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Field and Laboratory Determinations of Hypoxic Effects on RNA-DNA Ratios of Bluegill

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ABSTRACT.—We used RNA-DNA ratios in both field and laboratory experiments to examine the effects of hypoxia on short-term growth of bluegill Lepomis macrochirus collected from the lower Atchafalaya River Basin, Louisiana. In the field experiment, RNA-DNA ratios of bluegill (17.8–52.3 g) from hypoxic habitats (DO < 2.0 mg L⁻¹; n = 26) were significantly lower than ratios of bluegill from normoxic habitats (DO > 4.0 mg L⁻¹; n = 31). In each of two laboratory experiments 40 bluegill (14.54–76.70 g) were individually placed in aquaria to test the effects of hypoxia on RNA-DNA ratios. Additionally, bluegill were fed at different rates in the first experiment to determine the effects of feeding level on RNA-DNA ratios. Results of the first experiment confirmed the sensitivity of RNA-DNA ratios to short-term changes in growth, as fed bluegill had significantly higher ratios than starved bluegill. In both experiments RNA-DNA ratios were not significantly different in bluegill subjected to hypoxia. Results of the study indicate that laboratory experiments did not adequately simulate increased bioenergetic demands and fluctuating DO levels found in hypoxic areas of the Basin.

INTRODUCTION

In many tropical and temperate rivers the annual flood pulse results in high productivity as nutrients and organic matter that accumulate on the floodplain during low-water periods are recycled (Holden and Green, 1960; Bonetto, 1975; Schmidt, 1976; Junk et al., 1989). However, due to increased biochemical oxygen demand of the aquatic community, these areas can also experience spatially extensive and temporally protracted periods of hypoxia (defined here as DO concentrations < 2.0 mg L⁻¹). Annual inundation of the Atchafalaya River Basin (hereafter Basin), a floodplain swamp of the Atchafalaya River in southcentral Louisiana, results in a mosaic of physicochemically complex aquatic habitats. During the latter stages of the flood pulse the Basin typically experiences extended periods (1–6 mo) of hypoxia that can affect thousands of hectares of inundated floodplain.

Fishes exposed to hypoxic and fluctuating DO concentrations (see Doudoroff and Shumway, 1970 for review) often exhibit changes in hematocrit, blood pH, cardiovascular volume and metabolic pathways (Heath, 1995). In addition, hypoxia-induced reductions in food consumption, food conversion efficiencies and growth have been reported for coho salmon Oncorhynchus kisutch (Herrmann et al., 1962), largemouth bass Micropterus salmoides (Stewart et al., 1967), brook trout Salvelinus fontinalis (Whitworth, 1968) and guppy Poecilia reticulata (Weber and Kramer, 1983). In the Basin stress-related behavior (e.g., increased fish respiration at the water surface and increased ventilation rates) and altered fish distributions due to seasonal hypoxia have been observed (Gelwicks, 1996). We developed this study to examine potential effects of these physiological and behavioral changes on short-term growth.

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The RNA-DNA ratio (see Bulow, 1987, for review) is an index of growth over short time periods up to several weeks (Busacker et al., 1990). Use of the RNA-DNA ratio as a growth index is based on the premise that the quantity of cellular DNA is constant, whereas RNA content changes in accordance with protein synthesis (Bulow, 1987). RNA-DNA ratios have been used in studies of short-term growth and condition of numerous fishes, including bluegill *Lepomis macrochirus* (Bulow et al., 1978; Bulow et al., 1981), Atlantic cod *Gadus morhua* (Buckley, 1979), black crappie *Pomoxis nigromaculatus* (Haines, 1980), channel catfish *Ictalurus punctatus* (Peterson and Brown-Peterson, 1992), Pacific herring *Clupea pallasi* (McGurk and Kusser, 1992) and common herring *Clupea harengus* (Clemmesen, 1994). Fishes fed restricted rations have decreased RNA-DNA ratios up to 30% (Wilder and Stanley, 1983). Starvation-induced decreases in RNA-DNA ratios can be reversed after feeding is resumed, increasing as much as 50% within 6 d (Bulow, 1970). Effects of environmental factors such as food availability (Bastrop et al., 1992), temperature (Mathers et al., 1993) and environmental toxins (Wilder and Stanley, 1983) on growth and condition of fishes have been successfully evaluated with RNA-DNA ratios.

However, few studies have demonstrated (Bulow et al., 1978; Bulow et al., 1981) or quantified (Peterson and Brown-Peterson, 1992) the relationship between RNA-DNA ratios and ambient DO concentration. Our research was designed to evaluate the relationship between RNA-DNA ratios of bluegill and DO concentrations under field conditions. We predicted low DO concentrations would cause reduced growth of bluegill, which would be reflected in lower RNA-DNA ratios. Additionally, we tested this hypothesis in controlled laboratory experiments designed to simulate field conditions. Our objectives were to contrast RNA-DNA ratios of bluegill in normoxic and hypoxic areas of the Basin and compare results of these field studies with laboratory experiments incorporating regulated DO levels and food rations.

### Study Area

The Basin is a 4662-km² floodplain of the Atchafalaya River located in south-central Louisiana. Discharge from the Atchafalaya River usually peaks during spring (Lambou, 1990) when overbank flooding produces physicochemically complex habitats throughout the Basin. During most years a large area of hypoxia covering hundreds of hectares (Sabo et al., in press) persists for several months near the southern boundary of the Basin. This area, a cypress-tupelo gum swamp, is characterized by numerous pipeline canals and bayous with high tannic acid concentrations which give the water a distinct black appearance. Phytoplankton abundance and primary productivity are low. Conversely, normoxic water typically persists throughout the year in Basin lakes and distributaries. Lakes are characterized by high primary productivity, abundant phytoplankton, high surface DO concentrations, and little overbank flooding.

### Methods

*Field collections.*—To identify long-term hypoxic and normoxic sampling localities, DO concentrations were monitored 2 wk before bluegill collections over a wide range of potential collection localities. Fifty-seven bluegill were collected in the southeastern Basin from 6–20 June 1996 by electrofishing in normoxic lakes and areas with chronic hypoxic conditions. To ensure bluegill from hypoxic waters were exposed to long-term low DO conditions, fish were only collected from areas that were several kilometers from any DO refuge. Upon capture fish were measured [mm total length (TL)], weighed (g) and sections of dorsal epaxial muscle (approximately 200 mg) were removed from both sides of each fish with a sterile disposable scalpel. Each muscle sample was placed in a 2.0-ml cryogenic stor-
age tube and submerged in liquid nitrogen. The remainder of the fish was wrapped in aluminum foil and placed on ice. When a fish was collected, temperature (C), dissolved oxygen (mg L\(^{-1}\)) and pH were measured with a Hydrolab Datasonde-3 submersible water quality monitor. These procedures allowed us to monitor water quality in hypoxic and normoxic areas before and throughout the field study.

**Laboratory experiment one.**—Forty bluegill (mean wt ± standard error; 40.9 ± 1.89 g) were collected from the Basin, acclimated to laboratory conditions for 4 wk and placed individually into 30-L aquaria. Each aquarium was equipped with an adjustable standpipe and an undergravel filter powered by an airstone. Four treatment levels were randomly applied to ten tanks each: (1) food and high DO; (2) food and low DO; (3) no food and high DO and (4) no food and low DO. Feeding levels were included in the experiment to test effects of starvation and food-DO interactions on bluegill RNA-DNA ratios. During the experiment bluegill were fed a complete diet of frozen adult brine shrimp *Artemia salina*, which provided high levels of protein (60%) and essential amino acids (Barnabé, 1990). Feeding levels were maintained at 4% body weight day\(^{-1}\) (wet weight) which corresponded to about 0.5 g of brine shrimp (dry weight) per day, per fish, or about 1000 cal fish\(^{-1}\) day\(^{-1}\) (Cummins and Wuycheck, 1971). This ration level was well above that required for standard metabolism by 45-g pumpkinsweed *Lepomis gibbosus* (about 140 cal day\(^{-1}\); Brett and Sutherland, 1965), and was at or above mean consumption levels reported from field studies on bluegill, pumpkinsseed, rock bass *Ambloplites rupestris* and black crappie at similar temperatures (1–4%; Gerking, 1954; Seaburg and Moyle, 1964; Keast and Welsh, 1968; Kolehmainen, 1974).

Aquaria subjected to the high oxygen treatment were kept at full saturation by bubbling air through the undergravel filter. Hypoxic conditions in each tank were controlled with mixtures of nitrogen and oxygen. Because hypoxic conditions can reduce pH in the system, carbon dioxide was used to maintain constant pH levels between normoxic and hypoxic tanks.

At the conclusion of the 7-d experiment fish were anesthetized with MS-222 and killed by severing the spinal cord. Similar to the field sampling procedure, fish were processed immediately, although muscle samples were stored in an ultra-cold freezer (−80 C).

**Laboratory experiment two.**—As in experiment one, forty fish (mean wt = 37.01 ± 2.95 g) were collected from the Basin, acclimated to laboratory conditions and placed individually into 30-L aquaria. Two treatments (high DO and low DO) were randomly applied to twenty tanks each. All fish were fed frozen brine shrimp equivalent to 3% body weight per day. This experiment was designed to remove the effects of feeding level differences and allowed close examination of hypoxic conditions on RNA-DNA ratios of bluegill. To better simulate hypoxic conditions in the Basin the gas mixer was adjusted to allow higher flows of nitrogen to hypoxic tanks. After 7 d muscle samples were processed and analyzed in the same manner as the first experiment.

**RNA-DNA ratios.**—RNA-DNA ratios were determined according to methods outlined in Buckley and Caldarone (1988), with modifications of centrifugation speed (3750 rpm) and processing temperature (10 C). All samples were run in replicate. Muscle tissue from each fish was homogenized for 60 s in 10 parts (by volume) distilled water with a hand-held homogenizer. Replicate 0.350-μL samples were removed from each muscle homogenate, and diluted with 1.4 ml distilled water. The following procedures were applied to each replicate sample. First, 0.7 ml of cold, 0.6N HClO₄ was added, the sample was incubated on ice for 15 min, centrifuged (all centrifugations were at 3750 rpm for 10 min at approximately 10 C) and the supernatant was discarded. The pellet was washed twice by adding 2 ml of cold 0.2N HClO₄, centrifuging and discarding the supernatant after each centrifugation. Next,
2.24 ml of 0.3N KOH was added and the sample was incubated at 37 C for 1 h. Following incubation, 1 ml of 1.32N HClO₄ was added, the sample was vortexed, incubated on ice for 30 min and then centrifuged. The supernatant, containing the RNA fraction, was warmed to room temperature and the absorbance was measured at 260 nm with a spectrophotometer. The pellet, containing the DNA fraction, was washed with 2 ml of cold 0.2N HClO₄ and centrifuged. Following centrifugation, the supernatant was discarded and 2.2 ml of 0.6N HClO₄ was added. The sample was vortexed and incubated at 85 C for 15 min. After incubation the sample was vortexed and incubated on ice for an additional 15 min. The sample was then centrifuged, the supernatant (containing the DNA fraction) was warmed to room temperature and the absorbance was measured at 260 nm. For each replicate quantities of RNA and DNA were determined from the following equation: Nucleic Acid (μg/mg) = (A260 fraction - A260 blank) × sample volume × dilution factor/extinction coefficient × volume of aliquot × 1000 (Buckley and Bulow, 1987). Mean RNA-DNA ratio for each specimen was calculated from the two replicate samples.

Statistical analysis.—We tested RNA-DNA ratios for univariate normality and homogeneity of variance (SAS Institute, Inc., 1989). Because data met assumptions for parametric analyses, differences in RNA-DNA ratio means were tested with analysis of variance (ANOVA) or analysis of covariance (ANCOVA). All tests were evaluated at α = 0.05. Single-factor (oxygen) ANOVA was used for analysis of the second laboratory experiment (PROC ANOVA, SAS Institute Inc., 1989), but because temperature was significantly different between normoxic and hypoxic areas in the field study and laboratory experiment one, we used ANCOVA with temperature as the covariate (PROC GLM, SAS Institute., Inc., 1989). For experiment one treatment means were compared with LSMEANS (SAS Institute, Inc., 1989). Because of the size range of bluegill in each experiment, regression analysis (PROC REG, SAS Institute Inc., 1989) was used to examine correlations between RNA-DNA ratios and fish weight.

RESULTS

Field data.—We collected 31 and 26 bluegill, respectively, from normoxic and hypoxic areas of the Basin. Because one muscle sample was contaminated, RNA-DNA analyses were conducted on 56 fish. Analyses showed no significant differences (both P > 0.9107) in length (hypoxia, 118.15 ± 1.59 mm; normoxia, 117.74 ± 1.30 mm) and weight (hypoxia, 32.03 ± 1.70 g; normoxia, 31.58 ± 1.51 g) of bluegill from normoxic and hypoxic areas.

During field sampling mean temperature was 1.19 C higher (P < 0.0001) in hypoxic areas (Table I) whereas mean pH was not significantly different (P > 0.9497) between normoxic (7.05 ± 0.03) and hypoxic (7.05 ± 0.02) habitats. In hypoxic areas DO concentrations ranged from 1.22–3.04 mg L⁻¹, but were always below 2 mg L⁻¹ at night. DO concentrations in normoxic areas ranged from 3.23–7.18 mg L⁻¹ (never falling below 3.23 mg L⁻¹ at night) and were significantly higher (P < 0.0001) than hypoxic sites (Table I).

Mean RNA-DNA ratios of bluegill collected from normoxic areas were significantly higher (P < 0.0001) than RNA-DNA ratios from hypoxic areas (Table I). Neither temperature (P > 0.1558) or the interaction term (temperature × oxygen; P > 0.5471) had a significant effect on RNA-DNA ratios, and there was no detectable relationship between RNA-DNA ratios and bluegill size (P > 0.5301). Low standard errors indicated a high level of precision for the laboratory RNA-DNA methodology.

Laboratory experiments.—In both experiments differences in mean temperatures between normoxic and hypoxic tanks were less than 0.26 C, although this difference was significant in experiment one (P < 0.0002). In experiment one DO concentrations in normoxic tanks were approximately 4.5 times greater than hypoxic tanks (Table I). In the second experi-
Table 1.—Mean fish weights, water temperatures, pH, dissolved oxygen concentrations and fish RNA-DNA ratios from field and laboratory experiments. Significant differences at $\alpha = 0.05$ are indicated by an asterisk. Significance tests refer to within experiment differences (hypoxic versus normoxic); no tests were conducted between experiments. Standard errors are given for all variables, but coefficients of variation, and ranges are only given for mean RNA-DNA ratios.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Water type</th>
<th>n</th>
<th>Weight</th>
<th>Temperature</th>
<th>Dissolved oxygen</th>
<th>RNA-DNA ratio (±SE; CV; range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field</td>
<td>Normoxic</td>
<td>30</td>
<td>31.57 ± 1.51</td>
<td>26.93 ± 0.05*</td>
<td>4.93 ± 0.16*</td>
<td>0.87* (±0.02; 5.91; 0.075)</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>26</td>
<td>32.03 ± 1.70</td>
<td>28.12 ± 0.16*</td>
<td>2.21 ± 0.11*</td>
<td>0.68* (±0.03; 8.69; 0.092)</td>
</tr>
<tr>
<td>Laboratory 1</td>
<td>Normoxic</td>
<td>20</td>
<td>40.85 ± 2.36</td>
<td>21.53 ± 0.05*</td>
<td>8.55 ± 0.02*</td>
<td>1.02 (±0.04; 5.99; 0.095)</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>20</td>
<td>40.95 ± 2.77</td>
<td>21.78 ± 0.03*</td>
<td>1.86 ± 0.03*</td>
<td>0.94 (±0.03; 9.97; 0.152)</td>
</tr>
<tr>
<td>Laboratory 2</td>
<td>Normoxic</td>
<td>20</td>
<td>36.72 ± 3.96</td>
<td>21.06 ± 0.03</td>
<td>8.34 ± 0.02*</td>
<td>0.78 (±0.02; 5.21; 0.058)</td>
</tr>
<tr>
<td></td>
<td>Hypoxic</td>
<td>20</td>
<td>37.30 ± 4.49</td>
<td>20.90 ± 0.09</td>
<td>1.11 ± 0.03*</td>
<td>0.77 (±0.02; 5.62; 0.068)</td>
</tr>
</tbody>
</table>
ment DO concentrations in normoxic tanks were similar to experiment one, but increased nitrogen levels in hypoxic tanks resulted in a lower mean DO concentration (Table 1). Differences between mean DO concentrations of normoxic and hypoxic tanks were highly significant in both experiments.

There was no significant relationship (P > 0.7057) between RNA-DNA ratios and mean weight of bluegill in either laboratory experiment. In the first experiment RNA-DNA ratios of fed bluegill (1.03 ± 0.04) were significantly higher (P = 0.0300) than those of starved bluegill (0.93 ± 0.02). Although trends in RNA-DNA ratios of bluegill in normoxic tanks and hypoxic tanks were similar to fed and unfed fish (Table 1), differences were not statistically significant (P = 0.1100). There were no significant temperature (P > 0.9725) or interaction (P > 0.4417) effects on RNA-DNA ratios. As expected, RNA-DNA ratios were highest in fed fish in normoxic tanks (1.06 ± 0.05), intermediate in fed fish in hypoxic tanks (1.00 ± 0.06) and starved fish in normoxic tanks (0.97 ± 0.02) and lowest in starved fish in hypoxic tanks (0.88 ± 0.04; Fig. 1). Similar to results of the first experiment, RNA-DNA ratios of bluegill in normoxic tanks were not significantly higher (P = 0.3200) than RNA-DNA ratios of bluegill in hypoxic tanks (Table 1).

**DISCUSSION**

Numerous studies on larval (Wright and Martin, 1985; Steinhart and Eckmann, 1992), juvenile (Peterson and Brown-Peterson, 1992; Malloy and Targett, 1994) and adult (Haines, 1980; Mathers et al., 1993) fishes have provided substantial evidence that RNA-DNA ratios are sensitive indicators of short-term changes in somatic growth. Our data further support studies (Wright and Martin, 1985; Peterson and Brown-Peterson, 1992) that indicate physiological impacts of hypoxia and starvation are reflected in fish RNA-DNA ratios. Our field research suggests a link between hypoxia and decreased RNA-DNA ratios in bluegill, and is the first field study to demonstrate the relationship between environmental hypoxia and reduced fish RNA-DNA ratios. Although only the field study yielded statistically significant differences in RNA-DNA ratios from bluegill in hypoxic and normoxic habitats, overall trends of higher RNA-DNA ratios in bluegill subjected to normoxic conditions were consistent between the field study and laboratory experiments.

Fishes exposed to low DO concentrations experience increased allocations of energy for respiration (e.g., ventilation and circulation) and feeding (e.g., prey capture, digestion and assimilation), which reduces energy available for growth (Herrmann et al., 1962; Kramer, 1983, 1987). Largemouth bass exhibit decreased food intake under moderate reductions in DO concentration and markedly reduced food conversion ratios at an oxygen saturation of less than 4 mg l⁻¹ (Stewart et al., 1967). A similar reduction in bluegill feeding or food conversion efficiency in hypoxic areas of the Basin was reflected in significantly lower RNA-DNA ratios, indicating a reduction in recent growth. These results support other studies that have documented reduced body weight (Papoutsoglou and Tzha, 1996), growth and condition of fishes exposed to hypoxia (Stewart et al., 1967; Andrews et al., 1973; Cech et al., 1984; Peterson and Brown-Peterson, 1992). The sensitivity of RNA-DNA ratios as indices of short-term changes in environmental conditions is evidenced by the first laboratory experiment, which demonstrated a reduction in RNA-DNA ratios in starved bluegill. These results are consistent with other studies reporting reduced RNA-DNA ratios in starved striped bass *Morone saxatilis* (Wright and Martin, 1985) and rainbow trout *Oncorhynchus mykiss* (Bastrop et al., 1992). The decreasing trend in bluegill RNA-DNA ratios from treatment one (high food, high DO) to treatment four (no food, low DO) also indicated that hypoxic conditions may result in growth reductions even in the presence of maintenance food rations.
There are several possible explanations for the lack of agreement between the field study and the laboratory studies. First, the absence of a statistically significant reduction in bluegill RNA-DNA ratios in hypoxic laboratory tanks may accurately reflect the relationship between DO and growth. However, this does not seem likely given our field results and numerous studies that indicate hypoxic conditions result in physiological stress that reduces growth and condition in fishes (Herrmann et al., 1962; Stewart et al., 1967; Andrews et al., 1973; Kramer, 1983, 1987; Cech et al., 1984; Peterson and Brown-Peterson, 1992; Papoutsoglou and Tziha, 1996). In addition, fish were fed a fixed ration which may have caused a reduction in daily metabolic requirements (Brett, 1979) and a concomitant reduction in oxygen demands of bluegill in hypoxic tanks. Bluegill may also have been able to digest and assimilate a limited food ration with equal efficiency under both hypoxic and normoxic conditions, as has been reported for largemouth bass (Stewart et al., 1967). Results of these studies are consistent with feeding and oxygen level experiments that demonstrated growth of channel catfish fed a constant ration (3.1% body weight/d) was less affected by low DO concentrations than growth of fish fed ad libitum (Andrews et al., 1973). Taken together, these studies imply that fish fed restricted rations at low DO levels have reduced metabolic requirements and, therefore, growth may be limited only under extreme hypoxia (Brett, 1979).
Another important factor that likely affected results of our study was the reduced bioenergetic demands of bluegill under laboratory vs. field conditions (Doudoroff and Shumway, 1970). Bluegill in our laboratory studies expended no energy interacting with predators or competitors and minimal energy acquiring food. These reductions in energy expenditures, combined with an artificial foraging regime and constant DO conditions, may explain why significant reductions in RNA-DNA ratios of bluegill exposed to hypoxia were observed only in the field study. In addition, although we used short-term experiments to investigate relationships between hypoxia and bluegill RNA-DNA ratios in the laboratory experiments, fish collected during the field studies had been exposed to low DO conditions for several weeks. These results suggest that extended (e.g., > 2 wk) exposure to hypoxic conditions is necessary to significantly affect bluegill RNA-DNA ratios.

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