Influence of diet on stable carbon isotope composition in otoliths of juvenile red drum *Sciaenops ocellatus*

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ABSTRACT: Stable carbon isotope values of otoliths can provide long-term records of the environmental conditions experienced by fish. However, because otoliths contain carbon from metabolic sources, it is necessary to determine the proportion of metabolically derived carbon to properly interpret otolith $\delta^{13}C$ values. To evaluate the relative influence of dietary carbon and the dissolved inorganic carbon (DIC) of ambient seawater on the $\delta^{13}C$ isotopic composition of fish otoliths, juvenile red drum *Sciaenops ocellatus* were raised for 6 and 9 mo in tanks with flow-through ambient seawater and fed diets differing by 2.1‰ (p < 0.001) in carbon isotope composition ($\delta^{13}C$). At the end of the experiment, muscle tissue from the 2 treatments reflected the isotopic composition of the diets and differed by 2.1‰ (p <0.001). Muscle tissue from both groups was enriched by 1.5‰ in ^{13}C relative to the respective diets. The carbon isotope values of otoliths were enriched by ~16 to 17‰ relative to the respective diets, indicating that DIC was the dominant source (85 to 92%) for otolith carbon relative to carbon derived from diet. However, otoliths from the 2 treatments differed by 1.26‰ and expressed 60% of the difference in the isotope values of the diets. This result indicates that the nutritional characteristics of food may be a factor influencing the incorporation of metabolic carbon in the otolith. This is one of the first studies to provide evidence that the nutritional value of food influences otolith $\delta^{13}C$ value.

KEY WORDS: Otoliths · Stable isotopes · Red drum · *Sciaenops ocellatus*

INTRODUCTION

Fish otoliths, or ear stones, are primarily composed of calcium carbonate (aragonite). They are found in the otic capsule of the cranium of fish and function in hearing, equilibrium, and angular acceleration. Otoliths grow by accretion, incorporating elements from the surrounding endolymph. Because these elements remain in the otolith throughout the life of the fish they are permanent records containing valuable information that can be used to infer life-history characteristics (Degens et al. 1969, Radtke et al. 1998a, Schwarcz et al. 1998).

The stable isotope composition of otoliths ($\delta^{13}C$ and $\delta^{18}O$) can provide valuable information on the environmental conditions experienced by fish (Radtke et al. 1996, 1998b, Das et al. 2000, Begg & Weidman 2001). The incorporation of $\delta^{18}O$ into the otoliths of fish is tem-

perature dependent; therefore, the $\delta^{18}O$ composition of otoliths can be used to reconstruct ambient seawater temperatures experienced during the lifetime of the fish (Høie et al. 2004). Many studies have used oxygen and carbon isotopes to delineate fish populations and stocks (Gao & Beamish 1999, Newman et al. 2000, Bastow et al. 2002). However, a number of factors complicate interpretation of $\delta^{13}C$ data from otoliths.

In reference to ^{13}C, seminal studies by Degens et al. (1969) suggest that otolith aragonite is deposited in isotopic equilibrium with surrounding seawater. But more recent work demonstrates the incorporation of aragonite into fish otoliths from 2 sources: endogenous and exogenous. Endogenous sources are derived from catabolic processes, and exogenous sources are derived from dissolved inorganic carbon (DIC) in the external milieu entering the blood through the gills and through

the digestive tract, mainly as bicarbonate (Høie et al. 2003, Solomon et al. 2006). A poor understanding of the proportional contribution of each source and associated enrichment factors has limited the usefulness of otolith $\delta^{13}C$ values in fish ecology. Several studies have quantified the proportion of each carbon source, DIC and food, and, in most cases, the $\delta^{13}C$ values of the otoliths reflect ~80% DIC and ~20% food (Weidman & Millner 2000, Høie et al. 2003, Solomon et al. 2006, Tohse & Mugiya 2008). To our knowledge, the only study that has estimated the associated fractionation factors from the carbon sources to the otolith (Solomon et al. 2006) found otoliths to be enriched by 2.7 to 3.6‰ relative to the fish's diet.

In the present study, we attempted to validate these estimates by holding red drum *Sciaenops ocellatus* (Pisces: Sciaenidae) under isotopically identical water conditions while feeding them isotopically distinct diets. To ensure the fish equilibrated to the diets, we determined the $\delta^{13}C$ values of red drum muscle tissue, which reflects the value of each treatment diet (DeNiro & Epstein 1976, Nelson et al. 2010). We hypothesized that the $\delta^{13}C$ values of the juvenile red drum otoliths reflect to some degree the differences in the $\delta^{13}C$ values of the food the fish were fed during the experiment. Our objectives were to quantify the degree to which $\delta^{13}C$ differences in diet would be reflected in otolith $\delta^{13}C$ and to use these differences to calculate the relative contributions of DIC and respired dietary carbon to otolith carbon.

MATERIALS AND METHODS

Experimental design. Approximately 500 red drum (39 d old) were obtained from the Stock Enhancement Research Facility (Florida Fish and Wildlife Conservation Commission) in Port Manatee, Florida, on August 10, 2001. Fish were acclimated to laboratory conditions at the Florida State University Coastal and Marine Laboratory (FSUMCL) in 16 rectangular flow-through saltwater tanks (210 l volume) for several weeks and fed a standard prepared diet until they were able to consume their prescribed diets (Fig. 1). During the first week of the acclimation period, the fish were fed their accustomed commercial diet (Ziegler Salmon Starter, Burris; $\delta^{13}C \approx -21$‰). Fish were then fed another commercial hatchery feed (AquaMax Fingerling Starter 300, Purina Nutritional International, $\delta^{13}C \approx -20$‰) for 39 d until they were able to consume ground fish-tissue. On September 17, 2001 (Day 1 of the experiment), the fish were given 2 diets differing in carbon stable isotope ratios. The fish in Treatment A continued with the AquaMax feed. The fish in Treatment B were switched to a diet that consisted of muscle tissue from

the pinfish *Lagodon rhomboides* (family: Sparidae, $\delta^{13}C \approx -17$‰), a common inshore fish in NW Florida. The majority of the pinfish used were collected from Apalachicola Bay. Some pinfish were purchased from local seafood and bait dealers who harvested in the bay and nearby waters. Fresh pinfish were kept frozen until feeding time, when they were thawed, filleted, and ground or cut into pieces. Fish were fed ad libitum with AquaMax or minced pinfish fillets once each day.

The 16 tanks used for this experiment had average flow rates of 782.0 l h^{-1} (range = 599.9 to 851.7 l h^{-1}) and nominal exchange rates of 3.7 times h^{-1} (range = 2.8 to 4.3 times h^{-1}). Temperature (°C) and salinity measurements were taken several times daily from seawater flowing into the aquaria. Seawater temperatures were not controlled, but followed the seasonal ambient seawater temperatures of the near-shore northeastern Gulf of Mexico.

Growth rates of red drum were calculated by subtracting the mean standard length (SL, mm) of the fish from each diet treatment at the start of the experiment (x_s) from the individual SLs at the time each fish was sampled (F_n) and divided by the number of days of treatment (T_n), i.e.:

$$\text{Growth rate} = (F_n - x_s) / T_n \tag{1}$$

$\delta^{13}C$ values of the diets. A Finnigan Mat isotope ratio mass spectrometer (IRMS) was used to measure all stable isotope ratios by comparing the heavy:light element ratio in a sample relative to a known standard. Isotopic ratios are typically expressed using the 'del' (δ) notation with the general equation:

$$\delta X \ (‰) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \tag{2}$$

where X is the sampled isotope ratio (e.g. ^{13}C:^{12}C), R_{sample} is the isotope ratio of the sample, and R_{standard} is the isotope ratio of the standard. Random samples (n = 4) were taken from the batch of AquaMax commercial feed during the course of the experiment. The AquaMax pellets were ground with a cleaned coffee grinder and a Crescent Wig-L-Bug. Processed samples were shipped to Isotope Services, in Los Alamos, New Mexico, for determination of the carbon stable isotopes.

Random samples of muscle tissue from each collection (n = 23) of pinfish were processed similar to the muscle tissue fed to the red drum; i.e. it was frozen, thawed, rinsed, and soaked in tap water. All bones and scales were removed. For sample analysis, the samples were then dried in an oven at ~70°C for several days until the muscle was dry. The drying time varied for each individual muscle sample depending on amount. Upon removal from the oven, the tissue was ground into a fine, homogenous material with a Crescent Wig-L-Bug. Processed samples were shipped to Isotope Services. The 2 diets, commercial and pinfish

muscle, differed significantly (*t*-test, p < 0.001) in carbon isotopic composition by 2.12‰. The $\delta^{13}C$ values of the commercial diet (n = 4) had a mean (±SD) $\delta^{13}C$ of −19.64 ± 0.21‰. Because of the very small variation in the $\delta^{13}C$ values, only 4 samples of the AquaMax diet were analyzed. The carbon isotopic values from the pinfish diet (n = 23) had a mean $\delta^{13}C$ of −17.52 ± 1.62‰.

Seawater samples from the tanks were collected to measure the $\delta^{13}C$ of DIC. Approximately 10 ml of seawater from 3 randomly selected tanks were injected into pre-evacuated glass vials once each month. The vials were refrigerated until further processing. Approximately 0.2 ml of 30% phosphoric acid was injected into each water sample. Head space (~0.2 ml) from the vials was then direct-injected into an IRMS at Florida State University.

Red drum were randomly sampled from each of the tanks on Days 160 and 261 of the experiment. On Day 160, 5 fish from each tank for a total of 20 fish from each diet treatment were randomly selected and measured. The fish were then overdosed with an anesthetic (MS-222) according to FSU Animal Care and Use Committee (ACUC) guidelines and frozen until further processing. All fish from 1 replicate in Treatment A died on Day 159 due to a malfunction in the aeration system and were used in the 160 d analysis. The remaining fish (n = 34) were sampled on Day 261: 15 fish in Treatment A and 19 in Treatment B.

Experimental red drum were measured (SL, mm) and weighed (to nearest 0.1 g), and a single fillet, minus bones and scales, was removed and weighed from each fish with the same precision. The muscle was then soaked in tap water for several minutes and dried at ~70°C for several days. Muscle tissue was then ground to a fine powder with a Crescent Wig-L-Bug. Carbon isotope ratios were determined at Isotope Services.

Sagittal otoliths were extracted with forceps, rinsed in tap water, and cleansed with a paper towel to remove tissue adhering to the otoliths. Right and left otoliths were held separately in evacuation vials, then flushed with nitrogen gas and dissolved with 30% phosphoric acid. The samples were then analyzed for isotopic carbon on an IRMS at Florida State University.

Data analysis. ANOVA and a mixed model with a fixed effect for treatment and a random effect for the tank the fish were held in were used. The mixed effect model was coded using the R LME4 (www.r-project.org) statistical packages. Linear regressions were performed using either Microsoft Excel or Minitab

Treatment A: AquaMax (commercial)
a = Shorter duration: 160 experiment days

| A1a n = 5 | A2a n = 5 | A3a n = 5 | A4a n = 5 |

Treatment B: Pinfish

| B1a n = 5 | B2a n = 5 | B3a n = 5 | B4a n = 5 |

b = Longer duration: 261 experiment days

| Died | A2b n = 5 | A3b n = 5 | A4b n = 5 |

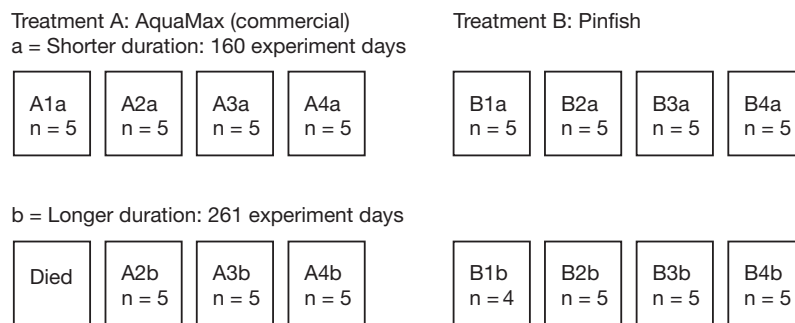| B1b n = 4 | B2b n = 5 | B3b n = 5 | B4b n = 5 |

Fig. 1. Schematic of the experimental setup showing 2 diet treatments and 2 exposure durations in 16 tanks. Each square represents an individual tank. All fish in 1 tank in the long-duration AquaMax treatment died early (on Day 160) and were used for the short-duration experiment instead

(V.2003). Grubbs test was used to test for outliers in the data set (Quinn & Keough 2002). All significance levels were set at α = 0.05.

RESULTS

Temperature and salinity of seawater

The monthly mean temperature (±SE) of the experimental tanks was 19.4 ± 5.1°C (Fig. 2). The highest monthly mean temperatures (26.9 ± 2.2°C) were in June, on Day 260, while the lowest (13.7 ± 3.8°C) were in January, on Day 120.

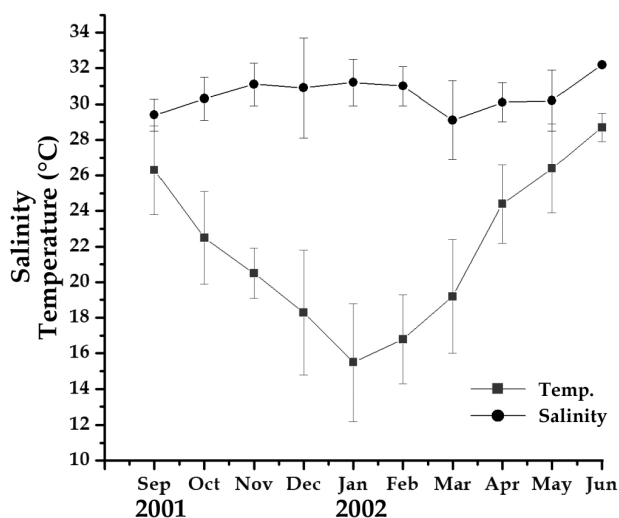The mean daily salinity in the experimental tanks was 30.1 ± 1.4 (Fig. 2).

Fig. 2. Mean monthly temperature and salinity (±SE) recordings at Florida State University Coastal and Marine Laboratory during the experiment

DIC isotopic composition

The $\delta^{13}C$ of DIC in the seawater was enriched relative to atmospheric CO_2 (–8‰) (Fig. 3). Monthly DIC isotopic compositions ranged from –4.82 to –2.59‰ and had a mean (±SE) of –3.83 ± 0.81‰. We did not observe a significant change in DIC values during the experiment (linear regression, R^2 = 0.18, p = 0.212). There was no difference between spring/summer and fall/winter DIC isotope values either (ANOVA, F = 0.735, p = 0.613).

Growth of red drum

The red drum exhibited near-linear growth throughout the experiment (Fig. 4). On Day 160 of the experiment, 5 specimens were sampled from each of the remaining replicates of both treatments. The mean SL overall was 138.1 mm (SE: 16.1; range: 103 to 172; n = 40). In fish sampled on Day 160 there was no significant difference (*t*-test, p = 0.43) between the mean SL from Treatment A (140.1 mm; SE: 16.6; range: 104 to 172) and the mean SL from Treatment B (134.2 mm; SE: 18.0; range: 103 to 162).

The remaining fish, sampled on Day 261 of the experiment, had a combined mean SL of 203.1 mm (SE: 24.9; range: 140 to 250). For fish sampled on Day 261, there was no significant difference (*t*-test, p = 0.42) between the mean SL from Treatment A (206.7 mm; SE: 15.5; range: 177 to 239; n = 15) and Treatment B (200.2 mm; SE: 30.5; range: 140 to 250; n = 19).
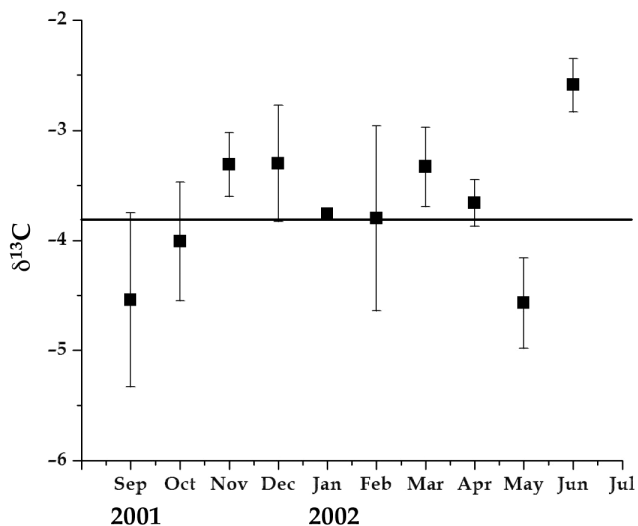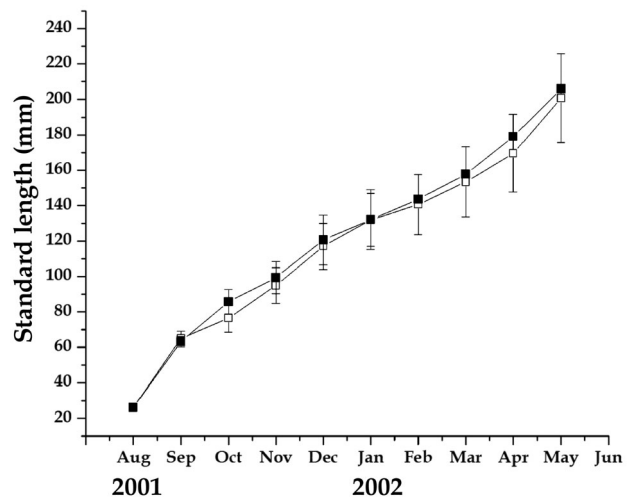

Fig. 4. *Sciaenops ocellatus.* Standard lengths of red drum from each diet treatment. ■: Treatment A, fish fed AquaMax; □: Treatment B, fish fed pinfish muscle

The mean growth rate of red drum from Treatment A (0.51 mm d^{-1}; SE: 0.09) was not significantly different (ANOVA, p = 0.88) from the mean growth rate of fish from Treatment B (0.49 mm d^{-1}; SE: 0.09). Growth rates calculated for the 160 d samples were not significantly different between treatments or among replicates (ANOVA, p = 0.88).

Carbon isotopic composition of red drum muscle tissue

Carbon isotope values of red drum muscle tissue were significantly different (p < 0.001) between the 2 treatments (Fig. 5a). The mean (±SD) $\delta^{13}C$ of Treatment A muscle tissue was –18.13 ± 0.15 (n = 32) and ranged from –18.36 to –17.63‰. Tissue from Treatment B (mean $\delta^{13}C$ = –16.01 ± 0.24; n = 38) ranged from –16.49 to –15.39‰. The difference in $\delta^{13}C$ values between the 2 treatments was 2.12‰. Three outliers (Grubbs test; α = 0.05) from treatment A1a, A5b, and B4b with values of –16.46, –16.29, and –16.88‰ were not included in the data set, and 1 muscle tissue sample from A4b was not processed.

Muscle tissue between the 2 diet treatments in both age classes was significantly different in isotopic value (Fig. 5a). Tissue from the fish in the short-duration experiment in Treatment A (mean (±SD) $\delta^{13}C$ = –18.18 ± 0.10‰) were depleted 2.03‰ relative to their Treatment B counterparts (mean $\delta^{13}C$ = –16.14 ± 0.17‰), and that difference was significant (mixed-effects model, p < 0.001). Likewise, in the long-duration experiments fish from the 2 treatments had significantly different carbon isotopic values (mixed-effects model, p < 0.001) where Treatment A (mean $\delta^{13}C$ = –18.06 ±

Fig. 3. Monthly carbon stable isotopic composition (mean ±SE) of the dissolved inorganic carbon (DIC) in seawater. The horizontal line represents the mean DIC $\delta^{13}C$ value of –3.83 ± 0.81‰
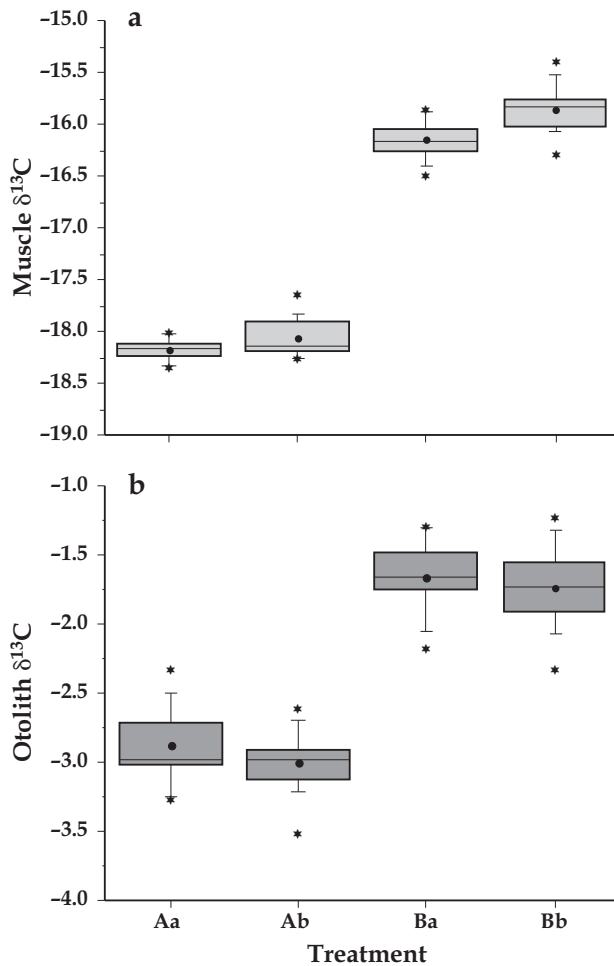
Fig. 5. *Sciaenops ocellatus.* Mean carbon isotopic composition of (a) muscle tissue and (b) otoliths from red drum fed different diets for short or long durations. Aa and Ab: AquaMax diet for 160 or 261 d, respectively; Ba and Bb: minced pinfish for 160 or 261 d, respectively. (●) Mean δ^{13}C; horizontal line: median; top and bottom of box: 1 SD from mean; whiskers: 25th and 75th percentile; *: data points below 25th and above 75th quartile. See Fig. 1 for experimental setup

0.20‰) was depleted 2.20‰ relative to Treatment B (mean δ^{13}C = −15.86 ± 0.21‰). Muscle tissue from both treatments was enriched by 1.51‰ relative to the respective diets.

Carbon isotopic values of red drum otoliths

The δ^{13}C values were measured for the sagittal otolith pairs from both diet treatments and both experimental durations. The mean (±SD) δ^{13}C value for the pairs of otoliths from the red drum raised from Treatment A (n = 66) was −2.94 ± 0.23‰, with a range of −3.50 to −2.44‰. Otoliths of fish from Treatment B (n = 76) had a mean δ^{13}C value of −1.68 ± 0.23‰ and

a range of −2.19 to −1.25‰. Two outliers from Treatment A (A1a-3, −1.29‰; A5b-4, −1.88‰) were not included in the otolith data set, and 1 outlier from Treatment B (B4b-2, −2.93‰) was not included in the data (Grubbs test, α = 0.05). These were the same outliers from the muscle data, and it was presumed that they were a result of processing error (Fig. 5b). This difference of 1.26‰ between the 2 pooled means was significantly different (mixed-effects model, p < 0.001; Fig. 5b). Each otolith pair between the 2 diet treatments exhibited similar disparity.

The otoliths from both diet treatments were similarly enriched in ^{13}C with respect to the isotopic values of their diet and muscle tissue. The otolith δ^{13}C values of fish in Treatment A were enriched by 16.70 ml^{−1} and Treatment B were enriched by 15.84 ml^{−1} relative to the δ^{13}C values of their respective diets. The otoliths from Treatment A were enriched 15.19‰ from the muscle carbon values, and Treatment B otoliths were enriched 14.33‰ with respect to the muscle tissue. These values were not significantly different (mixed-effects model, p = 0.21).

DISCUSSION

The results of this experiment and the experiments of others clearly demonstrate some influence of metabolically derived carbon on otolith δ^{13}C value (Kalish 1991, Radtke et al. 1998b, Schwarcz et al. 1998, Høie et al. 2003, Solomon et al. 2006, Tohse & Mugiya 2008). But, in order to properly interpret carbon stable isotope results for use in ecological studies, it is necessary to know the relative contribution of DIC and diet to the observed otolith δ^{13}C value. To that end, studies with various fish species have demonstrated a dietary contribution ranging from 17 to 35% (Kalish 1991, Høie et al. 2003, Solomon et al. 2006, Tohse & Mugiya 2008). Solomon et al. (2006), in a detailed experimental study on rainbow trout, estimated fractionation factors associated with otolith formation by manipulating the ambient water DIC δ^{13}C and the dietary δ^{13}C and, subsequently, by measuring the δ^{13}C of the otolith, the endolymph DIC, and the blood DIC. They found that metabolic carbon accounted for ~17% (±3% SE) of the otolith δ^{13}C value, while ambient DIC accounted for >80%. Also, they found a 2.7 to 3.6‰ net fractionation from the carbon sources to the otolith.

In our experiment, fish from both dietary treatments were held under the same water flow and DIC regimes and fed a diet that differed in isotopic content and nutritional characteristics. The mean difference in the δ^{13}C of the diets was 2.12‰, and this difference was reflected in the muscle tissue (Fig. 5a). However, we observed a difference of only 1.26‰ between the

mean $\delta^{13}C$ values of the otoliths for both treatments (Fig. 5b). Otolith $\delta^{13}C$ values ranged from -2.94 to -1.68‰ between the 2 treatments and were only slightly enriched relative to the mean $\delta^{13}C$ of seawater DIC, which was -3.8 ± 0.8‰. These differences from seawater DIC $\delta^{13}C$ were caused by isotopic fractionation during carbonate incorporation into the otolith and by the admixture of respired carbon derived from diet. The similarity between ambient seawater DIC $\delta^{13}C$ and the otolith $\delta^{13}C$ of both treatments indicates that the bulk of the carbon making up the otoliths was derived from seawater DIC. It is difficult to rationalize then, how the relatively small difference in the diet $\delta^{13}C$ value is expressed in the otoliths (1.26‰, some 60% of the difference between the diet and tissue), since the contribution of diet to otolith carbon appears to be small. Possible explanations for the differences in the observed otolith $\delta^{13}C$ between treatments include different degrees of fractionation at the gills or at the gut, or else food quality may have somehow influenced the otolith $\delta^{13}C$ by changing the relative contribution of dietary $\delta^{13}C$ relative to ambient DIC $\delta^{13}C$.

To determine the percent contribution of food to the $\delta^{13}C$ value of the otoliths from the observed results we used a simple 2 source mixing model, such that:

$$(\delta_{DIC} \times P_{DIC}) + (\delta_{Food} \times P_{Food}) + \varepsilon = \delta_{OTO} \qquad (3)$$

and

$$P_{DIC} + P_{Food} = 1 \qquad (4)$$

where δ_{DIC} is the mean $\delta^{13}C$ value of the DIC during the experiment, δ_{Food} is the mean $\delta^{13}C$ value of the food, P_{DIC} and P_{Food} are the percent contribution of the DIC and food, respectively, to the final otolith $\delta^{13}C$ value (δ_{OTO}), and ε is the fractionation factor between the dissolved carbon sources and the final otolith $\delta^{13}C$ value. Although we did not determine the fractionation factor independently we can use the values from Solomon et al. (2006) as a baseline to calculate the hypothetical values for the percent contribution of metabolic carbon to the diet. Because these fish are of the same species and were held under identical conditions, we assume that ε should be the same in both treatments. The value of ε based on the data by Solomon et al. (2006) is 3.15. Using Eqs. 3 & 4 we calculate that the percent contributions of DIC $\delta^{13}C$ and food $\delta^{13}C$ to otolith $\delta^{13}C$ were significantly different between treatments (mixed-effects model, $p < 0.00001$). For Treatment A, the mean ($\pm SD$) contribution of DIC $\delta^{13}C$ was 85% ($\pm 1\%$) and the contribution of dietary $\delta^{13}C$ was 15% ($\pm 1\%$). For Treatment B, the mean contribution of the $\delta^{13}C$ value of DIC was 92% ($\pm 2\%$) and the contribution of the food $\delta^{13}C$ value was 8% ($\pm 2\%$).

Given that both treatments were held under identical conditions, we suggest that the differences in the otolith $\delta^{13}C$ values were caused by differences in the nutritional characteristics of the food that caused the pinfish-fed red drum (Treatment B) to incorporate more DIC-derived carbon into the otoliths. It seems unlikely that the fractionation from carbon sources would be different because conditions in the experiment were identical (same species, juveniles of the same size and age, same ambient water sources), except for the 2 food treatments. If this hypothesis is correct, then the nutritional characteristics of food may also affect otolith $\delta^{13}C$ values, besides ambient water and food $\delta^{13}C$. This would make calculating a universal or species value for the contribution of metabolic carbon to otoliths extremely difficult. Researchers who conduct experiments on the contribution of metabolic carbon to otolith $\delta^{13}C$ values should consider varying the nutritional value of the experimental diet to determine if our hypothesis is correct.

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