter, LLC; Bend, Oregon). Fry were acclimated to pond water temperatures (19.45–19.67°C) and pH (8.20–8.74) by using an air-lift pump system to exchange the water in each vat at a rate of 1 L/min for 1 h. Fry were stocked between 2000 and 2200 hours. Fish were offered high protein salmon feed (50% protein; Nelson and Sons, Inc., Murray, Utah) at 7.5–10 kg/ha per day in two rations beginning 14 d after stocking and continuing for the next 26 d (i.e., through 40 d after stocking). Feed particle size was increased from numbers 00 to 1 to 2 as the fish grew. Fish were harvested 42 d after stocking. At harvest, ponds were drained to allow fish to collect in the harvest basins from where a sample of 100–200 fingerlings was collected for each pond. These fish were counted and weighed to calculate number of fish per kilogram, then a subsample of 40 fish were individually measured for total length. All fish from each pond were harvested and weighed. Total number of fingerlings from each pond was estimated from total weight and fish/ per kilogram. Fish survival (percent return) was calculated for each pond.

Data analysis.—All data, except fish production data, were analyzed with PROC MIXED (SAS 2002). Because these data were collected repeatedly from each pond, we modeled the data assuming a repeated measures construct with ponds as subjects. We tested a variety of covariance constructs (none, variance component, compound symmetry, first-order autoregressive, and spatial power structure) until we found the one that provided the best fit to the data for each response variable. Akaike's information criterion and the null model likelihood ratio test were used to determine the model of best fit for the data (Littell et al. 2000). We tested the effects of treatment, sampling date, and the treatment × sampling date interaction on each variable. *Prymnesium parvum* cell density data were square-root ($X + 0.5$)-transformed and all nutrients data, except NH_3-N, were $\log_{10}(X + 1)$-transformed before analysis due to skewness and zeros in the data. Fish production data were analyzed with a *t*-test. For all analyses, differences were considered significant at $\alpha = 0.05$.

RESULTS

Alum Treatment

The pH criterion (pH ≥ 9) for alum treatment was triggered beginning on day -6, and alum treatments were applied 20–35 times or at 1-d to 5-d intervals (mean = 2.5-d) for 41 to 45 d (mean = 43 d). Mean phenolphthalein alkalinity was 6.73 mg/L in alum ponds, and the quantities of alum required for treatments averaged 7.75 kg or 7.23 mg/L per treatment (Table 1). Phenolphthalein alkalinity declined over time, and the steepness of the trend lines differed among ponds (Figure 1).

TABLE 1. Mean ± SD values of pH, alkalinity, alum, and application frequency for ponds fertilized with N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when afternoon pH was ≥9.0 at the Dundee Fish Hatchery, Texas, in spring 2008. Values in parentheses are the ranges.

Pond	Afternoon pH	Phenolphthalein alkalinity (mg/L)	Alum (mg/L)	Alum (kg)	Frequency
8	8.8 ± 0.5 (7.4–9.6)	9.05 ± 4.20 (3.85–18.15)	9.73 ± 4.50 (4.10–19.9)	10.44 ± 4.89 (4.4–21.36)	20
9	8.9 ± 0.4 (7.8–9.6)	6.89 ± 3.30 (1.65–14.85)	7.40 ± 3.5 (1.80–15.9)	7.94 ± 3.79 (1.93–17.06)	28
12	9.0 ± 0.5 (7.9–9.9)	6.23 ± 2.70 (2.20–15.40)	6.66 ± 3.76 (2.40–26.50)	7.15 ± 4.03 (2.57–15.13)	30
13	9.0 ± 0.4 (7.9–9.7)	6.23 ± 2.70 (2.75–11.60)	6.69 ± 2.90 (3.00–12.40)	7.18 ± 3.11 (3.22–13.31)	33
15	9.1 ± 0.5 (7.9–9.8)	6.21 ± 3.00 (3.30–12.10)	6.65 ± 3.20 (3.50–13.60)	7.13 ± 3.40 (3.76–14.59)	35
All	9.0 ± 0.5 (7.4–9.9)	6.73 ± 3.37 (1.65–18.15)	7.23 ± 3.63 (1.80–19.90)	7.75 ± 3.90 (1.93–21.36)	29.2 ± 5.8 (20–35)

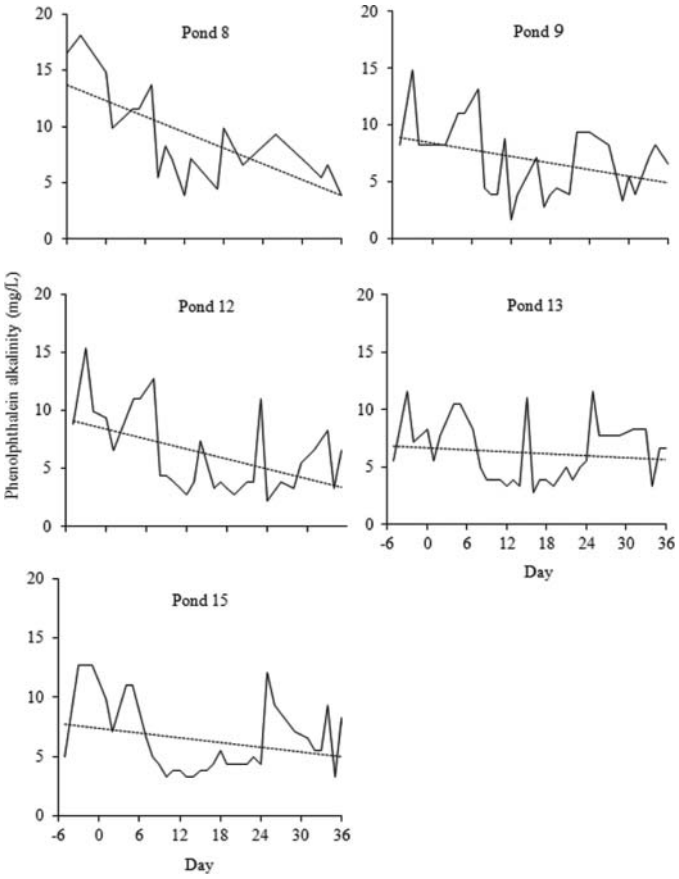

FIGURE 1. Trends of mean phenolphthalein alkalinity equivalents of alum applied to ponds fertilized with N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when pH was ≥9.0 at the Dundee Fish Hatchery in spring 2008. Dotted lines are linear trends. Day 0 is the date of treatment.

Water Quality

Mean morning or afternoon pH was significantly lower in alum ponds than in control ponds on most sampling days and over the course of the study (Figure 2; Table 2). Mean morning pH for alum ponds was 8.2 compared with 9.4 for control ponds (13% decline), and mean afternoon pH for alum ponds was 9.0 compared to 9.8 for control ponds (8% decline). After about day −5, morning pH in the alum ponds was below the threshold (pH 9) on all but 1 d, whereas afternoon pH exceeded the threshold on most days (Figure 2). By day 10, when stocked fish were 14-d old, mean morning pH was 8.5 (8.1–9.0) for alum and 9.8 (9.4–10.2) for control ponds, and mean afternoon pH was 9.2 (8.8–9.5) for alum and 10.1 (9.7–10.5) for control ponds.

Mean DO concentrations differed statistically between alum and control ponds (Table 2). Similarly, mean DO concentrations significantly differed between alum treatment and control over time, but the pattern was inconsistent, being higher in alum than control ponds on some days and lower on other days. Concentrations of morning DO were lower and below 5 mg/L in control ponds on a few sampling days toward the end of the study. Pond water temperatures were reduced from 22°C to 16°C by a cold front shortly after pond filling (day −12). Subsequently, water temperatures rose gradually to approximately 19°C on the day of fry stocking and to a maximum of 31°C on day 37. The difference in morning or afternoon water temperature was not statistically significant between alum and control ponds (Table 2).

Nutrients

Mean concentrations of NO_{2+3}-N and TKN were significantly lower in alum ponds than in control ponds and varied over time (Table 2; Figure 3). Beginning on day 5, mean

TABLE 2. *P*-values for model effects of repeated measures analysis of variance and mean values of variables measured in ponds fertilized with N at 300 μg/L and P at 30 μg/L three times weekly (control) or N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when afternoon pH was ≥9.0 (alum) at the Dundee Fish Hatchery in spring 2008. Values for organic and inorganic N and P were derived and not analyzed using ANOVA. Differences were considered significant at $\alpha = 0.05$.

	Model effects			Treatment: mean (SE)	
Variables	Treatment	Day	Day × treatment	Alum	Control
Morning temperature	0.7033	<0.0001	0.4290	20.0 (3.4)	20.0 (3.4)
Afternoon temperature	0.0965	<0.0001	<0.0001	22.5 (3.7)	22.7 (3.7)
Morning dissolved oxygen (mg/L)	<0.0001	<0.0001	<0.0001	8.5 (1.7)	8.0 (2.4)
Afternoon dissolved oxygen (mg/L)	0.0343	<0.0001	<0.0001	10.8 (1.5)	11.2 (2.1)
Morning pH	<0.0001	<0.0001	<0.0001	8.2 (0.6)	9.4 (0.8)
Afternoon pH	<0.0001	<0.0001	<0.0001	9.0 (0.5)	9.8 (0.8)
P. parvum density (cells/mL)	0.2474	<0.0001	0.0044	49.7 (141.8)	27.4 (90.9)
NO_{2+3}-N (mg/L)	0.0010	<0.0001	<0.0001	0.50 (0.33)	1.16 (0.78)
NH_3-N (mg/L)	0.4611	<0.0001	<0.0001	0.04 (0.01)	0.04 (0.01)
Total Kjeldahl N (mg/L)	<0.0001	<0.0001	<0.0001	2.5 (1.12)	3.00 (1.23)
Total N (mg/L)	<0.0001	<0.0001	<0.0001	3.0 (1.09)	4.16 (1.80)
Total P (mg/L)	<0.0001	<0.0001	<0.0001	0.11 (0.09)	0.24 (0.20)
PO_4-P (mg/L)	<0.0001	<0.0001	<0.0001	0.04 (0.02)	0.10 (0.5)
Inorganic N (mg/L)	—	—	—	0.53 (0.33)	1.20 (0.78)
Organic N (mg/L)	—	—	—	2.4 (1.13)	2.96 (1.23)
Organic P (mg/L)	—	—	—	0.08 (0.09)	0.14 (0.17)

concentrations were significantly lower in alum than in control ponds on 6 of 10 sampling days for NO_{2+3}-N and 2 of 10 sampling days for TKN. Mean concentrations of NH_3-N did not significantly differ between alum and control ponds on any sampling day or over the course of the study. Mean concentrations of PO_4-P and TP were significantly lower in alum than in control ponds (Table 2); differences were significant on 7 of 10 sampling days for PO_4-P and 5 of 10 sampling days for TP (Figure 4).

Density and Toxicity of *P. parvum*

Overall mean cell densities of *P. parvum* were 49.7 cells/mL for alum and 27.4 cells/mL for control ponds, but the difference was not statistically significant (Table 2). Cell densities peaked in both alum (330 cell/mL) and control (275 cells/mL) ponds by day −7 before declining to undetectable levels by day 7 in control ponds and by day 15 in alum ponds. *Prymnesium parvum* ichthyotoxicity was never detected in either alum or control ponds.

Fingerling Production

All ponds were drained to harvest the fingerlings on 42 d after stocking. Fingerling production was poor in both alum and control ponds. Alum ponds had average survival of 4.13% (0–13.7%) and production of 6.4 kg/ha (0–22 kg/ha), whereas there was no survival in control ponds. Average total length of fish was 27.93 mm (25.4–30.4 mm).

DISCUSSION

Alum reduced phenolphthalein alkalinity and pH, which supports previous studies (e.g., Boyd 1979a; Masuda and Boyd 1994). However, at the concentrations used in this study, alum was ineffective in maintaining afternoon pH below 9. The reductions in pH of alum ponds compared with the control were statistically significant but were probably biologically inconsequential. Afternoon pH remained consistently near or higher than 9.0, which is considered unsuitable for successful production of phase-1 *Morone* fingerlings from fry (Anderson 1994b; Barkoh 1996). Alum application rates of 15–25 mg/L, each applied once, have been demonstrated to decrease pH by 0.4–1.5 units, followed by gradual increases in pH to pretreatment levels in 30–60 d (Boyd 1979a; Mandal and Boyd 1980; Masuda and Boyd 1994). In our study, alum applications (mean rate, 7.2 mg/L) reduced pH by 0.8–1.2 units, followed by relatively rapid increases in pH to 9 or greater in about 2.5 d (1–5 d). Fertilization of alum ponds with inorganic N and P fertilizers could have accelerated the return of pH to levels above 9. Alum treatments cause declines not only in alkalinity and pH but also of soluble P and N (Boyd 1979a; Boyd 1990; Masuda and Boyd 1994; Holz and Hoagland 1999; Rishel and Ebeling 2006). Alum reduces these nutrients through coagulation and precipitation with aluminum ions, whereas alkalinity and pH are reduced when the hydrogen ions from alum react with alkalinity ions to produce carbon dioxide (CO_2) and water (Boyd 1979a, 1990). Limited or reduced nutrient availability decreases phytoplankton growth and CO_2 uptake (Boyd 1979b; Hecky and

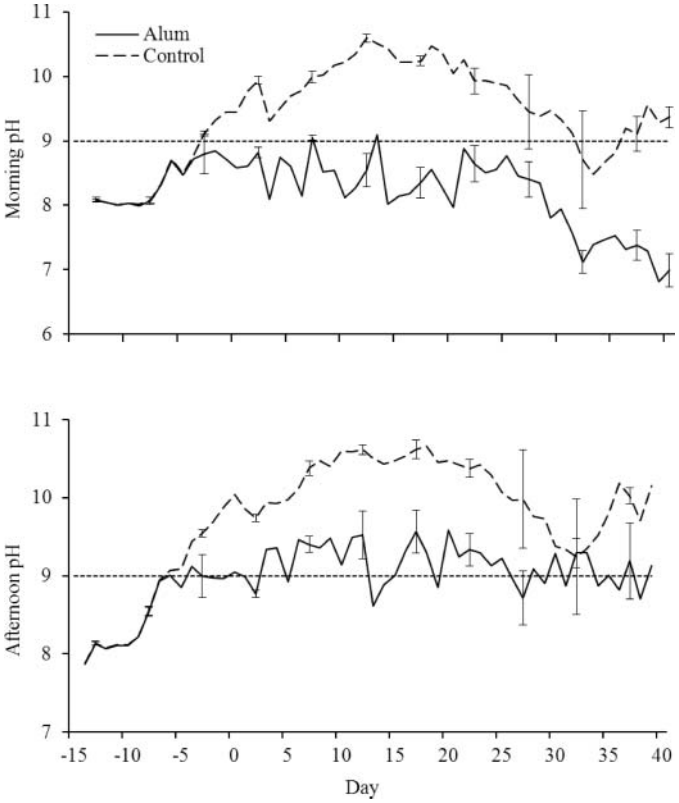

FIGURE 2. Trends of mean morning and afternoon pH in ponds fertilized with N at 300 μg/L and P at 30 μg/L three times weekly (control) or N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when pH was ≥9.0 (alum) at the Dundee Fish Hatchery in spring 2008. Vertical bars are standard errors; horizontal dotted line is the pH threshold (9.0) for managing pH in fingerling *Morone* production ponds. Day 0 is the date of treatment.

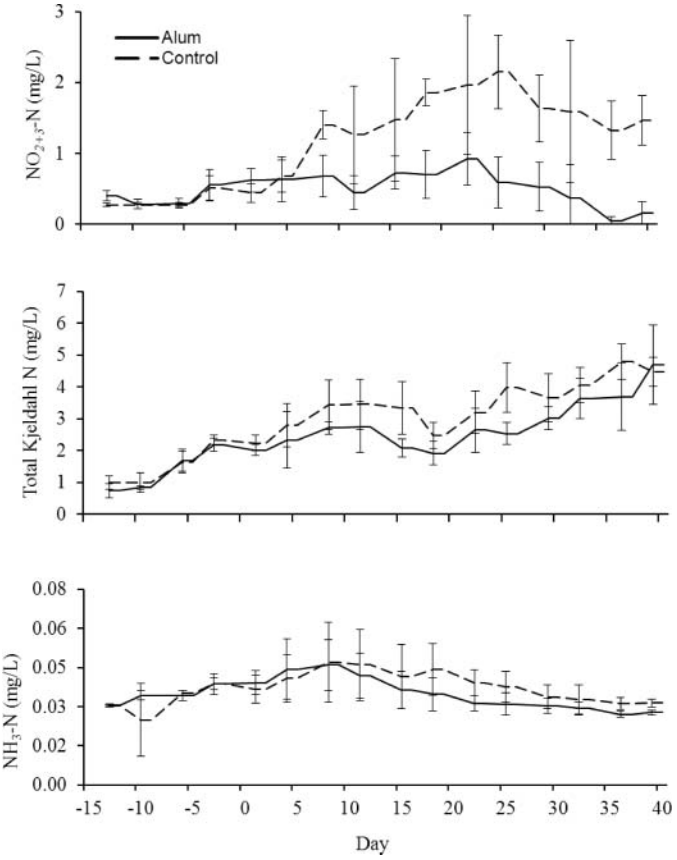

FIGURE 3. Trends of mean nitrogen concentrations in ponds fertilized with N at 300 μg/L and P at 30 μg/L three times weekly (control) or N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when pH was ≥9.0 (alum) at the Dundee Fish Hatchery in spring 2008. Vertical bars are standard errors. Day 0 is the date of treatment.

Kilham 1988; Boyd 1997; Kurten et al. 1999). Under these conditions, it takes relatively more time for the reduced pH to return to pretreatment levels as observed by Boyd (1990) and Masuda and Boyd (1994). Conversely, our fertilization of alum ponds replaced some of the nutrients removed by alum, which may have supported phytoplankton growth and higher rates of CO_2 uptake than otherwise. Consequently, pH increased quickly from the low levels achieved by alum applications.

Alum reduced concentrations of measured N (NO_{2+3}-N, TKN, and TN) and P (PO_4-P and TP) compounds, except NH_4-N, which agrees with results of previous studies (Malhotra et al. 1964; Malecki-Brown et al. 2009). Thus, alum application counteracted our fertilization strategy and resulted in less P and N to control *P. parvum* than otherwise. Though *P. parvum* density did not statistically differ between alum and control ponds, probably because of the high variability in the data, the density in alum ponds was almost twice that in control ponds, which is biologically significant. Further, *P. parvum* was undetectable in control ponds approximately 8 d before becoming undetectable in alum ponds. Thus, *P. parvum* control was delayed in alum ponds, which could potentially results in fish mortality if conditions that promoted production or release of toxins existed during that time. Ichthyotoxicity of *P. parvum* was never an issue during this study, probably because cell densities did not reach bloom levels or nutrients (P and N) were not limiting or both and, consequently, resulted in no stress on these algae. Phosphorus and nitrogen deficiency conditions have been demonstrated as sources of stress to *P. parvum*, resulting in the production or release of toxins (Granéli and Johansson 2003).

Striped Bass survival, and consequently production, was poor in both alum and control ponds probably due to high pH (≥9) levels. Anderson (1994b) reported no survival in Striped Bass culture ponds with a pH >9 before Striped Bass were 14 d old, and suggested a pH >8.5 be avoided during this period for successful fingerling Striped Bass production. Barkoh (1996) achieved better (≥80%) fingerling Striped Bass survival in ponds with a mean pH ≤8.5. These conditions were not achieved in the our study and, thus, may explain the poor fingerling Striped Bass survival and production. Another factor that may have contributed to the poor production was poor growth of the surviving fish, also probably caused by high pH. The mean length of 27.9 mm for the 42-d culture period was substantially less than the long-term mean length of 38 mm for fish produced

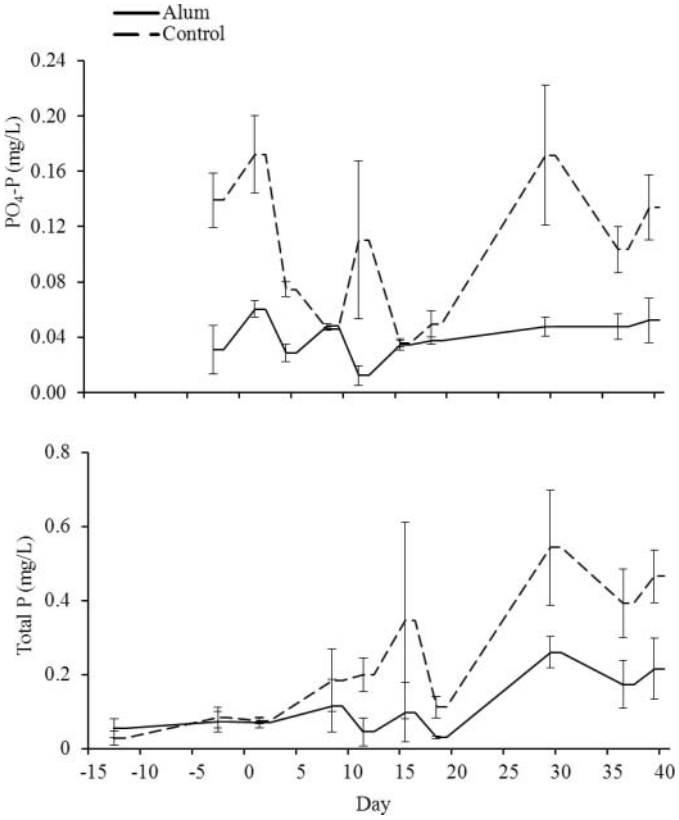

FIGURE 4. Trends of mean phosphorus concentrations in ponds fertilized with N at 300 μg/L and P at30 μg/L three times weekly (control) or N at 300 μg/L and P at 30 μg/L three times weekly plus alum treatments when pH was ≥9.0 (alum) at the Dundee Fish Hatchery in spring 2008. Vertical bars are standard errors. Day 0 is the date of treatment.

in 40 d at 35% survival rate at DFH (D. Smith, Dundee Fish Hatchery, Electra, Texas, personal communications)

Our results support reports that alum can reduce pH (Mandal and Boyd 1980; Rishel and Ebeling 2006) and thus has promise as a tool for managing pH levels in fish culture ponds (Boyd 1990; Tucker and D'Abramo 2008). However, the concurrent use of alum to control pH of ponds receiving fertilization as a strategy for controlling *P. parvum* during culture of Striped Bass needs further investigation to refine protocols. We suggest future studies investigate higher alum treatment rates (e.g., phenolphthalein alkalinity : alum = 1:1.5 or 1:2) along with a modification of the current fertilization regimen. We theorize that a higher alum treatment rate would cause a larger reduction in pH since the extent of pH depression by alum is related to total alkalinity (Boyd 1979a). Because of the high alkalinity of the DFH water, increasing alum application rates beyond the phenolphthalein alkalinity demand may achieve lower pH levels. Alternatively, alum treatments may be based on total alkalinity. Discontinuing inorganic fertilization (or reducing the application frequency) after *P. parvum* cells are eliminated or drastically reduced, for example, would minimize phytoplankton growth and CO_2 uptake. Such a strategy could lengthen the time for pH to return to pretreatment levels and thereby allow the fish to reach life stages that can tolerate the ambient pH levels.

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Efficacy of Aquaflor (50% Florfenicol)–Medicated Feed to Control Mortality Associated with Flavobacterium columnare Infection in Florida Largemouth Bass and Bluegill

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ARTICLE

Efficacy of Aquaflor (50% Florfenicol)–Medicated Feed to Control Mortality Associated with *Flavobacterium columnare* Infection in Florida Largemouth Bass and Bluegill

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Abstract

Aquaflor (florfenicol, 50% with/without) is a potent, broad-spectrum, antibacterial agent with bacteriostatic properties that are active against a variety of Gram-positive and Gram-negative bacteria. This product is approved by the U. S. Food and Drug Administration for use on several fish species to control mortality associated with a variety of diseases, including columnaris (causative agent, *Flavobacterium columnare).* Two independent experimental trials were separately conducted to evaluate the effectiveness of Aquaflor to control mortality associated with columnaris disease. Aquaflor was administered in feed at a targeted daily florfenicol dosage of 10 mg/kg of body weight for 10 consecutive days. Test species were fingerling Florida Largemouth Bass *Micropterus salmoides floridanus* and Bluegill *Lepomis macrochirus*. In each trial, 8 or 10 test tanks (4 or 5 treated, 4 or 5 control) were stocked with either approximately 473 bass (mean length = 6.4 mm; mean weight = 3.3 g) or 100 Bluegills (length = 10.3; weight = 25.2 g). At the end of the 14-d posttreatment periods, mean cumulative mortality of bass in treated tanks was 5.7% per tank, which was significantly less than that in control tanks (12.0%). Mean cumulative mortality of Bluegills in treated tanks was 19% per tank, which was significantly less than that in control tanks (38%). Analysis of treated feed samples at the start of each trial verified the initial targeted dose of florfenicol was within 11% of the target dose for both bass and Bluegills. Based on these results, we concluded that Aquaflor-medicated feed homogeneously mixed to provide florfenicol at a daily dose of10 mg/kg of body weight fed for 10 d was effective in controlling mortality in bass and Bluegill fingerlings, exposed to columnaris disease.

Flavobacterium represent a group of Gram-negative bacteria that can be associated with high levels of mortality in a variety of freshwater fish species. *Flavobacterium columnare* is the causative agent of columnaris disease, which can be a chronic or acute disease that can infect virtually any wild or cultured fish (Plumb 1999). The worldwide distribution of columnaris makes it one of the most important diseases affecting aquaculture (Morrison et al. 1981; Post 1987; Wagner et al. 2002; Thomas-Jinu and Goodwin 2004). Although columnaris is primarily an external disease, it can become systemic with or without advanced skin and gill necrosis (Noga 2000). There is no difference in the causative Columnaris bacteria between an internal or

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external infection. Differing treatment options dictate the classification of internal of external, not the disease. Establishing and maintaining suitable fish culture conditions and procedures can reduce the occurrence and severity of disease outbreaks (Post 1987; Jeney and Jeney 1995).

Florfenicol is a potent, broad-spectrum, antibacterial agent with bacteriostatic properties and is active against a variety of Gram-positive and Gram-negative bacteria (Horsberg et al. 1996). Because of its palatability to fish and high potency, florfenicol has become an important veterinary therapeutic drug, especially when administered orally in feed (Samuelsen et al. 1998; Gaunt et al. 2003; Wang et al. 2009; T. E. Powers, K. J. Varma, and J. D. Powers [abstract presented at the European Association for Veterinary Pharmacology and Toxicology meeting, 1988]). Aquaflor is an aquaculture feed premix (white powder) containing 50% florfenicol. Worldwide, Aquaflor has been approved in more than 20 countries (e.g., Norway, Japan, Chile, Canada, and the USA) to control disease mortality associated with infectious pathogens in a variety of cultured fish. The U.S. Food and Drug Administration Center for Veterinary Medicine (CVM) has approved Aquaflor as a Veterinary Feed Directive drug for use to control mortality from (1) enteric septicemia (ESC) in catfish associated with *Edwardsiella ictaluri*, (2) coldwater disease in freshwater-reared salmonids associated with *F. psychrophilum*, (3) furunculosis in freshwater-reared salmonids associated with *Aeromonas salmonicida* (post April 2012), (4) columnaris disease in freshwater-reared finfish (including Florida Largemouth Bass *Micropterus salmoides floridanus* and Bluegill *Lepomis macrochirus*) associated with *F. columnare*, and (5) streptococcal septicemia in freshwater-reared warmwater finfish associated with *Streptococcus iniae*. Leading up to approval in April 2012, public and private aquaculture groups in the USA sought to expand the approved Aquaflor label to include additional freshwater-reared finfish species and pathogens susceptible to florfenicol. Florfenicol has been shown to be efficacious against a number of fish pathogens, including *Aeromonas salmonicida* and *Vibrio salmonicida* (Fukui et al. 1987; Inglis and Richards 1991; Nordmo et al. 1998; Samuelsen et al. 1998; Bruun et al. 2000; Schmidt et al. 2001), *Edwardsiella ictaluri* (McGinnis et al. 2003), *F. columnare* (Gaunt et al. 2010b), and *Streptococcus iniae* (Bowker et al. 2010).

In many cases, when evidence of a pathogen is detected, diagnosis and administration of treatment must be rapid to control mortality and prevent an epizootic (Klontz 1987; Alderman 1988; Plumb 1999). Three oral antibiotics are approved by the U. S. Food and Drug Administration (FDA) for use to control mortality associated with various diseases in cultured fish populations. It is important to clarify that these treatment stipulations pertain to cultured food fish and for stocked recreational species (still considered food fish) when regulations allow for harvest prior to satisfying withdraw periods mandated by the FDA. None of these antibiotics were approved for use on Largemouth Bass *Micropterus salmoides* or Bluegill *Lepomis macrochirus* to control mortality associated with columnaris. Terramycin 200 for Fish (44% oxytetracycline dihydrate; Phibro Animal Health, Corp., Ridgefield Park, New Jersey) is approved for use on all freshwater-reared rainbow trout *Oncorhynchus mykiss* and Steelhead (anadromous Rainbow Trout) infected with *F. columnare*. Romet 30 (25% sulfadimethoxine, 5% ormetoprim) and Romet TC (16.7% sulfadimethoxine, 3.3% ormetoprim; Aquatic Health Resources, Minnetonka, Minnesota) are approved for salmonids and catfish to treat *Aeromonas salmonicida* and *Edwardsiella ictaluri*. Before April 2012, Aquaflor was conditionally approved for use only on channel catfish *Ictalurus punctatus* to control mortality associated with columnaris disease.

Freshwater sport fish hatchery programs, including those for centrarchid species, continually demand development and approval of new therapeutic treatments for cultured species (Matthews et al. 2012; U.S. Fish and Wildlife Service, unpublished data). To help expand the FDA-approved label for Aquaflor (Lot number 030905, Intervet/Schering-Plow Animal Health Corp., Roseland, New Jersey) to include cultured sport fish of high demand for recreational stocking and restoration, two separate experimental trials were conducted in May and November of 2009 to evaluate the efficacy of Aquaflor-medicated feed to control mortality in freshwater-reared Florida Largemouth Bass and Bluegills diagnosed with columnaris infections. Each trial was conducted under an FDA-accepted research protocol at a targeted daily florfenicol dosage of 10 mg/kg of body weight for 10 d. The goal of the trials was to test the effectiveness of florfenicol to control mortality in Florida Largemouth Bass and Bluegills caused by natural infections of *F. columnare* and to provide evidence to support expanding the Aquaflor label to allow treatment of all freshwater-reared finfish for this claim.

METHODS

Two separate warmwater efficacy trials—one with Florida Largemouth Bass and the second with Bluegill—were conducted at the Florida Bass Conservation Center (FBCC), Richloam Fish Hatchery, Webster, Florida. Each trial lasted 25 d and consisted of a 1-d pretreatment period, a 10-d treatment period, and 14-d posttreatment period. Treatment conditions (Aquaflor-treated versus nontreated control) were allocated among tanks by using a completely randomized design. During the treatment period, Aquaflor-medicated feed was administered to treated tanks, and nonmedicated feed was administered to control tanks. During the posttreatment period, fish in all tanks received nonmedicated feed. The reference populations in both trials consisted of "Phase II" or advanced fingerling production slated for stocking into public assess water bodies. Feed amount for a specific tank was reduced if $\geq$25% mortality occurred in that tank during the treatment period.

Test tanks were 378 L (based on stand pipe height) and made of dark green fiberglass (183 $\times$ 46 cm, 54 cm deep). Dark colored tanks were used to help calm excitable species like Florida

Largemouth Bass and Bluegill and can be used without tank covers that hinder observation, cleaning, and feed distribution. Aquaflor-medicated feed was administered at a target daily florfenicol dosage of 10 mg/kg of body weight for 10 consecutive days. Using a Marion Mixer, Aquaflor premix (Merck Animal Heath, Summit, New Jersey) was top-coated with Menhaden fish oil (Omega Protein, Inc. Houston, Texas; 0.5% with/without) onto Silver Cup #4, 42% protein Salmon/Trout Crumbles (Nelson & Sons, Inc., Murray, Utah) for the bass trial and Silver Cup 2.0 mm, 42% protein Salmon extruded slow-sinking pellets for the Bluegill trial. Medicated and nonmedicated feed samples were collected from which florfenicol concentrations were analytically verified by Eurofins/AvTech Laboratories Inc., Portage, Michigan (Hayes 2005). Analysis of three, 200-g medicated-feed samples collected from the top, middle, and bottom of the feed bag was used to verify actual florfenicol concentration (mg/kg feed) of the mixed feed and to determine whether medicated feed had been mixed homogeneously during top-coating. Daily doses of the top-coated florfenicol feed were not analyzed over the 10-d treatment period. Two, 200-g nonmedicated feed samples were collected to ensure that feed had not been contaminated with florfenicol during manufacturing.

Disease onset in both trials were natural outbreaks. Before each trial began, columnaris was presumptively diagnosed in the reference populations of fingerling bass and Bluegills. Diagnosis was based on observance of skin lesions commonly characterized as "saddleback" in columnaris infections (Noga 2000) and detection of bacteria from posterior kidney tissue streaked on dilute Mueller–Hinton or Shieh's media. All plates were cultured at 26.0°C (Plumb 1999) for 5 d; colonies were visually noticeable between 48 and 72 h. Plates were visually examined every 24 h. Colonies cultured on the media that were yellow, had rhizoid edges, and adhered tightly to the media were examined microscopically. A diagnosis was deemed positive if the bacteria were (1) long, slender rods, (2) exhibited flexing and gliding motility, and (3) aggregated into stacked columns (hay stacks). All cultures meeting these criteria were additionally viewed via hanging drop procedures (Whitman 2004). Cultures from the pretrial evaluations were sent to the U. S. Fish and Wildlife Service (USFWS) La Crosse Fish Health Center (FHC), Onalaska, Wisconsin, for confirmation by polymerase chain reaction (PCR; Bader et al. 2003). During treatment and posttreatment periods, external and internal gross necropsies were performed on selected moribund fish (up to five fish per tank during each period). In addition, skin scrapes and kidney tissue from each fish necropsied were cultured and examined microscopically to presumptively identify cause of mortality via the same protocol used in pretrial diagnosis.

Fish that died during the 1-d pretreatment period were subtracted from the total number of fish transferred to each tank and not used to calculate cumulative mortality at the end of each trial. Mortality, general fish behavior, and fish feeding behavior were recorded daily during the treatment and posttreatment periods. Normal and abnormal behaviors were documented, and feeding behavior was characterized as aggressive (all feed consumped), semiaggressive (most feed consumed), or nonaggressive (little to no feed consumed). The targeted dose was presumed by the rapid consumption of the feed and lack of residual feed removed daily from the bottom of each tank.

Dissolved oxygen (DO) and water temperature were recorded daily with an Oxyguard Handy Polaris Portable DO meter (Oxyguard International A/S, Birkerød, Denmark). Water alkalinity, hardness, and pH were measured once in water samples collected from each reference population tank and once from a water sample collected from a single test tank during the posttreatment period. These water quality variables were measured with a Hach Advanced portable laboratory CEL 890 digital titrator (Model 16900) and a SensIon1 portable pH meter (Hach Co., Loveland, Colorado). Carbon dioxide was monitored daily with an Oxyguard CO_2 Analyzer.

Each trial focused on the proportional risk of death versus survival. Probability of death was modeled with a mixed-effects logistic model fitted with SAS Proc GLIMMIX (logit link; SAS Institute 2007; Wolfinger and O'Connell 1993). The random effect of a test tank was modeled with an R-side covariance structure. Percent cumulative mortality in each test tank during the treatment and posttreatment periods only was calculated by using the total number of fish in each test tank at the beginning of the treatment period as the denominator (i.e., total mortality in test tank + number of live fish hand-counted from the test tank at end of trial). Mean percent cumulative mortality was compared between treated tanks and control tanks for all treatment and posttreatment days. Treatment levels were considered statistically significant at $\alpha = 0.05$.

Each trial was single-blinded to minimize the potential for data-collection bias. Nonblinded personnel who were aware of treatment conditions, stocked fish into tanks and weighed feed during the treatment period. Blinded participants collected and recorded all other data.

Florida Largemouth Bass trial.—The trial was conducted May 20 to June 13, 2009, on fingerling bass (mean length = 6.4 cm [SD = 0.6]; mean weight = 3.3 g [SD not available as only group weight was measured]). Mean weight and length of fish determined before the start of the trial was based on weighing three aliquots of fish (about 100 fish per aliquot) and measuring the total length of 20 fish. Fish were collected from the reference population (76,000 Florida Largemouth Bass fingerlings intensively cultured on pelleted feed in a 45,000-L concrete raceway) and stocked into 10 test tanks, 5 of which were randomly designated as treated and 5 as control. A total of 1,625 g of bass fingerlings (approximately 500 fish) were transferred to each tank to achieve fish densities similar to the reference population. To minimize the potential for bias, the transfer was completed in two rounds. In the first round, approximately 812 g of bass (approximately 250 fish) were weighed and transferred to each test tank; in the second round, approximately 813 g of bass (approximately 250 fish) were weighed and transferred to each test tank. To facilitate fish transfer, fish in

the reference population were crowded to one end of a raceway with a seine and then collected with dip nets. Excess water on fish and net was allowed to drain. For light sedation, the fish in the net were placed into a tarred bucket of water containing tricaine methanesulfonate (Tricaine-S, Western Chemical, Inc., Ferndale, Washington) at 25 mg/L of water and weighed to the nearest 0.1 g on a Denver Instruments balance (Denver MXX-5001 5,000 g, Denver, Colorado). Test tanks were supplied with first-pass well water (mean, 23.9°C) at an inflow of approximately 18.9 L/min (three exchanges/h).

Aquaflor-medicated feed was presumptively administered at a target daily florfenicol dosage of 10 mg/kg of body weight for 10 consecutive days. Actual florfenicol concentrations in the feed were not tested during the 10-d treatment. The medicated feed contained a calculated florfenicol dose of 0.2 g/kg of feed (0.04% florfenicol) and was fed at 5.0% initial mean body weight to achieve the target dose. During the treatment and posttreatment periods, feeding rate was not adjusted for growth. Feed was administered to tanks every 5 h by automatic feeders (Loudon style feeders, EMF Metal Fabrication, Centerville, Iowa), for a daily total of 81.3 g of feed per tank (500 bass/tank × 3.25 g/bass × 0.05 body weight). Additionally, medicated feed, florfenicol at 10 mg/kg body weight, was fed daily at 5.0% of body weight to the reference population (76,000 bass; average weight, 3.0 g) in a 45,420-L concrete raceway (U.S. Fish and Wildlife Service Investigational New Animal Drug Identification Number 10-697-09-10) in an effort to reduce mortality of our production bass. The results were used for visual comparison of large-scale field application to the trial tanks for observation of reduced mortality on a production level.

Bluegill trial.—The trial was conducted November 20 to December 14, 2009, on subadult Bluegills (mean length = 10.3 cm [SD = 1.1]; mean weight = 25.2 g [SD not available, only group weight measured]). We were randomly allocated 100 Bluegills to each of eight test tanks (four treated, four control). The remaining Bluegills in the 4,920-L raceway reference population (produced for stocking kids fishing ponds in state managed lakes) numbered less that 150 and were not treated with medicated feed. To minimize the potential for bias, all fish were hand-counted into a bucket and transferred to tanks in two rounds of 50 fish per round. Tanks were supplied with first-pass well water, 22.3°C, at an inflow of approximately 15.1 L/min (2.4 exchanges/h).

Aquaflor-medicated feed was presumptively administered at a daily target florfenicol dosage of 10 mg/kg of body weight for 10 consecutive days. Medicated feed in this trial contained a calculated florfenicol dose of 0.5 g/kg of feed (0.1% florfenicol) that was fed at 2.0% initial mean body weight to achieve the target dose. During the treatment and posttreatment periods, the feeding rate was not adjusted for growth. Feed was administered to tanks every 5 h by automatic feeder, for a total of 50.4 g of feed per tank per day (100 Bluegills/tank × 25.2 g/Bluegill × 0.02 body weight).

RESULTS

Florida Largemouth Bass

At trial completion, mean percent cumulative mortality in treated tanks (5.7% [SD = 1.6] per tank) was significantly ($P = 0.0004$) less than that in control tanks (12.0% [SD = 2.2]; Figure 1). A significant difference in mean percent cumulative mortality between treated and control tanks was detected and remained in effect from treatment day 4 through the end of the trial (posttreatment day 14). Mean cumulative mortality in control tanks (255 fish) was about twice that of treated tanks (126) during the 10 d treatment period, and nearly six times that during the 14 d posttreatment period (35 versus 6 fish). Results of PCR assay of cultured bacterial isolates (pretrial) positively confirmed the presence of *F. columnare* on three of three plates tested. Necropsy results were also consistent with columnaris and indicated no concomitant pathogens were present, but no generalized screening on TSA with blood was completed to verify. *Tetrahymena* and *Ichthyobodo* were found in low prevalence externally on several of the control bass, but we judged these parasites to be secondary invaders targeting the lesions initially caused by *F. columnare*. Mortality decreased concurrently in the treated reference population over the 10-day treatment period, 977 mortalities counted on day 2 and 32 on day 10. Mean percent cumulative mortality closely mimicked the trial tanks (Figure 1).

Predetermined sampling protocols utilized for this trial did not mandate large numbers of bacterial cultures during any phase of the trial. During the 10-d treatment period, 12 treatment and 16 moribund bass from control tanks (range, 1–4 per tank) were examined for columnaris, of which 6 treated and 11 control fish were plated. There were 11 fish (5 treated and 6

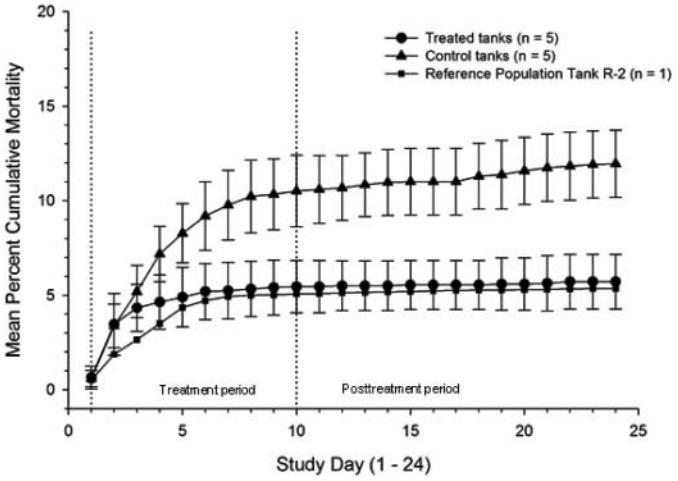

FIGURE 1. Mean (±1 SD) percent cumulative mortality of Florida Largemouth Bass in florfenicol-treated and control tanks, as well as in the reference population tank that was similarly treated with florfenicol. Treatments were to assess efficacy in control of mortality caused by columnaris.

control, posttreatment) examined for columnaris, of which two treated and one control were plated. Plates that exhibited no growth after 5 d were considered not infected with *F. columnare* and discarded.

General behavior of fish in treated and control tanks appeared to improve during the treatment period. Normal behavior was characterized by constant mid-level swimming with adequate response to stimuli such as feed addition and tank cleaning. The predominant abnormal behavior observed during this period was lethargy (resting near or on the bottom). Hyperactivity (elevated gilling and fin movements and repeated swimming end to end in the tank not resulting from stimulus) was noted among fish in one treated tank during the first few days of the treatment period but was not observed thereafter. During the posttreatment period, fish behavior in all tanks was characterized as normal. Throughout the trial, feeding behavior was characterized as aggressive (all food consumed) in all control tanks and in four of five treated tanks. Fish in one treated tank exhibited semiaggressive (majority of feed consumed) feeding behavior during the first 7 d of the treatment period. Feed analysis confirmed the mean florfenicol concentration from three samples was homogeneously mixed at 10.5 mg/kg of body weight per day, and no florfenicol contamination was measured in the control feed. Florfenicol dose consumed by each fish was presumptively based on the initial verified dose and total feed consumption. Actual amount of feed consumed per fish was unknown. No visual difference between treatment and control fish feeding behavior was observed during the 10-d treatment period, indicating no effects concerning florfenicol on feed consumption. At the end of trial, we discovered that about 473 fish had been stocked into each tank at the beginning of the trial in contrast with our assumption of 500 fish/tank. Consequently, the initial daily dose of florfenicol administered to fish in treated tanks was 11.1 mg/kg of body weight (111% of target).

Mean water temperature during the trial was 23.9°C (range, 22.6–24.9°C), and dissolved oxygen concentration was13.7 mg/L (range, 12.0–14.6 mg/L). Carbon dioxide levels averaged 9 mg/L (range, 8–11 mg/L). Ranges depict daily measurements observed over the 25-d trial period. Mean water hardness (353 mg/L as $CaCO_3$), alkalinity (380 mg/L as $CaCO_3$), and pH 7.8 were within ranges suitable for rearing bass at the hatchery. Each of these three water chemistry metrics were recorded twice.

Bluegill

At the end of this trial, mean percent cumulative Bluegill mortality in treated tanks (18.5% [SD = 6.6] per tank) was significantly ($P = 0.0098$) less than that in control tanks, (37.5% [SD = 7.2] per tank; Figure 2). A significant difference in mean cumulative mortality between treated and control tanks was detected and remained in effect from treatment day 8 through the end of the trial. Mean cumulative mortality in control tanks (129 fish) was nearly double that of the treated tanks (71) during the treatment period and was three times that of treated tanks (21versus 7 fish) during the posttreatment period. Approximately 86% of the total mortality in control tanks and approximately 96% of total mortality in treated tanks occurred during the 10-d treatment period. Results of PCR assay of cultured bacterial isolates (pretrial) positively confirmed the pathogen as *F. columnare* on six of nine plates tested.

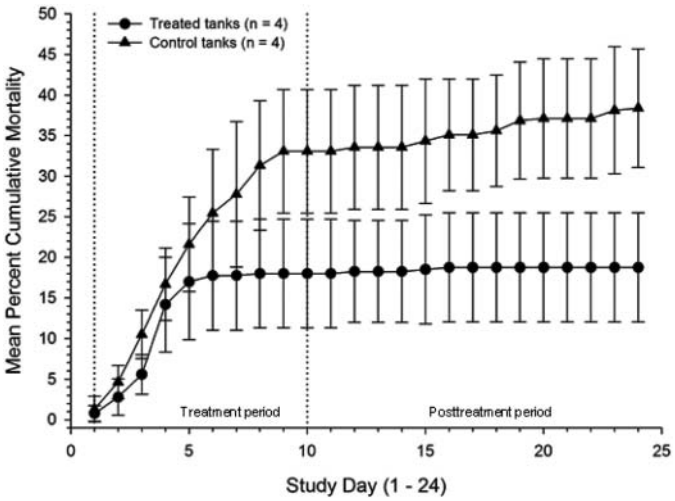

FIGURE 2. Mean (±1 SD) percent cumulative mortality of Bluegills diagnosed with columnaris disease in florfenicol-treated and control tanks. Treatments were to assess efficacy in control of mortality caused by columnaris.

During the 10-d treatment period 32 moribund Bluegills, 16 treated fish (4/tank) and 16 control fish (4/tank) were examined. Kidney tissue streaked on Shieh's media produced *F. columnare* in 15 of the 16 cultures from the treated tanks and 16 of the 16 cultures from the control tanks. Results from 11 fish sampled during the posttreatment period showed no confirmed *F. columnare* cultures (posterior kidney) from three moribund Bluegills collected from three separate treatment tanks. However, cultured kidney tissue from seven of the eight moribund Bluegills from control tanks (1–3 fish/tank) showed an *F. columnare* infection. Low sample size during the posttreatment period reflects low numbers of moribund fish available to sample. Plates that exhibited no growth after 5 d were considered negative for *F. columnare* infection and discarded. Colonies not identified as columnaris bacteria were randomly examined specifically for *Aeromonas hydrophilla* on Tryptic soy agar with blood, which resulted in no positive identification. Colony identification methods performed were hanging drop slide, cytochrome oxidase test, 3% KOH test, and gram stain. No other bacterial identifications were completed or required by FDA protocol at the time of these trials.

General feeding behavior in treatment and control tanks ranged from nonaggressive (small amounts of feed consumed) to aggressive during the first 6 d of the treatment period and was aggressive thereafter. Throughout the trial, general fish behavior was characterized as normal. The analytically verified initial daily dose of florfenicol administered to fish in treated tanks was 10.0 mg/kg of body weight (100% of target), and no

florfenicol contamination was measured in the control feed. Florfenicol dose consumed by each fish was presumptively based on the initial verified dose and total feed consumption. Actual amount of feed consumed per fish was unknown. No visual difference between treatment and control fish feeding behavior was observed during the 10-d treatment period, indicating no effects concerning florfenicol on feed consumption.

Mean water temperature during the trial was 22.3°C (range, 20.3–23.8°C), and dissolved oxygen concentration was 10.5 mg/L (range, 9.1–11.1 mg/L). Carbon dioxide levels averaged 11 mg/L (range, 8–15 mg/L). Ranges depict differences observed over the 25-d trial period. Mean water hardness (365 as mg/L $CaCO_3$), alkalinity (340 mg/L as $CaCO_3$), and pH 7.9 were within ranges suitable for rearing Bluegills at the hatchery. Each of these three water chemistry metrics were recorded twice, as protocol requested.

DISCUSSION

In both trials, the targeted concentration of Aquaflor top-coated feed (florfenicol at 10 mg/kg of fish body weight) was effective in controlling mortality caused by confirmed columnaris infections in two representative warmwater fish populations. No further analysis of the medicated feed was collected during the 10-d treatment period. The lack of monitoring florfenicol dose concentration over the treatment period allows for changes in medication concentrations, if any, to go unreported. Evaluation of florfenicol concentrations at three doses (10, 30, and 50 mg/kg of body weight per day) in channel catfish feed showed no significant change in medication concentrations at any level after a 19-d interval (Gaikowski et al. 2003). Initial florfenicol concentrations were analyzed in both FBCC trials and homogenously mixed feed concentrations were established. Gaunt et al. (2003) reported florfenicol remained stable in homogenously mixed catfish feeds. Concern of reduced florfenicol concentration leaching from the medicated feed was minimized based on pharmacokinetic studies that showed considerable loss of florfenicol from uneaten feed in water after 5 min (Yanong et al. 2005). In both of our trials, the majority of the feed was consumed in the treated tanks within seconds of the feed hitting the water. Uneaten feed was removed along with waste products after each feeding.

Prevalence of the infection was only visually observed and not statistically evaluated in the bass and Bluegill reference populations. Daily mortality counts from the bass reference population showed a 97% decline in mortality from day 2 to day 10 of the treatment period. Data were not reported from the Bluegill reference population due to the diminished population size after stocking the trial tanks. To our knowledge, there is no other published information reporting the effectiveness of florfenicol treatments on Florida Largemouth Bass and Bluegills for this claim. However, there is considerable evidence showing that florfenicol is effective against *F. columnare* in other fish species, as well as against other bacterial pathogens. Gaunt et al. (2010b) reported that florfenicol was effective in controlling mortality caused by columnaris in channel catfish (mean weight range, 6.8–9.2 g) when administered at the same dosage used in our trials. The authors reported that mean cumulative mortality following a 14-d posttreatment period was 8% in treated tanks and 54% in control tanks. Others have reported that florfenicol treatments reduced mortality in catfish caused by ESC associated with *Edwardsiella ictaluri* (Gaunt et al. 2003, 2004, 2006), in Atlantic Salmon *Salmo solar* caused by furunculosis associated with *Aeromonas salmonicida* (Samuelsen et al. 1998), and in sunshine bass (female White Bass *Morone chrysops* × male Striped Bass *M. saxatilis*) associated with *Streptococcus iniae* (Darwish 2007; Bowker et al. 2010). Bowker et al. (2010) reported that the analytically verified florfenicol dose administered was 8.3 mg/kg body weight per day. Regardless, these authors reported that mean percent cumulative mortality in treated tanks (19%) was significantly different from that in control tanks (52%).

Animal safety studies, dose determination, and residue depletion have not been conducted on Largemouth Bass or Bluegill but have been conducted on other warmwater and coolwater species. Gaikowski et al. (2003) reported that no detrimental histological effects were detected in channel catfish fed medicated feed at daily florfenicol dosages of 10, 30 or 50 mg/kg of body weight for 20 d. Straus et al. (2012) reported that no significant clinical histological lesions were detected when sunshine bass were fed florfenicol-medicated feed at daily dosages of 15, 45, or 75 mg/kg of body weight for 20 d. Similar results have been observed in Yellow Perch *Perca flavescens* (J. Bowker, unpublished) and tilapia (M. Gaikowski, USGS personal communication). Gaunt et al. (2004, 2010a) reported that Aquaflor was palatable, safe, and efficacious for controlling mortality caused by ESC in Channel Catfish and *Streptococcus iniae* in Nile Tilapia *Oreochromis niloticus* at florfenicol concentrations of 10–15 mg/kg of body weight per day. Florfenicol administered daily at 10 mg/kg of body weight to cultured Atlantic Salmon was also reported efficacious for the treatment of furunculosis (Inglis et al. 1991; Nordmo et al. 1994; Samuelsen et al. 1998).

One concern of fish culturists, fish health biologists, and veterinarians involved in fish culture is the withdrawal period, which is the number of days treated fish must be held before they can be harvested for market or released into the wild. For economic and logistic reasons, shorter withdrawal times are desirable. The tolerance for florfenicol has been established by the FDA at 1,000 parts/10^9 (or 1 μg/g) in muscle tissue and skin for Channel Catfish and salmonids (Bowser et al. 2009). This information, in part, was used by FDA to establish a withdrawal period of 12 d for the initial approval of Aquaflor for approved use in Channel Catfish (before 2009). This withdrawal period is considerably shorter than the withdrawal periods established for Terramycin 200 for Fish (21 d) and Romet30 and RometTC on salmonids (42 d) but considerably longer than for Romet30 and RometTC (3 d) on Channel Catfish (USFWS 2011). Recent trials conducted to evaluate depletion of florfenicol residues over

time in a variety of coolwater and warmwater fish species fed at a daily target dosage of 10–15 mg/kg of body weight for 10 d showed that water temperature and fish size are factors affecting the rate of drug residue depletion from fish tissues. Wrzesinski et al. (2006) reported for Channel Catfish (mean weight, about 900 g) florfenicol residue levels in muscle tissue remained below tolerance levels at 4 d following a 12-d treatment at a daily dose of 9.3 mg/kg of body weight and remained below tolerance levels thereafter. Kosoff et al. (2009) reported that florfenicol residues reached the 1,000 parts/10^9 (1 μg/g) tolerance level in (1) Nile Tilapia between 4.1 and 6.1 d when fish were tested at a water temperature of 30°C, (2) Walleye *Sander vitreus* between 9.7 and 12.6 d when tested at 25°C, and (3) sunshine bass between 0.7 and 2.6 d when tested at 25°C and 20°C. Bowser et al. (2009) reported that when florfenicol was administered at a daily dose of 15 mg/kg body weight to three different sizes of Nile Tilapia that elimination was slightly quicker for smaller fish: 9.2 d for 100-g fish, 8.6 d for 250-g fish, and 12.7 d for 500-g fish. Based on this information, the FDA added 3 d to the withdrawal period, making it15-d. This ensured that florfenicol residues declined to acceptable FDA standards for freshwater species (including Largemouth Bass and Bluegills treated at daily florfenicol doses up to 15 mg/kg of body weight reared or held at water temperatures ≥20°C. As a result of our trials and other associated literature, Aquaflor use in the USA has been expanded and approved (April 9th, 2012) to treat all freshwater-reared finfish to control mortality caused by columnaris; fish may be treated at a florfenicol dose of 10–15 mg/kg of body weight per day with a withdrawal period of 15 d (still under the Veterinary Feed Directive).

Evaluating efficacy is not only dependent on proper conduct of a trial, but perhaps more importantly on the early diagnosis of the disease, timely administration of treatment, and definitive confirmation of the causative infectious fish pathogen. In the Florida Largemouth Bass trial, the actual initial florfenicol dose administered to fish in treated tanks was 11.1 mg/kg of body weight per day. Despite evidence that the bass were initially slightly overmedicated, the therapeutic effects of treatment became evident midway through the treatment period when mortality in treated tanks decreased to neglibible levels. Further support of treatment efficacy was evident by the pattern of mortality observed in the bass reference population, where mean percent cumulative mortality mimicked that observed in test tanks treated with Aquaflor in our trial.

Fish disease management strategies, such as those described by Piper et al. (1982), emphasize that it is incumbent upon the fish culturist to maintain the best possible environmental conditions and minimize stress. However, even under the best environmental conditions, disease outbreaks can occur. In such situations, it is imperative for fish culturists to have legal access to antibiotics such as florfenicol to help control disease-related mortality. In the Bluegill trial, cultured posterior kidney tissue identified *F. columnare* in 94% and 100% of the samples for both treatment and control tanks during the treatment period and 0% and 88% during the posttreatment period. Similar patterns were found for the bass trial, but small numbers of plate cultures made comparisons difficult. Reduced mortality in treated tanks restricted numbers of moribund fish sampled during the posttreatment period in both trials. Control tanks continued to experience columnaris-related mortality during the posttreatment period, while no bass or Bluegill mortalities from a treated tank were diagnosed positive for columnaris during the same time. Based on these trial results, we consider Aquaflor-medicated feed effective in controlling columnaris-caused mortality in Largemouth Bass and Bluegill fingerlings. Both trials were initiated in an attempt to supply supporting evidence to gain approval for the use of Florfenicol on all freshwater species. Our efforts successfully aided in the approval process, and the now-approved use of florfenicol will help culturists reduce mortality levels caused by columnaris infections on freshwater species in the USA. Our trials were not designed to address the susceptibility of *F. columnare* to florfenicol. Minimum inhibitory concentrations (MIC) of 0.5–1.0 μg/mL were reported from *F. columnare* isolates against florfenicol from infected Channel Catfish (Gaunt et al. 2010b). Future *F. columnare* studies that include MIC testing should utilize methods detailed by Darwish et al. (2008).

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Influence of Lineage, Broodstock Conditioning, and Hormone Injection on Gila Trout Reproductive Success and Egg Fatty Acid Composition

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ARTICLE

Influence of Lineage, Broodstock Conditioning, and Hormone Injection on Gila Trout Reproductive Success and Egg Fatty Acid Composition

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Abstract

Successful reproduction of captive-reared fishes depends on hatchery practices and proper broodstock nutrition. Nutrient requirements and environmental conditions stimulating reproductive development are unknown for many threatened and endangered species and may differ between lineages within species. Accordingly, prior to spawning, Main Diamond Gila Trout *Oncorhynchus gilae* broodstock were exposed to different thermal cycles and hormonal injection to improve reproductive success. Additionally, egg fatty acid composition and hatching success of Main and South Diamond Gila Trout were evaluated to determine the extent of phenotypic differences between lineages. Thermal conditioning of broodstock accompanied with injections of exogenous luteinizing hormone releasing hormone analog (LHRHa) prior to spawning improved progeny survival over historic hatchery means as well as means for broodstock reared in raceways at constant temperatures without LHRHa. Egg n-6 fatty acid content appears to be associated with this improvement. Eggs from Main and South Diamond broodstock fed the same feeds had similar hatch rates but could be differentiated on the basis of fatty acid profile. Future trials should further evaluate the influence of dietary fatty acids on egg deposition and hatch rate, while acknowledging that changes in reproductive performance may differ across lineages. Broodstocks should continue to be conditioned with thermal cycling and use of LHRHa to induce ovulation, as these techniques resulted in significantly better hatch rates in the current study than use of only photoperiod manipulation to stimulate reproductive development.

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The Gila Trout *Oncorhynchus gilae* is a native southwestern trout species found only in the high desert and mountain watersheds of the Gila, Salt, and Verde river drainages in New Mexico and Arizona. By the middle of the 20th century, however, habitat degradation caused by poor livestock management, pollution, logging, forest fires, and hybridization with nonnative trout has reduced the Gila Trout's range to only four streams in the Gila River headwaters in New Mexico. When the Federal Endangered Species Preservation Act (ESPA) of 1966 was enacted, the Gila Trout was placed on the initial endangered species list. When the ESPA was expanded into the Endangered Species Act of 1973, the Gila Trout was one of the first species listed and protected.

The four genetically distinct relict populations of Gila Trout are known by the streams in which they were originally found. The lineages are South Diamond (South Diamond Creek), Main Diamond (Main Diamond Creek), Spruce (Spruce Creek), and the smallest and rarest population, Whiskey (Whiskey Creek). All source populations are located in the Gila National Forest. Since 1999, Gila Trout have been reared at the Mora National Fish Hatchery (NFH), the only facility that currently cultures this rare fish. Populations in active culture are lineages from the Main Diamond and South Diamond watersheds.

Culture information for this species is scarce, and improvements in culture practices are needed to increase egg viability from a hatchery standard of 20%. The poor hatch rate and viability of Gila Trout eggs at Mora NFH have affected the recovery of this rare species. When production goals were not met from 2003 to 2009, the biological staff at the NFH began examining different methods to increase viability above 20%

Investigations have approached the problem from several directions, including temperature conditioning, injection of luteinizing hormone releasing hormone analog (LHRHa), and most recently, egg fatty acid analysis and nutrition. Successful reproduction of female fishes depends on production of steroid hormones and eicosanoids that regulate oocyte development and maturation, ovulation, fertilization, and embryogenesis. Environmental conditions influence a hormonal feedback loop involving the hypothalamus, pituitary, and gonad, which regulates female reproductive development and oocyte maturation through to ovulation. After ovulation, survival of the egg depends on the successful union of egg and sperm to allow fertilization and embryogenesis to proceed; in addition, embryonic survival and growth are largely influenced by maternal deposition of resources within the egg during earlier stages of reproductive development (Brooks et al. 1997). Disturbances at any point during the reproductive cycle can lead to reproductive dysfunction, as is often witnessed in captive-reared fishes. Altering hatchery practices to simulate the natural environment and use of exogenous hormone therapies can improve reproductive success of broodstocks, enabling successful spawning of many fish species in captivity (Mañanós et al. 2009). Staff at Mora NFH are refining spawning techniques to improve Gila Trout reproductive success. Thermal conditioning is used to more closely simulate a natural temperature regime. Because Mora NFH has an enclosed recirculation system, water temperatures generally do not respond as quickly or drop as low as a natural or outside system would. Thermal conditioning has been used (generally in coordination with light cycles) by the NFH System with great success to extend fish spawning times. However, the mechanism by which thermal conditioning and hormone therapy directly influence egg viability is unknown.

With regards to nutritional improvements for the broodstock, fatty acid profiles can identify critical nutritional components that may allow the hatchery to develop a specialized feed. Across all salmonid species, little is known about nutritional requirements of broodstocks (Hardy 1989, Izquierdo et al. 2001). Really, a paucity of information exists for broodstock nutrition across all fish species, generally related to the added expense and difficulty of conducting long-term feeding trials with large fish. The limited amount of research that has been reported indicates lipid and fatty acid concentrations of broodstock diets are important factors in reproductive success (Izquierdo et al. 2001). Egg concentrations of specific long-chain polyunsaturated fatty acids (LC-PUFAs; e.g., 20:4n-6, 20:5n-3, and 22:6n-3) may be important predictors of hatching success and early survival in fishes (Sargent et al. 2002). Egg fatty acid profiles differ between wild and cultured fish (Ashton et al. 1993; Czesny et al. 2000), largely due to differences in the fatty acid profile of commercial diets compared with that of natural prey items. Hatching success for eggs produced by cultured Arctic Char *Salvelinus alpinus* fed commercial aquaculture feeds was lower than for eggs from wild fish (Pickova et al. 2007). Eggs from the wild char contained higher concentrations of 20:4n-6 and 20:5n-3 fatty acids and lower concentrations of 22:6n-3 than were in eggs produced by the cultured char. Egg 22:6n-3 concentrations can be used as a diagnostic tool to predict egg viability in some marine species, being positively correlated to fertilization rate, hatching success, and larval survival of Common Snook *Centropomus undecimalis*, for example (Yanes-Roca et al. 2009). The relationship between egg fatty acid composition and hatching success seems to be largely species-specific; moreover, most nutritional research indicates that egg fatty acid profile reflects the maternal diet (Izquierdo et al. 2001). With regards to Gila Trout, no information is available describing fatty acid composition of eggs or how the composition correlates to hatching success.

Our objectives with this study were to provide preliminary information about the two lineages of Gila Trout reared at Mora NFH and how their genetic differences might affect nutrient requirements of broodstocks, egg fatty acid composition, and reproductive success. We also wanted to provide a biological reason why egg viability might be improved by thermal conditioning of females and using hormones to induce spawning. By identifying differences in the composition of eggs produced from females with low and high egg viability, we may be able to identify nutrients that should be included in future formulations to improve viability through modifications in feed composition.

Accordingly, we analyzed eggs produced by Main and South Diamond Gila Trout broodstock with identical nutritional histories to determine whether the profile of egg fatty acids differed between lineages. We also cultured and spawned Main Diamond broodstock with and without thermal conditioning, and with and without LHRHa injections, to evaluate the effects of these hatchery practices on egg viability. Across both experiments we related egg fatty acid concentrations to progeny survival to identify which fatty acids were associated with the best progeny survival and thus might appropriately be incorporated into future broodstock diet formulations.

METHODS

Broodstock husbandry and egg collection; lineage comparison.—Hatchery-reared Gila Trout broodstock (Main and South Diamond lineages; approximately age 3 years) were randomly placed into two separate recirculating aquaculture systems (one for each lineage) in November–December of the year prior to spawning (2010 and 2011). Since this is a threatened species, the staff took care not to artificially influence selection of broodstock. No attempts were made to select, grade, or otherwise artificially influence the size, shape, or physical characteristics of the broodstock for these experiments. After the fish were moved to the systems, males and females were segregated into separate tanks ($\sim$9.5 m^3 in volume). Water was recirculated through each tank at $\sim$132.5 L/min; make-up water, supplied from a larger recirculation system at Mora NFH, was added at 3.8 L/min. Both systems contained a parabolic screen filter, ultraviolet filtration, air-stirred bead filters, and a cooling unit. Overhead fluorescent fixtures were controlled to provide a natural photoperiod combined with supplemental light from windows. All fish were fed a commercially available 5.5-mm-pellet, low-phosphorus feed intended for trout. In 2010, the fish were fed a low-phosphorus feed manufactured by Nelson and Sons; in 2011, they were fed a low-phosphorus feed manufactured by Rangen, Inc. (formulations were proprietary for both manufacturers). Within a year, feeds and feeding practices (i.e., transition through lots of feed, duration of time on each feed, and feeding regime) were identical for Main and South Diamond lineages. Fish were fed in accordance with their daily growth as determined with Haskell's feed equation (Haskell 1959),

$$\text{body weight (\%)} = 3 * \text{Conversion} * \Delta L * 100/L.$$

Historical data from Mora NFH were used, which indicated that for fish with an initial length of 15–17 in the change in length (ΔL) and feed conversion were 0.01 and 2.0, respectively; these values remained constant throughout the trial. The length in the denominator is the current length of the fish, which changed throughout the study. The estimated changes in length were calculated using the cube root of the ratio of the weight to the condition factor. The condition factor used, $C = 0.0004$ (value commonly used for trout), was a compromise between wild source Gila Trout (0.00039; J. Brooks, Project Leader New Mexico Fish and Wildlife Conservation Office, USFWS, personal communication) and hatchery-reared Gila Trout (0.00044; Jeff Conway, unpublished data obtained during tagging of fish with passive integrated transponders (PIT), spawning, and fish health monitoring).

Starting on approximately January 1 of each year, the water temperature in each separate recirculating system (originally 10.5°C) was lowered 1°C every 6 days until the mechanical lower limit of the cooling unit ($\sim$7.2°C) was reached (after $\sim$18 d). The temperature was maintained at $\sim$7.2°C until the third week of February, after which the process was reversed to raise water temperatures 1°C every 6 days until water temperatures reached ambient conditions (maximum 11.6°C in 2010 and 12.7°C in 2011; this took $\sim$30 d). During the second week of March each year, broodstock were checked for ovulation: soft abdomen and free-flowing eggs after slight pressure was applied to the abdomen. Because very few fish had ovulated by that time in either year, they were injected with 10 μg of LHRHa per kilogram of body weight and then checked every 3 to 4 days afterwards for ovulation. Females who had not ovulated were given one additional injection of LHRHa at least 7 days after the first dose. When females were determined to have ovulated, they were weighed, measured, and spawned via manual stripping. Prior to fertilization, a sample of unfertilized eggs was collected from each female for fatty acid analysis as described below.

In 2010, the eggs from each female were fertilized by males from the same lineage in a 2 × 2 diallele mating in accordance with Kincaid and Reisenbichler (2002). In 2011, eggs from each female were fertilized with a hatchery-reared male from the same lineage in a 1 × 1 cross due to insufficient numbers of sexually mature wild-source fish. All fish were PIT-tagged and fin-clipped at the Mora NFH for genetic analysis at the Southwestern Native Aquatic Resources and Recovery Center (SNARC, Dexter, New Mexico; formally Dexter NFH and Technology Center) for pair-wise relatedness. The samples were analyzed using the Relationship coefficient (*Rxy*), where coefficients of 0–0.1 indicate the parent fish were unrelated, 0.1–0.18 indicates cousins, 0.19–0.38 half-siblings, and 0.39 full siblings. Viable crosses had coefficients of 0.18 and below. All eggs were incubated in Heath trays modified to contain chambers to keep the families separate. The incubation temperature ranged from 10.6°C to 12.2°C, and eggs hatched in approximately 30 d. All eggs and alevins were hand-counted to determine fecundity and progeny survival. Fecundity is represented as eggs produced per body weight of the female (no./kg).

Broodstock husbandry and egg collection; reproductive conditioning and hormone application.—Hatchery-reared Gila Trout broodstock from the Main Diamond lineage were randomly separated into two groups in 2011. Again, special care was taken to not grade fish, to avoid artificial selection of broodstock. One

group (THERMAL + LHRH) was placed into the recirculation system described above for thermal reproductive conditioning. Animal husbandry, spawning, and egg collection methods were exactly the same as described for the first objective. The other group (CONTROL) was placed in another recirculation system consisting of 28 m^3 raceways without thermal manipulation. The raceway system was also supplied with filtered water from a larger recirculation reuse system utilized at Mora NFH. Water in the system without thermal manipulation was recirculated through each tank at ~627 L/min with make-up water added at ~28.4 L/min. The raceway system contained a rotating drum microscreen filter for removal of suspended solids, a propeller-washed bead filter for nitrification of ammonia, and a stripping tower to remove carbon dioxide and aerate the water. Overhead fluorescent fixtures and high bay windows provided light controlled to provide a seasonal light cycle that was similar for both treatments. Fish in both treatments were fed the same 5.5-mm-pellet low-phosphorus commercial diet (manufactured by Rangen, Inc.; proprietary formulation) according to daily growth requirements determined using Haskell's feed equation (Haskell 1959) and historical growth data from Mora NFH.

CONTROL broodstock were checked for ovulation on February 22, 2011, and spawned on February 24, February 25, and March 3, 2011, without the use of LHRHa. A sample of eggs from each female was collected for fatty acid analysis, and the remaining eggs were fertilized using 1 × 1 crosses with Main Diamond males. All eggs were incubated by family in Heath trays. Water temperature ranged from 10.6°C to 12.2°C with eggs hatching in approximately 30 d. Eggs and alevins were also hand-counted to determine fecundity and hatch rates for this objective.

Lipid and fatty acid analyses.—Prior to fertilization, approximately 10 eggs were collected from each female for fatty acid analysis. Briefly, all eggs were collected from the female into a Ziplock bag. Ten eggs were randomly removed from each bag and placed in 15-mL glass test tubes, covered in nitrogen gas, capped, frozen at -80°C, and shipped to Abernathy Fish Technology Center (AFTC, Longview, Washington) on dry ice for fatty acid analysis. As this is a threatened species, a minimum number of eggs were removed for these analyses. Eggs were stored at -80°C at AFTC prior to analysis. Total lipid was extracted from these unfertilized eggs by the method of Folch et al. (1957) and quantified gravimetrically in 2011; lipid content was not determined in 2010. Fatty acid methyl esters (FAMEs) were prepared from total lipid following the method developed by Li and Watkins (2001). The resulting FAMEs were then separated using a Varian CP-3800 gas chromatograph (Agilent Technologies, Santa Clara, California) equipped with a flame ionization detector fitted with a fused silica capillary column (CP-Select for FAME, 100 m × 0.25 mm I.D.). The injection volume was 1.0 μL in helium as the carrier gas (2.5 mL/min) with an injector temperature of 230°C. A split injection technique (200:1) was used, and the temperature program was as follows: 60°C held for 1 min, increased to 150°C at 30°C/min, then increased to 230°C at 1.5°C/min, and held at 230°C for 2 min. Individual FAMEs were identified by comparison with retention times of external standards (Supelco 37 Component FAME Mix and PUFA-3; Supelco, Bellefonte, Pennsylvania).

Statistical analyses.—For lineage comparisons, only samples from Main Diamond females that had been thermally conditioned and given LHRHa in 2010 and 2011 were used. To assess both lineage and lipid and fatty acid profiles, all reproductive and fatty acid data were evaluated for each parameter by individual one-way analysis of variance (ANOVA) with Kenward Rogers degrees of freedom and female as the experimental unit, using the mixed procedure in SAS version 9.0 (Cary, North Carolina). Data were not transformed prior to statistical analysis because the assumptions of the ANOVA were met. Individual fatty acids were considered individual dependent variables, and no alpha adjustments were used for analyses of these variables. Canonical discriminant analysis was used to determine whether the lineages differed statistically according to egg fatty acid composition. Discriminant functional analysis was used to determine the number of females for which lineage could be correctly identified according to the canonical coefficients determined during the canonical discriminant analysis. For the canonical discriminant and discriminant functional analyses, all fatty acids constituting more than 1% of the total FAMEs or involved in n-3 and n-6 fatty acid chain elongation and desaturation pathways were included. Fatty acids with concentrations below detectable analytical limits were treated as missing values, eliminating that female's fatty acid profile from the analyses. In total, 15% (25) of the females were excluded from the canonical discriminant and discriminant functional analyses due to missing values. All analyses described in this section were evaluated with $\alpha = 0.05$.

RESULTS

Thermal Conditioning and Hormone Application Prior to Spawning

CONTROL females were significantly larger than THERMAL + LHRH females, by measures of both weight and length at spawning (Table 1). No differences in condition factor, total egg production, or fecundity were observed, although survival of progeny produced by THERMAL + LHRH was significantly better than those from CONTROL.

Egg fatty acid profiles differed between THERMAL + LHRH and CONTROL treatments (Table 2). THERMAL + LHRH–treated females produced eggs with higher concentrations of 18:2n-6, 20:3n-6, and total n-6, C_{18} PUFAs, and n-6 LC-PUFAs, whereas CONTROL females produced eggs with significantly higher concentrations of 22:5n-3 and n-3 to n-6 fatty acid ratios.

TABLE 1. Broodstock characteristics at spawning and reproductive output (mean ± SD) of Main Diamond Gila Trout broodstock either reared at constant temperatures and spawned naturally (CONTROL) or held in thermal conditioning tanks and given LHRHa prior to spawning (THERMAL + LHRH) at Mora National Fish Hatchery in 2011. Significance designations are as follows: not significant (NS), $P < 0.05$(*), $P < 0.01$(**), and $P < 0.001$(***).

Parameter	THERMAL + LHRH	CONTROL	Statistical significance
Weight (kg)	811.4 ± 411.4	1061.4 ± 485.7	*
Length (cm)	389.3 ± 64.3	423.2 ± 52.8	*
Condition factor	1.3 ± 0.1	1.4 ± 0.6	NS
Total eggs	552.5 ± 360.5	514.6 ± 187.5	NS
Fecundity (eggs/kg)	763.9 ± 389.6	601.0 ± 303.2	NS
Progeny survival (%)	44.7 ± 23.0	22.0 ± 29.3	**
	n = 45	n = 15	

TABLE 2. Fatty acid methyl ester (FAME; % total) concentrations determined in Gila Trout eggs from Main Diamond lineage either reared at constant temperatures and spawned naturally (CONTROL) or in thermal conditioning tanks and given LHRHa prior to spawning (THERMAL + LHRH) at Mora National Fish Hatchery in 2011. Significance designations are as follows: not significant (NS), $P < 0.05$(*), $P < 0.01$(**), and $P < 0.001$(***).

FAME	THERMAL + LHRH[a]	CONTROL[a]	Statistical significance
14:0	1.9 ± 0.2	2.0 ± 0.2	NS
16:0	14.1 ± 0.8	13.8 ± 0.8	NS
18:0	6.0 ± 0.7	6.3 ± 0.6	NS
Total SFAs[b]	22.5 ± 1.1	22.6 ± 0.9	NS
16:1n-7	4.8 ± 0.4	4.9 ± 0.5	NS
18:1n-9	13.6 ± 1.2	13.5 ± 1.4	NS
18:1n-7	3.9 ± 0.3	4.0 ± 0.5	NS
Total MUFAs[c]	23.9 ± 1.8	24.2 ± 2.4	NS
18:2n-6	4.7 ± 0.7	4.0 ± 0.6	***
20:2n-6	1.4 ± 0.2	1.4 ± 0.2	NS
20:3n-6	0.6 ± 0.1	0.5 ± 0.1	***
20:4n-6	2.0 ± 0.1	2.0 ± 0.2	NS
Total n-6[d]	8.7 ± 0.9	7.8 ± 0.6	***
18:3n-3	0.6 ± 0.1	0.6 ± 0.1	NS
18:4n-3	0.3 ± 0.1	0.3 ± 0.1	NS
20:4n-3	0.6 ± 0.1	0.6 ± 0.1	NS
20:5n-3	9.2 ± 0.9	9.3 ± 1.5	NS
22:5n-3	4.5 ± 0.4	4.7 ± 0.5	*
22:6n-3	26.5 ± 1.3	26.5 ± 1.2	NS
Total n-3[e]	41.5 ± 1.5	41.7 ± 2.0	NS
Total 18-C PUFAs[f]	5.8 ± 0.9	5.0 ± 0.7	***
Total n-6 LC-PUFAs	4.0 ± 0.3	3.8 ± 0.2	***
Total n-3 LC-PUFAs[e]	40.7 ± 1.6	40.9 ± 1.9	NS
Total LC-PUFAs[e]	44.7 ± 1.7	44.7 ± 2.0	NS
EPA/ARA	4.5 ± 0.3	4.7 ± 0.4	NS
DHA/EPA	2.9 ± 0.4	2.9 ± 0.5	NS
n-3/n-6	4.8 ± 0.5	5.4 ± 0.3	***
Lipid (% wet weight)	8.5 ± 1.3	8.9 ± 1.4	NS
Egg weight (mg)	83 ± 2	88 ± 2	NS

[a]% total; mean ± SD.
[b]Also includes 12:0, 15:0, 17:0, 20:0, and 24:0.
[c]Also includes 14:1n-5, 17: 1, 20:1n-9, and 24:1n-9.
[d]Also includes 18:3n-6.
[e]Also includes 20:3n-3.
[f]Also includes 18:3n-6 and 18:3n-4.

TABLE 3. Broodstock characteristics at spawning and reproductive output (mean ± SD) of Gila Trout from conditioning tanks at Mora National Fish Hatchery in brood years (BY) 2010 and 2011. Significance designations are as follows: not significant (NS), $P < 0.05$(*), $P < 0.01$(**), and $P < 0.001$(***).

Parameter	Main Diamond	South Diamond	Statistical significance
	BY 2007 spawned in 2010		
Weight (g)	725.7 ± 272.6	729.2 ± 408.4	NS
Length (mm)	383.0 ± 45.7	379.0 ± 69.5	NS
Condition factor	1.2 ± 0.2	1.2 ± 0.1	NS
Total eggs	709.1 ± 308.1	778.7 ± 455.6	NS
Fecundity (eggs/kg)	1016.4 ± 302.1	1116.1 ± 362.9	NS
Progeny survival (%)	38.6 ± 22.6	37.2 ± 24.2	NS
n	36	50	
	BY 2008 spawned in 2011		
Weight (g)	811.4 ± 411.4	671.3 ± 420.7	NS
Length (mm)	389.3 ± 64.3	363.9 ± 72.3	NS
Condition factor	1.3 ± 0.1	1.2 ± 0.1	NS
Total eggs	552.5 ± 360.5	606.9 ± 360.0	NS
Fecundity (eggs/kg)	763.9 ± 389.6	1031.6 ± 417.2	**
Progeny survival (%)	44.7 ± 23.0	40.2 ± 21.0	NS
n	45	48	

Lineage Comparison

Physical characteristics of female Gila Trout broodstock at spawning did not vary significantly between lineages in either year (Table 3). Total egg production and progeny survival were similar between lineages for both years of the study. Fecundity was significantly higher in South Diamond stock than in Main Diamond stock in 2011 but did not differ between lineages in 2010. Although egg weight was not quantified in 2010, eggs produced by the Main Diamond stock were significantly heavier than South Diamond stock in 2011 (Table 4). Lipid content of the eggs was not different between lineages in 2011, although lipid content did decrease as individual egg weight increased (Figure 1) in both lineages.

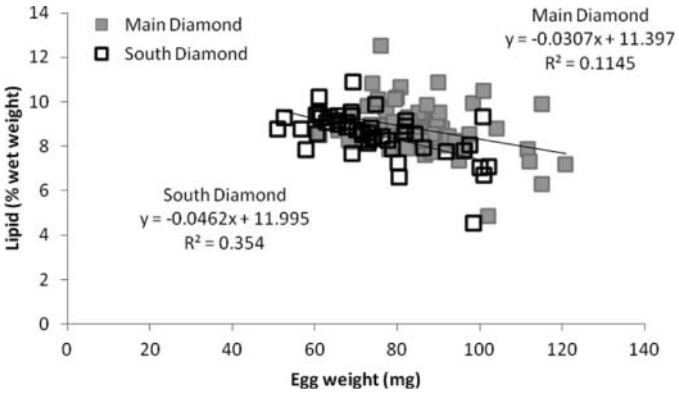

FIGURE 1. Relationship between egg weight and lipid content by Gila Trout lineage reared in conditioning tanks and spawned in 2011. Linear regression lines are statistically significant ($P < 0.01$), but regression coefficients between lineages do not differ significantly ($P = 0.2783$).

Egg fatty acid profiles varied between lineages in both years of the study (Table 4). Consistently across both years, 18:1n-7, 20:3n-6, 22:6n-3, and total n-6 LC-PUFA concentrations were higher and 18:3n-3 was lower in eggs produced by South Diamond stock. Main Diamond stock produced eggs with higher concentrations of 18:1n-9, 18:2n-6, total n-6, and total C_{18} PUFAs and lower concentrations of total n-3, n-3 LC-PUFAs, and LC-PUFAs; moreover, in 2010, Main Diamond eggs had a lower n-3/n-6 ratio than South Diamond stock's eggs did. In 2011, Main Diamond eggs had higher concentrations of 20:5n-3 and 22:5n-3 and a higher 20:5n-3–20:4n-6 ratio than did South Diamond eggs. However, South Diamond eggs contained greater concentrations of 14:0, 18:0, 20:2n-6, and 20:4n-3 and a higher 22:6n-3–20:5n-3 ratio than did Main Diamond eggs in 2011.

Egg fatty acid profiles were distinct between year-classes and lineages. Using canonical discriminant analysis, we determined differences in fatty acid profile between years and lineages to be most dependent on comparisons of egg concentrations of 18:1n-7 and n-6 fatty acids to remaining monounsaturated fatty acids (MUFAs), saturated fatty acids, and n-3 fatty acids (Table 5; 97.0% variation explained). The addition of the second canonical, primarily comparing egg concentrations of 18:3n-3, the 20:3n-6–18:2n-6 ratio, 20:2n-6, 20:4n-3, and 22:6n-3, significantly improved separation between groups, explaining 99.4% of total data variability (Figure 2). Using discriminant function analysis based on the canonicals described above, all broodstock eggs were correctly classified to spawning year (Table 6). Discriminating between lineages was marginally more successful in 2011 than 2010:81% and 76%, respectively (Table 6).

TABLE 4. Fatty acid methyl ester (FAME) concentrations determined in Gila Trout eggs from Mora National Fish Hatchery females that were thermally conditioned, injected with LHRHa, and spawned in brood years (BY) 2010 and 2011. Significance designations are as follows: not significant (NS), $P < 0.05$(*), $P < 0.01$(**), and $P < 0.001$(***); not determined (nd).

	BY2007 spawned in 2010			BY2008 spawned in 2011		
FAME	Main Diamond[a]	South Diamond[a]	Statistical significance	Main Diamond[a]	South Diamond[a]	Statistical significance
14:0	1.4 ± 0.2	1.4 ± 0.1	NS	1.9 ± 0.2	2.0 ± 0.2	*
16:0	14.7 ± 1.1	14.3 ± 0.7	NS	14.1 ± 0.8	14.4 ± 0.6	NS
18:0	5.8 ± 0.8	5.7 ± 0.6	NS	6.0 ± 0.7	5.7 ± 0.6	*
Total SFAs[b]	22.6 ± 1.9	22.1 ± 0.8	NS	22.5 ± 1.1	22.6 ± 0.7	NS
16:1n-7	4.5 ± 0.3	4.7 ± 0.6	NS	4.8 ± 0.4	4.9 ± 0.5	NS
18:1n-9	19.1 ± 1.4	18.0 ± 1.2	***	13.6 ± 1.2	13.2 ± 0.9	NS
18:1n-7	3.5 ± 0.3	3.7 ± 0.4	**	3.9 ± 0.3	4.1 ± 0.3	***
Total MUFAs[c]	28.8 ± 1.9	28.3 ± 2.1	NS	23.9 ± 1.8	23.8 ± 1.7	NS
18:2n-6	7.8 ± 0.6	7.0 ± 0.6	***	4.7 ± 0.7	4.7 ± 0.6	NS
18:3n-6	0.3 ± 0.1	0.3 ± 0.1	NS	nd[e]	nd	NS
20:2n-6	2.1 ± 0.2	2.2 ± 0.2	NS	1.4 ± 0.2	1.5 ± 0.1	*
20:3n-6	1.6 ± 0.2	1.7 ± 0.2	*	0.6 ± 0.1	0.8 ± 0.2	***
20:4n-6	2.8 ± 0.3	2.9 ± 0.3	NS	2.0 ± 0.1	2.0 ± 0.2	NS
Total n-6	14.7 ± 0.6	14.0 ± 0.8	***	8.7 ± 0.9	8.9 ± 0.9	NS
18:3n-3	0.5 ± 0.0	0.4 ± 0.1	***	0.63 ± 0.1	0.58 ± 0.1	*
18:4n-3	0.2 ± 0.1	0.2 ± 0.1	NS	0.3 ± 0.1	0.3 ± 0.1	NS
20:4n-3	0.3 ± 0.0	0.3 ± 0.0	NS	0.58 ± 0.1	0.63 ± 0.1	***
20:5n-3	5.7 ± 0.9	5.7 ± 0.7	NS	9.2 ± 0.9	8.6 ± 1.1	**
22:5n-3	2.6 ± 0.4	2.6 ± 0.3	NS	4.5 ± 0.4	4.2 ± 0.6	*
22:6n-3	20.8 ± 2.2	22.4 ± 1.4	***	26.5 ± 1.3	27.2 ± 1.2	*
Total n-3[d]	30.1 ± 2.9	31.7 ± 1.9	**	41.5 ± 1.5	41.4 ± 1.3	NS
Total 18-C PUFAs[e]	9.0 ± 0.7	8.2 ± 0.8	***	5.8 ± 0.9	5.8 ± 0.8	NS
Total n-6 LC-PUFAs	6.6 ± 0.3	6.8 ± 0.5	*	4.0 ± 0.3	4.2 ± 0.3	**
Total n-3 LC-PUFAs[d]	29.5 ± 2.9	31.0 ± 1.9	**	40.7 ± 1.6	40.6 ± 1.3	NS
Total LC-PUFAs[d]	36.0 ± 3.1	37.8 ± 2.0	**	44.7 ± 1.7	44.9 ± 1.3	NS
EPA/ARA	2.0 ± 0.2	2.0 ± 0.3	NS	4.5 ± 0.3	4.3 ± 0.4	**
DHA/EPA	3.7 ± 0.6	4.0 ± 0.5	NS	2.9 ± 0.4	3.2 ± 0.5	**
n-3/n-6	2.1 ± 0.2	2.3 ± 0.2	***	4.8 ± 0.5	4.7 ± 0.5	NS
Egg lipid (% as is)	nd	nd		8.6 ± 0.1	8.4 ± 0.2	NS
Egg weight (mg)	nd	nd		86 ± 1.5	74 ± 1.8	***

[a]% total; mean ± SD.
[b]Also includes 12:0, 15:0, 17:0, 20:0, and 24:0.
[c]Also includes 14:1n-5, 17:1, 20:1n-9, and 24:1n-9.
[d]Also includes 20:3n-3.
[e]Also includes 18:3n-6 and 18:3n-4.

DISCUSSION

In the current study, Gila Trout broodstock in the THERMAL + LHRH treatment produced progeny in 2011 with significantly better survival than CONTROL fish or the hatchery standard of ~20%(J. Conway, unpublished data). Egg production and ovulation were not affected by thermal conditioning and LHRHa application, as broodstock from both treatments produced similar quantities of eggs with comparable fecundities. This suggests that the thermal conditioning of broodstock and their treatment with LHRHa influenced oocyte development and final oocyte maturation more than they did ovulation. Enhanced survival of progeny from THERMAL + LHRH females may indicate enhanced deposition of resources within the oocyte during previtellogenic and vitellogenic growth, facilitating better fertilization success and embryonic development after fertilization. The larger size of the CONTROL broodstock may be due to more resources being used to sustain somatic growth of females rather than reproductive development, although this cannot be confirmed since the initial weights of females in both treatments were not recorded.

Mimicking natural environmental conditions in female broodstock conditioning tanks stimulates natural production of

TABLE 5. Total sample–standardized canonical coefficients for brood year 2007 and 2008 Gila Trout of both Main and South Diamond lineages. The overall relationship is statistically significant ($P < 0.0001$). Canonical 1 describes 97.0% of the variation in the data ($P < 0.0001$). The addition of canonical 2 raises the degree of description to 99.4% of the variation in the data and further separates the brood years and lineages statistically ($P < 0.0001$). Graphical expression of the canonical analysis is provided in Figure 2.

Fatty acid	Canonical 1 coefficient	Canonical 2 coefficient
18:2n-6	−2.05	2.56
20:2n-6	−1.17	1.24
20:3n-6	−0.55	−1.45
16:1n-7	−0.43	0.64
20:4n-6	−0.35	0.62
16:0	0.14	0.67
18:0	0.18	0.09
20:1n-9	0.2	−0.46
18:4n-3	0.29	0.47
20:5n-3	0.37	0.36
22:5n-3	0.43	−0.36
14:0	0.78	−0.16
18:1n-9	0.8	0.39
18:1n-7	1.01	0.34
22:6n-3	1.03	1.47
20:4n-3	1.14	3.75
18:3n-3	1.57	−3.16

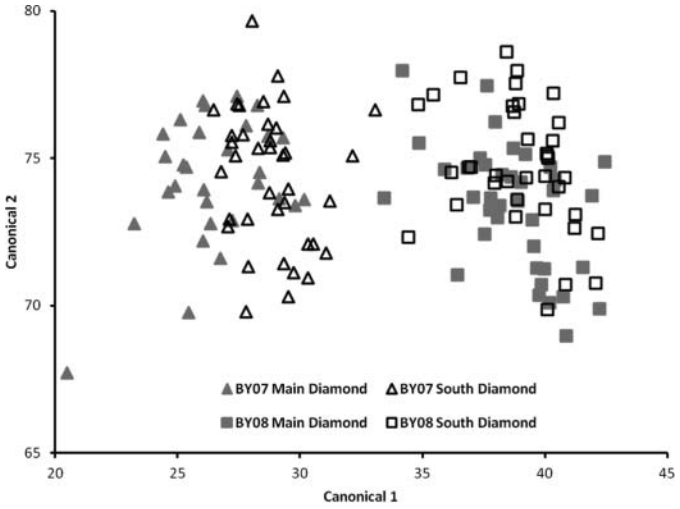

FIGURE 2. Canonical plot of Gila Trout egg fatty acid profiles from fish reared in conditioning tanks and spawned in 2010 and 2011 at Mora National Fish Hatchery. The overall relationship is statistically significant ($P < 0.0001$). Canonical 1 describes 97.0% of the variation in the data ($P < 0.0001$).

the steroids that prompt previtellogenic and vitellogenic oocyte growth and development (Mañanós et al. 2009). Administration of LHRHa prior to spawning stimulates the endogenous release of luteinizing hormone from the pituitary, which acts on the gonad to induce final oocyte maturation through steroid and eicosanoid production by ovarian tissue (Mañanós et al. 2009). Prostaglandins produced from 20:4n-6 fatty acids along the cyclooxygenase pathway (Sorbera et al. 2001) are among the most influential eicosanoids regulating final oocyte maturation in fishes (Stacey and Goetz 1982). These 2-series prostaglandins derived from 20:4n-6 are biologically more effective at inducing final oocyte maturation than are the 1- and 3-series prostaglandins derived from 20:3n-6 or 20:5n-3, respectively (Sorbera et al. 2001); which series of eicosanoids is produced is ultimately controlled by which fatty acid precursor is most prevalent within the tissue (Wathes et al. 2007). When present in tissues at high enough concentrations, 20:3n-6 and 20:5n-3 can also compete with 20:4n-6 for binding sites and inhibit 2-series eicosanoid production from 20:4n-6 (Willis 1981). Although the concentration of 20:4n-6 was not significantly elevated within eggs produced by either group of broodstock, eggs produced by THERMAL + LHRH broodstock contained higher concentrations of 18:2n-6 and 20:3n-6. Collectively, salmonids have the ability to produce LC-PUFA from C_{18} precursors (Tocher 2003), using 18:2n-6 as the initial substrate in the n-6 pathway and 20:3n-6 as an intermediate to 20:4n-6 production. This process of fatty acid chain elongation and desaturation occurs primarily within the liver, though elongase and desaturase genes are also actively expressed in intestine, brain, kidney, heart, gill, muscle, spleen, and adipose tissues (Zheng et al. 2005). Furthermore, although not confirmed in salmonids, fatty acid elongase and desaturase genes are actively expressed in Zebrafish *Danio rerio* oocytes, suggesting that some fish are

TABLE 6. Classification summary for discriminant analysis based on Gila Trout egg fatty acid profiles for brood years (BY) 2007 and 2008 of both Main and South Diamond lineages.

	Number (and %) of observations classified into BY and lineage				
	BY07 Main Diamond	BY07 South Diamond	BY08 Main Diamond	BY08 South Diamond	Total
BY07 Main Diamond	18 (60.0)	12 (40.0)	0 (0)	0 (0)	30 (100)
BY07 South Diamond	4 (10.5)	34 (89.5)	0 (0)	0 (0)	38 (100)
BY08 Main Diamond	0 (0)	0 (0)	31 (81.6)	7 (18.4)	38 (100)
BY08 South Diamond	0 (0)	0 (0)	7 (18.9)	30 (81.1)	37 (100)

capable of biotransforming C_{18} PUFAs into LC-PUFAs within oocytes at all developmental stages (Ishak et al. 2008). Both 18:2n-6 and 20:3n-6 concentrations were elevated in eggs from THERMAL + LHRH broodstock, from which Gila Trout may actively produce 20:4n-6 within the oocyte prior to and after spawning. Perhaps rearing Gila Trout in thermal conditioning tanks allows for more complete oocyte development, resulting in enhanced deposition of 18:2n-6 within the eggs for future use by the developing embryo. Enhanced progeny survival may be due to this enrichment of n-6 precursors that provide substrate for eicosanoid production during embryogenesis, which supports embryonic survival and development better within the THERMAL + LHRH group than in the CONTROL group. The exact mechanism by which the increased concentrations of 18:2n-6 and 20:3n-6 act to improve progeny survival is still unknown, whether it is augmentation of eicosanoid synthesis through enhanced production of 1-series eicosanoids from 20:3n-6; inhibition of 2-series eicosanoid production by 20:3n-6, if 20:3n-6 was desaturated to form 20:4n-6 within the developing egg for enhanced production of 2-series eicosanoids during fertilization or embryogenesis; or another mechanism independent of eicosanoid synthesis. Regardless, rearing broodstock in thermal conditioning tanks and supplying exogenous LHRHa prior to spawning did improve progeny survival, and n-6 fatty acids appear to be associated with this improvement.

Among broodstock reared in conditioning tanks and given LHRHa, the profiles of egg fatty acids also varied by brood year and lineage. All Gila Trout reared at Mora NFH were fed commercial diets under standard hatchery protocols, which necessitated changes in pellet size and diet composition according to growth and life stage. Although all fish within brood years were transitioned to new diets at the same time, slight differences in diet formulations (Izquierdo et al. 2001; Vassallo-Agius et al. 2001; Lewis et al. 2010) or duration of time on the respective diets (Almansa et al. 1999) between brood years may have contributed to the significant distinction in egg fatty acid profiles between 2010 and 2011. The feed formulation for all diets fed at Mora NFH is proprietary; therefore, we have not been able to discuss specifically or speculatively what impact the different ingredients may have had on these differences in egg fatty acid profiles. Notably, although feeding practices for both Main and South Diamond lineages were identical within years, differences in egg fatty acid profiles between lineages were apparent. This suggests that fatty acid metabolism with regards to deposition into or synthesis within the egg is under genetic influence. The canonicals representing Main and South Diamond lineages overlap substantially within years, perhaps because of the close relationship and genetic similarity between lineages, even though Main and South Diamond lineages each have distinct genetic identities (Loudenslager et al. 1986; Riddle et al. 1998). Genetic variability influences many aspects of metabolism and reproduction (Kinghorn 1983; Martyniuk et al. 2003), often with the result that some lineages or families are more adapted to success under specific conditions (Mousseau and Roff 1987). With regards to nutrition and metabolism, growth (Palti et al. 2006; Pierce et al. 2008), lipid deposition (Refstie and Austreng 1981; Quinton et al. 2007), diet digestibility, condition factor, and carcass proximate composition (Refstie and Austreng 1981) are heritable traits. Given the similarities in egg production and progeny survival between Main and South Diamond Gila Trout broodstock fed the same diets, slight differences in genetic makeup driving the observed changes in fatty acid metabolism are not distinct enough at this time to necessitate separate diets for Main and South Diamond broodstock. Future research should be initiated to verify this assumption and optimize diets for these broodstocks.

No physical differences were observed between the Main and South Diamond Gila Trout broodstock in either year. In 2011, fecundity was higher in South Diamond than in Main Diamond stocks. Egg mass and number are often divergent between fish populations: the females partition resources into producing either a greater number of small eggs or fewer large eggs (Svärdson 1949; Scott 1962; Elgar 1990; Fleming and Gross 1990; Jonsson and Jonsson 1999). This paradigm was true for the 2011 South Diamond broodstock. They produced more eggs relative to their body weight than Main Diamond stock did; however, South Diamond eggs were significantly smaller than those produced by Main Diamond females. Overall, lipid content of the South Diamond eggs was similar to those produced by the Main Diamond stock, even though lipid content did decline as egg weight increased in both stocks. Hatch rate was similar between lineages in each year and independent ($P > 0.05$) of egg weight and lipid content for both lineages in 2011. Hatch rate (means 37–44%) of the captive Gila Trout eggs in the current study was appreciably lower than that reported for rainbow trout (means, >65%) broodstock reared on diets either meeting recommended amounts or deficient in essential fatty acids (Vassallo-Agius et al. 2001); this was slightly lower than that reported for wild Gila Trout (~50%; USFWS 1993). However, survival of progeny was 85% to 120% better when broodstocks were reared in conditioning tanks and synchronized by using LHRHa prior to spawning compared with historic averages at Mora NFH (~20%, J. Conway, unpublished data) or with the average for broodstock reared under constant temperatures without LHRHa.

In conclusion, Main Diamond Gila Trout broodstock reared in conditioning tanks simulating the natural thermal environment and given LHRHa prior to spawning produced eggs with significantly better survival to hatch than did broodstock reared in raceways. By implementing new hatchery practices, such as the thermal conditioning of broodstock, Mora NFH has exceeded production goals for the years 2010–2013. Improvement in progeny survival may be linked to increased concentrations of 18:2n-6 and 20:3n-6 found within conditioned broodstock eggs. Although fatty acid profiles differed between Main and South Diamond lineages, total egg production and progeny survival

were similar for these two lineages. At this time, it appears that broodstock from both lineages can be reared on the same diet since hatch rate was similar across lineage; however, experimenting with different diets—perhaps containing plant oils rich in 18:2n-6—may improve reproductive output by female Gila Trout or result in interactive responses between the lineages.

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Potential of Asian Carp from the Illinois River as a Source of Raw Materials for Fish Meal Production

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ARTICLE

Potential of Asian Carp from the Illinois River as a Source of Raw Materials for Fish Meal Production

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Abstract

Incentivizing overfishing through the creation of high-value markets for Silver Carp *Hypophthalmichthys molitrix* and Bighead Carp *H. nobilis* has been proposed as a management strategy for controlling Asian carp in the Illinois River. Asian carp may be rendered into a protein-dense fish meal, and one of the most attractive, high-value end uses for such a product is aquafeed manufacturing. However, the nutritional content and shelf stability of Asian carp as a raw material must be determined to assess its suitability for rendering and subsequent use as a protein source in aquafeeds, which was the focus of this study. To determine seasonal, geographical, and species variation in body composition, fatty acids, and oxidative stability, Asian carp were collected from five reaches of the Illinois River during the fall, spring, and summer (up to 12 individuals of each species per reach per season) and analyzed. Slight geographical, seasonal, and species variation exists in the body composition and fatty acid profiles of adult Asian carp from the Illinois River. There was an apparent trend of increasing lipid content from lower to upper reaches and from fall to summer. Bighead Carp tended to be leaner (higher protein, moisture, and ash content) than Silver Carp (higher lipid content). Although Asian carp generally appear to be a good source of long-chain polyunsaturated fatty acids regardless of capture location or season, the concentration of these nutrients was highest in the fall. Oxidative stability analysis indicated Asian carp meal will need stabilizers to increase shelf life. Collectively, our results suggest Asian carp-based fish meals would be nutritionally suitable for use in aquafeeds, if precautions are taken to stabilize the product during storage. Creating demand for carp meal in the aquafeed manufacturing sector may prove a valuable strategy for aiding in the control of Asian carp populations in the Illinois River.

Following the rapid expansion of populations of Silver Carp *Hypophthalmichthys molitrix* and Bighead Carp *H. nobilis* (Chick and Pegg 2001), hereafter referred to as Asian carp, in the Mississippi River basin, a variety of management strategies aimed at controlling their movement and reducing their density have been suggested. These strategies include behavioral barriers (e.g., strobe lights, acoustic deterrents, air bubble curtains, electrical barriers), physical barriers (e.g., vertical drops, rotating drums, floating curtains), and chemical barriers (e.g., low oxygen, carbon dioxide; Conover et al. 2007; Rach et al. 2009). All of these approaches have been considered for controlling Asian carp in the Illinois River, a focal point for Asian carp control because of the hydrologic connection formed between the Mississippi River basin and the Great Lakes basin via the Des Plaines River and Chicago Area Waterway System (CAWS). A series of electrical deterrence barriers are currently in place in the CAWS (Moy et al. 2011), and an intensive surveillance program is in place to monitor the system for Asian carp (ACRCC 2012). Additional control efforts in the Illinois River are primarily focused on harvest and chemical eradication (Rach et al. 2009; ACRCC 2012). Although this "all-of-the-above" management approach appears diffuse rather than strategic, a redundant multistrategy approach is likely necessary to effectively suppress Asian carp populations in the Illinois River.

Harvest-based control efforts can be augmented by harnessing potential market forces to incentivize overfishing, but subsidies, low interest loans, or contract fisheries may be necessary until the market is fully developed (Conover et al. 2007). Asian

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carp are not favored food fish in the United States and, therefore, industrial end uses and markets are likely to generate greater demand. A considerable volume of Asian carp is currently being exported from Illinois to China for human consumption, but transportation costs and logistics limit this approach and its ability to drive harvest pressure to control carp populations. Like other fishes with limited seafood market potential (e.g., menhadens, anchovies, herrings), Asian carp can be rendered to produce nutrient-dense fish meals. Fish meals are rendered protein products derived from fish carcasses or offal. These products are primarily used as fertilizers or as feed ingredients in companion animal and livestock feeds. At this time, Asian carp are primarily processed into fertilizers, but it may be possible to target the higher value uses in the animal feeds industry, in particular aquafeeds. An Asian carp meal has several advantages over other alternative feedstuffs in aquafeed production because of its similarity to traditional, marine-origin fish meals that are considered ideal proteins in many ways. Fish meal replacement in aquafeeds presents a variety of challenges. Fish have a higher protein demand relative to other livestock (Keembiyehetty and Gatlin 1992), and protein tends to be the most expensive dietary component. Aquafeed manufacturers have relied on fish meal as a protein-dense ingredient to meet this demand, but the rising cost of fish meal has forced feed manufactures to explore alternative sources.

Fish meal replacement can be difficult depending on the qualities of the alternative feedstuff. For example, soybean meal is one of the most promising plant protein sources as a fish meal alternative because of its favorable amino acid profile and relatively protein content compared with other plant-derived protein meals (Gatlin et al. 2007). Despite the routine use of soy products in fish feeds (Hendricks 2002), high inclusion levels can confer undesired attributes to the feeds (Kaushik 2008), resulting in poor palatability (Adelizi et al. 1998), low feed conversion efficiency (Davies and Morris 1997), reduced mineral availability (Trushenski et al. 2006), and intestinal antinutritional effects (Ostaszewska et al. 2005; Heikkinen et al. 2006; Iwashita et al. 2008; Santigosa et al. 2008). In addition to soy products, a variety of other plant-based (e.g., canola, corn, wheat) and animal-based (e.g., poultry, blood, feather) feedstuffs have been explored. However, the chemical composition, digestibility, palatability, antinutritional factors, nutrient utilization, or functional inclusion of these feedstuffs pose challenges to incorporating them into aquafeeds (Glencross et al. 2007). An Asian carp meal could be an ideal alternative because it mimics traditional fish meal sources and is unlikely to exhibit the undesirable attributes of other alternatives. Furthermore, the ecological integrity of the Mississippi River basin would also benefit from the increased removal of these invasive fish.

The global supply of fish meal remains static at 5–6 million metric tons/year, and the aquaculture sector is the largest consumer (approximately 60%) of fish meal in the world, even though fish meal use in aquafeeds (in terms of dietary inclusion rates) has slowly decreased since 2006 (FAO 2012). As the aquaculture industry continues to grow, the combination of a static supply, high demand, and overdependence of aquafeed manufacturers on fish meal has led to a dramatic increase in feed cost. Fish meal prices doubled from US$694 to $1,379/metric ton between March 2007 and March 2008 (Tacon and Metian 2008), and current prices are approaching $2,000/metric ton (FAO 2013). Although the use of fish meal in aquafeeds is predicted to decrease in the future due to an improved knowledge of the digestive processes and nutritional requirements of many farm-raised fishes (FAO 2012), fish meal will continue to play a critical role in aquafeed production, particularly for carnivorous species. Thus, the aquaculture industry could benefit from an underutilized alternative fish meal source such as Asian carp.

Increased demand for fish meal developed from Asian carp would provide an incentive for commercial fishers to capture large quantities of these fishes, facilitating population control (Conover et al. 2007). Modeling predictions have suggested that harvest of both small and large Asian carp is essential to their control (Garvey et al. 2007, 2012). Given that current fishing efforts are aimed primarily at large fish for human consumption markets, Asian carp meal would also incentivize harvest of smaller and younger fish. Harvest enhancement is also attractive in that it could reduce the need for costly and unpopular chemical eradication strategies (e.g., Vasquez et al. 2012) while simultaneously revitalizing the regional commercial fishing industry. It has also been suggested that the only viable solution to control established populations of Asian carp in the short term is through enhanced harvest efforts (Conover et al. 2007). However, demand for Asian carp meal is dependent on the ability to produce a stable supply and quality rendered product. The nutritional composition and associated value of traditional reduction fisheries (i.e., fisheries that process their catch into fish meal or fish oil) landings are known to vary by species as well as harvest location and season (Bragadóttir et al. 2004; Boran et al. 2008). Furthermore, fish meal is more susceptible to oxidative spoilage than other protein meals due to its high surface-area-to-volume ratio and higher concentrations of unsaturated fatty acids (El-Lakany and March 1974; Boran et al. 2008). Therefore, the variability in nutritional value and protection against oxidation are critical factors in determining the market value of Asian carp meal as a feedstuff as well as its potential to facilitate the control of Asian carp populations and to support the aquafeed industry.

This study was designed to determine the suitability of rendering Asian carp into fish meal as a feedstuff for aquafeeds and briefly examine its potential to control carp populations through overharvest to support fish meal production. To assess the appropriateness of Asian carp as a feedstuff for aquafeed production, the species-related, seasonal, and geographic variation in body composition and storage stability were evaluated.

METHODS

To assess taxonomic, seasonal, and geographic variation in the composition of Asian carp, Bighead Carp and Silver Carp were collected seasonally during fall (2010), spring (2011), and

TABLE 1. Numbers of Silver and Bighead carps harvested from each reach of the Illinois River in fall 2010, spring 2011, and summer 2011.

Season	Species	Harvest location				
		Alton	La Grange	Peoria	Starved Rock	Marseilles
Fall	Silver	12	12	12	12	0
	Bighead	0	2	12	0	0
Spring	Silver	12	12	12	12	10
	Bighead	0	0	0	12	12
Summer	Silver	12	12	12	12	12
	Bighead	10	10	12	12	12

summer (2011) from five reaches of the Illinois River (up to 12 fish of each species per reach per season; Table 1). The reaches were separated by lock and dam complexes, and consisted of Alton, La Grange, Peoria, Starved Rock, and Marseilles. Fish were harvested using trammel nets and pulsed DC electrofishing, and commercial fishers assisted with several collections. Fish were transported on ice to the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University Carbondale (Carbondale, Illinois), where the carcasses were frozen (–20°C) prior to compositional analysis.

Carcasses were individually homogenized prior to analysis. Due to the large body size and thick bones of Asian carp, frozen carcasses were first cut into pieces (~5-cm cubes) using a butcher's bone saw. The partially thawed pieces were then homogenized in a knife mill (Knife Mill GM 300; Retsch, Haan, Germany). A 200-g subsample of the resulting homogenate was collected and stored frozen until compositional analysis. Some fish had to be homogenized in batches due to their large size. In these cases, batches were combined in a large bowl and mixed thoroughly by hand prior to subsample collection.

The subsamples were lyophilized (Freezone 6; Labconco, Kansas City, Missouri) to determine moisture content and subsequently pulverized using a coffee–spice grinder for determination of protein, ash, and lipid content. Total lipid was determined gravimetrically following chloroform–methanol extraction modified from Folch et al. (1957). Resultant lipid fractions were analyzed for fatty acid composition according to the procedures described by Laporte and Trushenski (2011). Briefly, samples were subjected to acid-catalyzed transmethylation performed overnight at 50°C as described by Christie (1982), and the resultant fatty acid methyl esters (FAME) were separated using a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a flame ionization detector fitted with a permanently bonded polyethylene glycol, fused silica capillary column (Omegawax 250, 30 m × 0.25 mm ID, 0.25-μm film; Sigma Aldrich, St. Louis, Missouri). The injection volume was 1.0 μL, with helium as the carrier gas (30 cm/s, 205°C), and an injector temperature of 250°C. A split injection technique (100:1) was used, with a temperature program as follows: 50°C held for 2 min, increased to 220°C at 4°C/min, and held at 220°C for 15 min. Individual FAME were identified by reference to external standards (Supelco 37 Component FAME Mix, PUFAs-1, and PUFAs-3; Supelco, Bellefonte, Pennsylvania). Ash content was determined gravimetrically after incineration in a muffle furnace for 4 h at 650°C. A LECO protein analyzer (FP-528; LECO Corporation, St. Joseph, Michigan) was used to determine protein content.

To estimate oxidative stability of Asian carp fish meal, the subsamples of lyophilized, ground Asian carp were placed in a refrigerator (0, 2, 4, and 8 weeks) and analyzed for the presence of peroxides and aldehydes with Peroxysafe and Aldesafe colorimetric assay kits (SafTest, Phoenix, Arizona). The samples were stored at –80°C until analysis, and all peroxide–aldehyde concentrations were standardized for whole-body lipid content (Folch et al. 1957). These values were compared with those of a commercially available, stabilized fish meal (Special Select menhaden fish meal; Omega Protein, Houston, Texas) stored under the same conditions.

Individual fish served as the experimental units for all statistical analyses ($n = 12$). A factorial analysis was not possible due to missing combinations of independent variables as a result of incomplete sampling (Table 1). As such, a nested ANOVA design was used within the generalized linear mixed-model framework (GLIMMIX procedures) of the Statistical Analysis System version 9.2 (SAS Institute, Cary, North Carolina) to determine whether there were differences in proximate composition among locations within each season as well as among seasons within a specific location; separate species-specific models were used for moisture, protein, lipid, and ash content. These data were also analyzed using a nested ANOVA within the mixed-model framework to determine whether differences in proximate composition existed between species within a specific location and harvest season. A one-way ANOVA was used to determine differences among species across seasons and locations. Linear regression was used to determine the relationship between total length and body composition of Asian carp. Individual fish collected during the fall sample season were used to determine oxidative stability of Asian carp meals compared with that of a stabilized fish meal control using repeated-measures mixed-model analysis. In all cases, differences were considered significant at an α-level of 0.05.

TABLE 2. Proximate composition (%) of Asian carp harvested seasonally from the Illinois River (fall 2010–summer 2011) and pooled across all five harvest locations (Alton, La Grange, Peoria, Starved Rock, and Marseilles). Numeric labels indicate mean percent composition of the intact carcasses (i.e., wet matter basis); numbers may not add to 100% because of minor amounts of carbohydrate not analyzed and rounding errors. Letters indicate significant differences within species across seasons at $P < 0.05$.

Species	Composition	Seasons		
		Fall	Spring	Summer
Silver Carp	Moisture	74.9 ± 0.4 z	71.0 ± 0.4 y	69.2 ± 0.4 x
	Protein	14.8 ± 0.8 y	15.7 ± 0.7 z	16.3 ± 0.7 z
	Lipid	3.9 ± 1.1 y	6.1 ± 1.0 z	7.4 ± 1.0 z
	Ash	5.6 ± 0.6 z	6.0 ± 0.5 z	5.6 ± 0.5 y
Bighead Carp	Moisture	80.4 ± 0.8 z	78.5 ± 0.4 z	76.7 ± 0.3 y
	Protein	13.1 ± 1.8 zy	13.4 ± 0.9 y	15.5 ± 0.6 z
	Lipid	1.3 ± 1.5	2.0 ± 0.8	2.3 ± 0.5
	Ash	5.0 ± 1.3 z	4.5 ± 0.7 y	4.8 ± 0.5 y

RESULTS

There were significant seasonal differences in body composition (Table 2) and FAME groups (Figure 1) in Asian carp from the Illinois River. Similar trends were observed for both Silver Carp and Bighead Carp: moisture, protein, and ash decreased across harvest seasons, fall to summer, while lipid increased. Saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) increased over the same time period, while polyunsaturated fatty acids (PUFAs), medium-chain polyunsaturated fatty acids (MC-PUFAs), long-chain polyunsaturated fatty acids (LC-PUFAs), n-3, n-6, and the ratio of n-3 to n-6 (n-3:n-6) decreased.

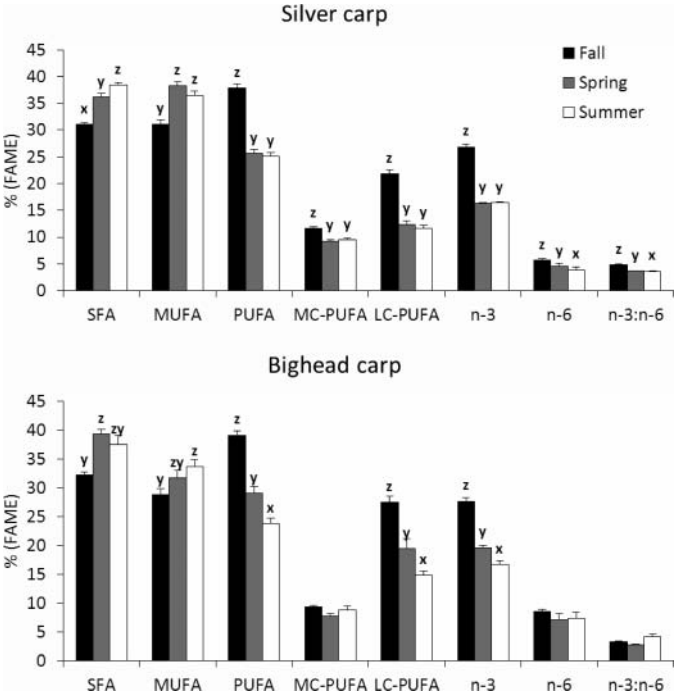

FIGURE 1. Fatty acid composition (% FAME) of Asian carp collected seasonally from the Illinois River (fall 2010–summer 2011). Data were pooled across all five harvest locations (Alton, La Grange, Peoria, Starved Rock, and Marseilles). SFAs = saturated fatty acids (no double bonds), MUFAs = monounsaturated fatty acids (one double bond), PUFAs = polyunsaturated fatty acids (two or more double bonds), MC-PUFAs = medium-chain polyunsaturated fatty acids (18 carbon atoms, two or more double bonds), LC-PUFAs = long-chain polyunsaturated fatty acids (20 or 22 carbon atoms, three or more double bonds). Different letters indicate significant differences within species across seasons at $P < 0.05$; error bars indicate SE.

Geographical differences in body composition and FAME groups within each season were also observed. Silver Carp demonstrated similar patterns among seasons in body composition: moisture and ash content of Silver Carp decreased from lower reaches to upper reaches (Alton to Marseilles), while lipid increased (Table 3). However, Silver Carp from the Peoria Reach did not follow these general trends in fall and spring. Protein content of Silver Carp within each season varied geographically with no apparent trends. The FAME groups for Silver Carp did not demonstrate any trends during fall, but spring and summer harvest periods showed similar geographic trends (Table 4). Saturated fatty acids decreased from lower to upper reaches of the Illinois River, while PUFAs, MC-PUFAs, LC-PUFAs, n-3, and n-6 increased moving up the river, peaking in Peoria before decreasing again (except for the n-6 group in spring, where there were no significant differences). Geographical differences for Bighead Carp were limited to the summer harvest period due to data gaps in fall and spring, and no trends were apparent in the body composition of Bighead Carp. However, Bighead Carp collected from the Marseilles Reach tended to be dissimilar (higher lipid content with lower moisture and ash) from the other reaches, except in protein content (Table 3). Few trends were apparent in Bighead Carp FAME groups (Table 5). Similar to Silver Carp, SFAs in Bighead Carp decreased in the upper reaches of the river. Additionally, n-3 fatty acids decreased in the upper reaches.

There were significant differences between Bighead and Silver carps within individual season–reach data sets and across all geographic locations and seasons. Overall, Bighead Carp were

TABLE 3. Proximate composition (%, mean ± SE) of Asian carp collected from the five reaches within the Illinois River (fall 2010–summer 2011). Lipid, protein, and ash are based on dry matter. Letters indicate significant geographical differences within each season at $P < 0.05$; ND = no data.

			Harvest location				
Species	Season	Composition	Alton	LaGrange	Peoria	Starved Rock	Marseilles
Silver Carp	Fall	Moisture	74.3 ± 0.9 z	74.5 ± 0.9 z	78.3 ± 0.9 z	72.5 ± 0.9 y	ND
		Lipid	11.9 ± 2.2 y	17.8 ± 2.2 zy	7.1 ± 2.2 y	25.0 ± 2.2 z	ND
		Protein	54.9 ± 1.6 y	60.0 ± 1.6 zy	65.7 ± 1.6 z	55.0 ± 1.6 y	ND
		Ash	27.0 ± 1.2 z	21.8 ± 1.2 z	25.2 ± 1.2 z	15.5 ± 1.2 y	ND
	Spring	Moisture	73.7 ± 0.9 z	70.2 ± 0.9 zy	73.7 ± 0.9 z	68.7 ± 0.9 y	68.5 ± 1.0 y
		Lipid	12.0 ± 2.2 y	24.8 ± 2.2 z	12.9 ± 2.2 y	25.7 ± 2.2 z	29.4 ± 2.5 z
		Protein	54.9 ± 1.6	52.8 ± 1.6	57.3 ± 1.6	51.6 ± 1.6	53.1 ± 1.8
		Ash	30.1 ± 1.2 z	18.1 ± 1.2 yx	23.5 ± 1.2 y	16.7 ± 1.2 x	14.7 ± 1.3 x
	Summer	Moisture	71.4 ± 0.9 z	70.5 ± 0.9 z	70.4 ± 0.9 z	68.1 ± 0.9 zy	65.6 ± 0.9 y
		Lipid	14.2 ± 2.2 y	18.6 ± 2.2 y	21.2 ± 2.2 y	29.9 ± 2.2 z	36.9 ± 2.2 z
		Protein	56.7 ± 1.6 z	54.1 ± 1.6 zy	53.7 ± 1.6 zy	51.1 ± 1.6 zy	48.8 ± 1.6 y
		Ash	25.8 ± 1.2 z	22.4 ± 1.2 zy	18.2 ± 1.2 y	14.6 ± 1.2 x	10.1 ± 1.2 x
Bighead Carp	Fall	Moisture	ND	79.1 ± 0.6	81.7 ± 1.4	ND	ND
		Lipid	ND	7.9 ± 1.1	4.9 ± 2.8	ND	ND
		Protein	ND	67.1 ± 1.3	66.0 ± 3.3	ND	ND
		Ash	ND	26.9 ± 1.0	24.2 ± 2.3	ND	ND
	Spring	Moisture	ND	ND	ND	79.9 ± 0.6	77.2 ± 0.6
		Lipid	ND	ND	ND	4.7 ± 1.1 y	14.4 ± 1.1 z
		Protein	ND	ND	ND	62.8 ± 1.3	62.0 ± 1.3
		Ash	ND	ND	ND	24.4 ± 1.0 z	17.8 ± 1.0 y
	Summer	Moisture	77.2 ± 0.6 z	77.9 ± 0.6 z	76.1 ± 0.6 zy	78.5 ± 0.6 z	73.7 ± 0.6 y
		Lipid	6.4 ± 1.2 y	7.5 ± 1.2 y	9.7 ± 1.2 y	7.2 ± 1.1 y	18.4 ± 1.1 z
		Protein	68.2 ± 1.5	67.4 ± 1.5	63.9 ± 1.5	68.9 ± 1.3	64.5 ± 1.3
		Ash	22.4 ± 1.0 z	21.8 ± 1.0 z	22.2 ± 1.0 z	21.0 ± 1.0 z	15.3 ± 1.0 y

leaner (higher moisture, protein, and ash, but lower lipid content) than Silver Carp in all reaches studied, regardless of season or geographic location (Table 6). The overall fatty acid profile of Asian carp (Table 7) demonstrated that Silver Carp had higher MUFAs, MC-PUFAs, and n-3:n-6, whereas Bighead Carp had higher levels of SFAs, n-6, and LC-PUFAs. Both species had similar levels of PUFAs and n-3.

Body composition was observed to be a function of total length in Silver Carp. Lipid had a strong positive relationship with length ($r^2 = 0.38$, $P = 0.0001$). Moisture ($r^2 = 0.42$, $P = 0.0005$) and protein ($r^2 = 0.39$, $P = 0.0001$) had strong negative relationships, whereas ash had a moderate negative relationship to total length ($r^2 = 0.20$, $P = 0.0001$; Figure 2). In Bighead Carp, only protein was moderately related to total length ($r^2 = 0.13$, $P = 0.0005$).

The test of parallelism (fish meal type × time) for spoilage indicators (aldehydes $P = 0.0078$ and peroxides $P = 0.0001$) was significant. Aldehydes ($P = 0.0015$) and peroxides ($P = 0.0010$) significantly increased over time, and there were significant differences between fish meal types for the presence of aldehydes ($P = 0.0289$) and peroxides ($P = 0.0047$). The divergent trend of both carp meals from the control was apparent, and the presence of spoilage indicators was substantially higher in the unstabilized carp meals than in the stabilized menhaden meal by 8 weeks (56 d; Figure 3). Additionally, aldehydes were more prevalent in both carp meals than peroxides.

DISCUSSION

Asian carp appear to be suitable for rendering and inclusion in aquafeeds based on their composition. Market economics and free-market access largely determine whether a fish is used for direct human consumption or rendered for feed production (Tacon et al. 2006; Tacon and Metian 2008), and given that Asian carp are not a favored food fish in the United States, this underutilized resource is better suited to other applications, including fish meal production. The proximate composition of Asian carp is similar to traditional fish meal sources such as menhaden (Rawles et al. 2010) and anchovy (IFFO 2006c; Glencross et al. 2007). However, the ash content of Asian carp is higher than traditional fish meal sources, but this may make it more attractive in aquafeeds that partially substitute fish meal with plant-based meals due to the higher levels of calcium and phosphorus in fish meals that tend to be low in plant protein sources.

TABLE 4. Fatty acid composition (% FAME, mean ± SE) for Silver Carp collected from five reaches within the Illinois River (fall 2010–summer 2011). Letters indicate significant geographical differences within each season at $P < 0.05$; ND = no data.

Season	Fatty acid group	Harvest location				
		Alton	LaGrange	Peoria	Starved Rock	Marseilles
Fall	SFAs	32.0 ± 0.9 zy	31.7 ± 0.8 zy	32.6 ± 0.8 z	28.2 ± 0.8 y	ND
	MUFAs	32.2 ± 1.3 zy	30.5 ± 1.2 yx	24.7 ± 1.3 x	36.7 ± 1.2 z	ND
	PUFAs	35.9 ± 1.2 y	37.8 ± 1.1 zy	42.7 ± 1.1 z	35.1 ± 1.1 y	ND
	MC-PUFAs	10.2 ± 0.5 y	13.3 ± 0.4 z	11.8 ± 0.4 zy	11.0 ± 0.4 y	ND
	LC-PUFAs	21.7 ± 0.9 y	21.0 ± 0.8 y	26.9 ± 0.9 z	18.2 ± 0.8 y	ND
	n-3	24.7 ± 0.9 yx	28.2 ± 0.9 zy	31.3 ± 0.9 z	23.1 ± 0.9 x	ND
	n-6	6.4 ± 0.3 zy	5.5 ± 0.2 yx	6.9 ± 0.3 z	4.3 ± 0.2 x	ND
	n-3:n-6	4.0 ± 0.2 y	5.2 ± 0.2 z	4.6 ± 0.2 zy	5.4 ± 0.2 z	ND
Spring	SFAs	43.4 ± 0.8 z	36.5 ± 0.8 y	34.3 ± 0.8 yx	32.0 ± 0.8 x	34.4 ± 0.9 yx
	MUFAs	37.1 ± 1.2 z	41.8 ± 1.2 z	31.9 ± 1.2 y	40.1 ± 1.2 z	40.7 ± 1.3 z
	PUFAs	19.4 ± 1.1 w	21.7 ± 1.1 xw	33.7 ± 1.1 z	28.0 ± 1.1 y	24.9 ± 1.2 yx
	MC-PUFAs	7.6 ± 0.4 y	8.7 ± 0.4 z	10.6 ± 0.4 z	9.1 ± 0.4 z	10.5 ± 0.5 z
	LC-PUFAs	8.5 ± 0.8 x	10.0 ± 0.8 yx	18.9 ± 0.8 z	13.4 ± 0.8 y	12.5 ± 0.9 yx
	n-3	10.8 ± 0.9 w	13.3 ± 0.9 xw	23.4 ± 0.9 z	16.9 ± 0.9 yx	17.6 ± 0.9 y
	n-6	4.2 ± 0.2	4.5 ± 0.2	5.1 ± 0.2	4.0 ± 0.2	4.7 ± 0.3
	n-3:n-6	2.6 ± 0.2 x	3.0 ± 0.2 yx	4.6 ± 0.2 z	4.2 ± 0.2 z	3.9 ± 0.2 zy
Summer	SFAs	42.6 ± 0.8 z	40.2 ± 0.8 zy	36.6 ± 0.8 y	35.7 ± 0.8 y	36.9 ± 0.8 y
	MUFAs	35.3 ± 1.2 yx	34.3 ± 1.2 x	30.6 ± 1.2 x	39.7 ± 1.2 zy	42.6 ± 1.2 z
	PUFAs	22.1 ± 1.1 y	25.5 ± 1.1 y	32.8 ± 1.1 z	24.5 ± 1.1 y	20.5 ± 1.1 y
	MC-PUFAs	8.7 ± 0.4 y	10.6 ± 0.4 zy	10.9 ± 0.4 z	9.0 ± 0.4zy	8.5 ± 0.4 y
	LC-PUFAs	10.1 ± 0.8 y	11.7 ± 0.8 y	15.9 ± 0.8 z	11.2 ± 0.8 y	9.5 ± 0.8 y
	n-3	14.9 ± 0.9 yx	17.8 ± 0.9 zy	20.5 ± 0.9 z	15.3 ± 0.9 yx	13.5 ± 0.9 x
	n-6	3.1 ± 0.2 y	3.8 ± 0.2 y	5.1 ± 0.2 z	3.8 ± 0.2 y	3.7 ± 0.2 y
	n-3:n-6	4.8 ± 0.2 z	4.8 ± 0.2 z	4.3 ± 0.2 zy	4.0 ± 0.2 zy	3.6 ± 0.2 y

The composition and quality of fish meal is based on fish condition at the time of harvest, which varies. The body condition of a fish and overall well-being is assumed to be adequate with sufficient lipid reserves, i.e., fish with higher fat content are in better condition (Anderson and Gutreuter 1983). The quality and chemical composition of fish meal within and among species depends on age, sex, diet, environment, and harvest season (Boran et al. 2008). Similar to many forage fish typically rendered into fish meal, we found that Asian carp carcass composition and quality varied seasonally, geographically, and by species. In general, Asian carp were leaner (low fat content) in the lower reaches of the Illinois River and during seasons with lower primary productivity such as fall. Additionally, Bighead Carp were leaner than Silver Carp across seasons and sample reaches, similar to results from the Missouri River (Orazio et al. 2011). Variation in the proximate composition of fish meal is closely related to the diet and feed intake of the species being rendered (Bragadóttir et al. 2004). Most reduction fisheries are based on plankton-eating species, such as menhaden and anchovy, which naturally experience seasonal variation in proximate body composition due to the seasonality of planktonic forage (Huss 1988). Given that Asian carp are also planktivorous (Williamson and Garvey 2005; Sampson et al. 2009), it is reasonable to observe similar seasonal variation in these species. During periods of ample food availability such as summer, protein content of the carcass will increase to a point, and then lipid deposition increases as feed intake surpasses energetic requirements, making a nutrient-rich fish for fish meal production (Bragadóttir et al. 2004; Boran et al. 2008). Following seasons with typically greater phyto- and zooplankton abundance, lipid deposition and overall body condition of Asian carp increased as well. However, seasonal availability of food does not explain all of the inconsistencies in body condition among reaches from season to season.

It would be expected that if primary production was the dominant factor affecting the composition of Asian carp, overall body condition should not vary appreciably by reach given that the entire Illinois River is subject to the same seasonal shifts in climate and broadly similar productivity cycles (though productivity may be somewhat higher in the downstream reaches). Several reaches did not follow this pattern, indicating that other factors are contributing to the condition of these fish. Just as fish are more nutritionally valuable during periods of ample food, they will be less nutritionally valuable as a raw material

TABLE 5. Fatty acid composition (% FAME, mean ± SE) for Bighead Carp collected from five reaches within the Illinois River (fall 2010–summer 2011). Letters indicate significant geographical differences within each season at $P < 0.05$; ND = no data.

		Harvest location				
Season	Fatty acid group	Alton	LaGrange	Peoria	Starved Rock	Marseilles
Fall	SFAs	ND	32.2 ± 2.6	32.2 ± 6.0	ND	ND
	MUFAs	ND	29.3 ± 1.8	25.9 ± 4.3	ND	ND
	PUFAs	ND	38.5 ± 1.5	42.0 ± 3.6	ND	ND
	MC-PUFAs	ND	9.3 ± 1.0	9.3 ± 2.3	ND	ND
	LC-PUFAs	ND	27.0 ± 1.2	30.3 ± 2.8	ND	ND
	n-3	ND	27.1 ± 1.1	30.5 ± 2.5	ND	ND
	n-6	ND	8.6 ± 1.7	8.4 ± 4.1	ND	ND
	n-3:n-6	ND	3.2 ± 0.7	3.6 ± 1.6	ND	ND
Spring	SFAs	ND	ND	ND	38.7 ± 2.5	40.5 ± 2.5
	MUFAs	ND	ND	ND	26.1 ± 1.7 y	35.9 ± 1.7 z
	PUFAs	ND	ND	ND	35.3 ± 1.5 z	23.7 ± 1.5 y
	MC-PUFAs	ND	ND	ND	5.7 ± 0.9 y	9.7 ± 9 z
	LC-PUFAs	ND	ND	ND	28.4 ± 1.1 z	12.4 ± 1.1 y
	n-3	ND	ND	ND	24.5 ± 1.0 z	15.8 ± 1.0 y
	n-6	ND	ND	ND	9.0 ± 1.7	5.6 ± 1.7
	n-3:n-6	ND	ND	ND	2.8 ± 0.6	2.8 ± 0.6
Summer	SFAs	42.8 ± 2.7 z	40.7 ± 2.7 z	40.0 ± 2.7 z	37.7 ± 2.5 zy	27.9 ± 2.5 y
	MUFAs	31.1 ± 1.9 yx	27.8 ± 1.9 w	29.9 ± 1.9 x	38.2 ± 1.7 zy	39.1 ± 1.7 z
	PUFAs	26.1 ± 1.6 zy	21.4 ± 1.6 yx	30.1 ± 1.6 z	24.1 ± 1.5 zyx	18.1 ± 1.5 x
	MC-PUFAs	8.3 ± 1.0 zy	7.1 ± 1.0 y	9.5 ± 1.0 zy	7.2 ± 0.9 y	11.9 ± 0.9 z
	LC-PUFAs	16.6 ± 1.2 zy	13.0 ± 1.2 zy	17.8 ± 1.2 z	15.6 ± 1.1 zy	11.9 ± 1.1 y
	n-3	20.0 ± 1.1 z	17.2 ± 1.1 zy	19.7 ± 1.1 zy	15.2 ± 1.0 yx	12.3 ± 1.0 x
	n-6	4.3 ± 1.8 y	4.5 ± 1.9 y	6.5 ± 1.8 zy	6.9 ± 1.7 zy	13.2 ± 1.7 z
	n-3:n-6	4.7 ± 0.7 zy	4.2 ± 0.7 zy	3.2 ± 0.7 y	2.2 ± 0.6 y	6.3 ± 0.6 z

during periods of starvation or high energy expenditure due to natural behavior (e.g., spawning, migration), stress (e.g., suboptimal temperature, low dissolved oxygen), food shortages (e.g., increased competition, reduced prey availability), and seasonal decreases in feed intake (e.g., overwintering). The lower body condition observed in Asian carp from the lower reaches of the Illinois River, and Bighead Carp in general, are likely attributed to competition. Bighead and Silver carps can have substantial diet overlap despite Silver Carp's broader feeding range, including the capacity to consume smaller food particles (Sampson et al. 2009). Additionally, Asian carp compete with native filter feeding fishes such as Gizzard Shad *Dorosoma cepedianum* and Bigmouth Buffalo *Ictiobus cyprinellus* (Irons et al. 2007; Sampson et al. 2009). Because of the similarities in diet composition among Asian carp and native planktivores within the Mississippi River basin, interspecific competition among species is highly probable in these systems (Sampson et al. 2009). Although there is no evidence of direct competition (i.e., limited food availability) in the Illinois River, the body condition of Gizzard Shad and Bigmouth Buffalo has decreased since the proliferation of Asian carp, and these changes were not strongly linked to complementary changes in other abiotic and biotic variables (Irons et al. 2007). Asian carp population densities are highest in the lower reaches of the Illinois River, particularly in Alton, LaGrange, and Peoria (Sass et al. 2010; Garvey et al. 2012), and higher densities probably contributed to lower overall body condition as a result of intraspecific competition for prey resources. This is supported by evidence that body condition improves in the upper reaches of the Illinois River as the population density of Asian carp decreases, which is contrary to expectations if productivity was the primary factor affecting body condition. Enhanced harvest of Asian carp should reduce both intra- and interspecific competition among planktivores, resulting in better body condition for Asian carp as well as native planktivorous fishes. However, gear selection and methods used to enhance harvest of Asian carp should be considered carefully to reduce the risk of native species bycatch, which may further affect the ecological integrity of the system.

Several lines of evidence suggest that Silver Carp can effectively outcompete Bighead Carp for prey resources in the Illinois River. Anecdotal evidence regarding Bighead Carp—such as the lower body condition (in this study and Orazio et al. 2011), narrower range of potential food particles (Sampson et al. 2009), lower abundance (Garvey et al. 2012), and the apparent faster

TABLE 6. Proximate composition (%) of Asian carp harvested from the Illinois River (fall 2010–summer 2011) across all five harvest locations (Alton, La Grange, Peoria, Starved Rock, and Marseilles) and seasons. Numeric labels indicate mean percent composition of the intact carcasses (i.e., wet matter basis); numbers may not add to 100% because of minor amounts of carbohydrates not analyzed and rounding errors.

	Species	
Composition	Silver Carp	Bighead Carp
Moisture	71.5 ± 0.3 y	77.5 ± 0.4 z
Protein	15.7 ± 0.5 z	14.7 ± 0.6 y
Lipid	5.8 ± 0.7 z	2.2 ± 1.0 y
Ash	5.8 ± 0.5	5.8 ± 0.6

TABLE 7. Fatty acid composition (% FAME, mean ± SE) of Asian carp collected from the Illinois River across all seasons (fall 2010–summer 2011) and harvest locations. Significant differences were determined at $P < 0.05$.

Fatty acid(s)	Silver Carp	Bighead Carp
12:0	0.15 ± 0.09 y	0.60 ± 0.12 z
13:0	0.06 ± 0.01 y	0.13 ± 0.01 z
14:0	6.53 ± 0.13 z	5.49 ± 0.17 y
15:0	1.04 ± 0.05 y	1.39 ± 0.07 z
16:0	22.10 ± 0.27 z	20.37 ± 0.35 y
17:0	0.50 ± 0.02 y	0.90 ± 0.02 z
18:0	4.93 ± 0.18 y	8.86 ± 0.23 z
20:0	0.25 ± 0.01 y	0.46 ± 0.01 z
22:0	0.02 ± 0.05 y	0.38 ± 0.06 z
24:0	0.01 ± 0.01 y	0.07 ± 0.02 z
SFAs	35.59 ± 0.51 y	37.67 ± 0.67 z
16:1n-7	14.74 ± 0.23 z	10.61 ± 0.30 y
18:1n-9	16.21 ± 0.47	15.21 ± 0.62
18:1n-7	3.63 ± 0.12 y	5.61 ± 0.16 z
20:1n-9	0.98 ± 0.03 y	1.20 ± 0.04 z
22:1n-11	0.01 ± 0.01 y	0.05 ± 0.01 z
22:1n-9	0.00 ± 0.03 y	0.22 ± 0.04 z
MUFAs	35.63 ± 0.52 z	32.73 ± 0.68 y
16:2n-4	1.91 ± 0.12 z	1.39 ± 0.15 y
16:3n-4	1.93 ± 0.05 z	0.70 ± 0.07 y
18:3n-4	1.07 ± 0.03 z	0.81 ± 0.04 y
18:2n-6	2.55 ± 0.05 y	3.05 ± 0.06 z
18:3n-6	0.28 ± 0.02	0.28 ± 0.03
20:2n-6	0.27 ± 0.01 y	0.42 ± 0.01 z
20:3n-6	0.34 ± 0.01 y	0.44 ± 0.01 z
20:4n-6	1.17 ± 0.11 y	2.33 ± 0.14 z
n-6	4.61 ± 0.13 y	6.36 ± 0.17 z
18:3n-3	4.41 ± 0.10 z	3.22 ± 0.13 y
18:4n-3	1.71 ± 0.05 z	0.94 ± 0.06 y
20:3n-3	0.31 ± 0.08 y	0.77 ± 0.10 z
20:4n-3	1.65 ± 0.04 z	1.28 ± 0.05 y
20:5n-3	6.37 ± 0.20	6.35 ± 0.26
22:5n-3	1.57 ± 0.07 y	2.04 ± 0.09 z
22:6n-3	3.23 ± 0.17 y	4.56 ± 0.23 z
n-3	19.26 ± 0.50	19.05 ± 0.65
PUFAs	28.78 ± 0.62	27.70 ± 0.81
LC-PUFAs	14.85 ± 0.51 y	18.24 ± 0.67 z
MC-PUFAs	10.01 ± 0.21 z	8.68 ± 0.27 y
n-3:n-6	4.21 ± 0.13 z	3.63 ± 0.18 y

rate of upstream movement (J. Garvey, Southern Illinois University Carbondale, personal communication)—compared with Silver Carp suggest they may be moving to less dense areas upstream in response to competitive exclusion. However, this hypothesis of competitive differences between these two Asian carp species needs to be explored further and is beyond the scope of this project. However, these variations in body composition have a fundamental importance to consumers, manufacturers, and wholesalers that demand a product equivalent to their expectations (Boran et al. 2008). Nevertheless, it should be noted that the geographic, seasonal, and species differences observed in this study were minor, and all size-classes of fish harvested would be deemed as valuable inputs for fish meal production.

To our knowledge this is the first attempt to process Asian carp into fish meal; these fish are typically marketed as seafood throughout the rest of the world. Much like the food fish industry, however, stabilizing the product is of critical importance. Freezing and frozen storage are important methods for preserving fish products such as fillets and fish meal (Asgharzadeh et al. 2010). One of the principal methods of predicting shelf life in processed food products is monitoring the level of lipid degradation by testing for the presence of peroxides and aldehydes (Sewald and DeVries 2012), and it is apparent that if an Asian carp meal product is to be used in aquafeed production, it will need to be stabilized to increase shelf life. The rate that spoilage occurs depends on product composition, particularly lipid composition in regards to chain length and hydrogen saturation, as well as environmental factors such as temperature, moisture, surface area, and light (Belitz et al. 2004; Sewald and DeVries 2012). Given that lipids present in fish meals contain a high proportion of unsaturated fatty acids and large surface area, they are particularly susceptible to spoilage (El-Lakany and March 1974). Silver Carp also have higher lipid content than Bighead Carp, which may make them more susceptible to spoilage. The rapid increase in the presence of peroxides and aldehydes in the Asian carp meals relative to the stable levels of the fish meal control demonstrates the need for antioxidants to increase the shelf life of an Asian carp meal product. A range of synthetic antioxidants are available and used in animal feeds, but ethoxyquin is the most frequently used synthetic stabilizer in fish meal because it is considerably more effective than other common feed additives (Lundebye et al. 2010). Given its effectiveness in other fish meals, it could easily be incorporated into Asian carp-based fish meals to extend shelf life.

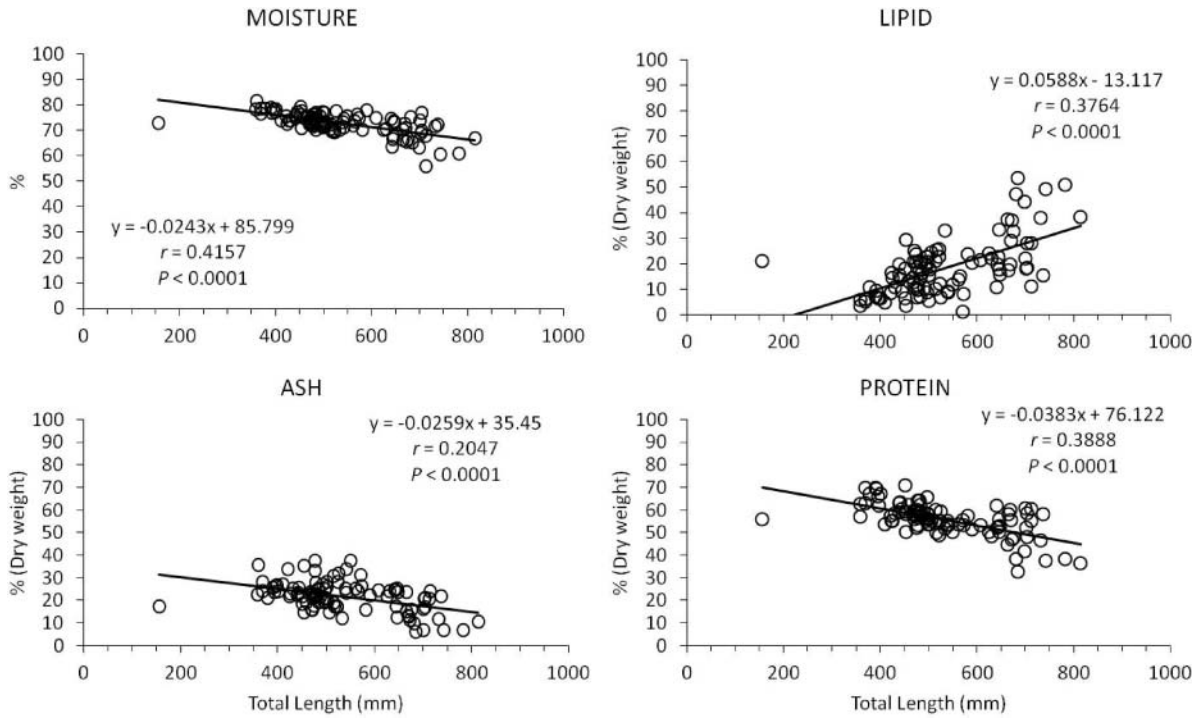

FIGURE 2. Relationship between total length and proximate composition of Silver Carp harvested from the Illinois River (fall 2010–summer 2011). Percent lipid, protein, and ash were determined on dry matter basis.

A stabilized Asian carp meal is a nutritionally suitable alternative for aquafeed production because of its similarity to traditional marine-based fish meals. The quality and concentration of essential nutrients in fish meal makes it a valuable component in the diets of most aquaculture species and many terrestrial farm-raised animals (IFFO 2006a) because it promotes normal growth, increases feed efficiency, enhances nutrient absorption, and improves palatability (Sullivan and Reigh 1995; Watanabe 2002; IFFO 2006b; Oyelese and Odubayo 2010). Additionally, the nutrients in fish meal are also effective for maintaining good health and improved disease resistance in fish through an enhanced immune response (Watanabe 2002; IFFO 2006b). One of the more important attributes of fish meal that are devoid in other alternative protein sources is the small, yet biological important, amount of LC-PUFAs (20 and 22 carbon, three or more double-bonded fatty acids). Given that many carnivorous species lack the ability to synthesize sufficient amounts of LC-PUFAs de novo, it is critical to provide LC-PUFAs in the diet. Adequate levels of LC-PUFAs are necessary for proper development and function. One of their many functions includes acting as precursors for eicosanoids that are involved in cardiovascular modulation, immunity and inflammatory response, renal and neural function, and reproduction (Sargent et al. 2002). An Asian carp-based fish meal would have similar levels of LC-PUFAs and benefits as those mentioned above from marine fish meal sources while also directing an otherwise unutilized resource to the human food chain, making it an attractive feedstuff for aquafeed manufacturers and the aquaculture industry.

Given the beneficial attributes of fish meal, feed manufacturers are willing to pay higher prices for fish meal (currently ~$1,900/metric ton) compared with lower quality alternative protein sources such as soybean meal (~$500/metric ton; FAO 2013). Currently, Asian carp meal is priced at $600–650/metric ton (P. Hitchens, Southern Illinois University Carbondale, personal communication), and fish markets along the Illinois River pay commercial fishers approximately $0.33/kg and $0.40/kg for Silver and Bighead carps, respectively (Schaffer's Fish Market, personal communication). Experienced commercial fishing crews (i.e., one to two boats and three to eight fishers) can routinely harvest > 10,000 kg of Asian carp/d from the Illinois River under optimal harvest conditions (R. Smith, Big River Fish, personal communication; Irons et al. 2007). Thus, Asian carp provide another source of income for commercial fishers while also providing a cost-effective alternative feedstuff to aquafeed producers. Additionally, the National Management and Control Plan for Bighead, Black, Grass, and Silver carps in the United States indicates that harvest enhancement is the only viable short-term control strategy for Asian carp where populations are already established (Conover et al. 2007). Given that Asian carp meal is a high-quality product like traditional marine fish meals but is currently priced similarly to lower-quality alternatives, this product may become an especially attractive ingredient for aquafeed manufacturers. However, it is expected that the price of Asian cap meal will increase to similar levels of traditional marine fish meal sources if a market is established. It is unclear what effects, if any, changes in

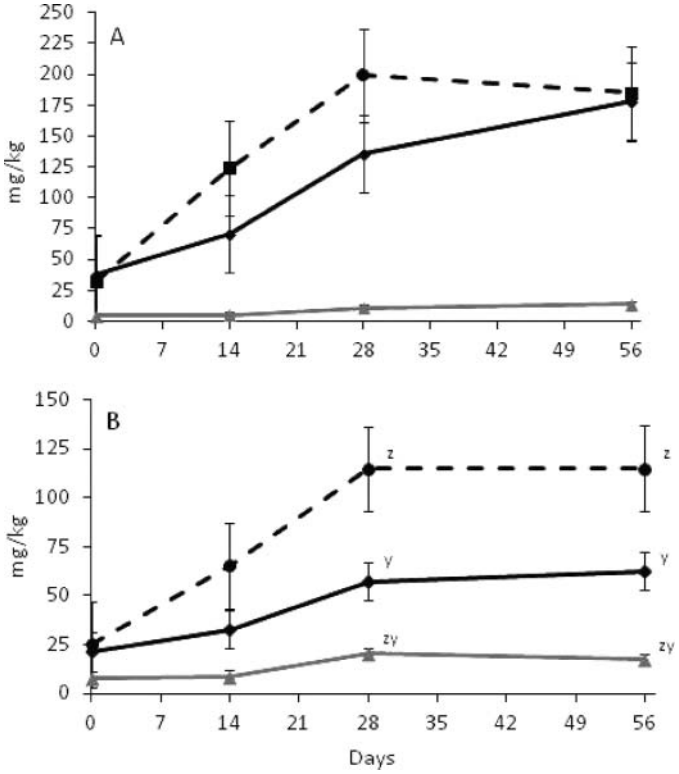

FIGURE 3. Oxidative stability of unstabilized Asian carp meals (Silver Carp = solid black line–diamonds, Bighead Carp = dashed black line–circles) compared with a commercially available stabilized menhaden fish meal control (gray line–triangles) based on the development of **(A)** aldehydes and **(B)** peroxides during refrigerated storage. Values were standardized for lipid content; error bars represent SE.

price structure may have on demand for Asian carp-derived fish meal.

The aquaculture industry continues to be the fastest-growing animal food producing sector, at a rate of roughly 8.8%/year (FAO 2012). To meet rising demand, the supply of feed inputs will also have to grow at similar rates (Tacon and Metian 2008). However, the aquaculture industry has not addressed its overdependence on fish meal in the production of aquafeeds. When the supply of fish meal became static, the aquaculture industry responded by paying higher prices and consuming larger portions of the global fish meal supply, while other industries found cheaper alternatives. The aquaculture industry now consumes the largest portion of the global supply of fish meal at approximately 60%, with little chance of consuming more of the overall supply (FAO 2012). The overdependence of aquaculture on fish meal to produce balanced and palatable aquafeeds for fishes makes them vulnerable to price fluctuations (Cheng et al. 2004). Thus, feed costs can be a significant variable cost in many aquaculture operations (Kaushik and Seiliez 2010). The industry could therefore benefit from an underutilized source of raw material such as Asian carp. An Asian carp meal is currently a more economical alternative feedstuff to marine-based fish meals and could ameliorate some of the effects of price shifts on aquafeed production while maintaining the overall quality of the feed. However, the ability of an Asian carp meal to ameliorate future price shifts maybe reduced given that its price is expected to increase because of its potential to be a high-value feedstuff.

In conclusion, reducing costs of feed can make aquaculture more profitable by substituting expensive ingredients (marine fish meal) for comparable, more cost-effective ingredients such as an Asian carp meal. The body composition and FAME profile of Asian carp from the Illinois River indicates that a rendered Asian carp meal is broadly similar to traditional marine fish meal sources but presently at a fraction of the cost. However, it is reasonable to expect that the price of Asian carp meal will increase to reflect the prices of similar products, such as traditional marine fish meal sources, but if the price does not increase to reflect this value, subsidies may be necessary to support the industry. Although subsidizing enhanced harvest to support this industry may be more cost effective than other control options. Given that the infrastructure, processing plants, transport, and commercial fishers to support an Asian carp meal industry is already being developed, a marketing strategy of Asian carp products is a logical approach to aid in the control of Asian carp populations in the Illinois River.

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