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Chemometric Discrimination among Wild and Cultured Age-0 Largemouth Bass, Black Crappies, and White Crappies Based on Fatty Acid Composition

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The potential to distinguish juvenile wild from cultured fishes and to discriminate among juvenile fishes by species based on fatty acid composition was demonstrated. Statistical approaches to data evaluation included analysis of variance, correlation analysis, principal component analysis (PCA), and quadratic discriminant analysis (QDA). Differences were determined between wild and cultured fishes both within and between species and between hatcheries. Fatty acid compositions were compared among individual (not composited) specimens of wild and cultured, age-0, freshwater species: largemouth bass *Micropterus salmoides*, black crappies *Pomoxis nigromaculatus*, white crappies *P. annularis*, and black-nose crappies. Four fatty acids were investigated: linoleic acid (18:2*n*−6), linolenic acid (18:3*n*−3), arachidonic acid (20:4*n*−6), and docosahexaenoic acid (22:6*n*−3). Linoleic acid was the primary fatty acid used to differentiate juvenile wild from cultured fishes. Concentrations of linoleic acid were significantly different ($p < 0.05$) in cultured largemouth bass and black crappies from the wild counterparts. Linolenic acid concentrations were not significantly different ($p < 0.05$) between wild and cultured largemouth bass but were significantly different between wild and cultured black crappies. Wild largemouth bass contained higher concentrations of arachidonic acid than the cultured bass, and concentrations of docosahexaenoic acid were twice as high in wild black crappies than cultured black crappies. On the basis of four signature fatty acids, 90 of 91 juvenile fishes were correctly classified as wild or cultured; 32 of 37 wild juvenile fishes originating from the same reservoir were differentiated by species. Data from the training set were used to classify a test set of fishes as to species, source, or origin with 100% accuracy.

KEYWORDS: Fishes; lipids; fatty acids; chemometric analysis

INTRODUCTION

Lipids are components of all living cells. The lipid content and fatty acid composition of an organism may vary within a species because of factors such as sex, location, season, or diet (*1*). Because the fatty acid composition of tissue lipids of animals often reflects that of the diet (*2*−*10*), analysis of fatty acids is a potential tool for distinguishing between wild and cultured species.

The purpose for establishing this line of research was to examine the forensic value of fatty acid profiling in distinguishing between wild and cultured fishes to discourage and prosecute commercial fraud occurring in this region. The goal of this study was to investigate the use of fatty acid composition in white-muscle tissue to distinguish cohorts of individual age-0 fishes. Unlike previous related studies, juvenile rather than adult fishes were examined. The analytical methodology thereby was adapted to accommodate small sample sizes. Also, unlike previous studies, individual specimens were examined rather than composite samples that might represent several fishes. Only the fillet or white-muscle tissue was analyzed. Other tissues may also indicate differences in fatty acid composition between wild and cultured fishes, but the fillet may be the only marketable evidence available to test in cases when endemic fishes are fraudulently portrayed for human consumption as cultured fishes.

Jahncke et al. (*8*) used linear discriminant analysis to successfully classify adult wild and cultured striped bass and hybrid striped bass based on the relative amounts of the fatty acids, linoleic acid (18:2*n*−6), linolenic acid (18:3*n*−3), arachidonic acid (20:4*n*−6), eicosapentaenoic acid (20:5*n*−3), and docosahexaenoic acid (22:6*n*−3). The flesh of wild individuals

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contained higher concentrations of 18:3*n*−3 and 20:4*n*−6. Cultured fishes contained higher concentrations of 18:2*n*−6 characteristically found in soybean meal, a major ingredient used in formulated fish food. Low concentrations of 20:4*n*−6 were detected in formulated fish diets and in cultured fishes that may be due to low level conversion of 18:2*n*−6 to longer chain, more unsaturated fatty acids. Over the 3-year study, as concentrations of 22:6*n*−3 varied in diets (ranging from a low of 4.7% to a high of 10.1%), concentrations in fishes correlated with increasing levels from a low of 5.8% to a high of 12.0% during the same time period. Jahncke et al. (*8*) attributed the observations to substitutions often made in commercial diets that provide the same nutritional value but are less expensive. Similarly, Suzuki et al. (*4*) determined that the muscle lipids of cultured carp and rainbow trout contained higher percentages of 18:2*n*−6 than those of the wild fish. High percentages of 20:4*n*−6 and 20:5*n*−3 in wild carp, 18:3*n*−3 in wild rainbow trout, and 22:6*n*−3 in cultured rainbow trout were observed.

Villarreal et al. (*11*) detected differences in fatty acid compositions between wild and cultured red drum. A major goal of the study was to aid law enforcement officials in differentiating endemic (wild) fishes from cultured (farm-reared) fishes to discourage illegal capture or commercial fraud. Concentrations of 20:4*n*−6, adrenic acid (22:4*n*−6), and docosapentaenoic acid (22:5*n*−6) were higher in wild than in cultured red drum. Because lower levels of the three fatty acids were commonly found in commercial diets, lower concentrations of each were found in cultured red drum. In comparison to wild stocks, cultured fishes fed only a formulated diet generally contain higher levels of *n*−6 fatty acids at the expense of *n*−3 series fatty acids (*12*, *13*).

Eight species of wild and cultured seawater fishes were investigated by Moon et al. (*14*). The *n*−3 polyunsaturated fatty acid content of the cultured fish was found to be approximately twice as much as that of the wild fish. Grigorakis et al. (*15*) studied wild and cultured gilthead sea bream and determined that cultured fishes were characterized by higher levels of monoenes, *n*−9, and 18:2*n*−6 fatty acids and wild fishes by higher levels of saturated fatty acids, 20:4*n*−6, *n*−3 fatty acids, and *n*−3/*n*−6 ratios. Jeong et al. (*16*) compared lipids and fatty acid compositions of wild and cultured sweet smelt and found that the wild fish muscles were rich in palmitoleic acid (16:1*n*−7), 18:3*n*−3, and 20:5*n*−3, whereas the cultured fish muscles were rich in oleic acid (18:1*n*−9), 18:2*n*−6, and 22:6*n*−3. The fatty acid proportions showed significant difference between fish farms because of different diets. Alasalvar et al. (*10*) reported that the lipids of cultured sea bass contained significantly higher proportions of myristic acid (14:0), eicosanoic acid (20:0), 18:1*n*−9, 11-eicosenoic acid (20:1*n*−9), 13-docosenoic acid (22:1*n*−9), 18:2*n*−6, and 8,11,14-eicosatrienoic acid (20:3*n*−6) and lower proportions of palmitic (16:0), stearic (18:0), 20:4*n*−6, 20:5*n*−3, 22:4*n*−3, DPA *n*−3 (22:5*n*−3), and 22:6*n*−3 fatty acid residues than wild sea bass. Differences observed between cultured and wild sea bass were attributed to the constituents of the diet of the fishes. Rueda et al. (*17*) reported that white-muscle fatty acid composition differences demonstrated that wild fishes displayed higher levels of 20:4*n*−6 and 22:6*n*−3, while cultured fishes exhibited higher levels of *n*−9 fatty acids. Additionally, lower 18:2*n*−6 content was observed in tissues from wild sharpsnout sea bream versus reared fish tissues, while 20:4*n*−6 content was higher in wild sharpsnout tissues. Ackman and Takeuchi (*12*) demonstrated that proportions of 20:4*n*−6 were 6 times greater in wild Atlantic salmon than in cultured salmon.

In this research, wild largemouth bass, black crappies, and white crappies were differentiated from cultured counterparts by examination of 18:2*n*−6, 18:3*n*−3, 20:4*n*−6, and 22:6*n*−3. The analytical and statistical methodology necessary to provide the basis for future studies of fatty acid composition in fishes was established.

MATERIALS AND METHODS

Study Sites. Age-0 wild largemouth bass *Micropterus salmoides*, black crappies *Pomoxis* nigromaculatus, and white crappies *Pomoxis annularis* were collected from the littoral zone of the Sale Creek embayment on Chickamauga Reservoir, TN, by electrofishing and shoreline rotenone sampling techniques. Chickamauga Reservoir is a main stem impoundment on the Tennessee River located at river km 781, elevation 208 m (*18*). Sale Creek embayment (2.7 km^2) is one of 15 major embayments of Chickamauga Reservoir. The mean depth is 2.23 m, with a maximum depth of 9.8 m (*19*).

Age-0 white crappies and black-nose crappies, a strain of black crappies, were received from Eagle Bend State Hatchery, Clinton, TN. Juvenile black crappies were received from Hopper−Stevens Hatchery, Lonoke, AR. Age-0 largemouth bass were received from American Sport Fish, Montgomery, AL. Samples of formulated fish diets were also obtained.

Preparation of Standards. Identification and quantitation of fatty acid methyl esters were determined by comparison of relative chromatographic retention times for single and multicomponent reference standards (Nu-Chek-Prep, Elysian, MN) and by standard addition techniques. Mass spectrometric analyses of fatty acids in fishes are described in detail in a separate paper (*20*). The single-component standards examined included stearic acid (18:0), linoleic acid (18:2*n*−6), linolenic acid (18:3*n*−3), arachidonic acid (20:4*n*−6), and docosahexaenoic acid (22:6*n*−3). Multicomponent reference standards were chromatographed with each group of sample fishes analyzed. Single- and multicomponent reference standards were weighed to about 5 mg, transferred to 1-mL glass stoppered volumetric flasks, and diluted to 1 mL with isooctane. A 1:10 dilution of the solution was also prepared.

Extraction Procedures. Fishes were immediately placed on ice and delivered to the laboratory for analysis. Total length and weight were recorded, and individuals were coded, placed in plastic bags, and frozen whole at −70 °C until analysis. Lipids were extracted from tissue samples using a chloroform/methanol (2:1, v/v) procedure based on the original methods of Folch et al. (*21*). The methods of Jahncke et al. (*8*) were designed for tissue samples weighing about 5 g and requiring 25 mg of pure lipid in chloroform/methanol, with a split injection ratio of 1:50 for gas chromatographic analysis. In the present study, the total body weight of juvenile fishes ranged from 2.78 to 48.18 g; therefore, tissue samples weighing 1 g were used to obtain aliquots of 5 mg of pure lipid in chloroform/methanol. The injector split ratio was 1:10. Partially frozen tissue samples, consisting of only the white portion of the flesh, were weighed to the nearest 0.001 g. Tissue samples were immediately transferred to 500-mL beakers and covered with about 30 mL of chloroform/methanol (2:1, v/v). The samples were homogenized (1−2 min) with a Cuisinart handheld blender equipped with a stainless steel base and blade. An additional 30 mL of solvent was added, and the tissue sample was homogenized (1−2 min). The sample remaining on the blender blade was washed into the beaker with approximately 10 mL of solvent. Beakers were covered with aluminum foil and allowed to stand for 10−15 min. Each sample was vacuum-filtered through a Coors 60240 funnel (Fisher Scientific) fitted with Whatman 1 filter paper (5.5 cm) attached to a 250-mL filter flask. A small amount of solvent was used to rinse the beaker and additionally poured through the funnel. The sample filtrate was transferred to a 100-mL glass-stoppered graduated cylinder. A sodium chloride (NaCl) solution (0.73 g of NaCl/100 mL of distilled water) corresponding to 20% of the filtrate volume was added to the cylinder and mixed by inverting the cylinder several times. Stoppered cylinders were refrigerated 12−48 h to promote phase separation. The volume of the lower phase (chloroform) was recorded. When the cylinder reached room temperature, the upper phase (methanol) was removed with a Pasteur pipet. Care was taken not to disturb the lower

phase. About 2 g of anhydrous sodium sulfate (Na_2SO_4) was added to the cylinder and mixed by gently inverting the cylinder. After the mixture was allowed to stand for 20 min, the contents of the cylinder were filtered through Whatman 1 filter paper into a 60-mL glass vial with a screw cap lid. Aluminum weighing pans (in duplicate) were weighed to the nearest 0.01 mg. A total of 5 mL of the chloroform extract were transferred to each weighing pan. Pans were placed in a drying oven for 30 min at 105 °C, transferred to a desiccator for an additional 30 min, and weighed. Proper precautions should be followed in a well-ventilated area when evaporating the chloroform extract in weighing pans in a drying oven for 30 min at 105 °C. A vacuum oven or alternative means of evaporating the sample should be utilized. Weights of the empty aluminum pans were subtracted from weights of the aluminum pans containing pure lipid. Duplicate analyses were averaged to yield the percent total lipid of each fillet. The data were used to determine the volume of chloroform extract required to achieve a 5-mg sample of lipid that was used in the subsequent derivatization process.

Derivatization Procedures. Hydrolysis of lipids to the fatty acids and derivatization of the fatty acids to fatty acid methyl esters (FAMEs) were made according to modifications by Jahncke et al. (*8*) based on the original work of Metcalfe et al. (*22*). Samples of pure lipid (5 mg) in chloroform extract were transferred to 60-mL culture tubes with Teflon-lined screw caps. All traces of chloroform were evaporated under a stream of dry nitrogen. Any unused portion of chloroform extract was blanketed with nitrogen and stored at −70 °C. Sodium hydroxide (1.5 mL of 0.5 M) in methanol was added to each culture tube. The tubes were vortexed (15−30 s), heated for 5 min in a 100 °C sand bath, and cooled in a beaker of tap water. Boron trifluoride in methanol, 2 mL of 12% w/w, was added to each tube. The tubes were again vortexed (15−30 s), returned to the sand bath at 100°C for 20 min, and cooled to 37 °C in a water bath. Isooctane (1 mL) was added to each culture tube. The tubes were vortexed (15−30 s), and 3 mL of saturated NaCl was added. Tubes were inverted repeatedly 180° by hand for 1 min, before centrifuging at 1300 rpm for 2 min to promote phase separation. The upper phase (isooctane) was transferred to another culture tube containing approximately a 2-mm layer of anhydrous Na_2SO_4. Tubes were shaken and allowed to stand for 20 min. After 20 min, the isooctane was transferred with a Pasteur pipet to suitable vials for GC analysis. Care was taken to avoid transferring the anhydrous Na_2SO_4.

Gas Chromatographic Analysis. Analysis for separation of FAMEs was conducted with a Hewlett−Packard (HP) 5880A gas chromatograph equipped with a flame ionization detector (FID) and an HP 7673A automatic sampler. Injections were made in a split mode (1:10 ratio) using a Supelco glass liner (cup design). An Omegawax 250 fused silica capillary column, 30 m × 0.25 mm ID, 0.25-μm film thickness (Supelco), was used to separate FAMEs for final analysis. Injector temperature was maintained at 250 °C, and 1 μL samples were injected. The FID temperature was maintained at 270 °C. Helium was the carrier gas used at a flow rate of 1 mL/min. Nitrogen was used as the makeup gas. A combined flow rate of carrier and makeup gas was 30 mL/min at the detector. The initial oven temperature was set at 170 °C and increased 1 °C/min until the temperature reached 225 °C. The temperature was held at 225 °C for 5 min, for a total run time of 1 h. Before each sample injection, the column was re-equilibrated to 170 °C.

Calculation of Weight Percent of Fatty Acids. The weight percent of each of the four selected fatty acids was calculated to scale the raw chromatographic data. The weight percent represents a percent of all fatty acids detected in the samples. Calculations were performed according to Jahncke et al. (*8*). An empirical correction factor, $K(t)$, was calculated relative to 18:0 in the multicomponent reference standard according to eq 1,

$$K(t) = \frac{A(s)W(t)}{W(s)A(t)} \quad (1)$$

where $K(t)$ is the correction factor of the particular methyl ester in question, $A(s)$ is the area of 18:0 in the reference chromatogram, $W(t)$ is the weight of the particular methyl ester in question, $W(s)$ is the weight of 18:0, and $A(t)$ is the area of the particular methyl ester in question. The empirical correction factor was used to calculate the weight percent of fatty acids in fish samples (eq 2),

$$\text{weight percent of fatty acids} = \frac{K(t)A(t)}{A}100 \quad (2)$$

where $A(t)$ is the area of the peak of a particular methyl ester and A is the sum of all peaks in the chromatogram excluding the solvent peak.

Validation of Procedures. To validate procedures, another researcher filleted eight fishes comprising a test data set for analysis as unknowns. Fishes were randomly selected and recoded. Because the unknowns might have been distinguished by size, fillets were finely chopped and prepared in similar proportions for analysis. Classifications of unknown fishes by species, origin, and source were recorded and withheld until final evaluation. Statistical analysis was used to identify the unknown samples based on the composition of linoleic (18:2n−6), linolenic (18:3n−3), arachidonic (20:4n−6), and docosahexaenoic (22:6n−3) fatty acids in the training set.

Statistical Analysis. Four statistical approaches to data evaluation were conducted involving correlation analysis, analysis of variance, principal component analysis (PCA), and quadratic discriminant analysis (QDA). The statistical analysis system (SAS) was used to analyze the data. An arcsine transformation was performed on the means of percent total lipids and weight percentages of fatty acid concentrations to more closely resemble a normal distribution (*23*). Duncan's analysis of variance for unequal sample sizes (PROC GLM) was performed on transformed data to determine significant differences between means at the $p = 0.05$ level. Correlation analysis (PROC CORR) compared pairwise relationships among measured lipids and fatty acids. PCA (PROC PRINCOMP) was used to generate the mapping of a linear combination of the original variables (weight percent of fatty acids) in an n-dimensional space. QDA (PROC DISCRIM) was used to predict classifications of known and unknown fishes into groups based on the species, origin, and source.

RESULTS AND DISCUSSION

Individual specimens were separated into seven different categories based on the species, origin, and source (**Table 1**). Means and standard deviations for total length, total weight, total lipid (% wet weight), and the four selected fatty acids were calculated for each category.

Relationships Observed among Lipids and Fatty Acids. By analysis of variance, no significant differences ($p \geq 0.05$) were observed among percent total lipids in all seven categories of specimens examined, although individual fatty acid compositions varied (**Table 1**). Wild largemouth bass, black crappies, and white crappies were significantly different ($p \leq 0.05$) in concentrations of linoleic acid (18:2n−6) than the cultured counterparts. Concentrations of linoleic acid were not significantly different ($p \geq 0.05$) among wild fishes collected from Chickamauga Reservoir, and concentrations of linoleic acid were significantly different ($p \leq 0.05$) for cultured largemouth bass from American Sport Fish than all other groups.

In general, freshwater fishes require dietary linoleic acid (18:2n−6) and/or linolenic acid (18:3n−3) (*24*, *25*). Fatty acids that cannot be biosynthesized by fishes and must be supplied by the diet are regarded as essential fatty acids. Linoleic acid (18:2n−6) and linolenic acid (18:3n−3) play an important role as precursors of n−3 and n−6 polyunsaturated fatty acids in freshwater species (*24*−*28*). The polyunsaturated fatty acids studied here are supplied by the diet or biosynthesized by sequential microsomal desaturation and elongation to produce docosahexaenoic acid (22:6n−3) from linolenic acid (18:3n−3) and arachidonic acid (20:4n−6) from linoleic acid (18:2n−6). Thereby, correlation analysis among the parameters measured is chemically significant for investigation of biosynthetic

Table 1. Total Lipid and Selected Fatty Acids in Test Fishes

origin[a]	source[b]	number of fishes	mean (±SD) total length (mm)	mean (±SD) weight (g)	total lipid (%)[c]	fatty acid (% weight) linoleic (18:2*n*–6)[c]	linolenic (18:3*n*–3)[c]	arachidonic (20:4*n*–6)[c]	docosahexaenoic (22:6*n*–3)[c]
					Largemouth Bass				
W	CH	23	125 ± 19	23.47 ± 10.57	1.27 ± 0.96 z	2.16 ± 0.72 y	1.23 ± 0.69 u	8.58 ± 1.38 q	26.55 ± 3.86 o
C	AS	22	137 ± 8	29.69 ± 6.01	1.26 ± 0.28 z	10.66 ± 1.71 x	0.95 ± 0.25 u	2.16 ± 1.66 p	24.23 ± 3.80 o
					Black Crappies				
W	CH	12	84 ± 20	8.12 ± 7.75	1.26 ± 0.28 z	2.86 ± 0.68 y	2.63 ± 1.33 t	7.05 ± 1.58 q	25.90 ± 4.25 o
C	HS	9	102 ± 8	13.72 ± 2.70	1.10 ± 0.09 z	5.89 ± 1.27 w	3.98 ± 0.80 s	8.43 ± 0.59 q	14.08 ± 1.98 n
					Black-Nose Crappies				
C	EB	11	89 ± 19	8.92 ± 4.91	1.27 ± 0.34 z	6.05 ± 1.28 w	4.05 ± 0.72 s	8.05 ± 1.84 q	13.50 ± 1.47 n
					White Crappies				
W	CH	2	91 ± 12	7.57 ± 3.39	1.24 ± 0.08 z	2.55 ± 1.02 y	2.57 ± 0.55 t	7.08 ± 0.77 q	25.04 ± 0.02 o
C	EB	12	94 ± 5	8.59 ± 0.62	1.81 ± 1.22 z	4.10 ± 0.20 v	6.93 ± 0.86 r	7.27 ± 0.72 q	13.99 ± 1.98 n

[a] Wild (W); Cultured (C). [b] Chickamauga Reservoir (CH); American Sport Fish Hatchery (AS); Hopper–Stevens Hatchery (HS); Eagle Bend State Hatchery (EB). [c] Means within each column followed by the same letter are not significantly different (Duncan's test; $\alpha = 0.05$).

pathways in endemic fishes and for investigating the influence of commercial diets upon cultured fishes.

Correlation analysis is meaningful in this research because individual and not composite samples were investigated. The correlation does not necessarily indicate a cause and effect between two variables but establishes that a trend exists when the variables are compared. The correlation tables are included in the Supporting Information. Correlation coefficients significant at $\alpha < 0.05$ are reported.

The correlation between total lipids and the individual fatty acids measured varied by species and origin (wild or cultured). In general, when correlation existed between total lipids and individual fatty acids, total lipids were positively correlated with linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3) and negatively correlated with arachidonic acid (20:4*n*−6) and docosahexaenoic acid (22:6*n*−3).

Correlation coefficient comparisons between the individual fatty acids indicated that in wild largemouth bass and black crappies, linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3) were each significantly ($p < 0.05$) negatively correlated with docosahexaenoic acid (22:6*n*−3) and that linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3) were positively correlated with each other. In wild specimens, arachidonic acid (20:4*n*−6) was not correlated with any of the other three fatty acids examined.

No significant correlations between individual fatty acids were observed for cultured black crappies or cultured black-nose crappies. In cultured white crappies, linolenic acid (18:3*n*−3) was negatively correlated with arachidonic acid (20:4*n*−6) and docosahexaenoic acid (22:6*n*−3), while arachidonic acid (20:4*n*−6) and docosahexaenoic acid (22:6*n*−3) were positively correlated with each other. In cultured largemouth bass, linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3) were each negatively correlated with docosahexaenoic acid (22:6*n*−3) and linoleic acid (18:2*n*−6) was negatively correlated with arachidonic acid (20:4*n*−6), while linolenic acid (18:3*n*−3) was positively correlated with arachidonic acid (20:4*n*−6).

Correlation analysis in this research produced results that were particularly different between cultured and endemic specimens, indicating the influence of dietary supplementation on the fatty acid profiles in fishes and the need for further study of the effects. Of the samples collected, only the wild samples were exposed to the same source water, i.e., the same water quality. Even so, a species difference existed between wild largemouth bass and black crappies.

Characterization of Fatty Acid Profiles among Fishes in a Training Set. Differentiation of wild fishes (spawned naturally and developed in a reservoir environment) from cultured fishes (spawned, reared, and managed in earthen ponds) could be determined based entirely on concentrations of linoleic acid (18:2*n*−6). Suzuki et al. (*4*), Jahncke et al. (*5*, *8*), Villarreal et al. (*11*), and Chanmugam et al. (*29*) also detected higher concentrations of linoleic acid in cultured fishes than in the wild counterparts.

Linolenic acid (18:3*n*−3) concentrations were higher in all cultured crappies than in all wild fishes and cultured largemouth bass received from American Sport Fish. Concentrations of linolenic acid were significantly different ($p \leq 0.05$) in white crappies received from Eagle Bend Hatchery than from all other wild or cultured fishes. Concentrations of linolenic acid (18:3*n*−3) were not significantly different ($p \geq 0.05$) between wild and cultured largemouth bass but were between wild and cultured black crappies.

Concentrations of arachidonic acid (20:4*n*−6) were significantly different ($p \leq 0.05$) in largemouth bass received from American Sport Fish than all other wild or cultured fishes. Villarreal et al. (*11*) found that concentrations of arachidonic acid were significantly different in wild than cultured red drum, with wild fishes having a higher concentration. The difference was attributed to the primary foraging habits of wild red drum. Shrimp, the primary forage, feed on red and brown marine algae that produce arachidonic acid.

Concentrations of docosahexaenoic acid (22:6*n*−3) were significantly different ($p \leq 0.05$) between cultured crappies and all other fishes analyzed. Concentrations of docosahexaenoic acid (22:6*n*−3) were not significantly different ($p \geq 0.05$) between wild and cultured largemouth bass; however, wild black crappies were significantly different ($p \leq 0.05$) than cultured black crappies with concentrations in the wild crappies almost twice as high. Jahncke et al. (*5*) found that concentrations of docosahexaenoic acid were also considerably higher in wild red drum than cultured drum. Chanmugam et al. (*29*) concluded that concentrations of docosahexaenoic acid were higher in wild catfishes than cultured catfishes. Wild fishes were assumed to consume diets consisting of plankton that are rich in omega-3 fatty acids, such as docosahexaenoic acid.

Wild Fishes. The similarities among the comparison for all wild fishes are striking; however, wild largemouth bass were significantly different ($p \leq 0.05$) than wild crappies in concentrations of linolenic acid (18:3*n*−3). A comparison of all wild fishes collected at Chickamauga Reservoir suggests that largemouth bass and black and white crappies were all ingesting the

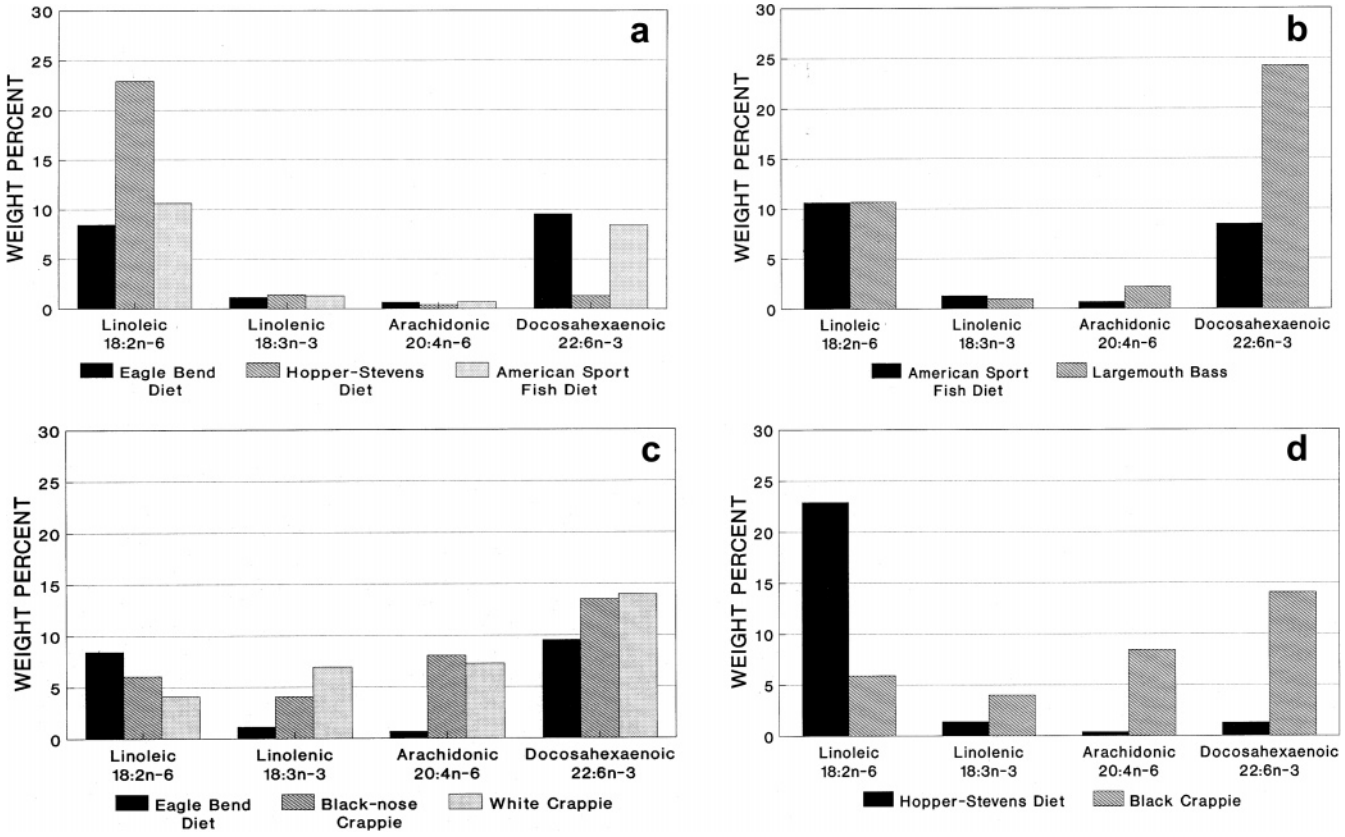

Figure 1. Comparison of mean fatty acid profiles of age 0 fishes expressed as the weight percent of total fatty acids for (a) supplementary diets, (b) diet and cultured largemouth bass, (c) diet and cultured black-nose and white crappies, and (d) diet and cultured black crappies.

same food. The potential exists for competition among the wild species for food resources.

Cultured Fishes. Cultured largemouth bass were significantly different ($p \leq 0.05$) from all cultured crappies in each of the four selected fatty acids. Black crappies and black-nose crappies were significantly different ($p \leq 0.05$) from white crappies in concentrations of linoleic acid (18:2*n*−6), and linolenic acid (18:3*n*−3) even though black-nose and white crappies came from the same hatchery (Eagle Bend). The black crappies from Hopper−Stevens Hatchery and the black-nose crappies from Eagle Bend Hatchery were not significantly different ($p \geq 0.05$) in any of the four fatty acids investigated even though the fish were reared in different hatcheries. Cultured black-nose crappies were significantly different ($p \leq 0.05$) from cultured white crappies (although the fish were reared at the same hatchery, Eagle Bend) in concentrations of linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3). Several explanations exist for the marked differences observed between cultured largemouth bass and all of the cultured crappies. Largemouth bass and crappies have different evolutionary histories that may result in different physiological strategies. Juveniles may still reflect the composition of the yolk sacs. Largemouth bass received from American Sport Fish were on a different diet than cultured crappies received from Hopper−Stevens or Eagle Bend, and largemouth bass came from a different geographic location than any of the cultured crappies. Water quality and chemistry more than likely varied between hatcheries. Fatty acid compositions were probably so similar between cultured black-nose and cultured black crappies simply because of the influence of species (black-nose crappies is a strain of black crappies). However, the ability to distinguish between the cultured black-nose crappies and the cultured white crappies is interesting because both came from the same hatchery (Eagle Bend). The variation may be due to the manner in which the different species process fatty acids or to differences in foraging for foods available in addition to the formulated fish diet provided.

Characterization of Fatty Acid Profiles of Formulated Fish Diets. Cultured black-nose and white crappies (Eagle Bend) were fed a commercial Salmon Starter diet (38-4763-92-50) (Zeigler Brothers, Gardners, PA) consisting of 55% crude protein, 15% crude fat (analysis of total lipid was 15.1%), and 2% crude fiber. Cultured black crappies (Hopper−Stevens) were fed Farmer's Choice, 36% Fingerling, Crumbles Catfish Food (Arkat Feeds, Dumus, AR) consisting of 36% crude protein, 3.5% crude fat (analysis of total lipid was 5%), and 5% crude fiber. Cultured largemouth bass (American Sport Fish) were fed Purina Trout Chow (5105) consisting of 40% crude protein, 10% crude fat (analysis of total lipid was 11%), and 5% crude fiber. The commercial diet from Hopper−Stevens contained more than twice as much linoleic acid (18:2*n*−6) than the commercial diets from Eagle Bend and American Sport Fish; when expressed as weight percent of total fatty acids contained in samples of equal mass (**Figure 1a**). Concentrations of docosahexaenoic acid (22:6*n*−3) were 7−8 times higher in the commercial diets fed at Eagle Bend and American Sport Fish than in the commercial diet fed at Hopper−Stevens. However, if the total lipid content of the diets is factored into the analysis, the available amount of 18:2*n*−6 is essentially the same for all diets when expressed as weight percent of total fatty acids per total percent lipids of the diet.

Fatty acid profiles for cultured age-0 largemouth bass (American Sport Fish), and the corresponding diet are compared in **Figure 1b**. The age-0 largemouth bass most closely reflect the diet in concentrations of linoleic acid (18:2*n*−6) and linolenic acid (18:3*n*−3); however, they differ greatly in the concentrations of docosahexaenoic acid (22:6*n*−3). Fatty acid

and diet profiles for cultured juvenile black-nose and white crappies (Eagle Bend) are presented in **Figure 1c**. The percentages of linoleic acid (18:2*n*−6) found in the diet were higher than in either taxa of crappies and were more than twice as high in the diet than in the white crappies. Relative percentages of linolenic acid (18:3*n*−3) and arachidonic acid (20:4*n*−6) ranged from approximately 3−7 times higher in the fishes than in the diet. Fatty acid concentrations for cultured juvenile black crappies (Hopper−Stevens) were compared to fatty acid concentrations in the diets (**Figure 1d**). None of the selected fatty acid concentrations in the diet was closely mirrored in the fishes. The fatty acid compositions of the age-0 cultured fishes analyzed did not directly mimic the fatty acid composition of the diets received as closely as reported for composited samples of adult cultured fishes (*8*). Stomach analysis of all cultured fishes revealed that cultured juveniles were also ingesting plankton and invertebrate species. Additionally, juveniles may be converting food directly into growth, whereas adults may use it for maintenance. Additional research is needed to determine how old a fish must be before it reflects its diet and whether this time varies among species. The analysis of fatty acid composition in juvenile fishes provides information valuable to the appropriate formulation of cultured fish diets.

Multivariate Examination of Relationships among the Training Set. PCA was used to map data calculated for each of the four selected fatty acids in all 91 known fishes analyzed, to differentiate wild from cultured fishes and further separate fishes by species and origin. The data were corrected for the mean and scaled by the standard deviation. PCA transforms original variables into uncorrelated variables or principal components. The new variables (principal components) had means equal to zero (mean centered) and variances equal to the corresponding eigenvalues.

The four fatty acids were measured in the same units; however, when all 91 known fishes are considered together, the fatty acids differ widely in variability (variance) across all categories of fishes as compared to the standard deviation measured within categories reported in **Table 1** (the variability resulted from marked differences in fatty acid composition among categories of fishes). The overall means and standard deviations for the 91 samples are linoleic acid (5.41 ± 3.46), linolenic acid (2.74 ± 2.15), arachidonic acid (6.54 ± 2.90), and docosahexaenoic acid (21.40 ± 6.50). Because the variables are not proportionate, the PCA was appropriately conducted using the correlation matrix instead of the covariance matrix.

The PCA developed on known fishes indicated that two or three principal components adequately describe the data. The first, second, and third principal components cumulatively explained 51, 91, and 98%, respectively, of the variation in the data.

A two-dimensional plot (**Figure 2**) created using the first two principal components clearly demonstrates a separation of fishes into three unique groups. Group I primarily represents all wild (W) fishes taken from Chickamauga Reservoir including largemouth bass, black crappies, and white crappies; Group II represents cultured (C) largemouth bass; and Group III primarily consists of all cultured (C) crappies including black crappies, black-nose crappies, and white crappies. On the basis of only two principal components, one cultured fish was misclassified into Group I (wild fishes) and one wild fish was misclassified into Group III (cultured crappies). The cultured fish misclassified into Group I is a largemouth bass, and the wild fish misclassified into Group III is a black crappie.

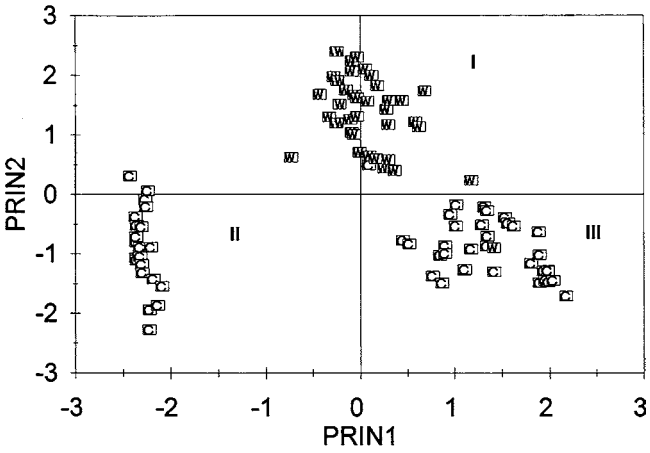

Figure 2. Principal component analysis demonstrating relationships among individual, age 0, wild (W) and cultured (C) fishes. Group I primarily represents all wild (W) fishes taken from Chickamauga Reservoir including largemouth bass, black crappies, and white crappies; Group II represents cultured (C) largemouth bass; and Group III primarily consists of all cultured (C) crappies including black crappies, black-nose crappies, and white crappies.

Relating the meaning of principal components back to the original variables is not always possible, but in this case, some conclusions about the influence of fatty acid composition can be drawn. **Figure 2** consists of four quadrants. The upper two quadrants contain primarily wild fishes, and the lower two quadrants contain primarily cultured fishes. The first principal component (PRIN1) discriminated between cultured largemouth bass, all wild fishes, and all cultured crappies. The second principal component (PRIN2) discriminated between wild and cultured fishes.

Cultured largemouth bass appeared primarily in the lower, left quadrant where negative loadings (i.e., eigenvectors) for linoleic acid (18:2*n*−6) occurred in both PRIN1 and PRIN2. Cultured largemouth bass were significantly higher (10.66%) in concentrations of linoleic acid than all other categories of fishes. PCA may have discriminated for cultured largemouth bass based solely on concentrations of linoleic acid. Cultured crappies appeared in the lower, right quadrant where concentrations of linolenic acid (18:3*n*−3) showed positive loadings on PRIN1 and negative loadings on PRIN2. Concentrations of linolenic acid were highest in all of the cultured crappies. All of the wild fishes were centrally distributed in the upper two quadrants. Negative loadings on PRIN1 and positive loadings on PRIN2 occurred for docosahexaenoic acid (22:6*n*−3) in the upper left quadrant. Positive loadings on both PRIN1 and PRIN2 occurred for arachidonic acid (20:4*n*−6) in the upper, right quadrant. PCA appeared to discriminate all wild fishes based on concentrations of arachidonic and docosahexaenoic acids. Concentrations of arachidonic and docosahexaenoic acids were not significantly different among any of the wild fishes. In addition, concentrations of docosahexaenoic acid were not significantly different between cultured largemouth bass and all of the wild fishes. Concentrations of arachidonic acid in the cultured bass were significantly different than all other categories of fishes and were discriminated based on concentrations of linoleic acid (lower, left quadrant).

Plots of component weights make it easier to visually interpret the pattern of weights of the eigenvectors (*30*). In parts **a** and **b** of **Figure 3**, each fatty acid is represented as a vector from the origin to the points whose coordinates are the weights on the two principal components of the plot. The correlation

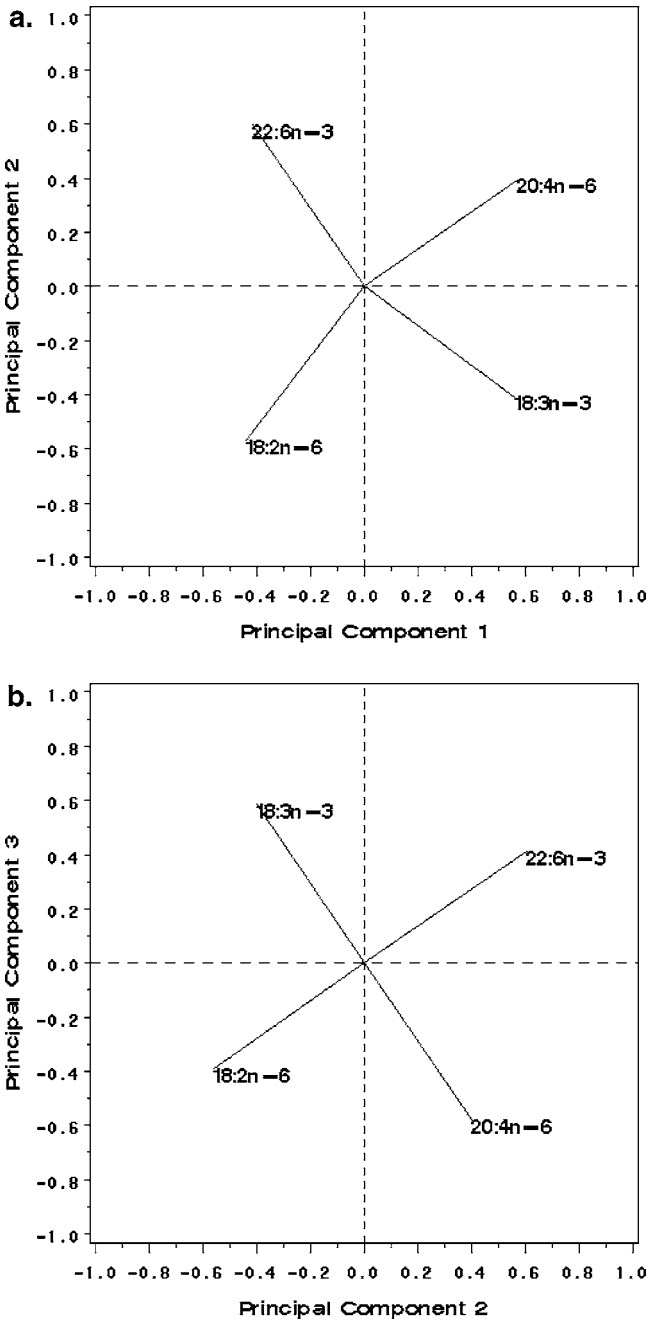

Figure 3. (a) Component weights on PRIN1 and PRIN2. (b) Component weights on PRIN2 and PRIN3.

between fatty acids is reflected approximately in the angle between the variable vectors in the first two dimensions (uncorrelated variables occur at right angles; positive correlations occur at small angles approaching 0°, while negative correlations occur at large angles approaching 180°). PRIN1 is composed of negative loadings on 18:2*n*−6 and 22:6*n*−3 and positive loadings on 18:3*n*−3 and 20:4*n*−6, primarily separating the cultured fishes by species. PRIN2 distinguishes between the essential fatty acids, i.e., 18:2*n*−6 and 18:3*n*−3, and the fatty acids that can be biosynthesized, i.e., 20:4*n*−6 and 22:6*n*−3. PRIN3 distinguishes between the *n*−3 and *n*−6 fatty acids.

Multivariate Classification of Training and Test Sets of Fishes. A discriminant function or classification criterion was determined for the known, training set of fishes and used to classify the unknown or test set of age-0 fishes as wild or cultured (**Table 2**) and into individual categories (**Table 3**) based on the percentages of linoleic acid (18:2*n*−6), linolenic acid (18:3*n*−3), arachidonic acid (20:4*n*−6), and docosahexaenoic acid (22:6*n*−3) extracted from individual specimens. The classification criterion can be based on either the individual within-group covariance matrixes (yielding a quadratic function) or the pooled covariance matrix (yielding a linear function). A test of the hypothesis that the covariance matrixes were equal in all groups indicated ($p < 0.0001$) that the variances and covariances differed across the groups examined. Therefore, homogeneity was rejected, and the covariance matrixes were not pooled. The individual within-group covariance matrixes were used to calculate the discriminant function, resulting in quadratic functions of the quantitative variables instead of linear functions (*30*). On the basis of the quadratic discriminant function developed for known fishes, the classification of fishes of unknown species, source, and origin was possible.

Table 2. Quadratic Discriminant Analysis Classification of Known and Unknown Fishes Based on Origin

	number of observations classified into origin		
from origin	C[a]	W[b]	N[c]
known fishes			
C	54	0	54
W	1	36	37
unknown fishes			
C	6	0	6
W	0	2	2

[a] Cultured fishes. [b] Wild fishes. [c] Total number of fishes in each group.

Table 3. Quadratic Discriminant Analysis Classification of Known Fishes into Categories 1–7

	number of observations classified into species, origin, and source							
from category	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	N[h]
1	21	1	1	0	0	0	0	23
2	3	9	0	0	0	0	0	12
3	0	0	8	0	1	0	0	9
4	0	0	0	22	0	0	0	22
5	0	0	3	0	8	0	0	11
6	0	0	0	0	0	12	0	12
7	0	0	0	0	0	0	2	2

[a] Largemouth bass from Chickamauga Reservoir. [b] Black crappies from Chickamauga Reservoir. [c] Black crappies from Hopper–Stevens Hatchery, Inc. [d] Largemouth bass from American Sport Fish. [e] Black-nose crappies from Eagle Bend State Hatchery. [f] White crappies from Eagle Bend State Hatchery. [g] White crappies from Chickamauga Reservoir. [h] Total number of fishes in each category.

Discrimination of Fishes According to Origin (Wild or Cultured). Of the 54 known cultured fishes analyzed (**Table 2**), all were classified correctly, and of the 37 known wild fishes, 36 were classified correctly. One wild fish was incorrectly identified as a cultured fish. Unknown fishes were classified in the same manner. Of the 8 unknown fishes, all were correctly classified.

Discrimination of Fishes According to Species, Origin, and Source. Of the known juvenile fishes (**Table 3**), 22 cultured largemouth bass, 12 cultured white crappies, and 2 wild white crappies were classified correctly. Of the 23 wild largemouth bass, 21 were classified correctly. One wild largemouth bass was incorrectly identified as a wild black crappie, and the other wild largemouth bass was incorrectly identified as a cultured black crappie. Of the 12 wild black crappies, 9 were classified correctly and 3 were misidentified as wild largemouth bass. A total of 8 of the 9 cultured black crappies, were classified

Table 4. Quadratic Discriminant Analysis Classification of Unknown Fishes into Categories 1, 4, 5, and 6

	number of observations classified into species, origin, and source				
from category	1[a]	4[b]	5[c]	6[d]	*N*[e]
1	2	0	0	0	2
4	0	2	0	0	2
5	0	0	2	0	2
6	0	0	0	2	2

[a] Largemouth bass from Chickamauga Reservoir. [b] Largemouth bass from American Sport Fish. [c] Black-nose crappies from Eagle Bend State Hatchery. [d] White crappies from Eagle Bend State Hatchery. [e] Total number of fishes in each category.

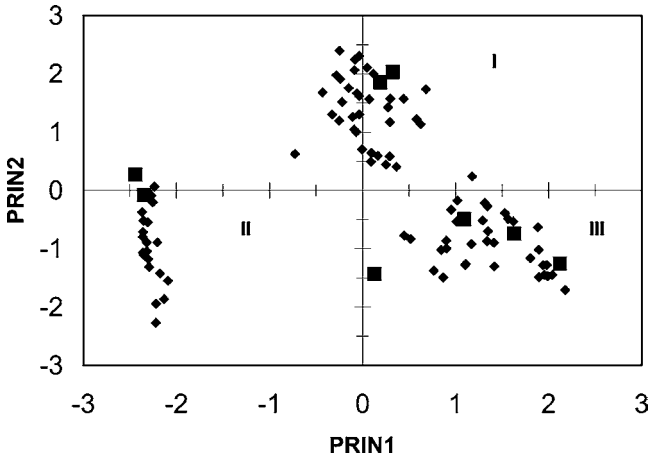

Figure 4. Principal component analysis demonstrating relationships among individual, age 0, known, training set fishes (◆) and unknown, test set fishes (■). Groups I, II, and III are defined in **Figure 2**.

correctly, while 1 was incorrectly identified as a cultured black-nose crappie. In addition, of the 11 cultured black-nose crappies, 8 were classified correctly and 3 were incorrectly identified as cultured black crappies.

Unknown juvenile fishes were categorized into groups 1, 4, 5, or 6 (**Table 4**) as to species, source, or origin, with 100% accuracy. Not all seven categories were represented in the unknown samples, but this was unknown to the analyst conducting the analytical and statistical evaluation of the unknown samples.

Cultured largemouth bass and cultured white crappies were classified correctly among the 91 known fishes assayed. All 8 wild and cultured unknown fishes analyzed were classified correctly. A total of 9 cultured black crappies from Hopper−Stevens were analyzed, and 8 were classified correctly. A total of 11 cultured black-nose crappies were analyzed, and 8 were classified correctly. A total of 1 cultured black crappie resembled a cultured black-nose crappie, and 3 cultured black-nose crappies resembled cultured black crappies. Differences between cultured black crappies and cultured black-nose crappies were difficult to detect. This was attributed to the fact that black-nose crappies are a strain of black crappies. Species of wild fishes were also distinguished with a high degree of accuracy. Of the 23 juvenile wild largemouth bass, 21 were classified correctly among all 91 known fishes sampled. Wild black crappies were more difficult to distinguish, and of the 12 analyzed, 9 were correctly classified among the 91 known fishes sampled. The ones misclassified were categorized as wild largemouth bass. Only 2 wild white crappies were collected, but both were classified correctly.

Even though wild fishes from a single location were more difficult to differentiate by species using the four fatty acids examined, all those misclassified by species were still correctly classified by origin as wild fishes with the exception of one cultured black crappie. The only cultured fishes misclassified were either black or black-nose crappies, and the misclassification occurred between the two categories.

Comparison of QDA with PCA for Predicting Unknowns. Although PCA is not primarily applied to predicting unknowns from statistics generated using a known or training data set as is discriminant analysis, the comparability of PCA and QDA results were tested by projecting the unknowns onto the PCA map (**Figure 4**). To do so, the principal components of the unknowns were calculated using the means and standard deviations generated by analysis of the known samples to represent the population. Using PCA, the same categorization results were achieved as with QDA, thereby reinforcing the conclusion that the information contained in the four fatty acids examined contains discriminatory information useful as taxonomic markers.

ABBREVIATIONS USED

CORR, correlation; DISCRIM, discriminant; FAME, fatty acid methyl ester; FID, flame ionization detector; GLM, general linear model; HP, Hewlett−Packard; ID, inside diameter; PCA, principal component analysis; PRIN1, first principal component; PRIN2, second principal component; PRIN3, third principal component; PRINCOMP, principal component; PROC, procedure; QDA, quadratic discriminant analysis; SAS, statistical analysis system.

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Supporting Information Available: Correlation tables for total lipids, linoleic acid (18:2*n*−6), linolenic acid (18:3*n*−3), arachidonic acid (20:4*n*−6), and docosahexaenoic acid (22:6*n*−3). This material is available free of charge via the Internet at http://pubs.acs.org.

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