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Variations in isotope incorporation rates and trophic discrimination factors of carbon and nitrogen stable isotopes in scales from three European sea bass (*Dicentrarchus labrax*) populations

Rodde Charles [1, 2, 3, 9] , De Verdal Hugues [1, 2, *], Lefebvre Sebastien [5], Menniti Christophe [6], Vandeputte Marc [7], Clota Frederic [7], Allal Francois [4], McKenzie David [9], Benzie John A.H. [3], Nahon Sarah [8]

[1] CIRAD, UMR ISEM, F-34398 Montpellier, France
[2] ISEM, Université de Montpellier, CNRS, EPHE, IRD, Montpellier, France
[3] Worldfish, Jalan Batu Maung, Bayan Lepas, 11960 Penang, Malaysia
[4] MARBEC, Université de Montpellier, CNRS, Ifremer, IRD, Palavas-les-Flots, France
[5] Université de Lille, CNRS, Université Littoral Côte d'Opale, UMR 8187, LOG Laboratoire d'Océanologie et Géosciences, station marine de Wimereux F-59000, France
[6] Centre de Formation et de Recherche sur les Environnements Méditerranéens (CEFREM), UMR 5110, Université de Perpignan Via Domitia, 66860 Perpignan, France
[7] Université Paris-Saclay, INRAE, AgroParisTech, GABI, F-78350 Jouy-en-Josas, France
[8] INRAE, Université de Pau et des Pays de l'Adour, E2S UPPA, UMR 1419, Nutrition, Métabolisme, Aquaculture, Saint Pée sur Nivelle F-64310, France
[9] MARBEC, Université de Montpellier, CNRS, Ifremer, IRD, Palavas-les-Flots, France

* Corresponding author : Hugues de Verdal, email address : hugues.deverdal@cirad.fr

charles.rodde@cirad.fr ; sebastien.lefebvre@univ-lille.fr ; menniti@univ-perp.fr ; marc.vandeputte@inrae.fr ; frederic.clota@inrae.fr ; Francois.Allal@ifremer.fr ; david.mckenzie@cnrs.fr ; j.benzie@cgiar.org ; sarah.nahon@inrae.fr

Abstract :

Carbon (δ13C) and nitrogen (δ15N) stable isotope analyses are used in marine ecology to study trophic relationships and migrations of species since they reflect dietary sources consumed which may vary geographically. However, better estimations of isotope incorporation rates and trophic discrimination factors (TDF) under controlled conditions are required. Moreover, variability of isotope incorporation rates and TDF among and within populations has been poorly described, especially in fish scales, whereas the use of non-lethal method is becoming a standard. This study aimed to experimentally assess whether carbon and nitrogen isotope incorporation rates (λC and λN, respectively) and TDF of scales vary in the European sea bass (Dicentrarchus labrax) among (1) Atlantic, West Mediterranean and East Mediterranean populations, (2) sexes and (3) individuals. Fish were reared under controlled conditions and switched from a diet 1 to a diet 2 with different δ13C and δ15N values. Scales were sampled

repeatedly on 16 fish within the three populations, from the day of diet change (day 0) to the end of the experiment (day 217). Isotope incorporation rates of scales and TDF were determined using a time-dependent model. Isotopic carbon and nitrogen half-lives (t50C and t50N) were similar among the three populations but males had significantly lower t50C and t50N than females (29 ± 2 and 35 ± 2 days vs. 53 ± 7 and 80 ± 11 days, respectively). Females had higher growth rates but lower catabolic rates than males. Variability of λC and λN was large within sexes: t50C ranged from 17 to 159 days and t50N ranged from 18 to 342 days among individuals. Thus, variability between sexes and among individuals must be considered to avoid misinterpretation in field-based studies. For the 48 fish, TDF were 4.91 ± 0.03 and 2.46 ± 0.06‰ for carbon and nitrogen, respectively, and similar between sexes and among populations. Besides, TDF varied among individuals from 2.95 to 5.59‰ and from 0.93 to 3.55‰ for carbon and nitrogen, respectively. Empirical mixing models were run to estimate how different TDF influenced estimation of the contributions of food sources to diet of their consumer. The output differed considerably when using TDF from fish literature or those estimated herein, which confirms that a tissue-specific TDF must be used to avoid misinterpretation in field-based studies. Individual variation in TDF did not, however, influence estimation of the contributions of food sources, confirming that scales are a valid tissue for non-lethal sampling.

Highlights

► We determined $\delta^{13}C$ and $\delta^{15}N$ values in scales of *Dicentrarchus labrax.* ► Each fish was sampled 17 times without deleterious effects on growth or survival. ► Incorporation rates were higher in males and strongly variable among individuals. ► Trophic discrimination factors were strongly variable among individuals. ► Individual variability should be taken into account in field-based studies.

Keywords : Fish, Metabolic pathway, Time-dependant model, Mixing model,non-lethal sampling

1. Introduction

Analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes has proven to be a powerful tool in marine ecology, to study trophic relationships and migrations of various species through time and space (Hansson et al., 1997; Perga and Gerdeaux, 2003; Dempson et al., 2010; Sweeting, 2010). The $\delta^{13}C$ and $\delta^{15}N$ values of organisms reflect those of assimilated dietary sources as it is generally accepted that consumers are enriched by 1.0 and 3.5‰ in ^{13}C and ^{15}N, respectively, relative to their diets (Fry and Arnold, 1982; Minagawa and Wada, 1984). However, these assumptions are not universal and reconstruction of diet history as well as quantification of trophic relationships of organisms in their environment require better estimations of both carbon and nitrogen isotope incorporation rates and trophic discrimination factors (TDF) under controlled conditions (Wolf et al., 2009; Martínez del Rio and Carleton, 2012).

Isotope incorporation rate is defined as the time required by an organism to acquire the isotopic composition of its new diet (Martínez del Rio and Carleton, 2012). This variable is essential to determine the temporal window in which stable isotope data can be used to elucidate the diet of an animal (Perga and Gerdeaux, 2005; Wolf et al., 2009). Stable isotope values of an organism in a situation of disequilibrium, after a change in diet, do not represent either the past or the present diet (Sweeting, 2010). It is well established that isotope incorporation rates are higher in metabolically active species, organisms or tissues, depending on both growth rate (i.e., anabolic rate or adjunction of new tissues) and catabolic rate (i.e., replacement of tissues; Hesslein et al., 1993; MacNeil et al., 2006). Generally, isotope incorporation rates are higher in liver and plasma than in muscle and red blood cells of fishes (Carleton and Martínez del Rio, 2010). Moreover, isotope incorporation rates vary with environmental conditions such as temperature, food quantity and quality as well as the

physiological state of the animal, such as ontogenetic stage or level of stress (Witting et al., 2004; Carleton and Martínez del Rio, 2010; Bloomfield et al., 2011; Carter et al., 2019).

Trophic discrimination factor, the difference between stable isotope values of a consumer and its diet when at isotopic equilibrium, also fluctuates considerably. For fishes, carbon and nitrogen TDF vary from 0.2 to 4.0‰ and from -0.4 to 5.5‰, respectively (Sweeting et al., 2007a; 2007b). Accurate values are required to interpret relationships across trophic levels. A robust estimation of TDF is also a fundamental requirement for mixing models that predict the proportional composition of consumers' diets from stable isotope data (Phillips et al., 2014). Physiological mechanisms underlying TDF are not thoroughly understood but result from the balance between processes of assimilation and excretion of light versus heavy elements acquired in the food (Minagawa and Wada, 1984; Ponsard and Averbuch, 1999; Olive et al., 2003). Thus, TDF is influenced by both dietary and non-dietary factors (Trueman et al., 2005; Barnes et al., 2007; Matley et al., 2016; Nuche-Pascual et al., 2018).

In isotope-based studies, variation among individuals has been evaluated as the variance of $\delta^{13}C$ and $\delta^{15}N$ values. For fishes, $\delta^{13}C$ and $\delta^{15}N$ values of tissues have been shown to vary within and among populations (Barnes et al., 2008), and especially with sex (Kim et al., 2012; Marcus et al., 2019). To date, however, studies of variation in isotope incorporation rates and TDF within and among populations are scarce. When distinct isotope values appear among individuals, the assumption commonly made is that they must have been feeding on distinct food sources (e.g. Grey, 2001). However, several studies revealed an inherent variability between individuals fed with a same diet (Matthews and Mazumder, 2004; Araújo et al., 2007; Barnes et al., 2008). These studies underscored the need to take this variability into account in field-studies before concluding that individuals have distinct feeding habits. Differences in individual physiology are suggested to be the cause of this inherent individual variability (Bearhop et al., 2004). Isotope incorporation rates are estimated by changing from

119 one isotopically distinct experimental diet to another, and then sampling tissues over time. In
120 fishes, most isotope incorporation rate studies have used dorsal white muscle, so individuals
121 must be sacrificed at each sampling point to monitor stable isotope values (Hesslein et al.,
122 1993; German and Miles, 2010; Madigan et al., 2012). This precludes the study of individual
123 variation in the kinetics of isotopic incorporation. Using non-lethal methods, such as sampling
124 of red blood cells, plasma, fins and scales, would permit multiple sequential samplings on the
125 same individuals, to study variation in isotope incorporation rates. Moreover, the development
126 of non-lethal methods is desirable from a perspective of animal welfare (European Union,
127 2010; Australian Government, 2013; US Government, 2015). To our knowledge, the few
128 studies that have sampled the same individuals over time after diet change have shown
129 marked variation in carbon and nitrogen isotope incorporation rates (Hilderbrand et al., 1996;
130 Voigt et al., 2003; Evans Ogden et al., 2004; Kim et al., 2012). Similarly, individual variation
131 in TDF was revealed within different species (Lecomte et al., 2011; Kurle et al., 2014; Galván
132 et al., 2016).

133 In the present study, we estimated isotope incorporation rates and TDF of carbon and
134 nitrogen stable isotopes in the scales of 48 European sea bass (*Dicentrarchus labrax)* from
135 three distinct populations (Atlantic AT, West Mediterranean WM and East Mediterranean
136 EM; Guinand et al., 2017) reared under controlled conditions. European sea bass is highly
137 prized by both commercial and sports fishermen, but a severe decline in stocks has recently
138 raised concern about the conservation status of the species (de Pontual et al., 2019). The use
139 of carbon and nitrogen stable isotope analyses is a good tool to improve the management of
140 this species because it can reveal its feeding habitats and migrations (Cambiè et al., 2016). We
141 assessed whether there were differences in isotope incorporation rates and TDF among (1) the
142 three populations, (2) sexes and (3) individuals. Finally, empirical stable isotope mixing
143 models were run with different carbon and nitrogen TDF to assess their influence on dietary

predictions in field-based studies. Scales are a superposition of an organic layer mainly composed of proteins (mostly collagen) and an inorganic layer which is a carbonate salt (Hutchinson and Trueman, 2006). However, scales are not an inalterable record: several studies support the existence of a catabolic activity in scales with the destruction and the renewal of collagen by cells within the scale structure, respectively named osteoclasts and osteoblasts (Sire et al., 1990; Suzuki et al., 2000). Thus, it can be hypothesized that isotope incorporation rate in scale might not be only driven by growth.

2. Material and methods

2.1. Ethics statement

This study was carried out in accordance with the recommendations of Directive 2010-63-EU on the protection of animals used for scientific purposes. Protocols were approved by C2EA−36 ("Comité d'éthique en expérimentation animale Languedoc-Roussillon") under the authorization APAFiS n° 2018081714549886 (version 2).

2.2. Animals and rearing conditions

A controlled feeding experiment was conducted on the three populations of European sea bass: AT, WM and EM. Fish were produced at the Ifremer Experimental Aquaculture Research Station in Palavas-les-Flots, France (43°31'13°N; 3°54'37°E). Fish were produced on the same day by artificial fertilization and each population reared in triplicate in nine separate tanks from birth to 188 days of age. At that stage, 51 AT, 46 WM and 51 EM fish were randomly selected from the different tanks, individually identified by injecting a passive integrated transponder tag (PIT-tag, Biolog-id®), then grouped in a 1500 L tank 21 days before the beginning of the diet change experiment. The tank was supplied with recirculated water treated by UV, sand filter and biological filter, renewal rate was 100% per hour. Water

167 temperature was 21.1 ± 0.9 °C and oxygen saturation rate was on average 11.9 mg L^{-1}. An
168 artificial photoperiod was set up to provide a light-dark ratio of 12:12 hours.

169 *2.3. Diet change experiment*

170 For 100 days until the beginning of the diet change experiment (at 209 days post
171 hatching), fish were fed *ad libitum* (approximatively 2.5% of their body weight per day) with
172 a commercial diet (diet 1, "Neo Supra-S", Le Gouessant Aquaculture®, Lamballe, France).
173 Then, fish were switched to a diet (diet 2) manufactured at the experimental fish farm of
174 Donzacq (INRAE, France, 43°39'20°N 0°47'24°W). Each diet was taken from a single bag of
175 feed, to avoid any potential variability in $\delta^{13}C$ and $\delta^{15}N$ values among feed batches. Diet 2
176 was formulated with a similar proximate composition to diet 1 (58% of crude protein, 13% of
177 fat, 8.4% of carbohydrates, 10% of ash and 0.5% of fiber) in order to minimize nutritional
178 stress, but it was formulated to have markedly different $\delta^{13}C$ and $\delta^{15}N$ values. The differences
179 in $\delta^{13}C$ and $\delta^{15}N$ values between the two diets were achieved by inclusion of ingredients from
180 different origins. The values of $\delta^{13}C$ were -22.24 ± 0.08‰ in diet 1 *vs.* -25.30 ± 0.07‰ in diet
181 2, while $\delta^{15}N$ was 7.98 ± 0.12‰ in diet 1 *vs.* 6.39 ± 0.15‰ in diet 2. Diets were supplied by
182 an automatic self-feeder that fish were able to activate as desired (Covès et al., 2006).

183 *2.4. Growth and scale sampling*

184 At the beginning of the diet change experiment (day 0, i.e. at 209 days post hatching), 16
185 tagged fish per population (48 fish from the 148 fish reared in total) were randomly selected.
186 Before any handling, all the fish were anaesthetized with benzocaine (37.5 g of ethyl 4-
187 aminobenzoate per m^3 of seawater). The fish were weighed once a week from day 0 to day 63,
188 once every three weeks from day 63 to day 126 and then at days 154, 161, 175 and 217. For
189 each individual fish, growth rate (*Kg*) was estimated by fitting an exponential growth model
190 to fish weight data as following:

191 $W_t = W_0 e^{Kg \times t}$ (eq. 1)

192 where W_t and W_0 are the fish weights at time t and at the beginning of the experiment
193 respectively, Kg expressed in day^{-1}.

194 At each weighing time, five to ten fully formed scales were sampled from the dorsal area
195 behind the head of each of the 48 selected fish, without taking the new scales that had
196 regenerated after the previous samplings. Scales were obtained gently with curved pliers,
197 taking great care not to cause any deep wounds. The fish were then treated with a povidone-
198 iodine gel to promote the healing process. Scales were carefully rinsed with ultra-pure water
199 (milli-Q®, Merck-Millipore, Molsheim, France), dried at 60 °C for 48 hours and stored in a
200 cool and dry place pending analysis. To assess whether the sampling protocol was stressful or
201 not, the weights of the 48 fish were compared to those of the other fish reared in the same
202 tank and treated identically without any scale sampling. At the end of the experiment, fish
203 were dissected to be sexed.

204 *2.5. Carbon and nitrogen stable isotope analysis*

205 Preliminary isotope analyses were performed to test the influence of carbonate on $\delta^{13}C$
206 values of scales (Perga and Gerdeaux, 2003). Several scales were rinsed with hydrochloric
207 acid (HCl, 2 mol L^{-1}), rinsed three times with ultra-pure water and finally dried for 12 hours at
208 45°C. The differences between $\delta^{13}C$ values of untreated and acid-washed scales was -0.25 ±
209 0.15‰ and thus inferior to analytical error as previously reported by Sinnatamby et al. (2008).
210 Consequently, scales samples were used in their raw form without treatment. Different scales
211 from the same part of a same fish, sampled a same day, had similar carbon and nitrogen stable
212 isotope values (-19.81 ± 0.18‰ and 9.95 ± 0.09‰, respectively). Furthermore, as C:N ratio of
213 scales was 3.0 ± 0.1, lipid extraction was not necessary (Skinner et al., 2016). The $\delta^{13}C$ and
214 $\delta^{15}N$ values were independent of the C:N ratio (R^2 = 0.1102 between $\delta^{13}C$ values and C:N
215 ratio; R^2 = 0.0015 between $\delta^{15}N$ values and C:N ratio). Between 0.3 and 2 mg of whole dried

scales per sample were packed into a tin capsule to determine $\delta^{13}C$ and $\delta^{15}N$ simultaneously (scales were never cut or ground). Moreover, $\delta^{13}C$ and $\delta^{15}N$ values of diets 1 and 2 were determined using pellets from different areas of each feed bag. Diets 1 and 2 were ground into a fine and homogeneous powder using a mortar and a pestle. Then, approximately 0.5 mg of powder was packed and also analysed.

Continuous-flow elemental analyser/isotope ratio mass spectrometry (EA/IRMS) was used to analyze $\delta^{13}C$ and $\delta^{15}N$ values of all samples using an Isoprime GVI IRMS (Elementar, Langenselbold, Germany) interfaced with an EuroEA 3000 elemental analyzer (Eurovector, Pavia, Italia). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were expressed in conventional delta (δ) notation in *per mille* (‰) relative to the levels of ^{13}C in Vienna Pee Dee Belemnite and ^{15}N in atmospheric air, according to the following equation:

$$\delta x = \frac{R_{sample} - R_{standard}}{R_{standard}}$$

where x is ^{13}C or ^{15}N and R is the ratio of heavy to light isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$). Repeated measurements on alanine exhibited a precision of ± 0.11‰ and ± 0.12‰ for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. Commercial standards, alanine, wheat flour and corn flour from IsoAnalytical Lab (Crew, United Kingdom), IAEA-N-1, IAEA-N-2, IAEA-CH3 cellulose and USGS24 graphite from National Institute of Standard and Technology (Gaithersburg, USA) were used for a multipoint calibration.

2.6. Estimation of isotope incorporation rates, catabolic rates and trophic discrimination factors

For each fish, isotope incorporation rates of carbon and nitrogen were estimated using a single-compartment and first-order kinetic time-dependent model (Hobson and Clark, 1992):

$$\delta x_t = \delta x_\infty + (\delta x_0 - \delta x_\infty) e^{-\lambda x \times t} \text{ (eq. 2)}$$

where x is carbon or nitrogen, δx_t is the isotopic value of fish scale at time t, δx_∞ is the estimated asymptotic stable isotope value that fish scales reach at the steady state with their new diet, δx_0 is the isotopic value of fish scale at the beginning of the diet change experiment, and λx is the isotope incorporation rate (expressed in day^{-1}). A one-compartment model was chosen as this is more relevant than a multi-compartment model for scales (Heady and Moore, 2013).

Isotopic half-life, i.e. the time needed for half of the carbon or nitrogen in the scales to be replaced by atoms from a new diet, was calculated as:

$$t_{50}x = \frac{ln(2)}{\lambda x} \text{ (eq. 3)}$$

where t_{50} is the isotopic half-life (expressed in days), x is carbon or nitrogen, and λx is the estimated value of isotope incorporation rate.

In order to estimate how long it takes to reach an equilibrium state, the time needed for 95% of the scale carbon or nitrogen to be replaced by atoms from a new diet was calculated as:

$$t_{95}x = \frac{ln(20)}{\lambda x} \text{ (eq. 4).}$$

The relative contribution of growth and catabolic rates to change in carbon and nitrogen stable isotope values were estimated using a time-dependent model. Isotope incorporation rate (λ) is the result of join contribution of growth rate (Kg) and catabolic rate (Kc, Hesslein et al., 1993). For each individual, catabolic rates of carbon and nitrogen were determined as:

$$\lambda x = Kg + Kcx \text{ (eq. 5)}$$

where x is carbon or nitrogen, Kg is estimated using eq. 1 and Kcx is the catabolic rate (expressed in day^{-1}).

Finally, carbon and nitrogen diet-to-fish trophic discrimination factors (TDF) was calculated for each fish as:

$$\Delta x = \delta x - \delta x_{diet}$$ (eq. 6)

where Δx is the TDF (expressed in ‰), x is carbon or nitrogen, δx is the stable isotope value measured in fish scales at the end of the experiment (δx at day 217) or at the steady-state (δx_{∞} estimated by eq. 2) and δx_{diet} is the stable isotope value of the diet.

2.7. Empirical mixing models

To further explore the importance of using an accurate TDF, empirical mixing models were run with six different sets of carbon and nitrogen TDF. Sets chosen were: (1) 1.5 and 2.75‰, (2) 5.01 and 2.50‰, (3) 4.83 and 2.56‰, (4) 4.90 and 2.36‰, (5) 2.95 and 0.93‰, (6) 5.59 and 3.55‰, for carbon and nitrogen respectively. Set (1) is from literature for fish tissues (Sweeting et al., 2007a; 2007b). Sets (2), (3) and (4) are corresponding to the average values estimated in this study for scales from AT, WM and EM, respectively. Sets (5) and (6) are corresponding to minimal and maximal values estimated in this study. These five sets reflect the variation in scale TDF at population and individual levels. The relevance of empirical mixing models was to determine (1) whether it was really necessary to use TDF of scales rather than those of others tissues from fish literature and (2) whether the variability measured among populations or individuals influenced the predictions of the mixing model. The contributions of the two hypothetical diets (source 1 and source 2) were determined using simmr package ("Stable Isotope Mixing Models in R", Parnell et al., 2013; Parnell, 2019). Empirical $\delta^{13}C$ and $\delta^{15}N$ values of fish scales, source 1 and source 2 were -15 and 8‰; -25 and 11‰ and -15 and 2‰, respectively. The carbon and nitrogen stable isotope values of sources 1 and 2 were chosen to have almost an equal contribution of 50%/50% to the diet when estimated with carbon and nitrogen TDFs obtained on the 48 fish.

2.8. Statistical analysis and modelling

The exponential growth model and time-dependent incorporation models were firstly applied to the combined data from all 48 fish, using iterative nonlinear regression with the "nlme()" function from the nlme package (Pinheiro et al., 2018) in R (version 3.5.2., R Core Team, 2018) assuming that individual effect was a random effect with a normal distribution. Growth rate (*Kg*), as carbon and nitrogen isotope incorporation rates (λx), catabolic rates (*Kcx*) and TDF (Δx) were thus estimated for the whole fish group while taking into account variability among individuals. Secondly, each parameter was estimated for each population and sex, and values were compared among populations and sexes (considered as covariates) using an analysis of variance (ANOVA; Pinheiro and Bates, 2000). Pairwise differences were then explored using post hoc Student tests. The standard error (SE) of the parameters, i.e. the accuracy of the estimations made by the models, was calculated for the whole group, the populations and sexes. We then estimated one value of each parameter for each individual, using the same models. The mean and the standard deviation (i.e. the variability) of each parameter were then calculated combining all individual estimations. The $\delta^{13}C$ and $\delta^{15}N$ values measured at day 217 were compared among populations and sexes with an ANOVA applied to a linear model, considering population or sex as a fixed effect. The assumptions of normality and homoscedasticity of residuals were tested with Shapiro-Wilk and Bartlett tests, respectively, for both nonlinear and linear models.

3. Results

3.1. Fish growth rates and survival

Fish survival was 100% throughout the experiment and no significant difference appeared when comparing growth rates between sampled and non-sampled fish. Among the sampled fish, the numbers of males and females were respectively 6 and 10 for AT, 5 and 11 for WM and 6 and 10 for EM.

310 During the experiment, fish grew exponentially from 22.5 ± 5.5 g to 167.2 ± 45.3 g for
311 AT, 22.3 ± 7.4 g to 183.6 ± 64.6 g for WM and 23.2 ± 7.2 g to 188.2 ± 80.7 g for EM (Fig. 1).
312 Growth rates (*Kg*) were similar among populations (Table 1, $p > 0.05$, ANOVA). In contrast,
313 *Kg* differed significantly according to sex (Fig. 1, Table 1, $p < 0.001$, ANOVA) with the 31
314 females having a higher *Kg* than the 17 males. Females grew from 25.5 ± 5.6 g to 188.9 ±
315 62.4 g (0.75 g day^{-1}) whereas males grew from 17.6 ± 4.8 g to 162.4 ± 61.8 g (0.67 g day^{-1}).
316 Individual growth rate varied from 0.0061 to 0.0126 (mean ± standard deviation: 0.0090 ±
317 0.0019) and from 0.0066 to 0.0136 day^{-1} (0.0101 ± 0.0016) for males and females,
318 respectively.

319 *3.2. Isotope incorporation rates*

320 To reach a new equilibrium with diet 2, $\delta^{13}C$ and $\delta^{15}N$ values in the scales of the 48 fish
321 rapidly changed with time from -17.72 ± 0.60 to -20.25 ± 0.28‰ and from 10.85 ± 0.36 to
322 9.15 ± 0.36‰, respectively (Fig. 2 and Fig. 3). Carbon and nitrogen isotope incorporation
323 rates (λC and λN, respectively) were accurately estimated using a single compartment first-
324 order kinetic time-dependent model. Neither λC nor λN were significantly different among the
325 three populations ($p > 0.05$, ANOVA, Table 1). The mean time necessary for half of the
326 carbon and nitrogen in the 48 fish scales to be replaced by new atoms following the diet
327 change was 33 days for carbon and 67 days for nitrogen. A diet steady state would be reached
328 after 143 days and 291 days for carbon and nitrogen, respectively (Table 1).
329 Both λC and λN were significantly different between sexes ($p < 0.01$, ANOVA, Table 1)
330 with males having higher λC and λN than females. Carbon and nitrogen half-lives differed
331 markedly among individuals. Carbon half-life varied from 17 to 159 days (mean ± standard
332 deviation: 34 ± 32 days) and from 15 to 143 days (45 ± 29 days), for males and females,
333 respectively. Nitrogen half-life varied from 18 to 107 days (54 ± 26 days) and from 34 to 342
334 days (87 ± 66 days) for males and females, respectively.

335 *3.3. Contribution of growth and catabolic rates to isotopic incorporation*

336 Carbon and nitrogen *Kc* of fish scales were similar among the three populations ($p > 0.05$,
337 ANOVA, Table 1). In contrast, *KcC* and *KcN* differed significantly between sexes, with males
338 having higher *Kc* than females ($p < 0.05$, ANOVA, Table 1). Regarding *KcN*, it was never
339 significantly different from zero, except for males ($p < 0.05$, Student test, Table 1). Carbon *Kc*
340 varied from -0.0028 to 0.0318 day^{-1} (mean ± standard deviation: 0.0189 ± 0.0093 day^{-1}) and
341 from -0.0060 to 0.0337 day^{-1} (0.0107 ± 0.0102 day^{-1}) for males and females, respectively.
342 Nitrogen *Kc* varied from -0.0058 to 0.0315 day^{-1} (0.0074 ± 0.0094 day^{-1}) and from -0.0076 to
343 0.0078 day^{-1} (0.0008 ± 0.0047 day^{-1}) for males and females, respectively.

344 *3.4. Sensitivity of empirical mixing models to TDF*

345 At day 217, scales from the 48 fish reached carbon and nitrogen steady-state with the new
346 diet. Fish scale $\delta^{13}C$ and $\delta^{15}N$ values measured at day 217 were close to the asymptotic values
347 estimated by the model. Indeed, the differences between $\delta^{13}C$ and $\delta^{15}N$ values measured at
348 day 217 and asymptotic values were 0.14‰ and 0.30‰, respectively (eq. 2, Table 1). Based
349 on $\delta^{13}C$ and $\delta^{15}N$ values measured at day 217, carbon and nitrogen TDF were not significantly
350 different among populations or sexes ($p > 0.05$, ANOVA, Table 1). However, when based on
351 the estimated asymptotic values, carbon TDF was significantly different among populations
352 ($p < 0.05$, ANOVA, Table 1). This difference was, however, inferior to the analytical error
353 (less than 0.2‰ between minimal and maximal TDF among populations). Carbon and
354 nitrogen TDF varied among individuals from 2.95 to 5.59‰ (mean ± standard deviation: 4.83
355 ± 0.44‰) and from 0.93 to 3.55‰ (2.63 ± 0.56‰), respectively.

356 In empirical mixing models, the use of average carbon and nitrogen TDF estimated for
357 each population, as well as minimal and maximal TDF, had no significant influence on the
358 relative contributions of source 1 and source 2 to fish diet (Fig. 4). Indeed, the different scale
359 TDF led to a predicted contribution of source 1 between 41.5 and 45.2% and a predicted

360 contribution of source 2 between 54.8 and 58.5%. On the other hand, the use of carbon and
361 nitrogen TDF estimates for fish tissues from the literature caused a large under-estimation of
362 the source 1 (24.4%) and over-estimation of the source 2 (75.6%), compared to the use of
363 specific fish scale carbon and nitrogen TDF.

364 **4. Discussion**

365 In the present study, non-lethal sampling of scales permitted estimation of how carbon
366 and nitrogen isotope incorporation rates, and TDF, differed among populations, sexes and
367 individuals of European sea bass. This revealed variation between sexes in both carbon and
368 nitrogen isotope incorporation rates, among populations in carbon TDF, and was particularly
369 marked among individuals. Empirical mixing models showed that (1) the specific TDF of
370 scales is needed to obtain accurate predictions, literature values for other tissues are not
371 satisfactory, and (2) the variability that existed among populations or individuals did not
372 influence mixing model predictions.

373 *4.1. Variation in growth rate*

374 Our results highlighted that growth rates of fish (*Kg*) were equivalent between fish from
375 AT, WM and EM populations. These results were not in accordance with Vandeputte et al.
376 (2014). The differences between both studies may be explained by the design of the
377 experimental system such as feed, temperature, rearing density, anaesthesia frequencies or
378 other uncontrolled effects. Fish multiplied their weight by eight after 217 days of
379 experimentation. The 48 fish sampled for stable isotope analyses had similar final weights to
380 non-sampled fish present in the rearing system indicating that frequent scale sampling does
381 not markedly impact fish welfare.

382 Our study confirmed that *Kg* of European sea bass is influenced by sex with the 31
383 females having 12% higher *Kg* than the 17 males. Previous studies have also shown that

females are larger than males at a given age, with differences ranging between 20 and 40% (Chatain et al., 1997; Gardeur et al., 2001; Saillant et al., 2001).

4.2. Variation in isotope incorporation rates

Isotopic carbon and nitrogen half-lives ($t_{50}C$ and $t_{50}N$) of fish scales were similar among the three fish populations. Carbon t_{50} was estimated to be 33 days whereas nitrogen t_{50} was estimated to be 67 days for whole scales of fish. It is important to note this result was obtained in juvenile fish and would probably be different in other development stages. Values estimated for nitrogen were relatively close to those reported in scales of *Oncorhynchus mykiss* (27.7 days for nitrogen, Heady and Moore, 2013) but different from those reported for *Barbus barbus* (145 days, Busst and Britton, 2018). To our knowledge, no estimation of $t_{50}C$ is available in literature for fish scales. In the present study, $t_{50}C$ was half the $t_{50}N$. Depending on species, tissues and environmental conditions, $t_{50}C$ and $t_{50}N$ have almost any relationship: they can be closely linked (Herzka and Holt, 2000; Vander Zanden et al., 2015), $t_{50}C$ can be higher than $t_{50}N$ (Church et al., 2009; Lefebvre and Dubois, 2016) or lower (Carleton and Martínez del Rio, 2005). Such differences between $t_{50}C$ *and* $t_{50}N$ indicate a decoupling of carbon and nitrogen metabolic pathways and are dependent upon the sources of carbon and nitrogen used for *de novo* synthesis of proteins in scales. Fish scale is composed of an organic layer (mainly collagen) and an inorganic layer (carbonate salt). However, carbonate content in the scales is very low so the measured $\delta^{13}C$ value of scales only represents the organic layer (Hutchinson and Trueman, 2006). Proteins are synthesized from dietary and non-dietary sources including proteins, lipids and carbohydrates. Firstly, the use of dietary carbohydrates and lipids rather than dietary proteins to build proteins of fish scales could explain such carbon and nitrogen decoupling (Hobson and Bairlein, 2003). Since neither carbohydrates nor lipids can provide the nitrogen in proteins, lower value of $t_{50}C$ than $t_{50}N$ can be explained by an increase of the contribution of endogenous nitrogen to synthesize amino acids, leading to a

409 decrease of dietary nitrogen incorporation. Conversely, it can be hypothesized that dietary *vs.*
410 non dietary contributions of carbon remain constant (Carleton and Martínez del Rio, 2005).
411 Secondly, Carleton and Martínez del Rio (2005) hypothesized that dietary nitrogen
412 incorporation is reduced compared to dietary carbon incorporation when nitrogen is used to
413 synthesize non-essential amino acids. Endogenous nitrogen is reused after amino acid
414 degradation to synthesize new non-essential amino acids, instead of being excreted. In
415 contrast, this process does not exist with essential amino acids because they cannot be
416 synthesized and are obtained exclusively from feed. As scale collagen is mainly composed of
417 non-essential amino acids (i.e. glycine, alanine and proline) with essentials estimated to
418 comprise less than 20% of scale collagen (Kimura et al., 1991; Kaushik, 1998), a decoupling
419 between carbon and nitrogen may occur.
420 Results of the time-dependent model indicated that *Kc* contributed to 53.3% and 5.8% to
421 λC and λN, respectively. Regarding nitrogen, previous studies reported that *KcN* was close to
422 zero in fish scales, but did not indicate the sex of the sampled fish (Heady and Moore, 2013;
423 Busst and Britton, 2018). Present results are consistent with previous studies, except for males
424 whose *KcN* contributed to 30.8% to λN. In the case of carbon, isotope incorporation rate
425 seems to be partly driven by catabolic rate. Thus, the hypothesis that isotope incorporation
426 rate in scales was not driven only by growth seems to be validated for carbon, but not for
427 nitrogen (except in the case of males). To estimate *KcC* and *KcN,* we assumed the change in
428 total weight of fish was a reliable proxy to estimate *Kg* of scales. This assumption is
429 supported by Leim (1924) and Heidarsson et al. (2006) who proved that specific growth rate
430 of whole fish was correlated to specific growth rates of scales with a 1:1 ratio. Moreover, the
431 elemental composition of scales (i.e. C:N ratio) was constant throughout the diet change
432 experiment (3.0 ± 0.1 for C:N ratio, 23.29 ± 2.04% and 7.68 ± 0.67% for C and N
433 percentages, respectively). Consequently, we concluded that no shift in scale composition

434 occurred over time and thus whole fish *Kg* could be used without bias to estimate both carbon
435 and nitrogen catabolic rates. However, whole fish *Kg* likely remains a rough estimation of the
436 true *Kg* of carbon and nitrogen in scales and other factors such as moisture concentration in
437 whole fish could be taken into account to improve *Kg* estimation in scales. Thus, further
438 investigation is required to validate our results.

439 It is interesting that males and females differed in their carbon and nitrogen half-lives.
440 Although females had higher *Kg* than males, lower *KcC* and *KcN* were estimated. These
441 results would support that *Kg* is inversely correlated with *Kc* in young fish with smaller males
442 having higher *Kc* (Rossignol et al., 2011). However, the variability of $t_{50}C$ and $t_{50}N$ was high
443 within each sex. For example, some females reached $t_{50}C$ after only 17 days whereas others
444 needed more than 150 days. Similar variations have been measured in muscle of leopard shark
445 with $t_{50}C$ varying from 150 to 792 days among individuals (Kim et al., 2012). As variability
446 of *Kg* within males and females was low (standard deviation:mean ratio was around 0.15 for
447 each sex), these results suggest that such variability of λC and λN was due to the high
448 variability of *KcC* and *KcN*, respectively. This level of variability in growth rate is similar to
449 previous reports for European sea bass (Gardeur et al., 2001; Vandeputte et al., 2014).

450 The results of our study provide important understanding of the isotopic clock, which is
451 essential to interpret diet and habitat shifts over time in field (Phillips and Eldridge, 2006;
452 Wolf et al., 2009; Sweeting, 2010). In field-based stable isotope studies, the identification of
453 the new food sources using scales of juvenile fish will require at least 143 and 291 days after
454 a diet change for carbon and nitrogen, respectively. Moreover, after a diet change, female
455 European sea bass will need 19% and 50% more time than males to reach an isotopic
456 equilibrium for carbon and nitrogen, respectively. The potential variability among individuals
457 will also be important to consider to avoid misinterpretation of field data. Our results
458 highlight that strong variation in individual stable isotope values can appear even if

459 individuals are fed with a similar diet. Thus, as already mentioned by Barnes et al. (2008), one
460 should be careful before concluding that individual variation in isotopic values is a proof that
461 individuals rely on distinct food sources. Variability in isotope incorporation rates was linked
462 to the intrinsic metabolism of each fish rather than to a measurement bias. Carbon and
463 nitrogen stable isotope values were similar among scales from the same part of a same fish.
464 Furthermore, neither carbonate nor lipid content of the scales could account for the variability.
465 The variability in isotope incorporations rates of scales does not preclude the use of this tissue
466 in field-based studies. Indeed, individual variability in isotope incorporations rates of scales
467 was less than that found for red blood cells and muscle, two tissues frequently used in field-
468 based studies (Kim et al., 2012). The use of scales to elucidate ecology of fish must consider
469 the time required to reach an equilibrium state with diet. As individual variability must be
470 high, interpretation of results needs to take into account this constraint that is true for other
471 tissues.

472 *4.3. Trophic discrimination factor*

473 Our values of carbon and nitrogen TDF of scales (4.91‰ and 2.46‰, respectively) are
474 within the range of TDF reported for fish scales in the literature (Heady and Moore, 2013;
475 Busst and Britton, 2016; 2018). For example, Busst and Britton (2016) reported carbon TDF
476 of 4.7 and 4.9‰ and nitrogen TDF of 2.4 and 2.4‰ for *Barbus barbus* and *Squalius cephalus*
477 scales, respectively. Accurate TDF of a species can only be estimated when equilibrium with
478 the new diet has been reached. Therefore, if the TDF estimated using $\delta^{13}C$ and $\delta^{15}N$ values
479 measured at day 217 included any vestiges of $\delta^{13}C$ and $\delta^{15}N$ values from the diet 1, prior to
480 the diet change, they would not be valid and reliable. The $\delta^{13}C$ and $\delta^{15}N$ values measured at
481 day 217 were, however, similar to those estimated by the model at the asymptote, indicating
482 that fish scales were indeed at equilibrium with their new diet; any influence of diet 1 was
483 negligible. The higher value of carbon relative to nitrogen contrasts with values reported for

484 other fish tissues such as white muscle (Sweeting et al., 2007a; 2007b). In fact, carbon and
485 nitrogen TDF of scales reflect the amino acid composition of collagen that differs from that of
486 muscle (Howland et al., 2003). The variability found among individuals for carbon and
487 nitrogen TDF (from 2.95 to 5.59‰ and from 0.93 to 3.55‰, respectively) is broader than that
488 previously reported for scale collagen (Guiry and Hunt, 2020). Individual variability of TDF
489 in scales may vary depending upon fish species and whether scales were analysed in their raw
490 form or if collagen, their main component, was extracted for separate analysis (e.g. Guiry and
491 Hunt, 2020). Our results highlighted that the output of the mixing model considerably differs
492 when using TDF of fish tissues from literature (Sweeting et al., 2007a; 2007b) or TDF
493 estimated from scales. In isotope field-based studies, we recommend to use scale-specific
494 TDF to avoid any bias in the estimation of the contributions to diet of different food sources.
495 Although TDF were variable in scales among populations and individuals, estimation of the
496 contribution of each food source to diet based upon the TDF showed almost no variability.
497 Consequently, variation in scale TDF is not an obstacle in field-based studies to accurately
498 determine the contributions to diet of various food sources.

499 To conclude, present results highlight the need to take into account individual variation in
500 field-based studies. In particular, individual variation can have a strong impact when
501 scheduling sampling campaigns, to ensure all fish have reached equilibrium after a diet
502 change, and when discussing whether fish rely on distinct or similar food sources.

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686

687 **Table Caption**

688 **Table 1.** Estimated parameters from equations 1 to 6 using iterative nonlinear regression: growth rate (*Kg*),
689 carbon and nitrogen isotope incorporation rates (λC and λN), carbon and nitrogen half-lives ($t_{50}C$ *and* $t_{50}N$), time
690 to reach carbon and nitrogen isotopic equilibria with the new diet ($t_{95}C$ *and* $t_{95}N$), carbon and nitrogen catabolic
691 rates (*KcC* and *KcN*), *KcC*/λC*100* and *KcN*/λN*100,* asymptotic values $\delta^{13}C_{\infty}$ and $\delta^{15}N_{\infty}$ as well as carbon and
692 nitrogen asymptotic trophic discrimination factors (ΔC_{∞}, ΔN_{∞}). Measured $\delta^{13}C$ and $\delta^{15}N$ values ($\delta^{13}C_{217}$ and
693 $\delta^{15}N_{217}$) as well as carbon and nitrogen trophic discrimination factor (ΔC_{217}, ΔN_{217}) at day 217. Mean values are
694 given with standard error (± *SE*, n = 48 for all fish, n = 18 for AT, WM and EM populations, n = 17 and 31 for
695 males and females, respectively). Standard error reflects the accuracy of the estimations provided by the models
696 fitted at whole group level as well as at population and sex levels.

697 **Figure Caption**

698 **Figure 1.** Fish growth according to the population: Atlantic (A, n = 16), West Mediterranean (B, n = 16) and
699 East Mediterranean (C, n = 16) populations as well as males (D, n = 17) and females (E, n = 31). Curved lines
700 indicate the mean experimental growth model fitted to measured data using iterative nonlinear regression. Points
701 indicate the weight measured for each fish.

702 **Figure 2.** Change in $\delta^{13}C$ values of fish scales following a diet change according to the population: Atlantic (A,
703 n = 16), West Mediterranean (B, n = 16) and East Mediterranean (C, n = 16) populations as well as males (D, n =
704 17) and females (E, n = 31). Straight lines represent the $\delta^{13}C$ value for the new diet (diet 2). Curved lines
705 represent the mean single-compartment first-order kinetic model fitted to measured data using iterative nonlinear
706 regression. Points indicate $\delta^{13}C$ values of scales measured for each fish.

707 **Figure 3.** Change in $\delta^{15}N$ values of fish scales following a diet change according to the population: Atlantic (A,
708 n = 16), West Mediterranean (B, n = 16) and East Mediterranean (C, n = 16) populations as well as males (D, n =
709 17) and females (E, n = 31). Straight lines represent the $\delta^{15}N$ value for the new diet (diet 2). Curved lines
710 represent the mean single-compartment first-order kinetic model fitted to measured data using iterative nonlinear
711 regression. Points indicate $\delta^{15}N$ values measured of scales for each fish.

712 **Figure 4.** Estimation of the contributions of two food sources to fish diet using mixing models with different
713 couples of carbon and nitrogen trophic discrimination factors (TDF). TDF 1 is 1.5 and 2.75‰ estimated for fish
714 tissues by Sweeting et al. (2007a; 2007b). TDF 2, 3 and 4 are: 5.01 and 2.50‰, 4.83 and 2.56‰, 4.90 and
715 2.36‰, corresponding to the average values estimated in this study for scales from respectively Atlantic, West

716 Mediterranean and East Mediterranean fish. TDF 5 and 6 are: 2.95 and 0.93‰, 5.59 and 3.55‰, corresponding
717 to the minimal and maximal values estimated in this study. Grey and white bars are the percentages of source 1
718 and source 2, respectively, estimated by the empirical mixing models.

Table 1

Estimated parameters from equations 1 to 6 using iterative nonlinear regression: growth rate (*Kg*), carbon and nitrogen isotope incorporation rates (λC and λN), carbon and nitrogen half-lives ($t_{50}C$ *and* $t_{50}N$), time to reach carbon and nitrogen isotopic equilibria with the new diet ($t_{95}C$ *and* $t_{95}N$), carbon and nitrogen catabolic rates (*KcC* and *KcN*), $KcC/\lambda C*100$ and $KcN/\lambda N*100$, asymptotic values $\delta^{13}C_\infty$ and $\delta^{15}N_\infty$ as well as carbon and nitrogen asymptotic trophic discrimination factors (ΔC_∞, ΔN_∞). Measured $\delta^{13}C$ and $\delta^{15}N$ values ($\delta^{13}C_{217}$ and $\delta^{15}N_{217}$) as well as carbon and nitrogen trophic discrimination factor (ΔC_{217}, ΔN_{217}) at day 217. Mean values are given with standard error (± *SE*, n = 48 for all fish, n = 18 for AT, WM and EM populations, n = 17 and 31 for males and females, respectively). Standard error reflects the accuracy of the estimations provided by the models fitted at whole group level as well as at population and sex levels.

	All fish	**AT**	**WM**	**EM**	**Males**	**Females**
***Kg* (day^{-1})**	0.0097±0.0003	0.0095±0.0005	0.0098±0.0005	0.0098±0.0005	0.0090±0.0004**b**	0.0101±0.0003**a**
Carbon						
λC (day^{-1})	0.0210±0.0010	0.0231±0.0019	0.0196±0.0018	0.0208±0.0018	0.0235±0.0017**a**	0.0198±0.0012**b**
$t_{50}C$ (day)	33±2	30±2	35±3	33±3	29±2**a**	35±2**b**
$t_{95}C$ (day)	143±7	130±11	153±14	144±13	127±9**a**	151±9**b**
***KcC* (day^{-1})**	0.0115±0.0011	0.0140±0.0019	0.0098±0.0018	0.0110±0.0018	0.0146±0.0018**a**	0.0098±0.0013**b**
$KcC/\lambda C*100$ (%)	53.3	60.6	50.0	52.9	62.1	49.5
$\delta^{13}C_\infty$ (‰)	-20.39±0.03	-20.29±0.05**A**	-20.47±0.06**B**	-20.40±0.05**A,B**	-20.42±0.05	-20.36±0.04
ΔC_∞ (‰)	4.91±0.03	5.01±0.05**A**	4.83±0.06**B**	4.90±0.05**A,B**	4.88±0.05	4.94±0.04
$\delta^{13}C_{217}$ (‰)	-20.25±0.04	-20.17±0.09	-20.29±0.06	-20.29±0.08	-20.31±0.08	-20.21±0.05
ΔC_{217} (‰)	5.05±0.04	5.13±0.09	5.01±0.06	5.01±0.08	4.99±0.08	5.09±0.05
Nitrogen						
λN (day^{-1})	0.0103±0.0010	0.0105±0.0017	0.0108±0.0017	0.0094±0.0017	0.0130±0.0017**a**	0.0087±0.0012**b**
$t_{50}N$ (day)	67±7	66±11	64±10	74±14	53±7**a**	80±11**b**
$t_{95}N$ (day)	291±29	285±47	277±45	319±60	230±31**a**	344±48**b**
***KcN* (day^{-1})**	0.0006±0.0010	0.0010±0.0017	0.0010±0.0017	-0.0004±0.0017	0.0040±0.0017**a**	-0.0014±0.0012**b**
$KcN/\lambda N*100$ (%)	5.8	9.5	9.3	0	30.8	0
$\delta^{15}N_\infty$ (‰)	8.85±0.06	8.89±0.09	8.95±0.09	8.75±0.10	8.86±0.08	8.82±0.08
ΔN_∞ (‰)	2.46±0.06	2.50±0.09	2.56±0.09	2.36±0.10	2.47±0.08	2.43±0.08

$\delta^{15}N_{217}$ (‰)	9.15±0.05	9.18±0.08	9.22±0.08	9.06±0.11	9.09±0.08	9.19±0.07
ΔN_{217} (‰)	2.76±0.05	2.79±0.08	2.83±0.08	2.67±0.11	2.70±0.08	2.80±0.07

729 Upper and lower case letters indicate significant differences among populations and sexes, respectively

730 (ANOVA, $p < 0.05$).

731

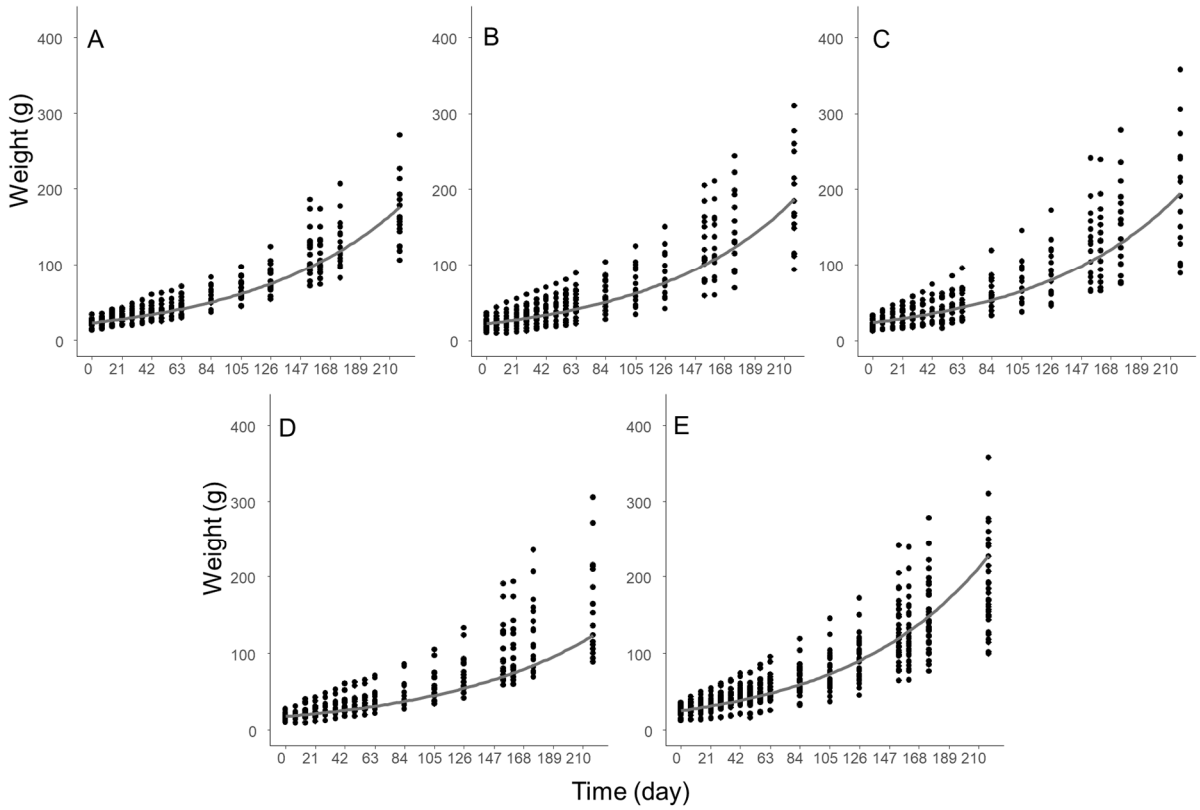
A
B
C
D
E
Weight (g)
Time (day)
0
100
200
300
400
0 21 42 63 84 105 126 147 168 189 210

Fig. 1.

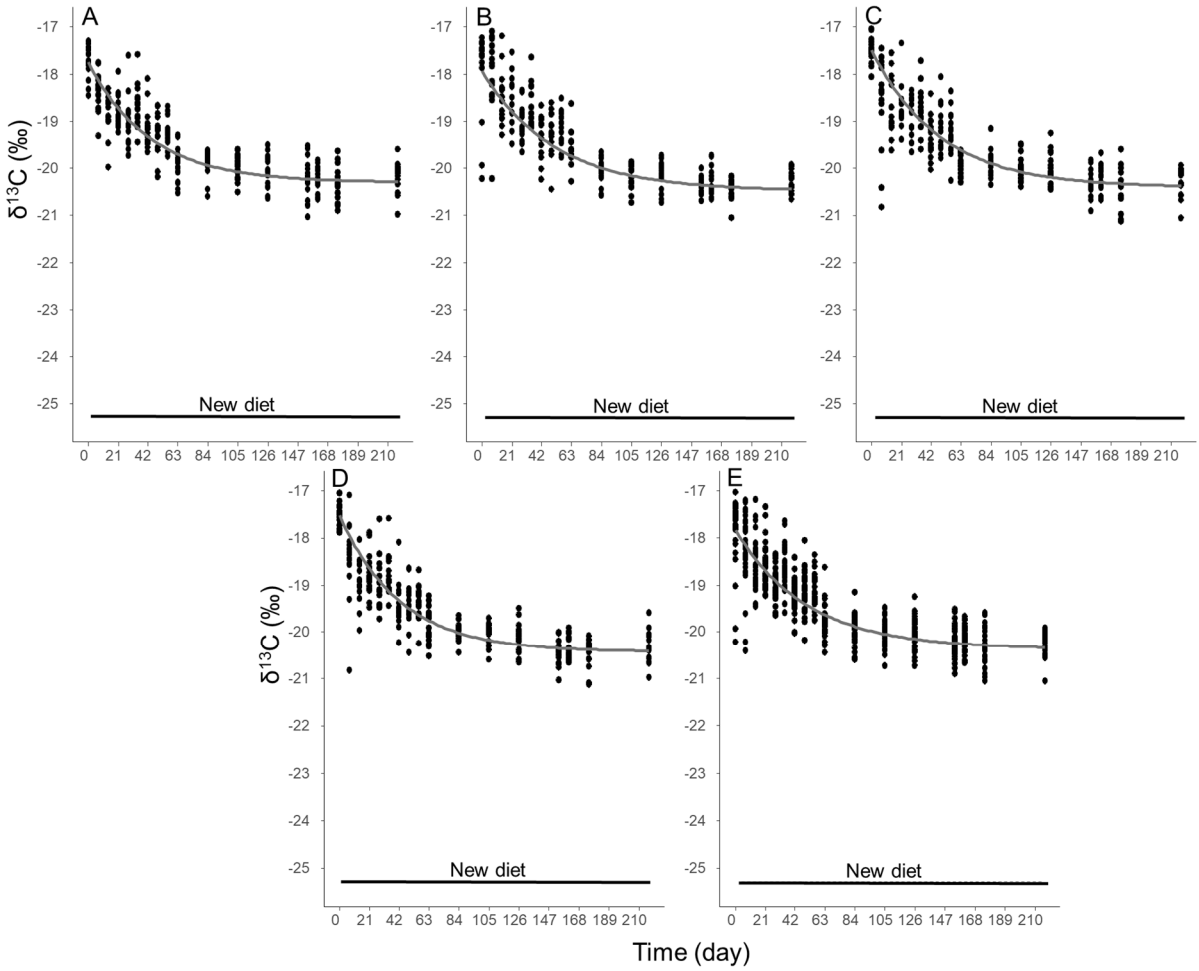
A
B
C
D
E
δ13C (‰)
-17
-18
-19
-20
-21
-22
-23
-24
-25
New diet
0 21 42 63 84 105 126 147 168 189 210
Time (day)

Fig. 2.

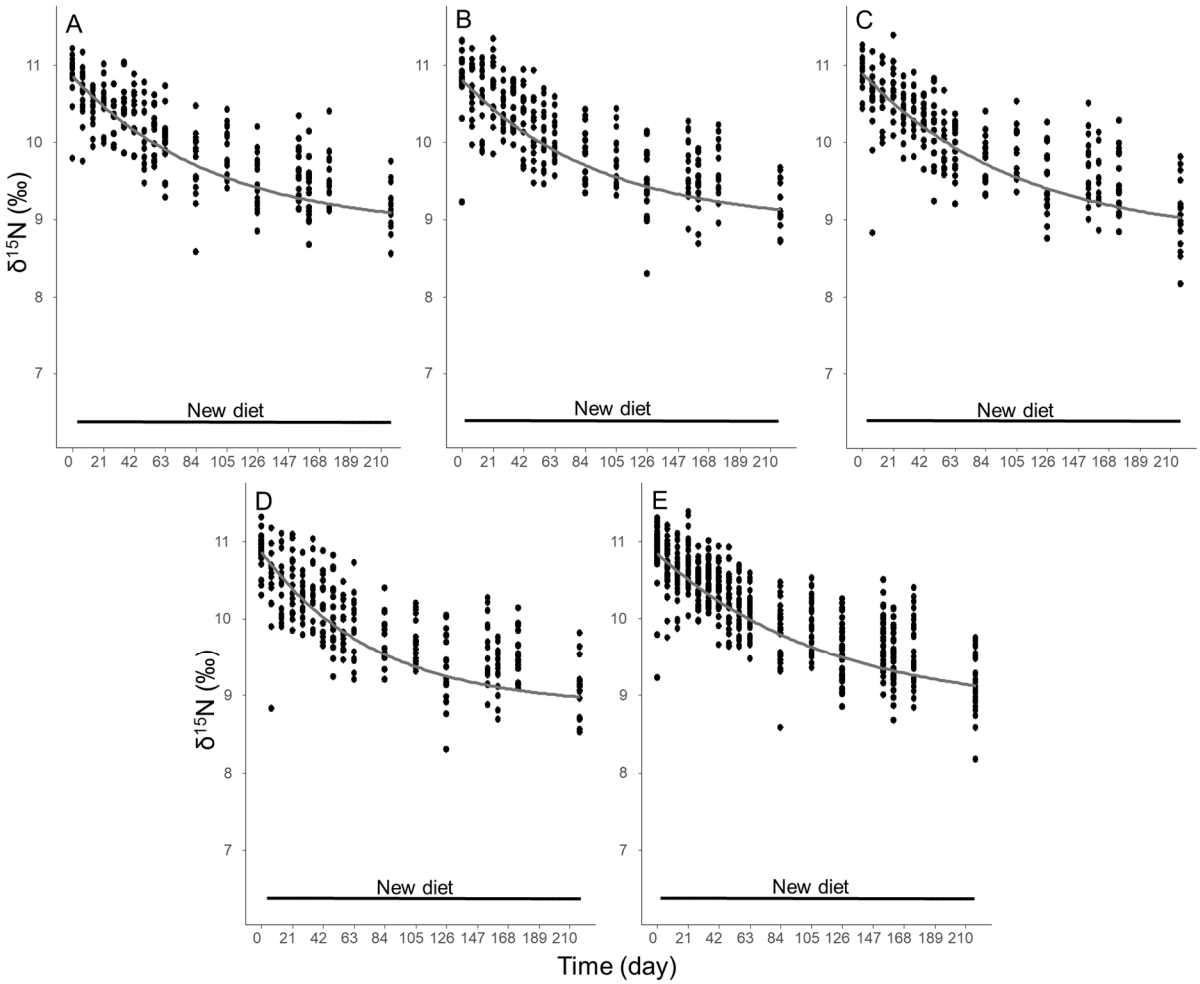
A
B
C
D
E
δ15N (‰)
11
10
9
8
7
New diet
0 21 42 63 84 105 126 147 168 189 210
Time (day)

Fig. 3.

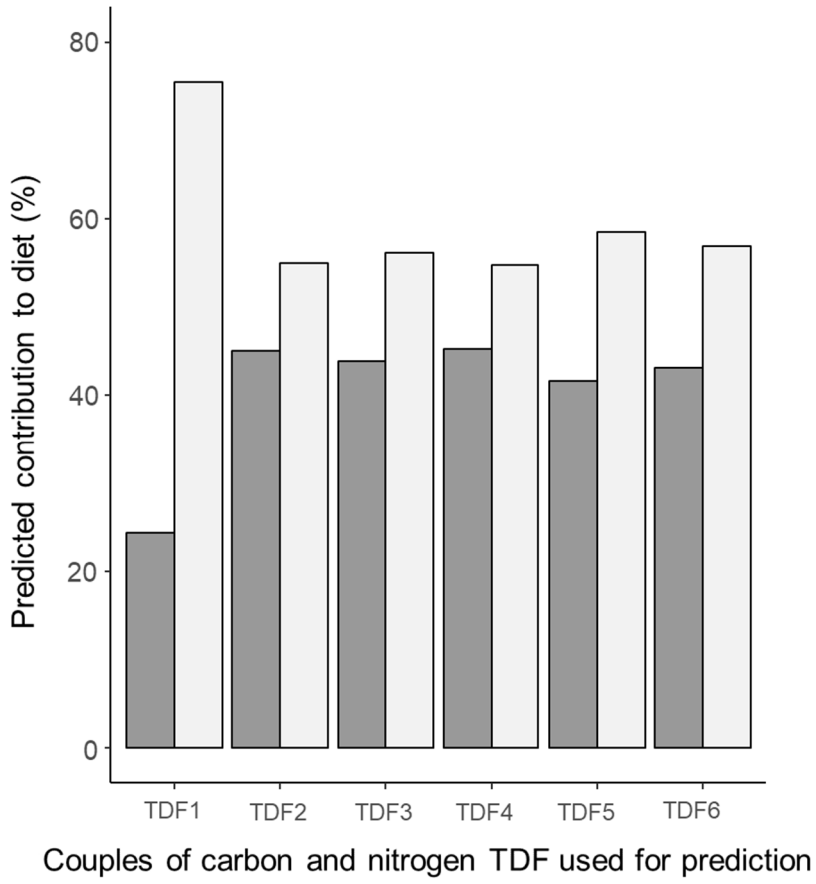
80
60
40
20
0
Predicted contribution to diet (%)
TDF1
TDF2
TDF3
TDF4
TDF5
TDF6
Couples of carbon and nitrogen TDF used for prediction

Fig. 4.