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RESEARCH ARTICLE

Experimental support towards a metabolic proxy in fish using otolith carbon isotopes

Jasmin C. Martino[1,*,‡], Zoë A. Doubleday[1,*], Ming-Tsung Chung[2] and Bronwyn M. Gillanders[1]

ABSTRACT

Metabolic rate underpins our understanding of how species survive, reproduce and interact with their environment, but can be difficult to measure in wild fish. Stable carbon isotopes ($\delta^{13}C$) in ear stones (otoliths) of fish may reflect lifetime metabolic signatures but experimental validation is required to advance our understanding of the relationship. To this end, we reared juvenile Australasian snapper (*Chrysophrys auratus*), an iconic fishery species, at different temperatures and used intermittent-flow respirometry to calculate standard metabolic rate (SMR), maximum metabolic rate (MMR) and absolute aerobic scope (AAS). Subsequently, we analysed $\delta^{13}C$ and oxygen isotopes ($\delta^{18}O$) in otoliths using isotope-ratio mass spectrometry. We found that under increasing temperatures, $\delta^{13}C$ and $\delta^{18}O$ significantly decreased, while SMR and MMR significantly increased. Negative logarithmic relationships were found between $\delta^{13}C$ in otoliths and both SMR and MMR, while exponential decay curves were observed between proportions of metabolically sourced carbon in otoliths (M_{oto}) and both measured and theoretical SMR. We show that basal energy for subsistence living and activity metabolism, both core components of field metabolic rates, contribute towards incorporation of $\delta^{13}C$ into otoliths and support the use of $\delta^{13}C$ as a metabolic proxy in field settings. The functional shapes of the logarithmic and exponential decay curves indicated that physiological thresholds regulate relationships between $\delta^{13}C$ and metabolic rates due to upper thresholds of M_{oto}. Here, we present quantitative experimental evidence to support the development of an otolith-based metabolic proxy, which could be a powerful tool in reconstructing lifetime biological trends in wild fish.

KEY WORDS: Chemical proxy, Otolith chemistry, Bioenergetics, Field metabolic rate, Teleost

INTRODUCTION

Metabolic rates reflect the energy used in a system for fuelling biological processes (Gillooly et al., 2001; Nagy, 2005) and are widely used to understand the biology of individuals (performance, growth, reproduction and excretion), populations (population dynamics and interactions) and ecosystems (trophic dynamics and food availability) (Brown et al., 2004; Chabot et al., 2016). As such, metabolic measures can address a wide range of important ecological questions. However, field metabolic rates can be difficult to measure in fish. The doubly labelled water method, which involves measuring the elimination of an introduced oxygen isotope signature, is used for most air-breathing animals but is unsuitable for fish (Nelson, 2016; Treberg et al., 2016). Metabolic rates have therefore been measured using electromyogram telemetry (Cooke et al., 2004; Quintella et al., 2009), heart rate monitoring (Clark et al., 2005; Thorarensen et al., 1996) or accelerometry (Metcalfe et al., 2016). However, these approaches only offer short-term 'snapshots' of metabolic rates in live fish. Records conserved in hard-calcified structures offer a valuable alternative to uncovering long-term and retrospective histories. Furthermore, biogeochemical techniques can reconstruct histories when direct measures are not possible, such as historical or extinct species using archaeological samples (Disspain et al., 2016; Wurster and Patterson, 2003), or inaccessible species, such as deep-sea fish (Shephard et al., 2007; Trueman et al., 2013). Otoliths (ear stones) are calcium carbonate ($CaCO_3$) structures in teleost fishes which permanently retain lifetime chemical signatures that can correspond to ambient environments and physiology (Campana, 1999; Izzo et al., 2018; Sturrock et al., 2015). Alternating translucent and opaque increments form annually in otoliths and arise from seasonal differences in growth rates. Otolith increments provide estimates of somatic growth rate and age, allowing chemical signatures to be matched to specific age and calendar years (Campana and Thorrold, 2001). Additionally, otolith archives are maintained in research institutes and museums worldwide, providing easy access to a wide range of species, locations and years.

Stable carbon isotopes ($\delta^{13}C$) in otoliths show potential as a biogeochemical tracer of metabolic rates in fish. Otolith carbon arises from two sources: (1) dissolved inorganic carbon (DIC) in environmental water incorporated via the gill and/or intestinal interfaces and (2) metabolically sourced carbon via cellular respiration of food (Chung et al., 2019a; Kalish, 1991a; McConnaughey et al., 1997). As metabolic demand increases, a concurrent increase in respiration and metabolic CO_2 increases the proportion of metabolically sourced carbon (M_{oto}) in the blood and endolymph, which is subsequently deposited onto the otolith. Metabolically sourced carbon is significantly depleted in ^{13}C compared with DIC in water, and so distinctive changes in $\delta^{13}C$ in otoliths are thought to primarily reflect shifts in the proportional contributions of carbon sources to otoliths (Dufour et al., 2007; Høie et al., 2003; Kalish, 1991a). The influence of environmental water and diet is considered minimal as $\delta^{13}C$ in ocean DIC is typically uniform and substantial changes to diet are required to significantly alter isotopic values in otoliths (Shephard et al., 2007; Sherwood and Rose, 2003; Trueman et al., 2016). Previous experimental studies uncovered that $\delta^{13}C$ correlates with M_{oto} (Høie et al., 2003; Kalish, 1991a; Solomon et al., 2006). Field studies have also revealed that $\delta^{13}C$ relates to measures of swimming capacity (Sherwood and Rose, 2003), temperature-driven metabolic cycles (Wurster and Patterson, 2003) and ontogenetic reductions in mass-specific metabolic rate (Trueman

[1]Southern Seas Ecology Laboratories, School of Biological Sciences, The University of Adelaide, Adelaide, SA 5005, Australia. [2]Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Chiba 277-8564, Japan. *Present address: Future Industries Institute, University of South Australia, Adelaide, SA 5095, Australia.

‡Author for correspondence (Jasmin.Martino@unisa.edu.au)

J.C.M., 0000-0002-3978-4362; Z.A.D., 0000-0002-6850-3507; M.-T.C., 0000-0003-3305-3339; B.M.G., 0000-0002-7680-2240

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et al., 2016; Wurster and Patterson, 2003). To date, only one study has directly related $\delta^{13}C$ in otoliths to measures of metabolic rate, uncovering a significant negative relationship between $\delta^{13}C$ and standard metabolic rate (SMR) in Atlantic cod (Chung et al., 2019b). Consequently, a gap exists in understanding how $\delta^{13}C$ relates to other metabolic parameters. SMR represents the minimum energy usage needed to sustain life, while maximum metabolic rate (MMR) represents the upper limit of metabolic capacity (Chabot et al., 2016, Treberg et al., 2016). Field metabolic rate falls in the middle of these two measures, and is composed of the energy totals from SMR, specific dynamic action (SDA, the postprandial increase in metabolism) and activity metabolism (Smith, 1980; Treberg et al., 2016). Quantifying the relationship between MMR and $\delta^{13}C$ may provide insight into the influence of activity metabolism on otolith values. Additionally, absolute aerobic scope (AAS) indicates fitness and performance capacity through calculating the difference between MMR and SMR. Linking $\delta^{13}C$ to MMR or AAS in addition to SMR could help pinpoint the exact metabolic drivers of changes to metabolically sourced carbon into otoliths. Furthermore, metabolic rates and biogeochemical incorporation are highly species-specific because of somatic characteristics and physiological thresholds, so a need exists to understand how this relationship co-varies in a range of species. Additionally, minimal work has been completed to understand $\delta^{13}C$ as a metabolic proxy across a wide thermal range, so little is known about the relationship under thermal stress.

Oxygen isotopes ($\delta^{18}O$) in otoliths are a potential proxy of environmental water temperatures. This proxy relationship arises as $\delta^{18}O$ in otoliths are primarily in equilibrium with $\delta^{18}O$ in ambient water, which is influenced by temperature and salinity (Darnaude et al., 2014; Kitagawa et al., 2013; Moreira et al., 2018). Species-specific validations are an important precursor for use of $\delta^{18}O$ as a temperature proxy in the field and have not been assessed in Australasian snapper. Additionally, as fish are ectothermic, temperature is a primary driver of metabolic rate (Clarke and Johnston, 1999; Gillooly et al., 2001). Consequently, there is an inherent relationship between temperature and metabolic rate that is frequently observed between $\delta^{13}C$ and $\delta^{18}O$ (Geffen, 2012; Kalish, 1991b; Wurster and Patterson, 2003). Reconstructing temperature histories using $\delta^{18}O$ can uncover environmental mechanisms driving metabolic shifts, and aids in our interpretation of $\delta^{13}C$ and M_{oto} as metabolic biomarkers.

To advance the development of a metabolic proxy in fish, we experimentally investigated the relationship between $\delta^{13}C$ and oxygen consumption (as a proxy measure of metabolic rate) in an iconic and valuable fishery species, Australasian snapper (*Chrysophrys auratus*). Our objectives were to (1) quantify values of $\delta^{13}O$ and $\delta^{13}C$ in otoliths and SMR, MMR and AAS of snapper under different temperature treatments, and (2) investigate the functional form and strength of the relationships between isotopic and metabolic parameters.

MATERIALS AND METHODS

Experimental design

Australasian snapper, *Chrysophrys auratus* (Forster 1801), is an iconic long-lived demersal species that supports important commercial and recreational fisheries in the Australasia and Indo-Pacific region (Fowler et al., 2016). Wild snapper eggs were collected in Cockburn Sound, WA, Australia, reared at 25°C and 35 psu at Challenger TAFE, WA, Australia, for 2 months and then transported to the University of Adelaide, SA, Australia, for experiments. Fish were batch marked via immersion in an alizarin complexone ($C_{19}H_{15}NO_8$) solution (40 mg l^{-1} of tank water) for 24 h (van der Walt and Faragher, 2003). Fingerlings were separated into 16 tanks (4 treatments×4 replicate tanks) which contained 40 l of locally sourced coastal seawater at 25°C and 40 psu as well as a submerged filter and aerator. After fish had acclimated to local conditions for 7 days, temperature was adjusted by 1.5°C per day until experimental temperatures (20, 24, 28 and 32°C) were reached. Water temperature was measured daily throughout the experiment with a handheld probe (HI-98127, Hanna Instruments, Keysborough, VIC, Australia). Salinity was tested twice weekly using a refractometer (RF-1, Vertex, Toronto, ON, Canada) and maintained at 35 psu via the addition of aged bore water. To maintain water quality, ammonia levels were regularly tested and kept below 0.25 ppm using commercial test kits (API, Chalfont, PA, USA) with 50% water changes occurring weekly. Fingerlings were fed to satiation twice daily with commercial fish food (Skretting Protec, Stavanger, Norway). Fish were exposed to experimental conditions for up to 61 days (Table 1). All animal procedures were approved by the University of Adelaide animal ethics committee (project no. S-2015-161).

Intermittent-flow respirometry

Intermittent-flow respirometry was used to measure fish oxygen consumption. Fish were fasted for 24 h prior to respirometry to limit the influence of specific dynamic action (Chabot et al., 2016). The respirometry system consisted of four chambers with a maximum of three chambers at a time used to measure fish oxygen consumption [mg l^{-1} (ppm)] and the fourth used to measure background respiration (Fig. S1). Each chamber was a closed system attached to a pump (approximately 250 ml s^{-1}), to recirculate water through the system loop, and a FireSting O_2 Optical Oxygen Meter (Pyroscience, Aachen, Germany), which was used to measure oxygen levels (Fig. S1). Logging software (Pyro Oxygen Logger v3.1) was used to record oxygen and time. The oxygen sensor system was calibrated prior to each run. To measure MMR, fish were exerted through a combined exhaustive chase and air exposure procedure (Gilmore et al., 2018; Roche et al., 2013). Fish were then immediately placed into the respirometry chamber and then left for 24 h to reach a resting state. Each respirometry cycle consisted of three stages: a 3 min flush stage where the pump pushed oxygenated water through each system; a 30 s wait stage where the system was closed, awaiting oxygen measurement; and a 12 min measuring period where the pump was off, the loop was closed and oxygen was measured.

The effective respirometer volume was calculated:

$$V_{RE} = V_{RT} - M_o, \quad (1)$$

where V_{RE} is the effective respirometer volume (l), V_{RT} is the total respirometer volume of the empty respirometer including the

Table 1. Mean (±s.e.m.) tank and Australasian snapper (*Chrysophrys auratus*) parameters for each temperature treatment

Treatment (°C)	Temperature (°C)	Water DIC (‰ VPDB)	Diet $\delta^{13}C$ (‰ VPDB)	Fork length (mm)	M_{oto} (%)	Sample size (*n*)
20	19.8±0.06	−6.7±0.9	−25.4±0.2	80.6±2.9	19.1±2.6	12
24	23.9±0.07	−7.4±0.6	−25.4±0.2	78.0±1.9	23.6±1.13	14
28	28.0±0.03	−7.8±0.6	−25.4±0.2	69.8±2.9	27.7±2.9	5

DIC, dissolved inorganic carbon; VPDB, Vienna Pee Dee Belemnite; M_{oto}, proportion of metabolically sourced carbon in otoliths.

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recirculation loop and M_o is the mass of the organism (kg) (Svendsen et al., 2016). As fish were assumed to be neutrally buoyant, the density of the organism was not used in the above equation (Svendsen et al., 2016). Rate of oxygen decline was determined using the slope of each measurement period, converted into hours and then mass-specific oxygen consumption ($\dot{M}_{O_2}$; mg O_2 kg^{-1} h^{-1}) was calculated:

$$\dot{M}_{O_2} = V_{RE} M_o^{-1} \frac{\delta CO_2}{\delta t}, \quad (2)$$

where $\delta CO_2/\delta t$ is the slope of the linear decrease in oxygen content over the measurement period (Steffensen, 1989; Svendsen et al., 2016). Mass-specific background respiration was accounted for by subtracting it from the whole-animal oxygen consumption rate using the following equation:

$$\dot{M}_{O_2,corr} = \dot{M}_{O_2} - \dot{M}_{O_2,B} V_{RT} V_{RE}^{-1}, \quad (3)$$

where $\dot{M}_{O_2,corr}$ is the corrected oxygen consumption and $\dot{M}_{O_2,B}$ (mg O_2 kg^{-1} h^{-1}) is the mass-specific background respiration calculated as if the organism was present in the chamber (Svendsen et al., 2016).

SMR is a key physiological trait representing the minimum amount of energy needed to sustain life and has important implications for maximum performance, growth rate, lifestyle and social interactions (Chabot et al., 2016). SMR was estimated as the lowest 20th percentile ($q_{0.20}$) of the recorded $\dot{M}_{O_2}$ values (Chabot et al., 2016). Theoretical SMR was also calculated using the metabolic theory of ecology to understand the relationship between $\delta^{13}C$ and average metabolic rates as predicted by size and temperature (Brown et al., 2004; Gillooly et al., 2001):

$$\text{Theoretical SMR} = B_0 \times M^{\alpha} \times e^{-(0.65/((8.62\times10^{-5})\times K))}, \quad (4)$$

where the B_0 is the normalised constant, α is the allometric scaling exponent of body mass (M), which is 0.79 for teleost fish (Clarke, 2006; Clarke and Johnston, 1999), and K is temperature in kelvin. MMR indicates the upper limit of metabolic capacity and highlights the peak potential of energy usage in the field (Norin and Clark, 2016; Treberg et al., 2016). MMR was calculated as an average of the $\dot{M}_{O_2}$ measured in the first respirometry cycle immediately after the combined exhaustive chase and air exposure procedure (Gilmore et al., 2018; Norin and Clark, 2016; Roche et al., 2013). AAS illustrates energy niches and fitness/performance windows (Clark et al., 2013). AAS was calculated as the difference between MMR and SMR (Clark et al., 2013):

$$\text{AAS} = \text{MMR} - \text{SMR}. \quad (5)$$

After intermittent-flow respirometry, fish were euthanised in an ice slurry and immediately frozen. Each fish was measured for fork length (FL; mm) and mass (M; g), and Fulton's condition factor (K), representing somatic condition, was calculated (Bolger and Connolly, 1989):

$$K = 10^5 \frac{M}{FL^3}. \quad (6)$$

Both sagittal otoliths were dissected from each fish, cleaned of adhering tissue and air dried. Experimental otolith material was distinguished from pre-experimental otolith material by a purple alizarin complexone band. Using a sterilised razor blade, approximately 1 mm of material was chipped from the edge of the otolith into filter paper and placed into acid-sterilised 4.5 ml vials.

Isotope analysis

Vials containing otolith material were purged for 2.5 min with nitrogen gas and injected with 100–120 µl of 103–104% phosphoric acid to digest the calcium carbonate and produce carbon dioxide. Otolith samples were analysed for carbon and oxygen isotopes using a GasPrep headspace analyser connected to a Nu Horizon (Nu Instruments) isotope-ratio mass spectrometer (IRMS). Powder standards (ANU-P3, UAC CaCO3 UniA, IAEA-CO8) were analysed to assess instrument drift and calculate precision. Isotope values were reported relative to Vienna Pee Dee Belemnite (VPDB) and expressed in standard delta (δ) parts per thousand (‰):

$$\delta = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times 1000, \quad (7)$$

where R is the ratio of ^{13}C:^{12}C or ^{18}O:^{16}O. Analytical errors were <0.18‰ for $\delta^{13}C$ and <0.33‰ for $\delta^{18}O$ values in otoliths. Water samples (50 ml) were collected at the beginning and end of the experiment and filtered through a 0.21 µm filter and refrigerated. DIC in water samples was measured using a GasBench II coupled with a Delta XL Mass Spectrometer in continuous flow mode (Thermo-Fisher Scientific). Fish food samples (n=3) were collected, ground to a fine powder using a mortar and pestle and analysed for carbon isotopes using a continuous flow system consisting of a Delta V Plus mass spectrometer connected with a Thermo Flush 1112 via Conflo IV (Thermo-Finnigan, Germany). Water and diet samples were both analysed at the West Australian Biogeochemistry Centre.

To calculate the proportional contribution from carbon sources to otoliths, the following isotopic mixing model was used (Jamieson et al., 2004):

$$\delta^{13}C_{oto} = M_{oto}\delta^{13}C_{diet} + (1-M_{oto})\delta^{13}C_{DIC-SW} + \Delta arag - HCO_3, \quad (8)$$

where M_{oto} is the fraction of carbon from metabolic sources, $\delta^{13}C_{oto}$ is the $\delta^{13}C$ of otolith, $\delta^{13}C_{diet}$ is the $\delta^{13}C$ of fish diet (metabolically sourced carbon), $\delta^{13}C_{DIC\text{-}SW}$ is the value of DIC in ambient water, and $\Delta arag–HCO_3$ is the isotopic fractionation between aragonite and bicarbonate, taken as ∼2.7‰ (Romanek et al., 1992).

Statistical analyses

Two-factor permutational univariate analysis of variance (ANOVA) was used to determine differences in metabolic rates and isotope values in snapper between temperature treatments (PRIMER v6.0/Permanova). Data were normalised prior to constructing resemblance matrices based on Euclidean distance dissimilarity and analysed by unrestricted permutation of the data for all tests. Within the two-factor design, temperature was a fixed factor and nested replicate tanks a random factor. When significant differences were detected, *post hoc* pair-wise comparisons were used to identify the source of differences in metabolic rates and isotopic ratios between temperature treatments.

Regressions were used to assess the relationships between isotopes in otoliths and metabolic parameters (R Studio v3.2.0, https://rstudio.com/products/rstudio/; Wright, 2012, Team, 2014). The relationships between $\delta^{13}C$ and SMR, MMR and AAS were modelled as a logarithmic relationship (Kalish, 1991a; Chung et al., 2019b):

$$\delta^{13}C = a + b \times \log(\text{metabolic rate}), \quad (9)$$

where a is an intercept when metabolic rate is 1 and b is the decreasing rate. The relationships of M_{oto} with measured SMR, measured MMR

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and theoretical SMR calculated using the metabolic theory of ecology were modelled as an increasing exponential decay model (Chung et al., 2019b):

$$M_{oto} = C(1 - e^{-k(\text{oxygen consumption})}), \quad (10)$$

where C is the upper bound and k is the decay constant. Exponential decay models were investigated using non-linear regressions (nls) in R using the stats package.

RESULTS

Rearing conditions remained stable throughout the experimental period, with desired temperature levels maintained for each treatment (Table 1). Mortality rate increased sharply at 32°C and no fish survived at this temperature, so were therefore not included in the respirometry experiments. DIC in tank water, fish size (mass and length) and Fulton's condition factor K did not vary among temperatures (Table 1, Table S1). Linear relationships were observed between physiological and isotopic markers (Fig. S2).

SMR ranged from 173.4 to 417.9 mg O_2 kg^{-1} h^{-1} (Fig. 1). Significant temperature effects were found with the differences occurring between the 20 and 24°C temperature treatments and the 28°C temperature treatment (Table S1). MMR ranged from 275.4 to 621.1 mg O_2 kg^{-1} h^{-1} (Fig. 1). MMR significantly increased with increasing temperature (Table S1). AAS ranged from 80.74 to 393.0 mg O_2 kg^{-1} h^{-1} with a mean of 178.7 mg O_2 kg^{-1} h^{-1} (Fig. 1). No significant differences between temperature treatments were detected for AAS (Table S1).

Values of $\delta^{13}C$ in otoliths significantly decreased with increasing temperature (Table S1). Carbon isotope values ranged from −8.13±0.09 at 20°C, to −8.97±0.09 at 24°C and −10.03±0.22 at 28°C (Fig. 2). Values of $\delta^{18}O$ in otoliths also significantly decreased with increasing temperature (Table S1). Oxygen isotope values ranged from −0.07±0.2 at 20°C, to −0.82±0.2 at 24°C and −1.78±0.15 at 28°C (Fig. 2).

A logarithmic curve was modelled between $\delta^{13}C$ and SMR (Fig. 3A), with a decreasing rate of −2.05±0.41 (t=−5.03, P<0.001) and an intercept (passing through SMR=1) of 2.60±2.27 (t=−1.14, P=0.26). A logarithmic curve was also modelled between $\delta^{13}C$ and MMR (Fig. 3B), with a decreasing rate of −2.54±0.45 (t=−5.60, P<0.001) and an intercept (passing through MMR=1) of 6.61±2.76

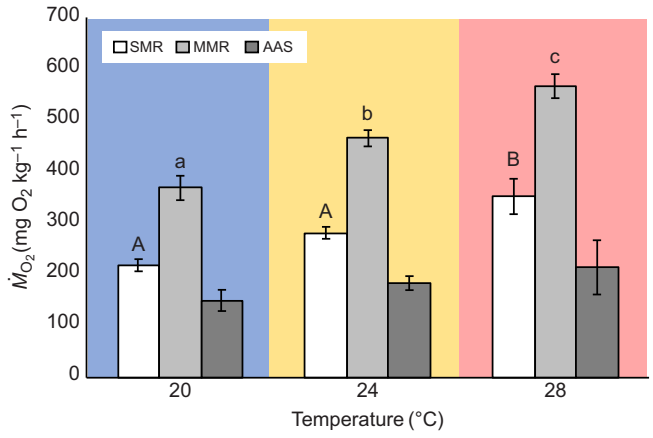

Fig. 1. Standard metabolic rate (SMR), maximum metabolic rate (MMR) and absolute aerobic scope (AAS) measured by intermittent-flow respirometry in Australasian snapper reared at different temperatures. Data are means±s.e.m. (n=31). Significant differences (P<0.05) between temperatures are represented by different letters.

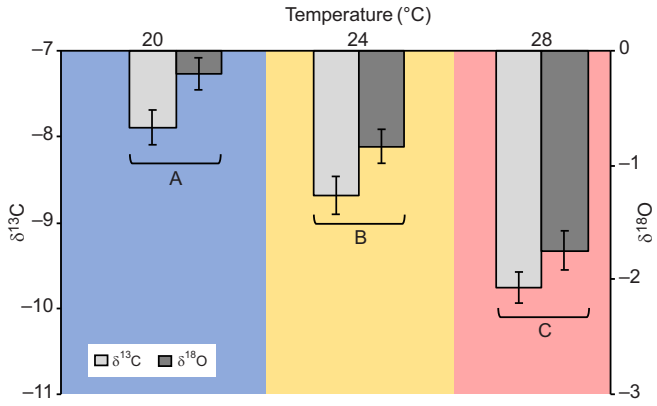

Fig. 2. Stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopes in otoliths of Australasian snapper reared at different temperatures. Data are means± s.e.m. (n=31). Significant differences (P<0.05) between temperatures for both isotopes are represented by different letters. Separate y-axes are used for each isotope.

(t=2.4, P<0.05). Logarithmic, linear, curvilinear and exponential models were investigated between $\delta^{13}C$ and AAS, but the relationship was found to not be suitable for statistical regressions. However, based on average means among temperature treatments, $\delta^{13}C$ was observed to decrease with an increase in aerobic scope (Fig. 3C). Although high variation was present in the 28°C temperature treatment, the lowest values of $\delta^{13}C$ were observed in this treatment and corresponded with the highest AAS values. Additionally, while M_{oto} did not significantly increase with temperature (Table S1, Fig. S3), strong relationships were seen with metabolic measurements. An exponential decay curve (increasing form) was observed between M_{oto} and measured SMR (Fig. 4A), with a decay constant of 0.007±0.004 (t=1.72, P=0.097) and an upper bound of 27.46±5.8 (t=4.75, P<0.001). However, a stronger exponential curve was observed between M_{oto} and theoretical SMR (Fig. 4B, Fig. S4), with a decay constant of 0.005±0.002 (t=2.07, P<0.05) and an upper bound of 32.02±7.5 (t=4.75, P<0.001). The relationship between M_{oto} and measured MMR was non-significant, with a decay constant of 0.002±0.002 (t=1.15, P=0.26; Fig. 4C).

DISCUSSION

Isotopes in otoliths display exciting potential as retrospective biomarkers in fish. Experimental research is critical to deepen our understanding of these isotopic relationships while minimising confounding factors. We experimentally reared Australasian snapper under different temperature regimes and found a significant negative logarithmic relationship between $\delta^{13}C$ and both SMR and MMR. Furthermore, an exponential decay curve was found between M_{oto} and SMR, which was stronger for theoretical SMR based on size and temperature, predicted from the metabolic theory of ecology. Also, $\delta^{18}O$ significantly decreased with increasing temperature, suggesting its potential as a temperature proxy in snapper. This study advances the use of carbon isotopes as a chemical proxy of metabolic rates, establishing confidence in its functioning across a wider range of species and thermal performance ranges.

Our results support previous studies that uncovered relationships between indirect metabolic parameters and $\delta^{13}C$ (Kalish, 1991a; Sherwood and Rose, 2003; Trueman et al., 2016; Wurster and Patterson, 2003). Carbon isotopes in otoliths are derived from two sources: (1) DIC in environmental water and (2) metabolically

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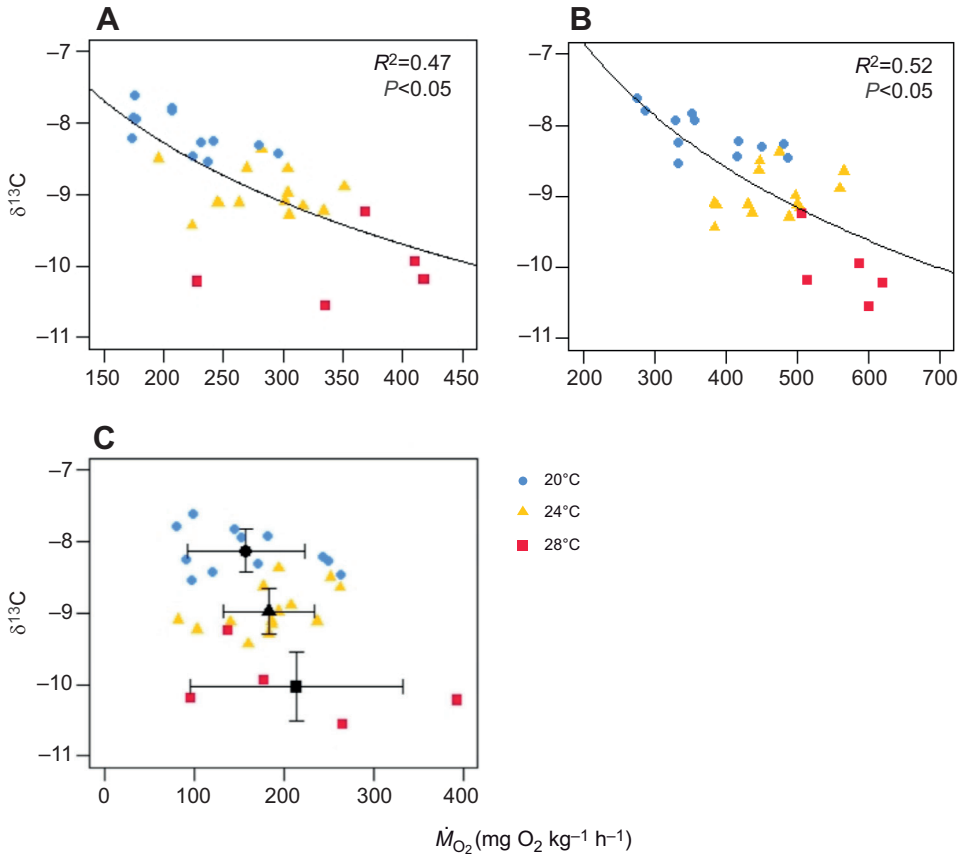

Fig. 3. Relationships between carbon isotopes ($\delta^{13}C$) in otoliths and metabolic parameters of Australasian snapper. (A) SMR, (B) MMR and (C) AAS (n=31). Also shown are logarithmic curves for SMR and MMR and treatment means (±s.d.) for AAS. Note the different scales on the x-axes.

sourced carbon from diet (Kalish, 1991a; Solomon et al., 2006; Tohse and Mugiya, 2008). The values of $\delta^{13}C$ in diet and water in our study remained constant across treatments yet $\delta^{13}C$ in otoliths were significantly altered, suggesting that physiology was driving the changes. As temperature increased, metabolic demands, higher respiration and food consumption likely increased M_{oto}, the proportion of metabolically sourced carbon into otoliths (Chung et al., 2019a). M_{oto} in Australasian snapper has been previously shown to be regulated by temperature and contribute 21–28% to the carbon pool in otoliths (Martino et al., 2019). As such, more negative values of $\delta^{13}C$ in snapper otoliths were indicative of a higher incorporation of M_{oto} in fish with higher metabolic rates. A logarithmic curve between $\delta^{13}C$ and oxygen consumption has been observed directly in Atlantic cod and modelled in other species (Chung et al., 2019b; Kalish, 1991b). The relationships assessed in this study were similarly revealed to be logarithmic curves. Our decreasing rate in logarithmic models of SMR (−2.05) and MMR (−2.54) were within the range found in the literature (−3.52, Kalish, 1991b; and −1.38, Chung et al., 2019b).

Metabolic performance and life history traits in ectotherms can often reflect a bell-shaped curve with increasing temperature (Neuheimer et al., 2011; Pörtner et al., 2017; Schulte, 2015; Pörtner and Farrell, 2008), with metabolic rate increasing until an optimal thermal maximum, after which biological functioning declines rapidly. Although it should be noted that there is debate on whether oxygen is the main limiting factor of ectothermic performance at high temperatures (Jutfelt et al., 2018). Previous experimental research investigating metabolic relationships with $\delta^{13}C$ focused on experimental and metabolic ranges before or approaching the optimum thermal apex, while field study estimates likely reflected fish functioning in their comfortable thermal ranges in natural environments (Chung et al., 2019b; Kalish, 1991b; Thorrold et al., 1997). Our study presents an investigation into $\delta^{13}C$ as a metabolic proxy across the thermal performance range, with the highest treatments likely representing conditions beyond the optimum thermal apex (pejus temperature). The distinctive shallowing of the metabolic and $\delta^{13}C$ relationship at higher metabolic values was likely caused by physiological thresholds. There is a likely upper maximum proportional contribution of metabolically sourced carbon that can be accommodated into otoliths and as the fish approach this value, the relationship between $\delta^{13}C$ and metabolic rates would evidently plateau (Chung et al., 2019b). The upper threshold of M_{oto} and, subsequently, the shape of the functional form between $\delta^{13}C$ and metabolic rates is likely to be species specific as a result of differences in life-history traits and physiology regulations. Consequently, this experimental study is important in corroborating not only the general utility of $\delta^{13}C$ as a metabolic proxy, but provides insight into species-specific variation through direct experimental measurements of only the second species to date, after Atlantic cod (Chung et al., 2019b). Further research is needed to understand the driving physiological or biochemical mechanism of carbon thresholds in otoliths.

Evidence of the highest temperature treatments (28 and 32°C) being beyond the pejus temperature for snapper is indicated by lower survival rates, suggesting a rapid depression in functioning as a result of thermal stress. Poor survival suggests that 28°C is beyond the optimum thermal range for the species but provides insight into carbon isotopes as a metabolic biomarker beyond the optimum thermal range; however, some intra-individual variability in the 28°C treatment was evident. It is unclear whether this variability is derived from the low sample size or whether fish in this treatment are more variable because of greater individual variability in the response to thermal stress.

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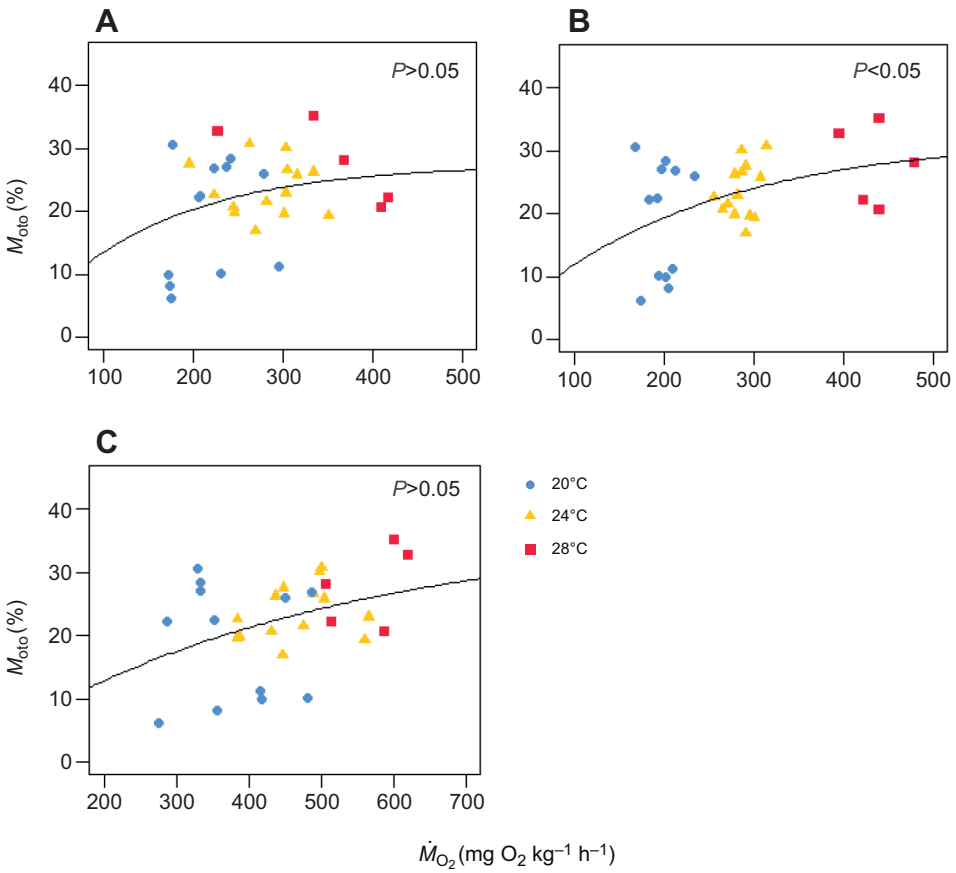

Fig. 4. Relationships between the proportion of metabolically sourced carbon in otoliths (M_{oto}) and measured and theoretical metabolic rates in Australasian snapper reared at different temperatures. (A) SMR, (B) theoretical SMR calculated using the metabolic theory of ecology and (C) measured MMR (n=31). Also shown are exponential decay curves (increasing form).

This is the first study to investigate how $\delta^{13}C$ relates to a range of metabolic measurements, which is important in separating out and identifying the best metabolic drivers of changes in M_{oto} into otoliths. Values of $\delta^{13}C$ were strongly related to both SMR and MMR, but not AAS. These relationships suggest that both basal energy needed for subsistence living and activity metabolism contribute towards incorporation of $\delta^{13}C$ into otoliths. As field metabolic rate is composed of SMR, activity metabolism and specific dynamic action, this study supports the appropriate use of $\delta^{13}C$ as a proxy of metabolic rate in field settings. More work is needed to understand the influence of specific dynamic action on incorporation of $\delta^{13}C$ into otoliths. Furthermore, while regressions were not suitable for the relationship between $\delta^{13}C$ and AAS, there was a general trend indicating lower metabolic rates accompanied higher metabolic scopes and providing evidence that our fish were at the higher end of thermal functioning. However, the functional form of the relationship between $\delta^{13}C$ and AAS is complex and context dependent (Chung et al., 2019b), and further experimental validation is needed to understand this relationship.

The relationship between M_{oto} and metabolic rates was stronger for theoretical SMR, obtained from estimates derived from the metabolic theory of ecology, than from measured SMR or MMR, derived from measured oxygen consumption of experimental fish. This may be due to measured oxygen consumption reflecting a short window of time (24 h) whereas isotope values reflect a longer period of time – ca. 2 months in this study. SMR may alter slightly between days due to natural fluctuations in behaviour or physiological responses. Fish are known to have different personalities, with individual differences in behaviour such as aggressiveness or shyness leading to differences in movement or acquisition of food (Mittelbach et al., 2014). Personality differences may contribute to differences in M_{oto}, which is reliant on oxygen and food consumption. Consequently, while we still indicate the strong exponential decay relationship between $\delta^{13}C$ and metabolic rates, theoretical metabolic rate may be a better indicator for the average fish per mass and temperature range when accounting for a longer time frame and averaging of personality differences between individuals. More precise estimates of metabolic rates could be determined using alternative analytical approaches, such as secondary-ion mass spectrometry (SIMS). Compared with micromilling, SIMS can target more precise regions in otoliths and can create high-resolution lifetime profiles of isotopes, which is particularly valuable when identification of incremental changes or high spatial resolution is warranted for the research question (Hanson et al., 2010; Matta et al., 2013; Weidel et al., 2007).

Additionally, our study suggests the suitability of $\delta^{18}O$ as a temperature proxy in snapper, with $\delta^{18}O$ in otoliths significantly decreasing with temperature. However, more experimental work is needed to explicitly validate this relationship in snapper, particularly with a greater manipulation and assessment of the influence of salinity. A validated $\delta^{18}O$ proxy of temperature would provide valuable insight into metabolic histories. Used in combination, metabolic and temperature histories could expose physiological changes in fish populations in response to environmental change, such as ocean warming. This could be particularly valuable for fish populations that cannot be directly monitored.

Using $\delta^{13}C$ as a proxy of field metabolic rate requires several considerations. We provide a brief summary of key limitations and solutions, while a comprehensive review is available in Chung et al.

(2019a). While significant changes in diet or $\delta^{13}C$ of ambient water are needed to significantly alter carbon isotopes in otoliths as indicated by simulations (Chung et al., 2019b), some effort can be made to improve the effectiveness of the metabolic tool. Confident use of the metabolic proxy is best for marine environments, particularly deep-sea environments, because of the general uniformity in DIC in these waters (Becker et al., 2016; Kroopnick, 1985; Schmittner et al., 2013; Tagliabue and Bopp, 2008). However, the Suess effect, which is an anthropogenic increase in carbon affecting carbon isotope values in the ocean since the 1800s, is an increasingly important consideration (Chung et al., 2019a; Eide et al., 2017; Keeling, 1979). Model calibrations to predict or reconstruct $\delta^{13}C$ in ocean waters may assist with using this metabolic tool (Chung et al., 2019a). A metabolic proxy in freshwater fish may be possible (Gerdeaux and Dufour, 2015; Solomon et al., 2006; Weidel et al., 2007), but further experimental work is needed to validate the functional form of the relationship between $\delta^{13}C$ and metabolic rates, and field studies will need to consider the high variability of $\delta^{13}C$ in freshwater systems (Bade et al., 2004). A further consideration is that metabolic rate in fish is subject to mass-specific decreases with age which will be observed as increasing $\delta^{13}C$ across the lifetime of most fish (Fidhiany and Winckler, 1998; Peck and Moyano, 2016; Rosenfeld et al., 2015). Consequently, intra-species comparisons may be limited to comparing rates of metabolic decline across a lifetime or with direct comparisons mostly restricted to within a life-history stage.

We have provided experimental evidence for the quantitative use of otolith $\delta^{13}C$ as a metabolic proxy in wild fish, by linking $\delta^{13}C$ in otoliths directly to oxygen consumption across a wide thermal performance range. This is also the first study to explore the relationship between $\delta^{13}C$ and both MMR and AAS, which was valuable in identifying metabolic drivers of changes to M_{oto}. We reveal that energy for basic functioning and activity metabolism, both core components of field metabolic rates, relates to incorporation of $\delta^{13}C$ into otoliths and consequently supports the use of $\delta^{13}C$ as a metabolic proxy in field settings. Biogeochemical reconstructions using hard-calcified structures like otoliths offer an inexpensive approach to develop long-term metabolic histories in wild fish. These chemical approaches have particular value for populations where direct monitoring is unfeasible, particularly historical or extinct species and inaccessible deep-sea species. Metabolic histories provide insight into physiological responses to environments and trophic interactions. This information will enable ecologists and fishery scientists to identify physiological stressors and, when necessary, to develop effective countermeasures (Wikelski and Cooke, 2006). Consequently, $\delta^{13}C$ in otoliths can be a powerful intrinsic proxy of field metabolic rate for wide use in conservation and management of fish populations.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.C.M., Z.A.D., B.M.G.; Methodology: J.C.M., Z.A.D., B.M.G.; Software: J.C.M., M.C.; Validation: J.C.M.; Formal analysis: J.C.M., M.C.; Investigation: J.C.M.; Resources: J.C.M., Z.A.D., B.M.G.; Data curation: J.C.M.; Writing - original draft: J.C.M.; Writing - review & editing: J.C.M., Z.A.D., M.C., B.M.G.; Visualization: J.C.M., M.C.; Supervision: Z.A.D., B.M.G.; Project administration: J.C.M.; Funding acquisition: B.M.G.

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