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Correlation between $\delta^{13}C$ and $\delta^{15}N$ in flying fish (*Exocoetus volitans*) muscle and scales from the South China Sea

by

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Abstract

We collected flying fish (*Exocoetus volitans*) from the South China Sea to determine whether fish scale isotope values correlate with those from muscle, and discuss relevant eco-environmental implications. A significant positive correlation was determined between fish scales and muscle $\delta^{13}C$ and $\delta^{15}N$, rendering a fish scale an alternative to muscle tissue for stable isotope analysis. However, muscle and scale isotopic offsets should be fully considered when using $\delta^{13}C$ and $\delta^{15}N$ to analyze the actual trophic level of fish and their food source. The average offsets of $\delta^{13}C$ and $\delta^{15}N$ between muscles and scales are -2.1 ± 0.5‰ and 2.3 ± 0.6‰, respectively, though these values vary slightly with fish mass. Weak correlations were found between $\delta^{13}C$ and $\delta^{15}N$, both in the flying fish muscle and scales, suggesting that other factors are influencing $\delta^{13}C$ and $\delta^{15}N$. Fish $\delta^{15}N$ also correlates with the size of individuals, while $\delta^{13}C$ reflects the marine habitat. Based on our data, it appears that more eco-environmental processes can be revealed from modern or ancient flying fish scales.

Key words: flying fish, muscle, scales, stable isotope analysis, South China Sea

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Introduction

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ is a well-known method to investigate the foraging ecology of far-ranging species. While $\delta^{13}C$ is useful to trace foraging sites and sources of primary production (e.g., Schoeninger et al. 1999; Hawke & Holdaway 2005), $\delta^{15}N$ is a robust marker for the trophic level (e.g., Vander Zanden et al. 1997; Amezcua et al. 2015). These analyses are especially applicable to fish ecology using muscle tissues (e.g., Pinnegar & Polunin 1999; Hoffman et al. 2015), but since the latter have not been preserved as fossils, we cannot directly analyze fish muscle from ancient times, and thus alternative materials are required to determine stable isotopic characteristics in paleoecological research.

To resolve this problem, fish scales of some bony fishes, in addition to fish fins (e.g., Smith et al. 2015), have been characterized for stable isotopic content. Strong correlations between stable isotope values of fish scales and muscle have been reported in many species, allowing scales to be used as an alternative to muscle when conducting stable isotope analyses (Syväranta et al. 2008; Ventura & Jeppesen 2010; Ramsay et al. 2012). Based on the relationships between scales and muscles, past eco-environmental processes such as eutrophication, reoligotrophication (Gerdeaux et al. 2006; Roussel et al. 2014), and dietary shifts (Pruell et al. 2003) are now possible to investigate from stable isotopic compositions in well-preserved ancient scales. Nevertheless, whether a fish scale can replace muscle tissue as an alternative for all bony fishes in stable isotope ecology remains unclear.

In this study, we focus on flying fish (*Exocoetus volitans*), which together with the squid (e.g., *Loligo chinensis*) are two most important food sources for many tropical seabirds. Flying fish live at the surface layer of warm ocean waters, between 40°N and 40°S worldwide. Normally, they school in groups and live in open waters, and are an important food source for epipelagic piscivorous fish (Wang 2011). Flying fish schools occur in inshore areas and spawn on seaweed and suspended matter between March and April every year. They then forage separately and return to the open waters (Zhang 1956). Flying fish are widely distributed around the South China Sea islands, with dense concentrations in the waters surrounding Xisha Islands, and also wide distributions to the west, north and northeast of Xisha Islands and Zhongsha Islands (Cao et al. 2003). Compared with squid, flying fish are the more important food source for seabirds, accounting for 70-90% of the total food of red-footed boobies (*Sula sula*) in the Xisha Islands (Cao 2005). However, flying fish have received less attention than squid in the studies of marine food webs (e.g., Navarro et al. 2013). Thus, we focus on flying fish in this study.

According to previous research on the paleoecology of the Xisha Islands, a large number of seabirds once inhabited these islands and numerous fish scales were recovered from coral-sand ornithogenic sediments (Xu et al. 2011). Muscle $\delta^{15}N$ of ancient flying fish was inferred using the value of ancient scale $\delta^{15}N$ estimated from a regression model between muscle and scale $\delta^{15}N$. These data were then used to estimate the food source of tropical seabirds in the past (Wu et al. 2017). However, $\delta^{13}C$ was overlooked. In this study, we comprehensively analyzed the relationships between $\delta^{13}C$ and $\delta^{15}N$ in flying fish muscle and scales, and discuss factors that influence the values of these two isotopes. Based on our data, it appears that numerous eco-environmental processes can be revealed from ancient flying fish scales.

Study area

The South China Sea (3°00′ – 23°37′N, 99°10′ – 122°10′E) is the third largest marginal sea in the world, located south of mainland China, and is mostly surrounded by the mainland, peninsulas and islands. It is located in the tropics where abundant resources occur, including a highly productive fishery (Wang 2011).

Materials and methods

Sample collection and preparation

A total of 82 modern flying fish samples were collected near the Xisha Islands, the South China Sea, including 10 samples from the Xisha Sea around Yongxing Island (17°N, 112°E) and 22 samples from the Southern Hainan Sea (18°N, 110°E, the southern Hainan Island) collected by fishermen in 2014 and 2015, respectively. Additional 50 samples were collected within the Western South China Sea (9° – 19°N, 111° – 115°E), using a small dredger during an open scientific expedition cruise in 2015. These samples represent different sizes of fish, from both nearshore and open water in the South China Sea. All fish samples were weighed and measured for their length (standard length, from the snout to distal caudal vertebrae); their scales and dorsal muscles without skin were then removed with forceps and a knife after defrosting and dried at 60°C.

Muscle samples were pretreated based on the methods of Logan and Lutcavage (2008) and Inamura

et al. (2012). Each 0.2 g sample was ground using a mortar and pestle and passed through a 0.90 mm mesh sieve. Samples were then placed twice into a 10 ml chloroform/methanol (1:1, v:v) solution for more than 12 h each time to extract and remove lipids. The remaining samples were then dried at 60°C and stored in tinfoil. Fish scale samples were pretreated based on the method of Estep and Vigg (1985) and Sinnatamby et al. (2007). We used a whole scale to represent the whole fish, and placed it into a clean plastic syringe. Then, 1.2 N HCl was drawn into the syringe to remove carbonates contained in the scale, which was then washed for 2 minutes using deionized water. The scales were air-dried and also stored in tinfoil.

Stable isotope analysis

Stable isotopic compositions $\delta^{13}C$ and $\delta^{15}N$ were determined at the State Key Laboratory of Atmospheric Boundary Layer Physics and Atmospheric Chemistry (LAPC), Institute of Atmospheric Physics, Chinese Academy of Sciences (Beijing, China). Well-treated samples and standards (urea) were fully combusted at 1000°C using a FLASH 2000 HT Elemental Analyzer (Thermo Fisher) and the gases were separated by a "purge and trap" adsorption column and then sent to IRMS (Isotope-Ratio Mass Spectrometry) MAT 253 (Thermo Fisher) for the isotope analysis. Stable isotope ratios were expressed in δ notation as the deviation from standards in parts per thousand (‰):

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000\ (‰),$$

where R refers to the ratio $^{13}C/^{12}C$, the $R_{standard}$ value is based on Vienna Pee Dee Belemnite (V-PDB), and

$$\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000\ (‰),$$

where R represents the ratio $^{15}N/^{14}N$, the $R_{standard}$ value is based on atmospheric air nitrogen (N_2-atm). Analytical precision (the standard deviation) for $\delta^{13}C$ and $\delta^{15}N$ is better than ±0.1‰ and ±0.2‰, respectively.

Results

The mass and length of flying fish collected in this study are relatively evenly distributed from near 0 to 350 g and 9 to 30 cm, respectively (Fig. 1), and are representative of our study area. In addition, there is a strong positive correlation between flying fish mass and length (Fig. 1). The exponent 2.75 is close to 3, consistent with the previous study (Inamura et al. 2012). Thus, both mass and length can represent the size of flying fish and their age. In this study, fish mass was used as a marker for flying fish size.

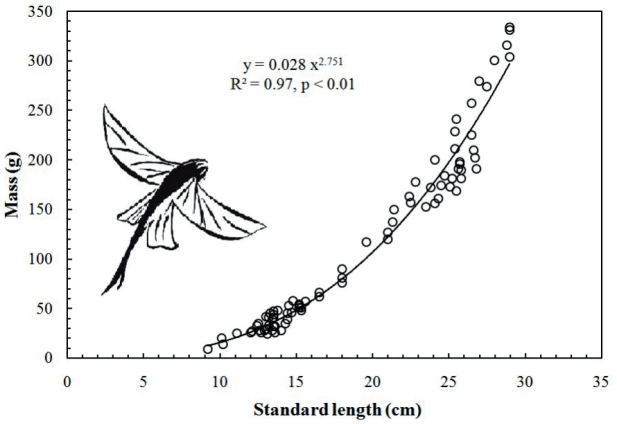

Figure 1

Relationship between mass and body length (standard length) of flying fish samples (n = 82)

Table 1

Statistics of $\delta^{13}C$ and $\delta^{15}N$ in flying fish muscle and scales (n = 82)

isotope		muscle	scale
$\delta^{13}C$ (‰)	min	-18.3	-17.5
	max	-16.9	-14.2
	mean ± SD	-17.7 ± 0.4	-15.6 ± 0.8
$\delta^{15}N$ (‰)	min	6.3	3.8
	max	11.4	9.2
	mean ± SD	8.6 ± 1.0	6.3 ± 0.8

Statistics of $\delta^{13}C$ and $\delta^{15}N$ in flying fish muscle and scales are given in Table 1, and detailed data are presented in Fig. 2. The correlations between flying fish scales and muscle $\delta^{15}N$ or $\delta^{13}C$ with fish mass are shown in Fig. 3. To explain the difference between $\delta^{15}N$ or $\delta^{13}C$ in different fish tissues, we calculated the offsets of $\delta^{13}C$ and $\delta^{15}N$ between muscle and scales and drew a scatter diagram of the isotope offset against fish mass (Fig. 4). To further define the factors affecting flying fish $\delta^{13}C$, correlations between $\delta^{13}C$ and $\delta^{15}N$ in fish muscle and scales were also determined (Fig. 5).

Discussion

Correlations between muscle and scale isotopes

Consistent with many other fish species (Perga & Gerdeaux 2003; Roussel et al. 2014), stable isotopic characteristics of flying fish scales are strongly

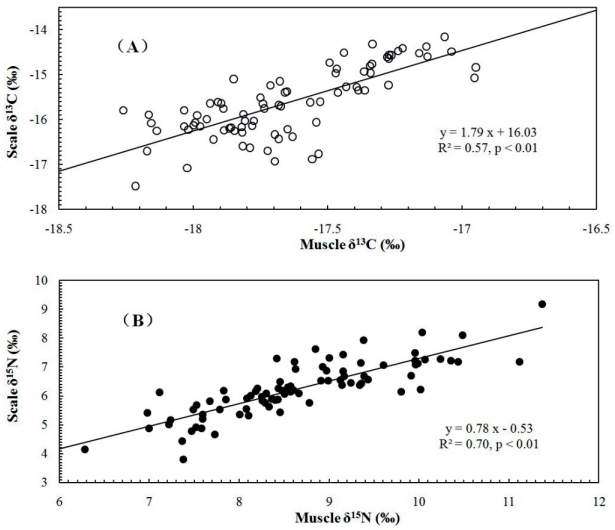

Figure 2

Correlations between flying fish scale and muscle $\delta^{13}C$ (A) and $\delta^{15}N$ (B)

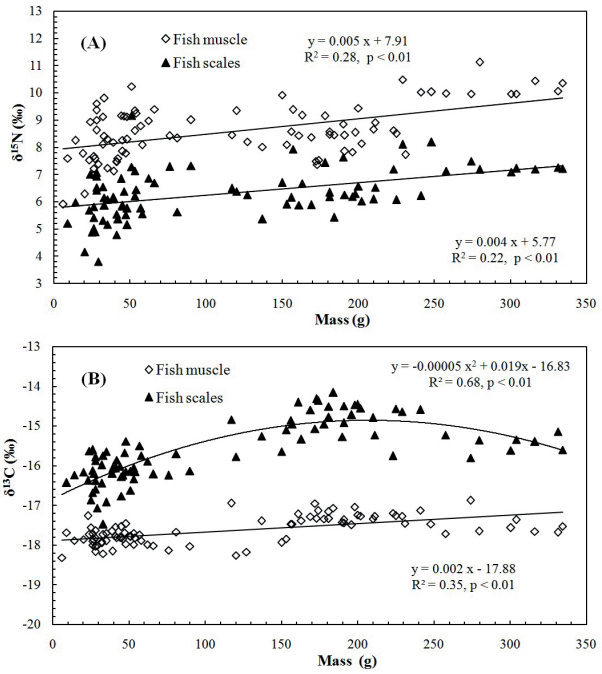

Figure 3

Correlations between flying fish scales (triangle) and muscle (rhombus) $\delta^{15}N$ (A) or $\delta^{13}C$ (B) with fish mass

correlated with the characteristics of muscle (Fig. 2). Therefore, the fish scale offers a non-lethal method to study the characteristics of stable isotopes of modern flying fish as in other fishes (Jardine et al. 2011). In addition, flying fish scales enable us to infer the stable isotopic ecology of ancient fish when their muscle tissues are not preserved but their scales are.

Correlations between muscle, scale and fish mass

The trophic level of an organism can be determined by $\delta^{15}N$ due to significant nitrogen isotope fractionation, which can cause an obvious increase in $\delta^{15}N$ in organisms from higher levels of the food chain (Schoeninger & DeNiro 1984; Wada et al. 1991; Hobson 1999; Post 2002). Similarly, $\delta^{15}N$ contained in organisms can increase as they age because they feed on different food sources with evidently different $\delta^{15}N$ values (Minagawa & Wada 1984; Xu et al. 2007). In this study with modern flying fish, $\delta^{15}N$ of both muscle and scales increased with mass (Fig. 3A), with flying fish muscle and scales exhibiting a $\delta^{15}N$ change of ~3‰ and a mass change of 350 g. Thus, we ascribe the variation of $\delta^{15}N$ of both flying fish muscle and scales to the size of fish (and thus age).

However, the relationship between muscle $\delta^{15}N$ and fish mass is evidently different from the relationship between scale $\delta^{15}N$ and fish mass (Fig. 3A). Given that both the slope and intercept of $\delta^{15}N_{muscle}$-mass regression are greater than those of $\delta^{15}N_{scale}$-mass regression, a larger fractionation of stable nitrogen isotope exists in muscle than in a scale, consistent with the previous studies (Blanco et al. 2009; Vašek et al. 2017). In addition, fish muscle $\delta^{15}N$ provides more information on recent food sources, whereas scale $\delta^{15}N$ records an average dietary composition over the lifetime (Perga & Gerdeaux 2003). Generally, an increase in one trophic level would result in enrichment of 3.4‰ in muscle tissues (DeNiro & Epstein 1981; Minagawa & Wada 1984; Post 2002). Fish scale $\delta^{15}N$ can also be used to calculate the actual trophic level, but only after considering the nitrogen isotopic offset; otherwise, the trophic level may be underestimated by more than half. In this study, the average values of $\delta^{15}N_{muscle}$ and $\delta^{15}N_{scale}$ are 8.6 ± 1.0‰ (n = 82) and 6.3 ± 0.8‰ (n = 82), respectively, and their difference ($\delta^{15}N_{muscle}$ - $\delta^{15}N_{scale}$ = 2.3 ± 0.6‰) can be applied as the muscle and scale nitrogen isotopic offset for flying fish.

In general, muscle $\delta^{13}C$ of prey animals (flying fish in our study) varies slightly, less than 1‰ during their lifetime if they do not migrate (DeNiro & Epstein 1978). Consistently, we found that the variation of $\delta^{13}C$ of fish muscle is less than 1‰. The muscle and scale carbon isotopic offset of flying fish, i.e. the difference between the average values of $\delta^{13}C_{muscle}$ and $\delta^{13}C_{scale}$ ($\Delta^{13}C$) is -2.1 ± 0.5‰. Several factors may cause scale $\delta^{13}C$ to be higher than that in muscle. For example, glycine is the predominant amino acid of scale collagen and it

is enriched by 8‰ in $\delta^{13}C$ compared to other amino acids (Cano-Rocabayera et al. 2015). Muscle $\delta^{13}C$ changes linearly with fish mass, while fish scale $\delta^{13}C$ changes nonlinearly (Fig. 3B). Consequently, the mass or the size is a factor that accounts for the change in scale $\delta^{13}C$, but may not be the predominant one. This carbon isotopic offset value, therefore, must be fully considered if $\delta^{13}C$ is used to infer the food source of fish (Saito et al. 2011).

Relationship between isotopic offsets and mass

Although the average offsets of $\delta^{13}C$ and $\delta^{15}N$ between muscle and scales can be calculated, they still vary with the increase in fish mass (Fig. 4). For $\delta^{15}N$, there is a weak correlation between the offset and fish mass, and the nitrogen isotopic offset between muscle and scales would increase from 2.17‰ to 2.47‰ during the growth from birth to a weight of 300 g (Fig. 4). Obviously, the variance of about 0.3‰ is small, so the average muscle and scale nitrogen isotopic offset of 2.3 ± 0.6‰ can apply to each flying fish, regardless their weight.

For $\delta^{13}C$, there is a strong negative correlation between its offset and fish mass (Fig. 4). For flying fish from birth to a weight of 300 g, the carbon isotopic offset between muscle and scales would change from -1.60‰ to -2.50‰. As flying fish age, the difference between their muscle and scale $\delta^{13}C$ would slightly increase, which we believe is attributed to fish scales recording the dietary information over their lifetime. Even so, the average carbon isotopic offset between flying muscle -2.1 ± 0.5‰ is still useful for estimating the actual diet in paleoecological studies when only fish scales are preserved in the fossil record.

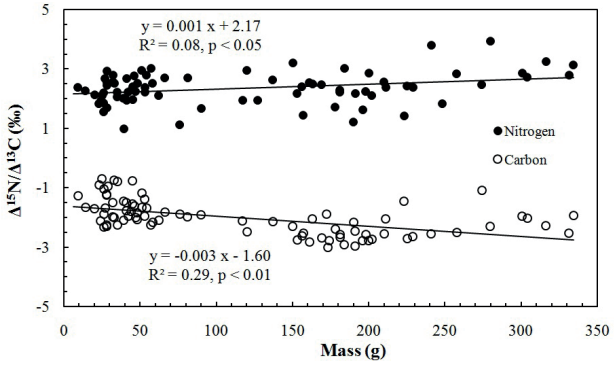

Figure 4

Correlations between nitrogen (solid circle) and carbon (empty circle) isotopic offset between flying fish muscle and scales ($\Delta^{15}N = \delta^{15}N_{muscle} - \delta^{15}N_{scale}$ and $\Delta^{13}C = \delta^{13}C_{muscle} - \delta^{13}C_{scale}$) with mass

Relationships between fish $\delta^{13}C$ and $\delta^{15}N$

$\delta^{15}N$ and $\delta^{13}C$ in fish muscle as well as in scales yielded a weak correlation (Fig. 5), suggesting that the fish size, the primary factor affecting $\delta^{15}N$, can impact $\delta^{13}C$ to some extent, but is not the most important factor.

In fact, $\delta^{13}C$ depends strongly on the geographic location. For example, $\delta^{13}C$ in organisms may increase with altitude in a mountainous area (Hobson et al. 2003), and dissolved inorganic carbon varies with the seawater depth (Sisma-Ventura et al. 2016). These variations are irrelevant to our study because flying fish live only in the surface layer of warm ocean waters. However, there are a number of geographical factors that may apply to this study: $\delta^{13}C$ of eupelagic plankton is much lower than that of coastal species (Kaehler et al. 2000; Xu et al. 2014), and $\delta^{13}C$ of organisms increases from polar areas to equatorial regions as a result of the latitude effect (Takai et al. 2000; Vaughn et al. 2010). Therefore, we attribute the variation of fish $\delta^{15}N$ to the change of fish mass and size, but the variation of fish $\delta^{13}C$ to the alteration of their geographic location.

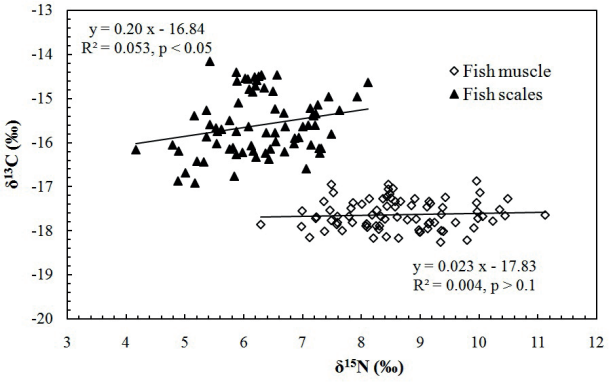

Figure 5

Correlations between $\delta^{13}C$ and $\delta^{15}N$ in the flying fish muscle (rhombus) and scales (triangle)

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