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Comparison of the isotopic composition of fish otolith-bound organic N with host tissue

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Abstract

The $^{15}N/^{14}N$ ratio of the fish-native organic matter preserved in fish otoliths (or $\delta^{15}N_{oto}$) may allow for reconstruction of fish trophic history and changes in food webs. To support this application, ground-truthing data are needed on the relationships among the $\delta^{15}N$ of diet, of fish tissue (e.g., white muscle tissue, $\delta^{15}N_{wmt}$), and $\delta^{15}N_{oto}$. Using a highly sensitive method for N isotope anlaysis, $\delta^{15}N_{oto}$ was compared to $\delta^{15}N_{wmt}$ in 24 teleost species. Within a species, the difference between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ ($\Delta\delta^{15}N_{o-w}$) varied little across individuals, confirming the utility of $\delta^{15}N_{oto}$ to reconstruct $\delta^{15}N_{wmt}$ changes for a given species. Across species, $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ were highly correlated. However, $\Delta\delta^{15}N_{o-w}$ varied systematically across species. Phylogeny, the concentrations of total N and amino acids, and life history were ruled out as the main cause for the observed variation in $\Delta\delta^{15}N_{o-w}$. $\delta^{15}N_{oto}$ was lowest relative to $\delta^{15}N_{wmt}$ in species producing larger otoliths. We propose that $\delta^{15}N_{oto}$ is elevated by isotopically fractionating metabolism of the organic matrix, which is less important when otolith growth is fast and thus the otolith is large.

Key words: Nitrogen isotopes, fish otoliths, trophic ecology, amino acids

1. Introduction

The nitrogen isotopic composition ($\delta^{15}N$) of fish tissues is an important tool for reconstructing fish behavior and ecology, due in large part to the predictable increase of $\delta^{15}N$ with trophic level (Minagawa & Wada 1984; Boecklen et al. 2011). This increase, referred to as the trophic discrimination factor, or TDF, derives from normal metabolic processes in tissues including synthesis and degradation, resulting in 2-5 ‰ higher $\delta^{15}N$ in the tissues of fish relative to their diet (Minagawa & Wada 1984; Pinnegar & Polunin 1999; Post 2002). Fish $\delta^{15}N$ is also

applied to identification of habitat use among habitats with differing $\delta^{15}N$ of baseline resources ($\delta^{15}N_{base}$; e.g., Lorrain et al. 2015). White muscle tissue (WMT), the most commonly measured tissue in fishes for ecological studies, is not preserved in the fossil record and even in modern tissue cannot be used to reconstruct $\delta^{15}N$ over the entire life of the fish because its turnover time is on the order of months to a year (e.g., Hesslein et al., 1993; Logan et al., 2006; Madigan et al., 2012). Otoliths are increasingly being used for $\delta^{15}N$ analysis of historical and fossil fishes (Vandermyde & Whitledge 2008; Grønkjær et al. 2013; Sirot et al. 2017; Lueders-Dumont et al. 2018; Cheng et al. 2018), and for reconstructing life history variability in $\delta^{15}N$ (Vane et al. 2018). However, widespread application of $\delta^{15}N$ in otolith-bound organic matter ($\delta^{15}N_{oto}$) to ecological studies of modern and past fish depends on validation of $\delta^{15}N_{oto}$ as a measure of $\delta^{15}N$ of WMT ($\delta^{15}N_{wmt}$) across diverse taxa.

Otoliths are composed of aragonite and a small fraction of organic matter (<1-10% by weight; Carlström 1963; Degens et al. 1969; Morales-Nin 1986). The organic matter (OM) is composed of collagens, non-collagenous proteins, glycoproteins, proteoglycans, and otopetrins (Asano & Mugiya 1993; Baba et al. 1991; Borelli et al. 2001). The OM is critical for the shape, physical properties, and overall mineral formation process in otoliths, forming the organic lattice onto which the calcium carbonate precipitates (Söllner et al. 2003; Tohse et al. 2008; Wojtas et al. 2012). This OM forms the substrate for $\delta^{15}N_{oto}$ analysis. Previous studies have found $\delta^{15}N$ of otoliths and muscle to be highly correlated within fish in the same population, but found $\delta^{15}N_{oto}$ to be lower than $\delta^{15}N_{wmt}$ by 1.1 ‰, 0.8 ‰, 0.7-3.7 ‰, 3 ‰, and 7.5 ‰ (respectively, Vandermyde & Whitledge 2008; Grønkjær et al. 2013; Sirot et al. 2017; Lueders-Dumont et al. 2018; Cheng et al. 2018). As otoliths are considered to be metabolically inert (Campana & Neilson 1985; Pereira et al. 1995), $\delta^{15}N_{oto}$ is expected to be lower than $\delta^{15}N_{wmt}$ due to the lack of the metabolic processes

63 that are known to produce $\delta^{15}N$ elevation in WMT and also because some component of otolith
64 OM was laid down during early life, which for many species is a period during which they feed at
65 a lower trophic level than in adult life. Better understanding of the relationship between $\delta^{15}N_{oto}$
66 and $\delta^{15}N_{wmt}$ would enable adaptation of $\delta^{15}N_{oto}$ to wider ecological and biogeochemical
67 applications using historical, fossil, and modern otoliths. Due to methodological improvements,
68 $\delta^{15}N_{oto}$ can now be analyzed in samples as small as 2 mg (Lueders-Dumont et al. 2018; Cheng et
69 al. 2018), adequate for the analysis of the smallest individual otoliths and of subsamples within
70 most otoliths.

71 Nitrogen isotopic measurements of other tissues, such as liver, fin clips, or scales, are
72 usually compared to that of white muscle tissue (e.g., Kelly et al., 2006; Willis et al., 2013;
73 Franssen et al., 2017), the preferred fish tissue for several reasons: $\delta^{15}N_{wmt}$ (1) exhibits the least
74 variable $\delta^{15}N$ relative to diet (Pinnegar & Polunin 1999; Jennings et al. 2001), (2) has the largest
75 TDF compared to other tissues, which results in greater confidence for determining fish trophic
76 level relative to background variability in $\delta^{15}N_{diet}$ and $\delta^{15}N_{base}$ (e.g., Buchheister and Latour, 2010;
77 Willis et al., 2013), and (3) is easily sampled from dorsal white muscle and then analyzed by
78 Dumas combustion and isotopic analysis of the N_2 produced, the most common approach in
79 ecological laboratories (Boecklen et al. 2011). However, historical archives of soft tissues are
80 exceedingly rare, precluding historical comparisons of fish $\delta^{15}N$. Otoliths, prevalent in historical,
81 archival, and sediment records (Brzobohaty & Nolf 1995; Ivany et al. 2000; Andrus et al. 2002),
82 are resistant to degradation in many conditions (Patterson 1999; Disspain et al. 2016) and protect
83 the aragonite-bound OM against diagenesis in sediments on centennial timescales (Lueders-
84 Dumont et al. 2018).

85 Using our high-sensitivity approach for measurement of $\delta^{15}N_{oto}$ (Lueders-Dumont et al.

2018), we analyze the $\delta^{15}N$ of the bulk OM in fish otoliths, which is then compared to $\delta^{15}N_{wmt}$ from 86 fish individuals from 24 species and seven orders. For four species raised in fish farms or laboratory settings (Atlantic cod, rainbow trout, brown trout, and Atlantic croaker), the $\delta^{15}N$ of diet ($\delta^{15}N_{diet}$) was used to calculate TDFs for both muscle and otolith. The resulting patterns in $\delta^{15}N$ were evaluated through comparisons of hydrolysable otolith amino acid concentrations of otoliths across a subset of 10 species and by modeling temporal differences in dietary averaging recorded by otolith (whole life) and muscle (months). From these experiments, we extract ground-truthing information for future investigations of fossil, historical, or archaeological fish otoliths.

2. Methods

2.1 Otolith $\delta^{15}N$ analysis

Fish heads or whole fish of 24 species were obtained from multiple sources (Table S1[1]). Only sagittal otoliths were used, except for catfish in which lapillus otoliths were analyzed as they were the largest of the three otoliths for this species. Whole otoliths were prepared for $\delta^{15}N$ analysis and $\delta^{15}N_{oto}$ analysis was conducted as previously described (Lueders-Dumont et al. 2018). The protocol included seven steps: external cleaning of the intact otoliths, crushing of the otolith to a fine powder in order to homogenize the otolith, cleaning of otolith grains to remove interstitial organic matter (leaving only OM that is intrinsic to otolith grains), dissolution of the clean otolith aragonite grains in order to expose grain-internal organic matter, oxidation of the freshly-exposed organic matter to nitrate, analysis of nitrate concentration, bacterial conversion of nitrate to nitrous oxide, and isotope analysis via a purpose-built, helium flow-based N_2O extraction and purification system on-line to a gas-source, stable isotope ratio mass spectrometer. Our previous work showed

[1] Refer to supplemental material

that OM within the cleaned grains of a ground otolith is between 13 and 40 % of OM contained within the externally cleaned, whole otolith, depending upon the species. Removal of grain-external OM, which is required for analysis of fossil otolith OM to avoid diagenetic artifacts and is applied here for consistency, resulted in no change in measured $\delta^{15}N_{oto}$ (Lueders-Dumont et al. 2018). The protocol is sufficiently sensitive for $\delta^{15}N_{oto}$ analysis of small (2 mg) otoliths with a long term precision of 0.3‰ (1σ) (Lueders-Dumont et al. 2018).

2.2 Muscle $\delta^{15}N$ analysis

White muscle tissue for $\delta^{15}N$ analysis was collected at the same time as otoliths from each fish. Approximately 1 cm^3 of dorsal white muscle tissue was removed and immediately frozen at -20ºC. Prior to freeze drying, tissue samples were transferred to -80ºC overnight and freeze dried for 24-48 hours until completely dry. Samples were homogenized with a mortar and pestle, then packed into tin capsules for combustion via elemental-analyzer isotope ratio mass spectrometry (EA-IRMS). Sample weights were 1 mg ± 0.2 mg. A pair of internal organic standards (ACA; Alfa Aesar) with concentrations bracketing the target N and C content of fish samples was run every 8 samples; and organic standard USGS-40 was run every 16 samples. An Isoprime 100TM isotope ratio mass spectrometer (IRMS) interfaced in continuous flow with an elemental analyzer (Vario ISOTOPE cubeTM, Elementar Analysensysteme GmbH, Hanau, Germany) was used for EA-IRMS analysis. One batch of samples was analyzed by the University of California Davis Stable Isotope Facility on an PDZ Europa ANCA-GSL elemental analyzer interfaced with a PDZ Europa 20-20 I.RMS (Sercon Ltd., Cheshire, UK). Average standard deviations of reference materials were 0.1 ‰ for the UC Davis samples and 0.04 ‰ for Isoprime samples analyzed at Princeton. Replicate samples from both labs resulted in 0.2 ‰ (1σ) differences after inter-lab calibration of

muscle $\delta^{15}N$ from the same individuals.

For wild cod, wild pollock, farmed cod, farmed rainbow trout, and farmed brown trout, cranial bone collagen $\delta^{15}N$ was also measured. Bone samples were demineralized to completion in 0.2 M HCl and rinsed extensively in reverse osmosis (RO) water. Samples were lyophilized, weighed to the closest 0.1 mg, and analyzed via EA-IRMS on a Costech ECS4010 Elemental Analyzer (EA) interfaced with a ThermoFinnigan Delta Plus Advantage IRMS in the Environmental Geochemistry Laboratory at Bates College. The analytical precision as measured by average standard deviations of internal reference standard materials (acetanilide, dried fish muscle and caffeine run every sixth sample) and replicates of subsamples was < 0.2 ‰ (1σ). The otolith saccular membrane was extracted and analyzed for pollock and farmed cod via EA-IRMS at Princeton University as per $\delta^{15}N_{wmt}$ methods above.

2.3 Derived variable: otolith-muscle offset ($\Delta\delta^{15}N_{o\text{-}w}$)

Otolith-muscle offset ($\Delta\delta^{15}N_{o\text{-}w}$) values were calculated by subtracting $\delta^{15}N_{wmt}$ from $\delta^{15}N_{oto}$ for each individual fish in order to quantify departures from the 1:1 line for each species. Average $\Delta\delta^{15}N_{o\text{-}w}$ for each species is reported as the mean ± 1σ of all individuals of the species.

2.4 Dietary $\delta^{15}N$ analysis and trophic discrimination factor (TDF) calculations

Four of the species investigated in this study were reared in fish farms or laboratory settings (laboratory-reared Atlantic croaker, farmed Atlantic cod, rainbow trout, brown trout) on known diets. These fish were used to investigate the relationship among $\delta^{15}N_{diet}$, $\delta^{15}N_{oto}$, and $\delta^{15}N_{wmt}$ and were chosen based on availability of dietary samples from each rearing facility. Cod (4 years old) and both species of trout (2 years old) were mature; croaker were juvenile (233 days old). All

154 species consumed formulated aquafeeds, the $\delta^{15}N$ of which was measured here for quantification
155 of TDF for otolith and muscle compared to diet (Table S2). Brown trout and rainbow trout, both
156 from the same farm, were reared on identical diets. Atlantic cod were reared on rotifers fed artemia
157 for 6 weeks, then fed a combination of formulated aquafeeds (Europa 18, Skretting ARC,
158 Stavanger, Norway; and Bio-Oregon Brood, Bio-Oregon, Westbrook, ME, USA). The $\delta^{15}N$ of
159 each cod aquafeed was measured, except for artemia, which the fish were fed for < 3 % of their
160 lifetime (Table S2). Atlantic croaker were reared on specially formulated experimental feed
161 optimized for Atlantic croaker (Mohan and Walther, 2016). Dietary samples were prepared for
162 $\delta^{15}N$ analysis as per *Section 2.2 Muscle $\delta^{15}N$ Analysis*. Trophic discrimination factors of otolith
163 (TDF_{oto}) and WMT (TDF_{wmt}) were calculated by subtracting the average diet $\delta^{15}N$ from the otolith
164 or muscle for each individual fish. For Atlantic cod, which had consumed rotifers and two
165 commercial diets, the $\delta^{15}N$ of diet ($\delta^{15}N_{diet}$) was calculated based on equal weighting by month for
166 comparison to $\delta^{15}N_{oto}$; the $\delta^{15}N_{diet}$ was calculated based only on the most recent $\delta^{15}N_{diet}$ for
167 comparison with $\delta^{15}N_{wmt}$.
168
169 ***2.5 Amino acid analysis in otoliths***
170 Amino acid (AA) concentrations were determined from a subset of otoliths that were
171 cleaned externally, powdered, and cleaned again as per *Section 2.1.* Species were chosen to obtain
172 amino acid data on each taxonomic order that was investigated for $\delta^{15}N_{oto}$ (with the exception of
173 Clupeiformes due to the sample mass requirement of 10 mg or greater for amino acid analysis).
174 Samples were sent to the Amino Acid Geochronology Lab at Northern Arizona University for
175 analysis of total hydrolysable amino acid composition and analyzed by fluorescence following
176 Bright and Kaufman (2011). Otolith process replicates had a mol % coefficient of variation of 1.5

177 % for amino acid concentration. Sample origins are the same as for Table S1 with the addition of
178 haddock (*Melanogrammus aeglefinus*) otoliths obtained from the Northeast Fishery Science
179 Center Fishery Biology Program (NEFSC FBP), Woods Hole, MA, USA. No muscle tissue was
180 available for these fish, so haddock were not included in otolith *vs.* muscle examination above;
181 however, haddock were included in the AA analysis due to their availability at the time of sampling
182 and to increase the representation within the Gadidae family. The amino acids routinely reported
183 are aspartic acid (Asp), glutamic acid (Glu), serine (Ser), alanine (Ala), valine (Val), phenylalanine
184 (Phe), leucine (Leu), and isoleucine (Ile) as they typically result in the best chromatographic
185 resolution. Under normal conditions for AA assays, both asparagine (Asn) and glutamine (Gln)
186 undergo irreversible deamination to form aspartic acid and glutamic acid, respectively (Hill 1965).
187 Asn and Asp are grouped together as Asp in the current study, and Glu and Gln are grouped
188 together as Glu.

189 The concentration of each amino acid was converted to a fraction of the total AA pool
190 because the proportion of each AA multiplied by its $\delta^{15}N$ influences the bulk $\delta^{15}N$ of any AA
191 mixture. The resulting AA profiles were used to examine inter-species differences in AA
192 composition using Ward's D2 dissimilarity index (Murtagh & Legendre 2014) in R (*Version*
193 *3.4.3*).

194

195 ***2.6 Modeling temporal averaging in otoliths and muscle tissue***

196 Otoliths and WMT have different dietary integration times, with otoliths recording whole-
197 life history and WMT recording recent life history. For adult fish, recent life history is often a
198 period of higher trophic level and $\delta^{15}N$ than early life history (Jennings, Pinnegar, et al. 2002;
199 Marsh et al. 2017). This would tend to lower the $\delta^{15}N$ bulk otolith N relative to WMT. The effect

of these differing integration times of otoliths compared to WMT was investigated quantitatively, for an illustrative range of patterns for dietary $\delta^{15}N$ over fish lifetime. Three dietary patterns were examined (summarized in Table S3). A logarithmic increase in $\delta^{15}N$ simulates the paradigm for gape-limited fish species, which consume small, low trophic level prey as juveniles then shift to larger, higher trophic level prey as they mature, eventually asymptoting in length and also trophic level (e.g., Kitagawa and Fujioka, 2017). A linear increase in $\delta^{15}N$ simulates the scenario in which diet $\delta^{15}N$ increases without asymptoting (e.g., a few species in Badalamenti et al., 2002), such as during discrete but protracted periods of fish life history in which a fish is growing quickly and with access to correspondingly large prey. Finally, a step-change decrease in $\delta^{15}N$ simulates fish movement from a high to low $\delta^{15}N_{base}$ or trophic level (e.g., Dale et al., 2011).

Diet progressed through time in units of weeks and was recorded in $\delta^{15}N_{wmt}$ and $\delta^{15}N_{oto}$, with each time point weighted equally in both otolith and muscle $\delta^{15}N$. Fish were "grown" for 8 years (415 time steps); muscle recorded only the preceding 3 months of diet while otolith recorded the entire life history of diet. Otolith or muscle $\delta^{15}N$ at time t was calculated by week-based averaging for weeks 1 to t, with equal weighting per week. Trophic discrimination factor, TDF, was held constant at 3.4‰ across all time points and diet types.

Equal otolith weighting (the differences in temporal integration between otolith and muscle) per week was assumed based on evidence that otolith mass accumulation is relatively linear with fish age (e.g., Anderson et al., 1992). We acknowledge that, for WMT, equal weighting per week is an oversimplification, as muscle turnover is a decay function as opposed to linear and, moreover, that muscle turnover time is usually reported as a half-life (T50) or a T95—the length of time required for 50% and 95% of tissue to record a diet switch, respectively (e.g., Fry & Arnold 1982; Hesslein et al. 1993; Herzka & Holt 2000). For simplicity, we aimed to investigate the effect

on $\Delta\delta^{15}N_{o\text{-}w}$ when otolith and muscle have the same ability to record diet $\delta^{15}N$ (i.e. both with equal weighting), the only difference being that one records a longer time frame (8 years for otolith compared to 3 months for WMT). In detail, muscle turnover time has been shown to vary by species, by diet within a species, and within life history (see Gannes et al., 1997; Vanderklift and Ponsard, 2003; Robbins et al., 2010; McMahon and McCarthy, 2016), but considering these effects is not critical for the first-order questions being investigated here.

3. Results and Discussion

3.1 Patterns in $\delta^{15}N_{wmt}$ and $\delta^{15}N_{oto}$

$\delta^{15}N_{wmt}$ ranged from 6.1 ‰ (farmed Mozambique tilapia, *Oreochromis mossambicus*) to 22.2 ‰ (wild caught red grouper, *Epinephelus morio*) for a total range of 16.1 ‰ (Fig. 1a). $\delta^{15}N_{oto}$ ranged from 5.9 ‰ (farmed Atlantic cod, *Gadus morhua*) to 19.9 ‰ (wild caught red grouper) for a total range of 14.0 ‰; the range of $\delta^{15}N_{oto}$ for farmed tilapia (6.3 ‰) to red grouper was 13.6 ‰. The contracted range of $\delta^{15}N_{oto}$, by 2.5 ‰, is consistent with the integrated nature of whole-otolith analysis, which has the potential to smooth over temporal variation in $\delta^{15}N_{diet}$.

3.1.1. Wild caught fish

$\delta^{15}N_{wmt}$ was highest for red grouper (*Epinephelus morio*) (22.2 ‰). White catfish (*Ameiurus catus*, 17.6 – 19.8 ‰) and bluefish (*Pomatomus saltatrix*, 16.7 – 17.5 ‰) had the second and third highest. $\delta^{15}N_{oto}$ was highest for the same species as for $\delta^{15}N_{wmt}$: red grouper (19.9 ‰), white catfish (17.2 – 19.3 ‰), and bluefish (17.2 – 17.5 ‰). $\delta^{15}N$ variations among species were generally consistent with known trophic or baseline information. Bluefish and red grouper, two of the highest $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ species, are high trophic level piscivorous species (Szczebak and

Taylor, 2011; Froese and Pauly, 2018). The high $\delta^{15}N$ values of both otolith and muscle from white catfish, an omnivorous fish, are unusual and may result from high $\delta^{15}N_{base}$ in agriculturally influenced river systems, which can have higher baseline $\delta^{15}N$ due to N losses associated with suboxia and denitrification (Harrington et al. 1998; Anderson & Cabana 2005; Vandermyde & Whitledge 2008; Diebel et al. 2009). The catfish used in the present study were caught in Maryland in the Chesapeake Bay watershed, a region known to be highly influenced by nutrient loading (Kemp et al. 2005).

3.1.2. Farmed fish

$\delta^{15}N_{wmt}$ was lowest overall for farmed fish. Among the farmed fish, $\delta^{15}N_{wmt}$ was lowest for tilapia (6.1 – 6.9 ‰), followed by brown trout (*Salmo trutta*, 8.9 – 9.3 ‰) and rainbow trout (*Oncorhynchus mykiss*, 9.0 – 9.4 ‰). For $\delta^{15}N_{oto}$, farmed Atlantic cod (5.9 - 6.4 ‰) was the lowest instead of tilapia, which was second lowest (6.3 – 7.0 ‰), while wild cod (6.7 - 9.3 ‰) was the third lowest. The lower $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ of farmed species compared to non-farmed fish was consistent with farmed fishes consuming formulated feeds containing protein derived from low trophic level fish (e.g., anchoveta commonly used for fishmeal) or plant-based protein (FAO 2016).

3.2 Relationship between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$

$\delta^{15}N_{oto}$ was highly correlated to $\delta^{15}N_{wmt}$, with data for most species falling along the 1:1 line (Fig. 1a). Large differences between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ ($\Delta\delta^{15}N_{o-w}$) occurred only among species in the Gadidae family (Fig. 1a, b). Smaller yet coherent species trends in $\Delta\delta^{15}N_{o-w}$ were observed across all species (Fig. 1b). The results can be split into two general categories: (1)

robustness and covariation between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$, and (2) coherent offsets in absolute values.

3.2.1. Robustness and covariation between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$

$\delta^{15}N_{oto}$ tended to covary with $\delta^{15}N_{wmt}$ for most species, and absolute values of $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ were very similar. For non-Gadidae species, a geometric mean linear least squares regression best described the relationship between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ of the form $y = 0.96x$ (± 0.06) + 0.70 (± 0.75) with $r^2 = 0.75$.

Wild cod, farmed cod, and pollock (*Pollachius pollachius*), all in the Gadidae family, exhibited anomalously low $\delta^{15}N_{oto}$ compared to $\delta^{15}N_{wmt}$. This finding was consistent with previous work reporting low $\delta^{15}N_{oto}$ for Atlantic cod (Grønkjær et al. 2013; Lueders-Dumont et al. 2018). Nonetheless, $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ were highly correlated for these anomalous species. Gadidae species data were described by a geometric mean linear least squares regression: $y = 0.91x$ (± 0.16) – 5.60 (± 2.34) with $r^2 = 0.65$. Gadidae were offset from the non-Gadidae regression by 6.3‰. Despite this offset, the regressions yielded similar slopes: m = 0.91 ± 0.16 and 0.96 ± 0.06 respectively. The similarly-high r^2 values for both Gadidae and non-Gadidae ($r^2 = 0.65$ and 0.75 respectively) datasets indicate at least some shared controls on $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ across diverse lineages.

To summarize, $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ values were similar for most fish. These relationships were in contrast to our hypothesis that $\delta^{15}N_{oto}$ would be lower than $\delta^{15}N_{wmt}$. The higher $\delta^{15}N_{oto}$ than expected suggests that the paradigm of N isotopic fractionation in WMT may be incomplete, that the amino acid pools are shared between muscle and the fish inner ear, or that the organic N in the otolith also undergoes isotopic alteration before its encapsulation. The specific mechanism

aside, the similar overall ranges in $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ indicate N isotope fractionation commonalities between otoliths and muscle.

3.2.2. Coherent offsets in absolute values

All individual fish within the same species tended to have similar $\Delta\delta^{15}N_{o\text{-}w}$, ranging from +3.1 ‰ for lake trout (*Salvelinus namaycush*) to -7.3 ‰ for wild Atlantic cod, with an average $\Delta\delta^{15}N_{o\text{-}w}$ of -0.73 ‰ across all species (Fig. 1b; Table S4). The lowest $\Delta\delta^{15}N_{o\text{-}w}$ in a non-Gadid species was -2.0 ‰ for red grouper (*Epinephelus morio*). Species-level $\Delta\delta^{15}N_{o\text{-}w}$ was consistent within Gadidae, Salmonidae, Serranidae, and Sparidae families, which had multiple species per family. There was no coherence, however, between $\Delta\delta^{15}N_{o\text{-}w}$ and phylogenetic relatedness among families (Fig. 1b). The surprisingly coherent patterns within species indicated that $\Delta\delta^{15}N_{o\text{-}w}$ variations among taxa were not due exclusively to noise around the 1:1 line. Lueders-Dumont et al. (2018) found that variations in $\delta^{15}N_{oto}$ among individuals within the same population consuming the same diet were negligible. The existence of coherent species patterns further supports the notion that $\delta^{15}N_{oto}$ is consistent within a population.

A few species exhibited relatively high standard deviations in $\Delta\delta^{15}N_{o\text{-}w}$ (Table S3). The Atlantic herring (*Clupea harengus*) plotted in Fig. 1 were captured from multiple regions in the Gulf of Maine, USA; the large standard deviation of $\Delta\delta^{15}N_{o\text{-}w}$ led us to plot fish by station, with the result being that $\delta^{15}N_{oto}$ varied by site whereas $\delta^{15}N_{wmt}$ recorded similar values among all stations (Fig. S1). The $\Delta\delta^{15}N_{o\text{-}w}$ within one station (n = 6 individual fish) was 2.3 ± 0.5 ‰, compared to 1.8 ± 1.0 ‰ for all fish (n = 10) across three stations. Thus, life history variability likely produces the relatively large $\Delta\delta^{15}N_{o\text{-}w}$ variations across the individuals measured, due either to prey availability or $\delta^{15}N_{base}$.

315

316 ***3.3 Origin of coherent species patterns in $\Delta\delta^{15}N_{o\text{-}w}$***

317 While the overarching relationship between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ was a 1:1 line except for
318 the species in the Gadidae family, signifying that $\delta^{15}N_{oto}$ recreates $\delta^{15}N_{wmt}$ in general, small but
319 significant species patterns in $\Delta\delta^{15}N_{o\text{-}w}$ led us to probe the possible factors underlying this
320 variation. To investigate coherent offsets among species, we measured $\delta^{15}N_{diet}$ for four species of
321 farm- or laboratory-reared fishes to calculate TDFs for muscle and otolith across species (*Section*
322 *3.3.1*), quantified the hydrolysable amino acid composition of otoliths from a wide range of species
323 (*Section 3.3.2*), investigated other parameters including N content that may affect $\Delta\delta^{15}N_{o\text{-}w}$
324 (*Section 3.3.3*), and developed a model to address the fact that otoliths integrate the whole life
325 history of the fish whereas muscle records a shorter period of life history (*Section 3.3.4*). Finally,
326 a N isotope fractionating process is proposed that explains the coherent, otolith size-based $\Delta\delta^{15}N_{o\text{-}w}$ patterns across species (*Section 3.4*).

328

329 ***3.3.1 Trophic discrimination factor***

330 Trophic discrimination factor (TDF), defined as $\delta^{15}N_{consumer} - \delta^{15}N_{diet}$, can only be directly
331 quantified for farmed or laboratory-reared fish and was investigated for both otolith and muscle
332 (Fig. 1c). TDF_{wmt} was 4.3 ‰, 4.3 ‰, 2.8 ‰ and 3.7 ‰, respectively for brown trout, rainbow
333 trout, juvenile Atlantic croaker, and Atlantic cod, which were within the previously reported range
334 (2-5 ‰; e.g., DeNiro & Epstein 1981; Minagawa & Wada 1984; Post 2002). Variations in TDF_{wmt}
335 among species tend to coincide with the nutritional amino acid matching of fish diet compared to
336 the nutritional needs of the fish for metabolism and growth, with lower quality feeds leading to
337 higher TDF (Gaye-Siessegger et al. 2004; McMahon & McCarthy 2016; McMahon et al. 2015).
338 In the current study, Atlantic croaker, with the lowest TDF_{wmt}, may have had a more optimal diet

for nutritional needs compared to trout and cod, which had higher TDF_{wmt}.

TDF_{oto} was 6.2 ‰, 6.0 ‰, 2.4 ‰, and -1.6 ‰ respectively for brown trout, rainbow trout, juvenile Atlantic croaker, and Atlantic cod. With the exception of cod, all values were higher than the previously reported values for TDF_{oto} which ranged -0.2-0.3 ‰ for laboratory-reared Atlantic cod across multiple diets (Grønkjær et al. 2013). The negative TDF_{oto} for cod is lower than values for cod reported in Grønkjær et al. (2013), which may be due to differences in dietary quality or other factors not yet determined. The anomalously low value for TDF_{oto}, while TDF_{wmt} was in the normal range (3.4 ‰, the mean TDF_{wmt} across all studies; Post, 2002), indicates that the low cod in $\Delta\delta^{15}N_{o-w}$ data results from an absence of an N isotope fractionating processes in otoliths and not due to processes occurring in WMT. The anomalously low value for $\delta^{15}N_{oto}$ was not observed in saccular membrane, bone collagen, or liver (Fig. S2), further suggesting that the anomalous behavior is restricted to the otolith and not found in other structures in cod.

This is the first study to compare otolith and diet for adult fishes and for multiple species. Previous natural abundance dietary studies have found that $\delta^{15}N_{oto}$ and $\delta^{15}N_{diet}$ were similar (Grønkjær et al. 2013; Cheng et al. 2018), in contrast to the findings from the present study that $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ tend to be similar (Fig. 1a). We do not know why juvenile fish, such as those in Grønkjær et al. (2013) and Cheng et al. (2018) showed otolith and diet $\delta^{15}N$ to be similar while our study showed otolith and muscle $\delta^{15}N$ to be similar. We can conceive of two possible explanations.

The first possibility is that the previous studies were of juvenile fish whereas the majority of fish in our study were adult fish. Consistent with this ontogenetic explanation, within our study of four farmed fish, Atlantic croaker had the lowest $\Delta\delta^{15}N_{o-w}$, and it was the only juvenile of the four (Fig. 1c). To provide a suggestion of the underlying processes that may be involved, the

relative proportion of total fish amino acids as circulating free amino acids as opposed to tissue protein (e.g., muscle) is allometric and known to decrease over fish life history. The whole-body free amino acid pool accounts for 30 percent of total amino acids in larval fish, compared to only 3 percent in juvenile fish (Houlihan et al. 1995). As a result, the $\delta^{15}N$ of circulating amino acids should be less easily altered by the metabolic processes in muscle or other tissues in larval fish. The second possible explanation relates to the N isotope fractionating process at the surface of the actively-forming otolith that we propose below to explain the variation in $\Delta\delta^{15}N_{o\text{-}w}$ observed among taxa (*Section 3.4*). During periods of rapidly accreting otolith mass, such as during juvenile growth, we would predict that a larger fraction of the organic layer is preserved in the otolith carbonate, and that this would yield a lower $\delta^{15}N$ for the otolith relative to the diet and muscle of the fish. The otoliths studied by Grønkjær et al. (2013) and Cheng et al. (2018) were much smaller than (roughly 1/10 the mass of) those in our study and thus presumably growing very rapidly to achieve adult size, adding plausibility to such an accretion rate-based explanation.

In any case, the distinction between our results and those of Grønkjær et al. (2013) and Cheng et al. (2018) call for controlled studies of diet, otolith, and WMT from multiple time points over fish development.

3.3.2 Amino acid concentration

To investigate species patterns in $\Delta\delta^{15}N_{o\text{-}w}$, the relative proportions of total hydrolysable amino acids (HAAs) were compared among a subset of ten species (Fig. 2a-c). HAA profiles are useful because: (1) they serve as a coarse assay for protein differences among species and (2) different amino acids have variable $\delta^{15}N$ values such that the weighted average of constituent HAAs determines the bulk $\delta^{15}N$ of proteins. Amino acid concentrations were normalized relative

385 to the total concentration of the eight AAs measured here because the fraction of each AA, not its
386 absolute concentration, drives the $\delta^{15}N$ of a given mixture of AAs. The acidic amino acids,
387 glutamic (Glu) and aspartic (Asp) acids, routinely contributed the highest fractions of any AA (Fig.
388 2b), consistent with reports that biomineralizing proteins are rich in Glu and Asp (e.g., Weiner,
389 1979; Lowenstam and Weiner, 1989; Robbins and Brew, 1990; Sarashina and Endo, 1998).
390 Phenylalanine (Phe) and isoleucine (Ile), both essential amino acids, tended to have the lowest
391 fractions relative to other amino acids in each species. The other four amino acids, valine (Val),
392 leucine (Leu), serine (Ser), and alanine (Ala) contributed intermediate proportions. This is
393 consistent with branched and non-polar amino acids contributing lower proportions to
394 biomineralizing proteins (Bright and Kaufman, 2011 and references therein). Glycine (Gly), not
395 measured in the present study, is common in structural proteins including bone collagen and tooth
396 enamel. Previous literature (Hüssy et al. 2004; McMahon et al. 2011) indicates that otolith Gly
397 concentrations are comparable to those of Phe, but less than Leu, Val, or Ser concentrations,
398 therefore contributing roughly 5-6 % of the total amino acid pool.

399 Applying a discriminant analysis to the scaled amino acid data resulted in three clusters
400 (Fig. 2a): Cluster A, typified by species with AA profiles dominated by Asp (>25% of total AA
401 pool); Cluster B, typified by species dominated by Glu (> 25%); and Cluster C, typified by species
402 with relatively lower Asp and Glu (< 22%) and also relatively higher Val, Leu, and Phe (14 %, 12
403 %, and 5 % compared to < 10 %, < 8 %, and 3 %) than other groups (Fig. 2c). Otolith proteins
404 may differ among taxa (Söllner et al. 2003; Tohse et al. 2008; Weigele et al. 2015; although in
405 many cases, proteins themselves may be functional homologs, Thomas et al. 2018), so the finding
406 that AA proportions, themselves the constituents of proteins in the otolith, differ was not
407 surprising. However, there was no relationship between the clusters and species groupings in

408 $\Delta\delta^{15}N_{o\text{-}w}$ (Fig. S3) implying that factors other than HAA produce the coherent species patterns
409 observed in $\Delta\delta^{15}N_{o\text{-}w}$.

410 Furthermore, no single amino acid appears to drive the observed patterns in $\Delta\delta^{15}N_{o\text{-}w}$ (Fig.
411 3a). This was surprising, as acidic AAs (Glu and Asp), the highest proportions in otoliths, are
412 known to exhibit elevated $\delta^{15}N$ (McClelland & Montoya 2002; Chikaraishi et al. 2009). Gadidae
413 otoliths tended to have lower Glu and Asp and higher Val, Leu, and Phe compared to other species,
414 yet these AA differences were insufficient to produce interspecies differences in $\Delta\delta^{15}N_{o\text{-}w}$. Mass
415 balance calculations of $\delta^{15}N_{oto}$ based on AA fractions separated Gadidae from non-Gadidae species
416 by 1.4 ‰ but did not recreate the 10.4 ‰ difference in $\Delta\delta^{15}N_{o\text{-}w}$ between Gadidae and non-Gadidae
417 species (Fig. S4). It is important to note these mass balance calculations rely on 8 amino acids
418 where others are likely present and contributing to the $\delta^{15}N_{oto}$. Glycine, proline and threonine, for
419 example, are present in moderate concentrations in otoliths of a reef associated snapper (McMahon
420 et al., 2011). Glycine (and threonine) are depleted in ^{15}N relative to many other amino acids
421 (McMahon et al., 2018; Whitney et al., 2019) and likely contribute to but cannot explain the
422 difference in $\Delta\delta^{15}N_{o\text{-}w}$ between Gadidae and non-Gadidae species. This exercise suggests that
423 variations of individual AA fractions cannot explain the data.
424
425 ***3.3.3 $\delta^{15}N_{wmt}$, N content, and otolith size***
426 A lack of correspondence of phylogeny, diet, or amino acids with $\Delta\delta^{15}N_{o\text{-}w}$ led us to
427 investigate $\Delta\delta^{15}N_{o\text{-}w}$ with other data that we had on hand: N content, $\delta^{15}N_{wmt}$, and otolith weight
428 were all investigated as possible correlates of $\Delta\delta^{15}N_{o\text{-}w}$. N content was correlated to $\Delta\delta^{15}N_{o\text{-}w}$ but
429 explained only a small fraction of the variation in $\Delta\delta^{15}N_{o\text{-}w}$ (Fig. 3b; Pearson correlation, *cor* =
430 0.39, $p < 0.001$). $\delta^{15}N_{wmt}$ was not correlated with $\Delta\delta^{15}N_{o\text{-}w}$ (Fig. S5; Pearson correlation, *cor* = -

0.59, $p = 0.30$).

$\Delta\delta^{15}N_{o\text{-}w}$ was negatively and significantly correlated with otolith weight (Fig. 3c; Pearson correlation, *cor* = 0.69, $p < 0.001$). Red snapper (*Lutjanus campechanus*), red grouper (*Epinephelus morio*), and snowy grouper (*Hyporthodus niveatus*), all of which have large, ventrally-flattened otoliths, were clear outliers (similarly, great northern tilefish, *Lopholatilus chamaeleonticeps*, have large, reticulate, ventrally flattened otoliths and a similar $\Delta\delta^{15}N_{o\text{-}w}$ as red snapper, red grouper, and snowy grouper; Fig. S6). Excluding red snapper, red grouper, and snowy grouper, the correlation improved from -0.69 to -0.93 ($p < 0.001$), with a geometric mean regression of $y = -0.018x + 1.63$ ($r^2 = 0.87$). Otolith weight per perimeter, a measure of the otolith weight standardized by how reticulate the otolith is (Fig. 3d) was strongly correlated with $\Delta\delta^{15}N_{o\text{-}w}$ and had only red snapper as an outlier (Pearson correlation, not including red snapper, *cor* = -0.94, $p < 0.001$, compared to red snapper-inclusive *cor* = -0.83, $p < 0.001$), with a geometric mean regression of $y = -0.99x + 2.19$). However, for the few species for which *n* was greater than 3, there was no correlation between otolith size and $\Delta\delta^{15}N_{o\text{-}w}$ within a species (Fig. S7), perhaps because of the small range of otolith weights for each species or because the otolith-size effect was obscured by inter-fish variations in life history (referred to as "otolith weighting" in *Section 3.3.4* below).

In summary, baseline or trophic factors may contribute to variability in the relationship between $\delta^{15}N_{wmt}$ and $\delta^{15}N_{oto}$; nevertheless, the 1:1 correlation is a dominant feature in the data reported here (Fig. 1a). Departures from the 1:1 line (a secondary signal) were related to otolith size (Fig. 3c, d). The specific effect of life history variation in $\delta^{15}N_{diet}$ on the 1:1 relationship was investigated with a model comparing otolith and muscle $\delta^{15}N$.

454 ***3.3.4 Model/data comparison***

455 Differences in temporal integration between otolith and muscle (henceforth "otolith
456 weighting") may introduce $\Delta\delta^{15}N_{o\text{-}w}$ variability due to changes in $\delta^{15}N_{diet}$ over life history. The
457 finding that the smallest differences between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ occurred in farmed species that
458 had spent the majority of their lives consuming a constant diet (Fig. 1a-b) indicates that the
459 temporal integration windows of otolith and muscle may affect $\Delta\delta^{15}N_{o\text{-}w}$ significantly. For wild
460 fish that undergo ontogenetic shifts in diet or habitat with concurrent changes in $\delta^{15}N$, $\delta^{15}N_{oto}$ and
461 $\delta^{15}N_{wmt}$ will record different $\delta^{15}N$ due to the shorter window of time captured by WMT.

462 Results of the model show that two requirements must be met for $\delta^{15}N_{oto}$ to equal $\delta^{15}N_{wmt}$,
463 i.e., to produce a $\Delta\delta^{15}N_{o\text{-}w}$ of 0 and a 1:1 relationship among multiple fish of the same species: (i)
464 temporally-integrated mean $\delta^{15}N_{diet}$ is the same for both muscle and otolith and (ii) intrinsic TDF_{oto}
465 and TDF_{wmt} must be the same. This can be seen for any age-1 fish in Fig. 4, which tend to plot
466 closest to the 1:1 line because otolith and WMT time averaging is relatively similar over the one-
467 year time period. On longer time periods (e.g., age-2 fish and older), fish tend to plot further away
468 from the 1;1 line due to the memory of previous $\delta^{15}N_{diet}$ retained by the otolith but not WMT.

469 For multiple fish individuals with similar lifetime trajectories in $\delta^{15}N_{diet}$ but which occupied
470 isotopically distinct environments, inter-fish variations in baseline $\delta^{15}N$ lead to a similar $\Delta\delta^{15}N_{o\text{-}w}$
471 among all same-age individuals (e.g., blue dashed line in each of Fig. 4a, b, c). Lower $\delta^{15}N_{diet}$ in
472 early life history (Fig. 4d, e) leads to $\Delta\delta^{15}N_{o\text{-}w} < 0$ (Fig. 4a, b); higher $\delta^{15}N_{diet}$ in early life history
473 (Fig. 4f) lead to $\Delta\delta^{15}N_{o\text{-}w} > 0$ (Fig. 4c). In scenarios for which dietary $\delta^{15}N$ reaches a plateau (a
474 realistic scenario for many species; e.g., Jennings, Greenstreet, et al. 2002; Marsh et al. 2017),
475 older fish have $\delta^{15}N_{oto}$ values that were closer to the 1:1 line than younger fish; that is, the $\delta^{15}N_{oto}$
476 of older fish is more likely to represent $\delta^{15}N_{wmt.}$ (Fig. 4a). This is due to, in older fish, the sequential

addition of invariant $\delta^{15}N_{oto}$ with each additional year.

The scenario of declining $\delta^{15}N_{diet}$ with age is unlikely to explain the roughly equal proportion of fish above and below the 1:1 line, as it would require that trophic level or baseline is higher for young compared to old fish for 50 % of fish individuals measured in Fig. 1. Most fish either increase in trophic level or stay the same over their life, so higher trophic level during early life history can be ruled out as the explanation in most cases. Baseline $\delta^{15}N$ may be higher in early life compared to later life in wild fish (e.g., Dale et al., 2011), and the possibility of baseline $\delta^{15}N$ differences of different life history stages should not be ignored. However, our measurements highlight that at least for some species, $\Delta\delta^{15}N_{o\text{-}w}$ results from differences between intrinsic TDF_{oto} and TDF_{wmt}, as shown by farmed fish reared on a constant diet.

In summary, TDF_{oto} is greater than TDF_{wmt} (e.g., rainbow trout, brown trout in Fig. 1c) or when $\delta^{15}N_{diet}$ is higher in early life history compared to adult life history (Fig. 4c), the result is $\Delta\delta^{15}N_{o\text{-}w}$ greater than or equal to 0; meanwhile, an increase in $\delta^{15}N_{diet}$ (common in nature) may explain some of the data with $\Delta\delta^{15}N_{o\text{-}w} < 0$ (Fig. 4 a-b). Lower TDF_{oto} than TDF_{wmt} (as in cod and Atlantic croaker, Fig. c) would also produce $\Delta\delta^{15}N_{o\text{-}w} < 0$.

As mentioned above (*Section 3.3.1*), $\Delta\delta^{15}N_{o\text{-}w}$ equates to $\Delta TDF_{o\text{-}w}$ if most of the otolith was grown under a constant $\delta^{15}N_{diet}$. This applies in situations where $\delta^{15}N_{diet}$ is unchanged over life history, or, as shown here, if $\delta^{15}N_{diet}$ has not changed recently, as for old fish, whose length has reached an asymptote but for whom otolith mass continues to accrue, thus adding $\delta^{15}N$-invariant material to both otolith and WMT. Many fish obtained for the current study were from retail fish markets and were adult fish of varying ages. Therefore, in many cases (e.g., most Perciformes), a species with $\Delta\delta^{15}N_{o\text{-}w}$ close to 0 may also equate to a species with $\Delta TDF_{o\text{-}w}$ close to 0. In theory, as the mean literature value for TDF_{wmt} is 3.4 ‰, TDF_{oto} may therefore also be

close to 3.4 ‰ in many cases. More precisely, the mean $\Delta\delta^{15}N_{o\text{-}w}$ of -0.7 ‰ would equate to a mean TDF_{oto} of 2.7 ‰.

We have determined through elimination of factors (including AA and N content, phylogeny, and life history modeling) that the coherent species patterns in $\Delta\delta^{15}N_{o\text{-}w}$ result largely from variations in intrinsic TDF_{oto}. Life history variations in $\delta^{15}N_{diet}$ do produce variability, seen most clearly in the herring data (Fig. 1, Fig. S1), and which is likely encompassed in the standard deviations of the $\Delta\delta^{15}N_{o\text{-}w}$ values for each species.

Other sources of variability that were not directly investigated in the current study include the effects of starvation and temperature on $\Delta\delta^{15}N_{o\text{-}w}$. Starvation is known to increase $\delta^{15}N_{wmt}$ (Gaye-Siessegger et al. 2004; McMahon & McCarthy 2016; McMahon et al. 2015), and a preferential effect on $\delta^{15}N_{wmt}$ could affect $\Delta\delta^{15}N_{o\text{-}w}$. Temperature can affect otolith amino acid profiles (Hüssy et al. 2004); as AAs have distinct $\delta^{15}N$, changes to AA composition have potential to alter $\delta^{15}N_{oto}$. and thus $\Delta\delta^{15}N_{o\text{-}w}$.

N content, amino acid content, and phylogeny were not major drivers of $\Delta\delta^{15}N_{o\text{-}w}$. Measurements of TDFs and a high degree of correlation with otolith size indicates that at least some of the variations in $\Delta\delta^{15}N_{o\text{-}w}$ arise from variations in TDF_{oto} (as opposed to life history, N content, or HAA). The main correlate of TDF_{oto} appears to be otolith size.

3.4 Hypothesis for the major cause of variation in $\Delta\delta^{15}N_{o\text{-}w}$

The $\delta^{15}N$ of the circulating pool of amino acids in blood sets the isotopic starting point for animal proteins, and nitrogen isotope fractionation does not appear to occur during protein synthesis (Sick et al. 1997; Schoeller 1999). Otolith proteins are formed in the Golgi apparati in macular cells of the otolith saccular membrane and then secreted or exocytosed into the

endolymph, the membrane-enclosed, ion- and organic-rich fluid from which the otolith precipitates in the fish inner ear. If all nitrogen reaching the otolith were incorporated into the otolith, the $\delta^{15}N$ would be similar to that of the arriving $\delta^{15}N$ pool supplied to the otolith, which would itself be strongly correlated to muscle $\delta^{15}N$. Thus, the taxonomic variation in the $\delta^{15}N$ relationship between otolith-bound N and muscle argues for N isotope fractionating processes in the endolymph, likely near or at the site of the actively-forming otolith.

In other biominerals, e.g., tooth enamel, an organic layer surrounding the forming mineral is degraded through post-secretory sequential degradation (PSSD; Robinson et al., 1998). PSSD is selective, targeting specific proteins, and is required for proper mineral formation in teeth (Robinson et al. 1978; Smith 1998; Simmer & Hu 2002) and bone (Wuthier 1969; Dean et al. 1985; Alini et al. 1992). In tooth enamel (Smith 1998; Simmer & Hu 2001) and bone collagen (Alini et al. 1992; Wuthier 1969; Stickens 2004), components of the organic matrix are removed through proteolytic processing, while the underlying proteinaceous organic matrix controls and directs mineral formation. In otoliths, previous work has identified proteases (Thomas et al. 2018) and protease inhibitors (Kang et al. 2008; Weigele et al. 2015; Thomas et al. 2018) in the otolith endolymph. Proteomic evidence suggests that proteolysis of Matrix metalloproteinase 2, an important protein in otolith formation and structure, regulates the timing of otolith mineralization (Thomas et al. 2018). We propose that such a protein degradation mechanism also controls the proportion of residual OM that becomes occluded by aragonite increment deposition, impacting the $\delta^{15}N$ of the occluded OM. In fish that make large otoliths, the faster accretion may result in more protein being capped by increment formation as opposed to being degraded, resulting in less ^{15}N enrichment in the occluded OM of faster-growing, larger otoliths.

This hypothesis was investigated using a one-box model (Fig. 5). The proportions of

accretion (with no isotope fractionation) and degradation (with isotope fractionation) of a layer of organic matrix were varied to achieve the full range of TDF_{oto}. A value of 10 ‰ was chosen for the isotope effect of degradation (ε_{degr}) based on the range in $\Delta\delta^{15}N_{o\text{-}w}$ (10.4 ‰ absolute difference between cod and lake trout, -7.3 ‰ and 3.1 ‰ respectively), allowing for the possibility of simulating the full range of values in TDF_{oto}.

The results suggest that a TDF_{oto} of 6 ‰, e.g., for rainbow trout, a small otolith species, could result from 80 % organic monolayer degradation and 20 % preservation in the otolith (Fig. 5). Cod, with a TDF_{oto} of -1.6 ‰, could result from 0.1 % degradation and 99.9 % preservation of organic matter. In contrast, most otoliths, with TDF_{oto} close to 4 ‰, result from 60% degradation of the organic envelope and 40 % preservation in the otolith.

The size dependency of $\Delta\delta^{15}N_{o\text{-}w}$ can be explained as follows: the smaller the otolith, and therefore the larger the surface area (SA) to volume (V) ratio, the greater the percentage of organic matter lost through PSSD, which occurs at the otolith surface, and the greater the $\delta^{15}N$ elevation of the OM incorporated into the otolith (see Fig. S8 for a rough calculation of SA:V). The larger the otolith, the smaller the SA:V and the lesser the percentage of OM that is degraded, resulting in a greater percentage of organic matter incorporated into the otolith (accretion) without N isotope fractionation. At a low proportional rate of PSSD relative to otolith incorporation, $\delta^{15}N_{oto}$ can be lower than $\delta^{15}N_{wmt}$, yielding a negative $\Delta\delta^{15}N_{o\text{-}w}$.

Given our hypothesis, changes in otolith shape could also impact $\delta^{15}N_{oto}$. For example, the weaker negative $\Delta\delta^{15}N_{o\text{-}w}$ vs. otolith mass trend (Fig. 3d) may derive from ontogenetic transitions in otolith shape (e.g., red snapper). However, we see minimal evidence for this within a species, at least for the small range of intra-specific otolith masses in the current study (Fig. S7). This will be an important question for future studies that may compare $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$ over a much

570 wider range of otolith sizes.

571 Previous work shows that a branching pathway, resulting in simultaneous $\delta^{15}N$ elevation
572 of protein and ^{15}N depletion of urea in the liver, occurs in mammals (Sutoh et al. 1993; Sick et al.
573 1997). The main products of these reactions, urea and protein, are offset by 4-10 ‰, and urea and
574 ammonia are ^{15}N-depleted relative to diet. Cod, with the lowest $\delta^{15}N_{oto}$, had $\delta^{15}N_{oto}$ that was lower
575 than $\delta^{15}N_{diet}$, resulting in negative TDF_{oto}. We hypothesize that a branching pathway provides
576 amino acids with low $\delta^{15}N$ ($\delta^{15}N < \delta^{15}N_{diet}$) to the otolith, and that the PSSD that usually elevates
577 $\delta^{15}N_{oto}$ is not occurring. It is not likely that cod are unique in having a branching pathway supplying
578 low-$\delta^{15}N$ amino acids to the otoliths, but simply that this low $\delta^{15}N$ organic matter pool can be
579 observed in cod due to lack of the normal, $\delta^{15}N$-elevating process (i.e. PSSD) in non-cod otoliths.

580 In conclusion, within individual fish species, the data indicate that variation in $\delta^{15}N_{oto}$
581 records $\delta^{15}N_{wmt}$ variation, with a relatively small offset ($\Delta\delta^{15}N_{o-w}$) in most cases but with important
582 exceptions (e.g., species in the Gadidae family). $\Delta\delta^{15}N_{o-w}$ appears to be dominated by variation in
583 TDF_{oto}, not TDF_{wmt}, based upon evidence from multiple tissues in farmed species and the otolith
584 size dependency of $\Delta\delta^{15}N_{o-w}$. Amino acid data showed the lowest proportions of Glu and Asp in
585 Gadidae species, but the low proportions alone were insufficient to explain the low $\delta^{15}N_{oto}$. N
586 content, phylogeny, and life history variations were also ruled out as important controls on $\Delta\delta^{15}N_{o-w}$.
587 Species with large otoliths have negative $\Delta\delta^{15}N_{o-w}$ whereas species that make small otoliths
588 have positive $\Delta\delta^{15}N_{o-w}$. We suggest that this effect derives from targeted proteolytic processing of
589 the organic envelope on the surface area of the otolith, producing elevated $\delta^{15}N$ in the organic
590 residuum that becomes incorporated into the otolith. For cross-taxonomic comparisons of $\delta^{15}N_{oto}$,
591 a potential way forward would be quantitative surface area analysis, for example, by N_2 gas
592 adsorption (Chiou et al. 1990; Pennell et al. 1995).

593 The tentative evidence for N isotope fractionating processes during otolith formation calls
594 for experiments on the interaction among organic molecules and between the otolith and mineral
595 interface in actively forming otoliths. However, one could that argue that more immediate need is
596 for controlled dietary studies of juvenile and adult fish, which would allow for direct comparison
597 of diet, otolith, and WMT from multiple time points over fish development.

598 Soft tissues such as WMT are not preserved, making it impossible to compare $\delta^{15}N_{wmt}$ of
599 historical or fossil fishes to modern fish, and bone collagen $\delta^{15}N$ has been found to be susceptible
600 to diagenesis (Serban et al. 1988; Tuross et al. 1988; Silfer et al. 1992). Otoliths provide robust
601 protection to fossil $\delta^{15}N$ even in suboptimal preservation environments, so long as pre-cleaning
602 treatments are conducted prior to analysis of otolith-bound OM (Lueders-Dumont et al. 2018). The
603 findings reported here provide ground-truthing information as well as broader guidance for the
604 interpretation of $\delta^{15}N_{oto}$ from historical and fossil fish assemblages.

605

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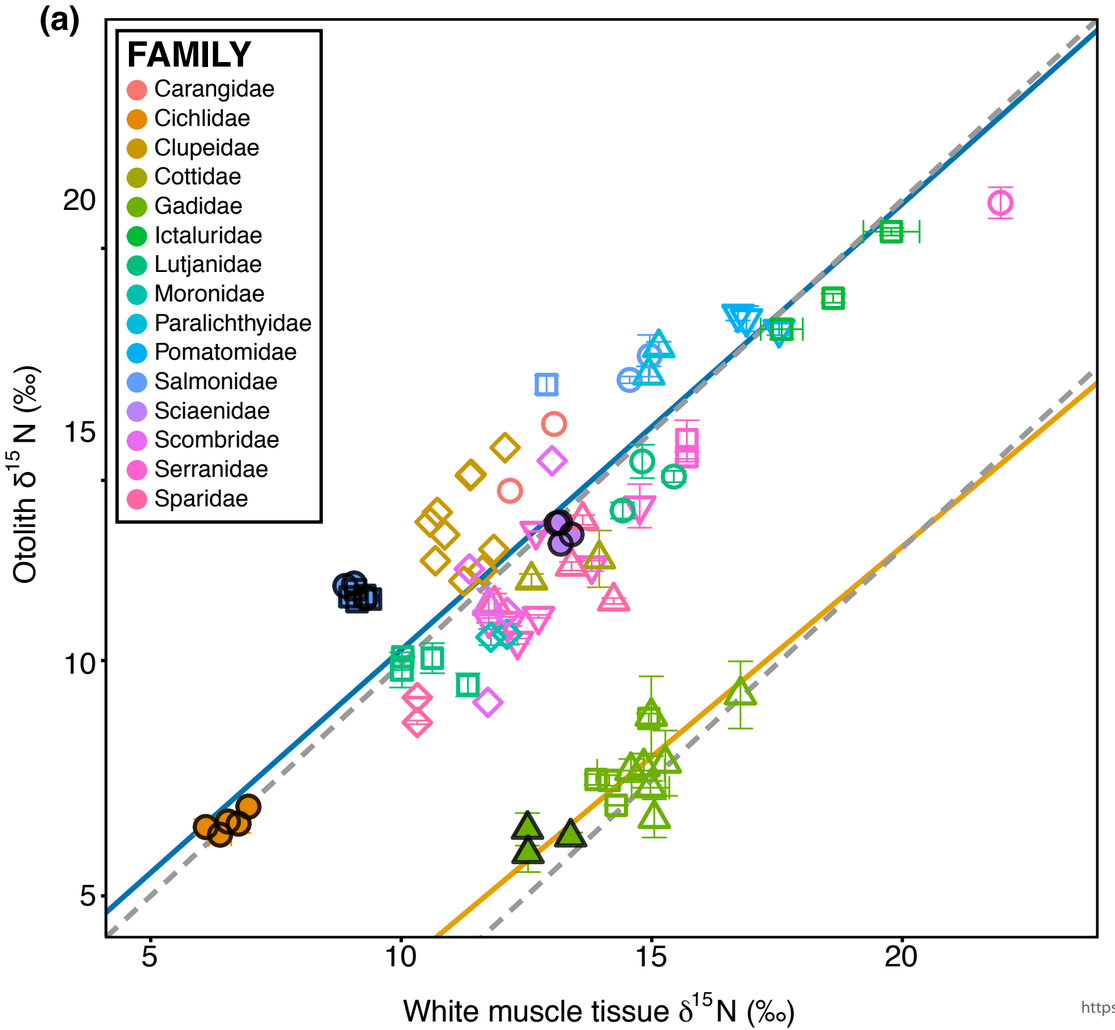
Figure captions

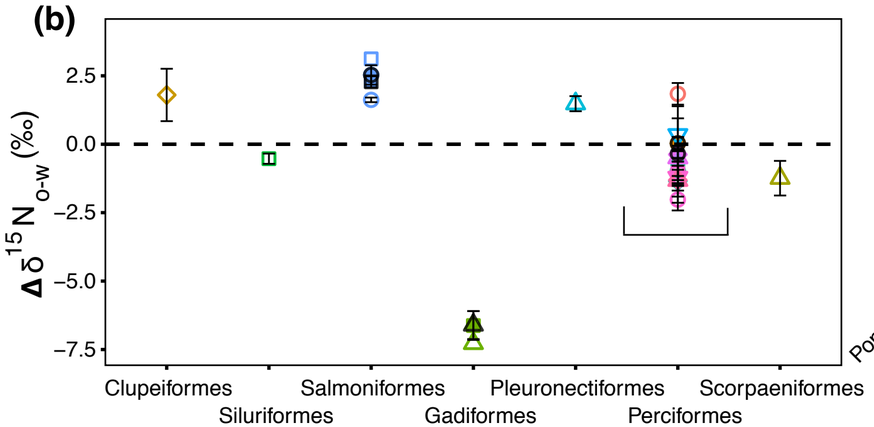
Fig. 1. Relationship between $\delta^{15}N_{oto}$ and $\delta^{15}N_{wmt}$. (a) Wild and farm-raised fish $\delta^{15}N_{oto}$ *vs.* $\delta^{15}N_{wmt}$ (‰ ± 1σ). Solid lines correspond to geometric mean regression for non-Gadidae (blue) and Gadidae (yellow) species. Dashed lines correspond to 1:1 lines with y-intercepts at 0 and -7.5. Filled symbols are for aquaculture species. (b) Otolith-muscle offset ($\Delta\delta^{15}N_{o-w}$) averages across species (± 1σ) for wild and farm raised fish**,** organized by phylogeny (shown from least evolutionarily derived to most derived) (Betancur-R. et al., 2013). Inset shows only families in the Perciforme order, again organized from least to most derived. (c) Trophic discrimination factors for otolith (TDF_{oto}) and muscle (TDF_{wmt})**.** TDF_{wmt} differed from TDF_{oto} for all four species (p-value < 0.05 in all cases).

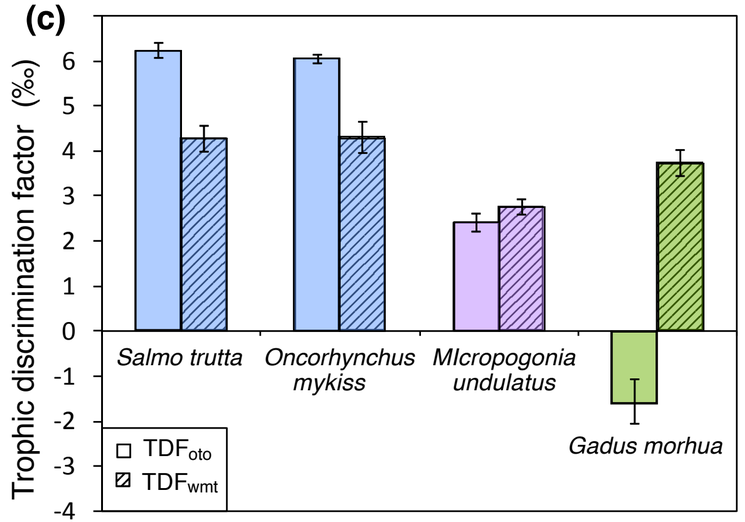
Fig. 2. Species differences in amino acid fractions. (a) Discriminant analysis resulted in three clusters based on amino acid fractions. (b) Amino acid fractions for all species for which AA data was obtained. (c) Mean (± 1 SD) amino acid fractions by cluster type. Colors and symbols same as for Fig. 1.

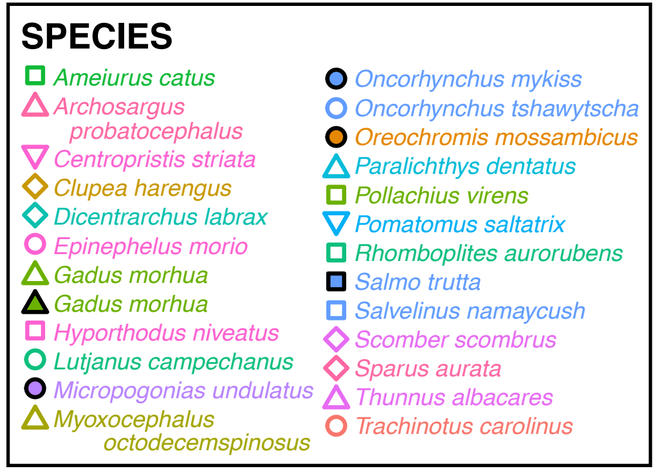
Fig. 3. Examination of possible drivers of $\Delta\delta^{15}N_{o-w}$. (a) amino acid fraction, shown in order of "source" AAs, aliphatic AAs, and acidic AAs; (b) N content per mg of otolith analyzed; (c) otolith weight (mg); and (d) otolith weight (mg) per perimeter (mm). The dashed line is the 1:1 line from Fig. 1 and equates to $\Delta\delta^{15}N_{o-w} = 0$. Symbols and colors are the same as for Fig. 1, where color corresponds to family, symbols corresponds to species, and filled symbols correspond to fish from aquaculture settings.

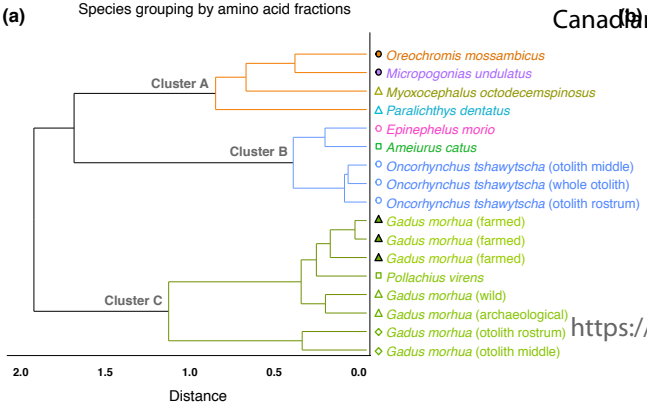
916 **Fig. 4. Model calculations of life history $\delta^{15}N_{diet}$, $\delta^{15}N_{oto}$, $\delta^{15}N_{wmt}$.** (a-c) show $\delta^{15}N_{oto}$ *vs.*
917 $\delta^{15}N_{wmt}$ resulting from (d-f) idealized life history variations in $\delta^{15}N_{diet}$. Age is indicated by
918 different symbols. Otolith integrates over entire life whereas muscle integrates only 3 months.
919 Gray dashed line shows 1:1 line through the plot origin; blue dashed line indicates linear best-fit
920 line across all age-8 fish. Arrows in (c) aid with visualization and refer to the direction of $\delta^{15}N_{wmt}$
921 and $\delta^{15}N_{oto}$ from ages 2 to 3.
922
923 **Fig. 5: Schematic of the one-box model view for nitrogen isotope fractionation in otoliths.**
924 The size of the otolith is representative, with small otoliths (a) corresponding to high TDF_{oto} and
925 large otoliths (c) corresponding to low TDF_{oto} as reported in Fig. 3d.

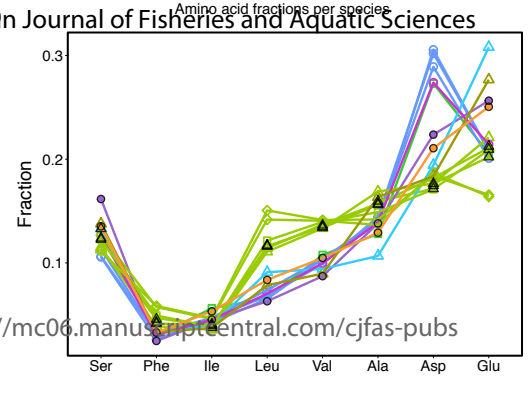

(a)
FAMILY
Carangidae
Cichlidae
Clupeidae
Cottidae
Gadidae
Ictaluridae
Lutjanidae
Moronidae
Paralichthyidae
Pomatomidae
Salmonidae
Sciaenidae
Scombridae
Serranidae
Sparidae
Otolith δ15N (‰)
White muscle tissue δ15N (‰)
5
10
15
20

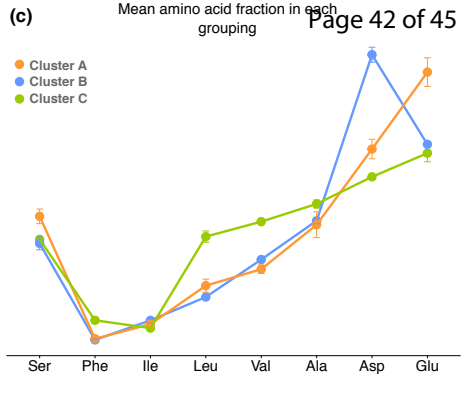

(b)
Δ δ15No-w (‰)
2.5
0.0
−2.5
−5.0
−7.5
Clupeiformes
Siluriformes
Salmoniformes
Gadiformes
Pleuronectiformes
Perciformes
Scorpaeniformes
2
1
0
−1
−2
Pomatomidae
Scombridae
Carangidae
Cichlidae
Lutjanidae
Moronidae
Sciaenidae
Sparidae
Serranidae
FAMILY (Perciformes)

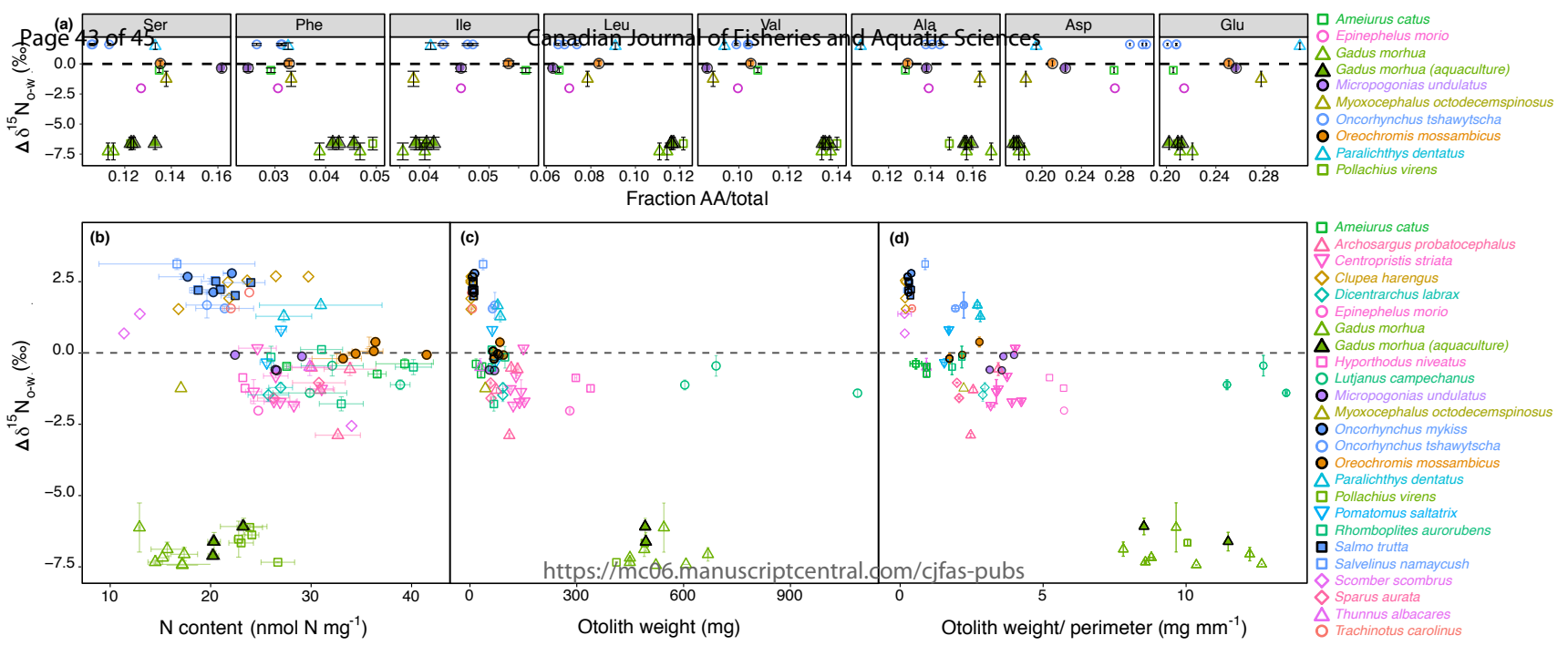

(c)
Trophic discrimination factor (‰)
6
5
4
3
2
1
0
-1
-2
-3
-4
Salmo trutta
Oncorhynchus mykiss
MIcropogonia undulatus
Gadus morhua
TDFoto
TDFwmt

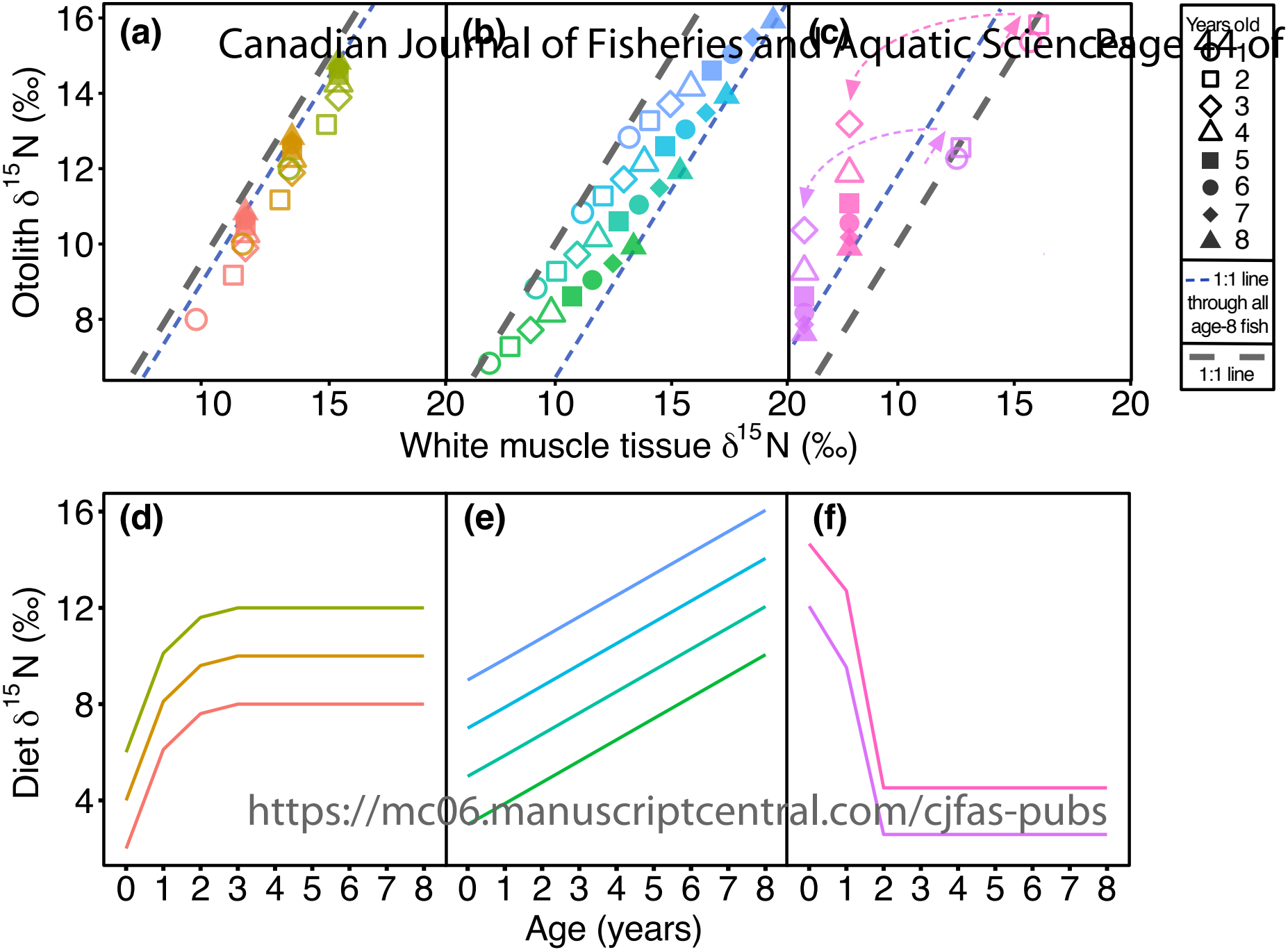

SPECIES
Ameiurus catus
Archosargus probatocephalus
Centropristis striata
Clupea harengus
Dicentrarchus labrax
Epinephelus morio
Gadus morhua
Gadus morhua
Hyporthodus niveatus
Lutjanus campechanus
Micropogonias undulatus
Myoxocephalus octodecemspinosus
Oncorhynchus mykiss
Oncorhynchus tshawytscha
Oreochromis mossambicus
Paralichthys dentatus
Pollachius virens
Pomatomus saltatrix
Rhomboplites aurorubens
Salmo trutta
Salvelinus namaycush
Scomber scombrus
Sparus aurata
Thunnus albacares
Trachinotus carolinus

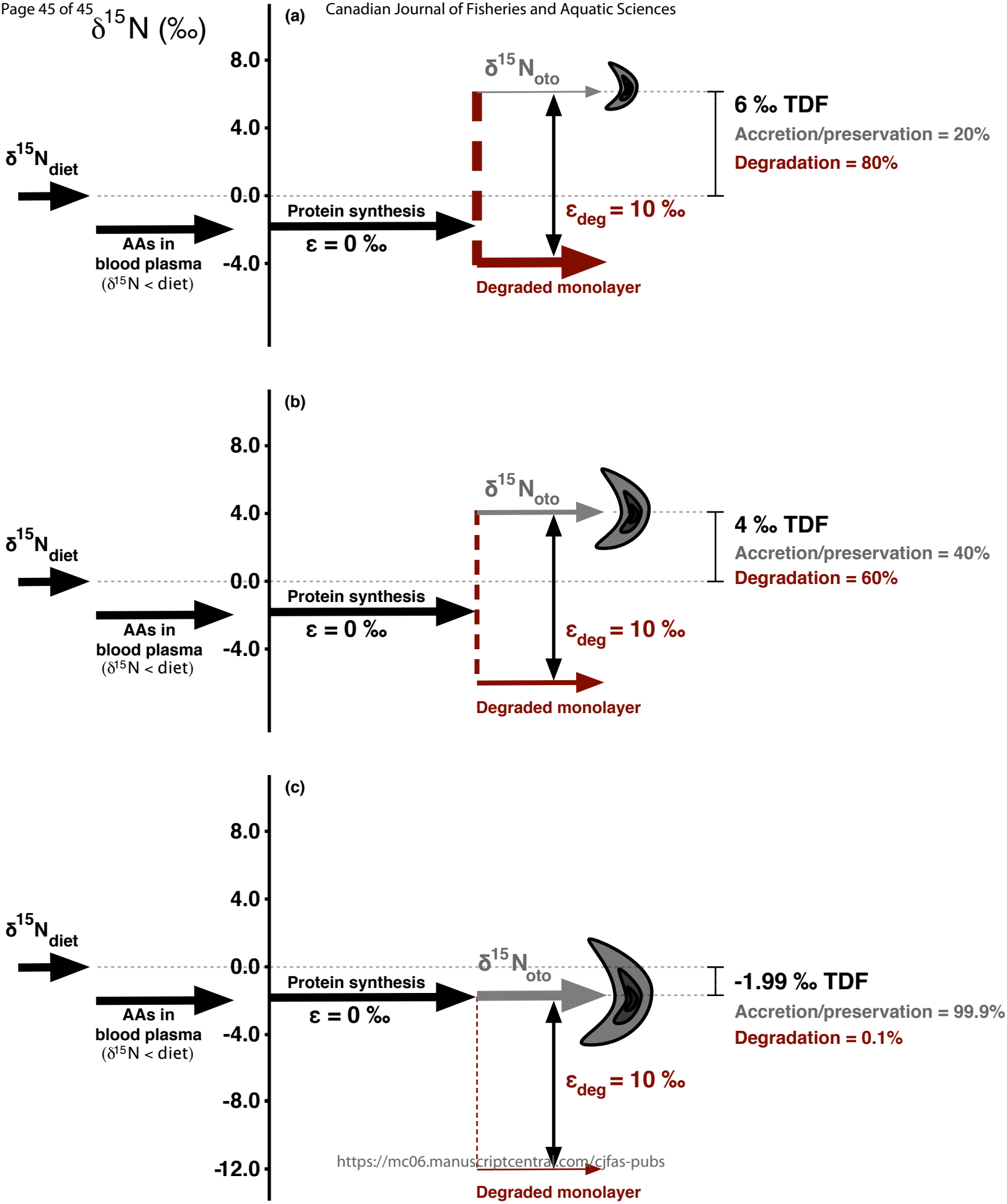









(a)
(b)
(c)
(d)
(e)
(f)
Otolith δ15N (‰)
White muscle tissue δ15N (‰)
Diet δ15N (‰)
Age (years)
Years old
1
2
3
4
5
6
7
8
1:1 line through all age-8 fish
1:1 line
https://mc06.manuscriptcentral.com/cjfas-pubs


δ15N (‰)
(a)
8.0
4.0
0.0
-4.0
δ15Ndiet
AAs in blood plasma (δ15N < diet)
Protein synthesis
ε = 0 ‰
δ15Noto
εdeg = 10 ‰
Degraded monolayer
6 ‰ TDF
Accretion/preservation = 20%
Degradation = 80%
(b)
8.0
4.0
0.0
-4.0
δ15Ndiet
AAs in blood plasma (δ15N < diet)
Protein synthesis
ε = 0 ‰
δ15Noto
εdeg = 10 ‰
Degraded monolayer
4 ‰ TDF
Accretion/preservation = 40%
Degradation = 60%
(c)
8.0
4.0
0.0
-4.0
-8.0
-12.0
δ15Ndiet
AAs in blood plasma (δ15N < diet)
Protein synthesis
ε = 0 ‰
δ15Noto
εdeg = 10 ‰
Degraded monolayer
-1.99 ‰ TDF
Accretion/preservation = 99.9%
Degradation = 0.1%