Ecological Indicators 97 (2019) 10–16

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Original Articles

Metabolic effects on carbon isotope biomarkers in fish

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ARTICLE INFO

Keywords:
Carbon isotopes
Stable isotopes
Metabolic rate
Biomarker
Biochemical tracers
Otoliths
Diet

ABSTRACT

Carbon stable isotopes ($\delta^{13}C$) in animal tissues are a powerful tool for tracking biological and environmental change. However, carbon isotope signatures can be altered by both physiological and environmental factors which can cloud interpretation in their use as biomarkers. We investigated metabolic effects (by varying temperatures) on $\delta^{13}C$ of three fish tissues (otolith, muscle and liver) and the proportional contributions of environmental water (dissolved inorganic carbon; DIC) and diet (metabolic sources). Juvenile Australasian snapper (*Chrysophrys auratus*) were laboratory-reared at four temperatures for up to two months and then $\delta^{13}C$ in otolith, liver and muscle were measured using isotope-ratio mass spectrometry (IRMS). Temperature significantly altered $\delta^{13}C$ signatures in all tissues. $\delta^{13}C$ in otoliths reflected carbon signatures from diet and water DIC, with values and variation of proportional contributions influenced by temperature. In muscle and liver, we found differences in $\delta^{13}C$ between tissues and across temperature treatments with concurrent high diet-to-tissue fractionation. We conclude that metabolic effects influenced carbon incorporation for all tissues, with otolith carbon providing valuable insights into field metabolic rates. However, metabolic effects complicated the use of soft-tissue to track diet. This study deepens our understanding of internal and external drivers of carbon isotopic signatures in fish tissues and enhances their utility as a biomarker in the field. Improved insight into biomarkers facilitates more accurate predictions of ecological and environmental change for better understanding and management of wild populations.

1. Introduction

Stable isotopes have been used to reconstruct trophic relationships and food webs (Gronkjaer et al., 2013; Wada et al., 1991), animal movements and migrations (Rubenstein and Hobson, 2004), environmental histories (Jones and Campana, 2009; Watanabe et al., 2009) and ecosystem fluxes of elements and water (Boecklen et al., 2011; Peterson and Fry, 1987). Carbon stable isotope values, denoted as the ratio between primary isotopes ^{12}C and ^{13}C ($\delta^{13}C$), can indicate key dietary and metabolic information through direct isotopic links between consumer tissues and diet. Incorporation of $\delta^{13}C$ into tissues can be altered by geophysical and physiological fractionation processes, creating distinctive signatures which can be retrospectively tracked.

Environmental data from aquatic systems are limited compared to terrestrial systems, largely due to the cost and resources required for direct monitoring (Richardson and Poloczanska, 2008), so geochemical analysis of tissues provides a cost-effective and efficient method of reconstructing ecological and environmental change. Hard calcified structures, such as bones or shells, have low metabolic activity and slow tissue turn-over, consequently incorporating long-term isotopic signatures. Hard calcified structures also show minimal degradation through time and extensive archives exist in research institutes and museums world-wide. This is particularly true for fish otoliths (ear bones) which are calcium carbonate ($CaCO_3$) structures in teleost fishes that form visible daily and annual increments. Otoliths are acellular and metabolically inert and trace elements are permanently locked into incrementally accreted tissue (Campana and Neilson, 1985). These chemical signatures can reflect environmental and physiological conditions allowing life-long and time resolved biomarkers to be recorded (Campana, 1999).

Stable carbon isotopes in otoliths may be a useful tracer of metabolic rates. Metabolic histories are important in understanding and managing fish species, being a fundamental predictor of behaviour, performance, growth, social and lifestyle interactions, and consumption of resources (Chabot et al., 2016; Hulbert and Else, 2000). Otolith carbon arises from two sources: dissolved inorganic carbon (DIC) in environmental water uptake via the gill and/or intestinal interfaces and metabolically sourced carbon via cellular respiration of food (Kalish, 1991a; McConnaughey et al., 1997; Trueman et al., 2016). Higher respiration and metabolic rates increase the proportion of metabolic carbon, which has a significantly depleted carbon signature (values of $\delta^{13}C$ are more negative) than the DIC proportion. Consequently,

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https://doi.org/10.1016/j.ecolind.2018.10.010
Received 2 July 2018; Received in revised form 2 October 2018; Accepted 3 October 2018

changes in $\delta^{13}C$ in otoliths are thought to predominately reflect metabolic shifts. The influence of other carbon sources is considered minimal as seawater DIC typically remains within a narrow range and considerable changes to diet are required to significantly alter values of $\delta^{13}C$ (Shephard et al., 2007; Sherwood and Rose, 2003; Trueman et al., 2016). However, further study, particularly controlled experimental work, is necessary to investigate the relationship between $\delta^{13}C$ and metabolic rates.

Carbon signatures in soft-tissues have been used extensively to track diet and uncover trophic and nutrient pathways (Boecklen et al., 2011; Pasquaud et al., 2007; Rau et al., 1983). However, diet-to-tissue carbon fractionation can vary between species, diets and tissues with recorded values from bulk isotope analysis ranging from 1‰ (DeNiro and Epstein, 1978) to 5‰ (Bloomfield et al., 2011; Gaston and Suthers, 2004). Variation in diet-to-tissue fractionation in soft-tissues can be influenced by the physiological and structural characteristics of the tissues. For instance, isotopic composition in tissues can be mediated by growth, whereby existing mass is diluted by newly synthesised mass, and metabolism, which can determine the rate of replacement or conversion of existing tissues (Buchheister and Latour, 2010; Hesslein et al., 1993). Metabolically active tissue, such as the liver, can also have faster tissue turnover but less newly synthesised mass than less metabolically active tissue, such as muscle, where growth is the primary determinant in isotopic incorporation (Buchheister and Latour, 2010; Carleton and Del Rio, 2010; Mont'Alverne et al., 2016). Furthermore, lipid content in soft-tissues can also affect carbon signatures, as lipids are typically 6–8‰ depleted in $\delta^{13}C$ compared to tissue protein or carbohydrates (DeNiro and Epstein, 1977). Temperature may also affect rates of carbon assimilation and levels of lipids in soft-tissues through metabolic effects. Limited studies have experimentally tested the effect of temperature on the relationship between $\delta^{13}C$ in fish soft-tissues and diet, with little work completed on liver tissue (Barnes et al., 2009; Bloomfield et al., 2011). Further work is required to understand species-specific carbon fractionation and to identify factors contributing to variation.

To increase our understanding of the factors controlling carbon isotope values in fish tissues, we performed an experiment on an iconic fishery species, Australasian snapper (*Chrysophrys auratus*). Temperature is known to directly influence metabolic rates of fish (Clarke and Johnston, 1999; Gillooly et al., 2001), therefore juvenile snapper were reared at four temperatures in controlled laboratory conditions. Values of $\delta^{13}C$ in carbon sources (DIC and diet) and fish tissues (otolith, liver and muscle) were then analysed. Our objectives were to 1) investigate the relationships between $\delta^{13}C$ in carbon sources and fish tissues and 2) assess the influence of metabolic effects on $\delta^{13}C$.

2. Methods

The study species, Australasian snapper (*Chrysophrys auratus*), is an iconic long-lived demersal species that supports important commercial and recreational fisheries in the Australasia and Indo-Pacific regions. Snapper culture is typically carried out in water temperatures of 13–28 °C, with optimum growth rate between 20 and 28 °C (Pecl et al., 2011).

Snapper eggs were collected from Cockburn Sound, Western Australia and reared at 25 °C and 35 psu at the Challenger Institute of Technology in Fremantle. Two-month old juvenile fish (approximately 40 mm, 1 g) were transported to the University of Adelaide, South Australia. To acclimatise to experimental conditions in the laboratory, fingerlings were kept in a 200 L holding tank at 25 °C. After this period the fingerlings were batch marked via immersion in an alizarin complexone ($C_{19}H_{15}NO_8$) solution (40 mg/L of tank water) for 24 h. Alizarin marks otoliths allowing pre-experimental and experimental otolith growth to be distinguished (van der Walt and Faragher, 2003). Acclimation period totalled 2 weeks. Fingerlings were then separated into 16 tanks with 5 fish stocked in each. Each tank contained 40 L of seawater (salinity: 35 psu), as well as a submerged filter and aerator. Seawater was locally sourced from coastal waters (Gulf St Vincent, South Australia). Light was administered under a 12 h light:12 h dark cycle from fluorescent tubes. For both acclimation and experimental periods, fingerlings were fed twice daily to satiation with commercial fish food (Skretting Protec FF) that was 50% protein, 17% lipid and made from a range of animal and plant protein. Fish were gradually acclimatised to experimental temperatures by increasing temperature at a rate of 1.5 °C per day. Tank temperatures were set at four levels (20 °C, 24 °C, 28 °C, 32 °C) with 4 replicates of each temperature controlled by external heating units (Teco Seachill) in surrounding water baths. Water temperature was measured daily throughout the experiment with a handheld probe (Hanna Instruments HI-98127). Salinity was tested twice weekly using a refractometer (Vertex RF-1) and maintained at 40 psu via the addition of aged bore water. To maintain water quality, 50% water changes occurred weekly and water was regularly tested for ammonia through commercial testing kits (API). Fish were exposed to experimental conditions for up to 2 months and then euthanised in an ice slurry. Weights and lengths of euthanised fish were measured. Sagittal otoliths were dissected, cleaned of adhering tissue and air-dried. Liver and muscle tissue (dorsal flank) was extracted and frozen.

Experimental otolith material, distinguished by a purple (alizarin) band, was chipped off into filter paper and placed in acid-sterilised 4.5 ml vials. Vials were purged for 2.5 min with nitrogen gas and injected with 100 µl – 120 µl of 103–104% phosphoric acid. Carbon isotopes in otolith samples were analysed using a GasPrep headspace analyser connected to Nu Horizon (Nu instruments) isotope-ratio mass spectrometer (IRMS). Powder standards (ANU-P3, UAC $CaCO_3$ UniA, IAEA-CO-8) were analysed to assess instrument drift and calculate precision. Analytical errors averaged 0·19‰ for $\delta^{13}C$ otolith values.

Liver and muscle tissue were freeze-dried (Dynavac, model FD-5) for 48 h and then 2 mg weighed into tin capsules. To analyse for $\delta^{13}C$, soft-tissue samples were then loaded into an EUROEA300 elemental analyser (Eurovector) connected to Nu Horizon (Nu instruments) IRMS. Powder standards (Glutamic acid, Glycin, TPA) and replicate tissue were analysed every 9th sample to assess instrument drift and calculate precision. Analytical errors averaged 0.10‰ for liver and 0.07‰ for muscle.

The influence of lipids on $\delta^{13}C$ in soft-tissue samples was mathematically accounted for, as lipids are typically 6–8‰ depleted compared to protein or carbohydrates (DeNiro and Epstein, 1977). To account for lipids in muscle, the optimum lipid-normalisation method for muscle was used (Post et al., 2007):

$$\Delta\delta^{13}C = -3.32 + 0.99xC{:}\,N \quad (1)$$

where C:N is the proportion of carbon to nitrogen in the sample. Subsequently, an estimate of normalised $\delta^{13}C$ was derived:

$$\delta^{13}C_{normalised} = \delta^{13}C_{untreated} + \Delta\delta^{13}C \quad (2)$$

There is an alternative optimum method to account for lipids in liver (Skinner et al., 2016). A percent lipid calculation was first used (Post et al., 2007):

$$\%lipid = -20.54 + 7.24xC{:}\,N \quad (3)$$

where % lipid is the lipid in the soft tissue. Secondly a mathematical lipid-normalisation model was used (Kiljunen et al., 2006):

$$\delta^{13}C_{normalised} = \delta^{13}C + Dx\left(I + \frac{3.9}{1 + 287/\%lipid}\right) \quad (4)$$

where D is 7.018 and represents the carbon isotopic difference between protein and lipid, while *I* is a constant at 0.048. The lipids in diet were not accounted for as fish consume and metabolise the entire feed (Bloomfield et al., 2011).

Water samples (50 ml) were taken at the beginning and end of the experiment, filtered through a 0.21 µm filter and refrigerated. DIC was