Individual variability in stable isotope turnover rates of epidermal mucus according to body size in an omnivorous fish

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Received: 3 December 2019 / Revised: 25 May 2020 / Accepted: 15 October 2020 / Published online: 29 October 2020 © The Author(s) 2020

Abstract Epidermal mucus (‘mucus’) is increasingly applied to fish ecological studies based on stable isotope analysis (SIA) due to its non-invasive collection. However, knowledge on mucus SI turnover rates of individual fish remains limited, including uncertainty over how they are influenced by fish body sizes. Here, a diet switch experiment predicted mucus SI turnover rates ($\delta^{13}C$ and $\delta^{15}N$) as a function of time using samples taken over 200 days from 10 individually tagged common carp $Cyprinus$ carpio covering two size groups. Non-linear mixed effects models revealed rapid turnover of both $\delta^{13}C$ and $\delta^{15}N$ ($T_{50}$: 2–5 days; $T_{95}$: 9–22 days); $\delta^{15}N$ turnover rates were slower for the larger cohort, while $\delta^{13}C$ turnover rates were independent of body size. Within size groups, turnover rates were not expected to vary between individuals. These experimental results suggest that due to these fast turnover rates, epidermal mucus can provide insights into the diets of fish over very short timeframes, although for $\delta^{15}N$ the body size of the fish needs consideration.

Keywords Half-life · Isotopic incorporation · Diet switch · Non-invasive sampling · Trophic discrimination factor

Introduction

The ecological application of stable isotope analyses (SIA) of consumer tissues has become a fundamental component of studies assessing the dietary sources and trophic levels of aquatic organisms (Peterson & Fry, 1987; Hobson, 1999). Integral to the ecological interpretation of SIA data is the understanding of stable isotope turnover rates, especially their variation across tissue-types within organisms (Tieszen et al., 1983; Winter et al., 2019a). For example, SI turnover rates in fish are considerably slower in scales than the more metabolically active dorsal muscle (e.g. Busst & Britton, 2018). Consequently, in SI studies aiming to quantify temporal prey resource use, understanding the dietary timescales represented by the analysed tissue(s) is crucial in the evaluation process (Vander Zanden et al., 2015).

Stable isotope turnover rates can also vary according to the body size of individuals (Thomas & Crowther, 2015; Vander Zanden et al., 2015). Multi-
taxa reviews suggest that the SI half-life, defined as the time to reach 50% equilibrium with the diet, increases with body mass (Weidel et al., 2011; Thomas & Crowther, 2015; Vander Zanden et al., 2015). This is because with increasing body size, rates of growth and catabolic tissue replacement generally decrease, leading to reduced SI turnover rates (Carleton & Martínez del Rio, 2005). Furthermore, in larger, older individuals, the incorporation of stable isotopes into tissues is increasingly reliant on catabolic turnover rather than somatic growth (e.g. Matley et al., 2016). Nevertheless, there has been little investigation into this variation within species, including fish (but see Kim et al., 2012). Moreover, for fish, SI turnover rate studies predominantly examine single cohorts of juveniles (Weidel et al., 2011) and tend to use these only within groups, where an individual is sampled for its SI data on one occasion only (Winter et al., 2019a, b). Consequently, knowledge is limited on the variability of SI turnover rates across individuals and how this is influenced by differences in body sizes.

In poikilotherms, the positive relationships between SI half-lives and body mass have been demonstrated for muscle, internal organs, blood and blood plasma (Vander Zanden et al., 2015). The same analysis has not been conducted for epidermal mucus, despite it being increasingly recognised as a metabolically active and non-lethal alternative to sampling muscle or blood for use in SIA of fish (Church et al., 2009; Maruyama et al., 2015; Shigeta et al., 2017; Burgess et al., 2018; Winter et al., 2019a, b). Its application within fish trophic studies remains relatively limited compared with other tissues, perhaps due to a paucity of knowledge of the relationships between body sizes and SI turnover rates. Mucus collection is non-invasive and with fish requiring minimal handling, it can be sampled from individuals on a relatively regular basis without compromising their welfare, unlike many other tissues. This facilitates the tracking of SI turnover rates at the individual level rather than within groups of fish, enabling more rigour in assessing the effects of body size on isotopic turnover. The aim of this study was thus to use an ex situ diet switch experiment using the omnivorous carp *Cyprinus carpio* Linnaeus, 1758 to predict the turnover rates of δ13C and δ15N as a function of time and assess how these rates are influenced by the body size of the individuals.

### Materials and methods

**Experimental design**

The experiment comprised of exposing two distinct size groups of *C. carpio* to a fixed diet over a 200-day period. Following the sourcing of pond-reared fish from a local hatchery (*N* = 10), the fish were housed in aquaria and acclimated for 10 days before being internally tagged with a 7 mm passive integrated transponder (PIT) tag and weighed (to 0.1 g). The mass of the fish enabled their grouping into two size categories (see Results). The fish were placed into three tanks of 40 l volume on a flow-through filtration system at 18°C and a light:dark regime of 12:12 h. The numbers of fish were equal per tank and their distribution by size was randomised. Fish remained in the same tanks and with the same individuals throughout the study. The fish were fed once per day for 100 days on a fixed diet of a formulated, pelletized feed whose protein source was marine fishmeal (45% protein, 10% fat, 1.4% crude fibre and 5.8% ash), where the daily ration approximated to 2.5% of mean starting body weight of the fish. At the end of this period, that was designed to standardise the SI values of the fish tissues, each individual fish was identified by their PIT tag, re-weighed (to 0.1 g) and a sample of epidermal mucus taken from their dorsal surface (above the lateral line and below the dorsal fin on both sides of the fish) using a sterile glass cover slip (as per Winter et al., 2019a). Although mucus collection can be particularly susceptible to contamination due to its adhesive properties (Kroska et al., 2019), this was minimised in a controlled laboratory setting. Furthermore, samples were cleaned of scales and debris under a microscope and rinsed in distilled water. The diet of the fish was then switched to a formulated, pelletized feed whose protein source was mainly vegetable based but also included some fishmeal (28% protein, 3.5% fat (as oil), 2.0% crude fibre and 7% ash), where the daily ration approximated to 2.5% of mean body weight at the diet switch. Mucus sampling and re-weighing was repeated every 10 days until day 50, with mucus always collected from the same part of the fish. Thereafter, samples were collected on days 75, 100, 150 and 200. All mucus samples were dried to constant mass at 60°C. All procedures were completed under UK Home Office licence 70/8083 and after
approval by the Animal Welfare and Ethical Review Body of Bournemouth University.

Stable isotope analysis

Samples of mucus and the two formulated feeds were analysed for their stable isotope ratios of $^{12}$C:$^{13}$C ($\delta^{13}$C) and $^{14}$N:$^{15}$N ($\delta^{15}$N) at the Cornell University Stable Isotope Laboratory, New York, USA. The samples were ground to powder and weighed to $\sim$1000 $\mu$g in tin capsules and analysed on a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) interfaced to a NC2500 elemental analyser (CE Elantech Inc., Lakewood, NJ, USA). The equipment was verified for accuracy against internationally known reference materials and calibrated against the primary reference scales for $\delta^{13}$C and $\delta^{15}$N values. Analytical precision of the $\delta^{13}$C and $\delta^{15}$N sample runs was estimated against an internal standard sample of animal (deer) material that was analysed every 10 samples, with the overall standard deviation estimated at 0.08 and 0.04%, respectively. Mucus C:N ratios were generally low (4.0), indicating low lipid content and no requirement for lipid correction of $\delta^{13}$C data.

Statistical analysis

The growth rates of the fish during the maximum time required to reach isotopic equilibrium ($T_{95}$; see Results) were expressed as the change in mass per day [(final mass − initial mass)/days] and the relative increase in mass (final mass/initial mass). The rates of mucus $\delta^{13}$C and $\delta^{15}$N turnover for the two fish size categories were modelled using an exponential decay function of time:

$$\delta Y_t = \delta Y_{eq} + \left(\delta Y_i - \delta Y_{eq}\right)e^{-exp(c)t}$$

where $\delta Y_t$ is the predicted $\delta^{13}$C or $\delta^{15}$N isotopic ratio at time $t$, $\delta Y_{eq}$ is the $\delta^{13}$C or $\delta^{15}$N isotopic ratio in equilibrium with the experimental diet, $\delta Y_i$ is the initial $\delta^{13}$C or $\delta^{15}$N isotopic ratio prior to the diet switch and $c$ is the turnover constant. The change in time required to attain tissue turnover ($T_z$) of 50% (half-life; $T_{50}$) or 95% (near-complete; $T_{95}$) was calculated as per (Tieszen et al., 1983):

$$T_z = -\ln\left(1 - \frac{z}{100}\right)/\exp(c)$$

The data were fitted to a non-linear mixed effects (NLME) model with fish size category as a covariate and fish ID as a random effect. Anomalous values in the data were removed if deviating from the mean by more than 3 × standard deviation (SD) and if clearly departing from the exponential decay function. Model selection was performed according to Zuur et al. (2009). Firstly, the model was set to contain the most complex fixed effect structure (fish size effect for $\delta Y_{eq}$, $\delta Y_i$ and $c$) and the optimal random effect structure (individual variation in $\delta Y_{eq}$, $\delta Y_i$, and/or $c$) was chosen using restricted maximum likelihood estimation (REML) and the minimisation of Akaike information criterion (AIC) values. Then, fixed effect structures were compared using maximum likelihood estimation (ML) and AIC. Finally, using the optimal fixed effect structure, random effect structures were verified using REML and AIC. Models with ΔAIC < 2 (compared to the optimal model) were considered to have substantial support (Burnham & Anderson, 2002). The final model(s) were presented using REML estimation.

While the relative contributions of growth and metabolism to isotopic turnover are easily calculated from traditional models of exponential isotopic decay (Hesslein et al., 1993), the process with regard to mixed effects modelling remains ambiguous and so was not performed here. Trophic discrimination factors (TDF) were generated by subtracting the mean $\delta^{13}$C or $\delta^{15}$N of the experimental diet from the mean $\delta^{13}$C or $\delta^{15}$N of mucus at Day 200. All error margins around the mean represent 95% CI unless stated otherwise. Analyses were conducted in R 3.6.1 (R Core Team, 2019) using the nlme package (Pinheiro et al., 2019).

Results

Fish size and growth rate

At the start of the experimental period, the mass of the fish enabled their grouping into two size categories: ‘small’ (mean mass = 20.5 ± 2.8 g; n = 5) and ‘large’ (mean mass = 43.5 ± 8.3 g; n = 5; Fig. 1). Over the experimental period, all fish increased in
mass, with their mean growth rates at Day 30 being 0.32 ± 0.05 g day⁻¹, where Day 30 represented the first re-weighing of the fish following their predicted dietary equilibrium according to the maximum estimate of $T_{95}$ (see below). Growth rate at Day 30 did not differ between size groups ($t$ test: $t_8 = 0.29, P = 0.78$), but relative increase in mass was significantly greater for the smaller group ($t_8 = 6.01, P < 0.001$).

Stable isotope data

The initial formulated diet had a mean $\delta^{13}$C of $-25.86 ± 0.11\%$, and mean $\delta^{15}$N of $3.60 ± 0.45\%$ ($n = 3$). The SI data for the experimental diet had a mean $\delta^{13}$C of $-23.92 ± 0.04\%$ and mean $\delta^{15}$N value of $5.63 ± 0.03\%$ ($n = 3$). Following the diet switch at Day 0 of the experimental period, temporal changes in $\delta^{13}$C and $\delta^{15}$N of the mucus samples were evident in all fish. For $\delta^{13}$C, the optimal exponential decay NLME model predicted a mean ($±$ SE) change of $0.41 ± 0.07\%$ for the large fish and $0.69 ± 0.10\%$ for the small fish, while for $\delta^{15}$N, the optimal model predicted a mean ($±$ SE) change of $1.92 ± 0.15\%$ for the large fish and $1.45 ± 0.21\%$ for the small fish (Table 1; Fig. 2). Note that one anomalous value of $\delta^{13}$C which deviated from the mean of the large fish by $4.4 ±$ SD was removed from analyses. In both models, the initial isotopic ratio ($\delta Y_i$) differed for large and small individuals. The isotopic ratio at equilibrium ($\delta Y_{eq}$) differed between cohorts for $\delta^{15}$N, but not for $\delta^{13}$C. In addition, $\delta Y_{eq}$ was expected to vary by 0.06–0.08% between individuals given the optimum random effect (RE) structures for predicting both $\delta^{13}$C and $\delta^{15}$N (Tables 1; 2a). Comparisons with more complex RE structures were constrained by a high incidence of over-parameterisation and therefore non-convergence (Table 2a). An alternative model to predict $\delta^{13}$C ($\Delta\text{AIC} = 1.6$) retained a group effect for $\delta Y_{eq}$, although the standard error of the estimates for large and small fish overlapped (Tables 2b; S1). At equilibrium, TDF values of mucus were $0.27 ± 0.21\%$ for $\delta^{13}$C and $2.09 ± 0.24\%$ for $\delta^{15}$N.

Stable isotope turnover rates

The isotopic turnover rate of mucus $\delta^{13}$C did not differ between large and small groups of fish, with the mean 95% turnover ($T_{95}$) occurring after 18 days (Table 1; Fig. 2). An alternative model for $\delta^{13}$C ($\Delta\text{AIC} = 1.4$) retained a group effect on the turnover constant $c$ and suggested slower turnover for the larger cohort of fish, although there was large uncertainty in these estimates (Tables 2b; S1). For mucus $\delta^{15}$N, the smaller fish had a faster turnover rate than the larger fish, with mean $T_{95}$ being 9 days in the small fish versus 22 days in the large (Table 1; Fig. 2). The SE margins for the values of $\delta^{15}$N $T_{95}$ did not overlap. Comparing within groups revealed that the rate of $\delta^{15}$N turnover was markedly faster than $\delta^{13}$C for the mean small fish (Table 1; Fig. 2). The optimal RE structures did not retain an effect of fish ID on $c$ (Table 2a), indicating turnover rates were not expected to vary consistently between individuals.

Discussion

The results revealed that the $\delta^{15}$N turnover rate of mucus in the juvenile C. carpio varied according to the starting size of the fish, with a more rapid rate in the smaller fish. In contrast, the $\delta^{13}$C turnover rate of mucus was independent of the starting size of the fish. The half-life of $\delta^{15}$N was longer in the larger fish, which is in line with predictions for the relationship between SI turnover rates and body mass (Vander Zanden et al., 2015) and observations for the Japanese catfish Silurus asotus Linnaeus, 1758 (Maruyama et al., 2017). While there was no significant difference in absolute growth rate between size-classes, relative increase in mass was greater for the smaller cohort, which could explain faster $\delta^{15}$N turnover, if relative growth is coupled with the rate of protein turnover in
tissues (Vander Zanden et al., 2015). Conversely, Kim et al. (2012) found no relationship between growth rate and $d^{13}$C or $d^{15}$N incorporation rates for leopard sharks *Triakis semifasciata* Girard, 1855, except for $d^{13}$C in blood plasma.

The NLME model for $d^{15}$N predicted differing values of the isotope ratio at equilibrium with the experimental diet (Day 200) for the large and small cohorts, indicating size-related differences in diet-tissue fractionation. This has also been observed for liver tissue in European sea bass *Dicentrarchus labrax* (Linnaeus, 1758), which, like mucus, typically has a higher lipid content than white muscle (Sweeting et al., 2007). Furthermore, the models revealed greater disparity in the initial $d^{13}$C and $d^{15}$N ratios at Day 0 for the large and small cohorts (0.28–0.77%), likely indicating a lack of isotopic equilibrium with the initial diet. This is perhaps confounded by the relatively short time taken for near-complete turnover (up to 22 days for the average individual) on the experimental diet; however, rates of isotopic incorporation can be diet-specific, dependent on, for example, digestibility of dietary items (Codron et al., 2011). Indeed, Winter et al. (2019a) observed that mucus $d^{13}$C half-lives for *C. carpio* were almost four times greater with a fishmeal-based diet compared to a plant-based diet. As the initial diet in the present study was fishmeal based for a period of 100 days, this might help explain the lack of isotopic equilibrium in the fish at the time of diet-switching (Day 0 of the experimental period). Consequently, these results suggest that in addition to body size, the composition of the diet of the fish also needs consideration when assessing rates of SI turnover, with plant-based diets potentially having faster turnover rates. With regard to between-individual variability in rates of turnover, this could be further explored by maintaining a control group on the initial diet or by conducting a mirrored diet switch as per Winter et al. (2019a).

Ecological experiments using SIA are often designed in relation to their duration rather than, for example, the extent of the change of mass in the experimental subjects. Therefore, the focus here was on estimating the SI turnover rates as a unit of time rather than as a function of growth of the fish. The predicted $d^{13}$C and $d^{15}$N half-lives (2–5 days) were considerably faster than in other studies examining mucus turnover of juvenile cyprinid fishes (17–144 days; Shigeta et al., 2017; Winter et al., 2019a). The results suggested that in both field and experimental settings, SIA of epidermal mucus will provide assessments of fish diets in the preceding days and weeks, rather than months, providing consideration is given to the body size of the individuals. In addition, the emphasis here on individual turnover rates meant that, although the sample size was relatively small, consistent individual differences in SI turnover were able to be analysed using a mixed

| Coefficients | $d^{13}$C | | $d^{15}$N | |
|-------------|-----------|-----------|-----------|
|              | Fixed effect | Random effect | Fixed effect | Random effect |
| $\delta Y_{eq}$ | $-23.77 \pm 0.02$ | $0.06$ | $-$ | $0.08$ |
| $\delta Y_{eq}$ (intercept) | $-$ | $-$ | $7.71 \pm 0.05$ | $-$ |
| $\delta Y_{eq}$: small | $-$ | $-$ | $-0.30 \pm 0.07$ | $-$ |
| $\delta Y_{i}$ (intercept) | $-23.36 \pm 0.05$ | $-$ | $9.63 \pm 0.10$ | $-$ |
| $\delta Y_{i}$: small | $0.28 \pm 0.08$ | $-$ | $0.77 \pm 0.14$ | $-$ |
| $c$ | $1.80 \pm 0.22$ | $-$ | $-$ | $-$ |
| $c$: (intercept) | $-$ | $-$ | $2.00 \pm 0.14$ | $-$ |
| $c$: small | $-$ | $-$ | $0.94 \pm 0.67$ | $-$ |
| $T_{50}$ (days) | $4.2$ (3.4 to 5.2) | $-$ | $-$ | $-$ |
| $T_{95}$ (days) | $18.1$ (14.5 to 22.7) | $-$ | $-$ | $-$ |
| $T_{50}$ (days): large | $-$ | $-$ | $5.1$ (4.4–5.9) | $-$ |
| $T_{50}$ (days): small | $-$ | $-$ | $2.0$ (1.0–3.9) | $-$ |
| $T_{95}$ (days): large | $-$ | $-$ | $22.1$ (19.1–25.5) | $-$ |
| $T_{95}$ (days): small | $-$ | $-$ | $8.6$ (4.4–16.9) | $-$ |

Where an effect of Group was included in the model, the ‘Large’ group is represented by the intercept. Random effects illustrate the variation between individuals (SD) according to the optimal random effects structure. Average isotopic half-life ($T_{50}$) and near-complete turnover ($T_{95}$) are displayed with SE limits in parentheses.
modelling approach. Elsewhere, invasive and/or lethal tissue sampling for SIA tends to prevent this type of study design in fish (but see Kim et al., 2012). Nevertheless, the most complex random effects structures in the NLME models did not converge, indicating a dataset with high residual variance (unstable isotopic change for some individuals) which compromised some model comparisons. Selection of

![Graphs showing changes in δ^{13}C and δ^{15}N for different groups over time.](image_url)

**Fig. 2** Changes to mucus δ^{13}C (a, c) and δ^{15}N (b, d) following the diet switch (Day 0) for large (a, b) and small (c, d) groups. Symbols represent individual fish. Curves display predictions for the average individual (bold line) with 95% CIs in grey. Dashed lines indicate the mean δ^{13}C or δ^{15}N isotopic signature of the experimental diet during the second feeding period.

the more simplified models revealed that δ^{13}C and δ^{15}N at equilibrium were expected to vary predictably among individuals by up to 0.08‰, which is in line with individual isotopic variability recorded by Heady & Moore (2013). By contrast, turnover rates were not expected to vary predictably among individuals.

The biochemical composition of fish mucus responds rapidly to physiological stressors. Notably,
Moreover, the rate of isotopic (particularly $\delta^{13}$C) turnover was unchanged. Furthermore, the rate of isotopic turnover may increase when mucus renewal is induced by external factors such as abrasion, as opposed to increased by frequent removal of the mucous layer itself (Fernández-Alacid et al., 2018). The repeated sampling of mucus here (at intervals of more than 24 h, their findings have implications regarding the influence of short-term animal movements in relation to diet composition.

### Acknowledgements
We gratefully acknowledge the support for ERW of the EU LIFE + Nature and Biodiversity Programme: LIFE14NAT/UK/000054.

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### Data availability
The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

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### Table 2 Comparisons of $\Delta$AIC among random effect (a) and fixed effect (b) structures of the NLME turnover model

|                      | $\delta^{13}$C | $\delta^{15}$N |
|----------------------|---------------|---------------|
| **Random effect structure** |               |               |
| $\delta Y_{eq} \sim$ 11ID | 0.0           | 0.0           |
| $\delta Y_i \sim$ 11ID | 7.7           | 5.3           |
| $c \sim$ 11ID         | –             | 5.3           |
| $\delta Y_{eq} + \delta Y_i \sim$ 11ID | –           | –             |
| $\delta Y_{eq} + c \sim$ 11ID | –           | –             |
| $\delta Y_i + c \sim$ 11ID | –           | –             |
| $\delta Y_{eq} + \delta Y_i + c \sim$ 11ID | –           | –             |

| **Fixed effect structure** | $\delta^{13}$C | $\delta^{15}$N |
|---------------------------|---------------|---------------|
| $\delta Y_{eq} \sim$ Group | 12.1          | 35.7          |
| $\delta Y_i \sim$ Group   | 0.0           | 18.0          |
| $c \sim$ Group            | 12.8          | 36.0          |
| $\delta Y_{eq} + \delta Y_i \sim$ Group | 1.6 | 7.0 |
| $\delta Y_{eq} + c \sim$ Group | 14.1          | 27.5          |
| $\delta Y_i + c \sim$ Group | 1.4           | 8.4           |
| $\delta Y_{eq} + \delta Y_i + c \sim$ Group | 2.7 | 0.0 |

Missing values represent model non-convergence. Models with substantial support ($\Delta$AIC < 2) are in bold.

Fernández-Alacid et al. (2018) reported increases in total volume of mucus, as well as glucose concentration of mucus, following exposure of fish to the air; however, protein concentration was unchanged. Furthermore, the rate of isotopic (particularly $\delta^{13}$C) turnover may increase when mucus renewal is induced by external factors such as abrasion, as opposed to under normal levels of exudation (Ibarz et al., 2019). While both studies used experiments lasting no more than 24 h, their findings have implications regarding the repeated sampling of mucus here (at intervals of 10 + days). Turnover rates may have been artificially increased by frequent removal of the mucous layer and/or by recurrent stress responses to handling and air exposure. Thus, future studies comparing the effects of repeated sampling versus single-occasion sampling of mucus on the rate of isotopic turnover could be highly informative.

In summary, $\delta^{13}$C and $\delta^{15}$N turnover rates in the epidermal mucus of the omnivorous $C$. carpio were rapid, while $\delta^{15}$N turnover rates were also size-dependent. Between-individual variation in $\delta^{13}$C and $\delta^{15}$N turnover warrants further investigation, including with multiple fish growth trajectories and tissue-types where this is possible and does not compromise welfare standards. The implications of this rapid SI turnover rate are potentially important, especially in the context of its application to studies examining the influence of short-term animal movements in relation to diet composition.

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

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