

Changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment

J. Guelinckx^{1,*}, J. Maes², P. Van Den Driessche¹, B. Geysen¹, F. Dehairs³, F. Ollevier¹

¹Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, C. Deberiotstraat, 3000 Leuven, Belgium

²VITO, Flemish Institute of Technological Research, Integrated Environmental Studies, Boeretang 200, 2400 Mol, Belgium

³Department of Analytical Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

ABSTRACT: Studies on diet or migration of organisms based on stable isotopes require precise estimates of how quickly stable isotope ratios change in the investigated tissues. Isotopic turnover rates in fish, however, are poorly understood. Prior to field applications of the stable isotope technique for investigating sand goby *Pomatoschistus minutus* migrations, a laboratory diet-switch experiment was conducted to (1) determine C and N isotopic turnover rates in sand goby muscle, liver and heart tissue, and (2) evaluate the relative contribution of growth and metabolic replacement to the total change in isotopic composition. Both time-based and growth-based models adequately described the carbon and nitrogen isotopic change in each tissue. The variation in isotopic turnover rates among the tissues and elements could be attributed to differences in metabolic activity. Muscle tissue had the slowest turnover rates, with half-lives of approximately 25 and 28 d for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. The shortest half-life value for $\delta^{15}\text{N}$ was in liver tissue (3 d) and for $\delta^{13}\text{C}$ in heart tissue (6 d). The rate of isotopic change in goby muscle tissue was mainly regulated by somatic growth, but metabolic replacement significantly accelerated the turnover rate for $\delta^{13}\text{C}$. In liver and heart tissue, basal metabolism contributed considerably to the isotopic shift. As a result, effects of short-term food deprivation were only found in liver and heart tissue. Although the observed trophic fractionation factors were within reported ranges, they were exceptionally large for $\delta^{13}\text{C}$ in muscle and liver tissue.

KEY WORDS: Stable isotopes · Carbon · Nitrogen · Isotopic turnover rate · Metabolism · Fasting · Trophic enrichment · Gobiidae

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INTRODUCTION

Stable C and N isotope ratios are widely used for reconstructing diets and elucidating migration dynamics. Coupling known spatial variation of stable C and N isotopes with tissue specific temporal variation allows researchers to track animal movement (Hobson 1999, Kurle & Worhty 2002, Herzka 2005). The principle is based on the fact that stable isotopic signatures of animal tissues reflect those of their diets. When an animal switches to an isotopically different food source the isotopic composition of its tissues will change as a consequence of 2 processes. For growing animals isotopic

turnover rate is predominantly regulated by simple dilution effects. As a result of switching to an isotopically different diet the initial isotopic composition of the animal (δ_i) will change with growth to a final value (δ_f), which is in equilibrium with the new diet. In this case the old δ_i is only diluted by addition of new tissue synthesized from the new diet during growth. Isotopic turnover rates, however, can be accelerated by the additional effect of tissue specific maintenance metabolism, i.e. the metabolic breakdown of old tissue synthesized during feeding on a previous diet and its subsequent replacement by tissue made from the new diet (Fry & Arnold 1982, Tieszen et al. 1983, Hobson &

*Email: jef.guelinckx@bio.kuleuven.be

Clark 1992). Although the contribution of metabolic replacement to the total isotopic turnover rate has generally been considered to be negligible or of minor importance in ectotherms, contrasting results have recently been found for fish tissues (Hesslein et al. 1993, Herzka & Holt 2000, MacAvoy et al. 2001, Bosley et al. 2002, Sakano et al. 2005, Suzuki et al. 2005, Logan et al. 2006, McIntyre & Flecker 2006).

Time lags associated with the change in stable isotope ratios are essential information for quantitatively analyzing shifts in food habits and habitats. Because isotopic turnover rates are specific to taxon, type of tissue being analyzed, ontogenetic stage and environmental conditions (Tieszen et al. 1983, Frazer et al. 1997, Bosley et al. 2002, McIntyre & Flecker 2006), it is crucial that studies using stable isotopes to infer dietary information and migration dynamics take tissue specific turnover rates for the organism and the system under study into account. Unfortunately, studies focussing on the rate of change in isotopic composition of various fish organ tissues after switching to a different food source are very scarce, especially for slow-growing fish or short-lived species. Recently, however, this particular research topic has become increasingly more popular. (See Herzka 2005 and McIntyre & Flecker 2006 for reviews of isotopic turnover rate studies on fishes; but also see Suzuki et al. 2005, Logan et al. 2006 and Miller 2006.)

When in equilibrium with the local food web, the isotopic composition of any organism closely resembles that of its food. Typically the $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios of a consumer are slightly higher than those in its diet, mainly due to the fact that the lighter isotope (^{12}C and ^{14}N) is preferred in enzymatic processes. In general, the stable C isotopic composition of the whole body of an animal is enriched in ^{13}C relative to its diet by about 0 to 1‰ (DeNiro & Epstein 1978). Corresponding trophic enrichment values for $\delta^{15}\text{N}$ are more variable but average around +3.4‰ (Fry & Sherr 1984, Minagawa & Wada 1984, Owens 1987). Although these trophic enrichments are commonly used in ecological studies they are actually mean values for a wide variety of animals. Isotopic shifts associated with trophic level can be quite variable and may depend on several possible factors such as taxon, main biochemical form of the nitrogenous waste and type of diet. Even within one individual there are considerable differences in trophic shift among tissues (Focken & Becker 1998, Vander Zanden & Rasmussen 2001, McCutchan et al. 2003, Vanderklift & Ponsard 2003). Taking these tissue- or component-specific differences into account can greatly improve the accuracy when predicting trophic levels or stable isotopic signatures of organisms based on known isotopic data.

This study is framed in a metapopulation research effort on the migration dynamics of sand goby *Poma-*

toschistus minutus between the North Sea and the Scheldt estuary. The sand goby is one of the most common fish species along the European Atlantic coast and its estuaries. *P. minutus* spawns in the North Sea between February and June. Growth rate is highest from July to October and negligible during winter. The sand goby has a short life span; most adults die in their second summer immediately after their first spawning (Fonds 1973, Hamerlynck 1990). As a marine species it presumably uses the Scheldt estuary as a nursery. Through immigration, maximum densities are generally reached in the upper estuary during September to October. Although this abundance pattern is highly consistent and predictable (Maes et al. 2004), the utilization of stable isotopes as tracers of individual recruitment to the estuary will reveal the migration dynamics on a finer temporal scale, thereby improving our understanding of the life history strategies of marine fish species (Herzka & Holt 2000).

The main purpose of this study was, firstly, to experimentally determine the isotopic turnover rate in dorsal muscle, liver and heart tissue of juvenile *Pomatoschistus minutus* and to identify the most appropriate tissue for subsequent use in a migration study. We hypothesized that these 3 goby tissues would have different isotopic turnover rates, although contrasting results for fish tissue have been published (e.g. Hesslein et al. 1993, MacAvoy et al. 2001, Suzuki et al. 2005, Logan et al. 2006). As a centre of metabolism, the liver is likely to be characterized by a more dynamic change in its biochemical composition than muscle tissue and possibly also heart tissue. When multiple tissues with different turnover rates are measured, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ can provide both short-term and long-term dietary information (Tieszen et al. 1983), ultimately increasing the temporal resolution of this technique in migration studies (Hobson 1999). Secondly, the relative contribution of growth and metabolic replacement to the total change in isotopic composition was considered. The observed isotopic change can be better understood with knowledge about the underlying physiological mechanisms. Thirdly, the influence of short-term food deprivation was investigated to infer possible confounding effects of fasting in the field. Finally, this experiment was used to shed some light on the differences in trophic enrichment among the 3 tissues.

MATERIALS AND METHODS

Experimental design. On August 24, 2004, sand gobies were sampled with a 2 m beam trawl in the Eastern Scheldt (a marine bay north of the Scheldt estuary), close to the Centre for Estuarine and Marine Ecology (NIOO-CEME) where this experiment was conducted

in a climate test chamber. Within the 1 h of sampling, individuals were transferred to 3 large polyethylene containers to acclimatize to laboratory conditions. Seven fish were randomly selected, measured and sacrificed to determine the initial isotopic composition (δ_i) for each tissue before the diet switch.

After 2 d of acclimatization, fish were randomly assigned to aquaria (capacity 30 l), at densities of 6 fish per aquarium. Aquaria were installed in a continuous flow-through system of filtered (to 45 μm) Eastern Scheldt water. At least 20% of the water volume was changed on a daily basis. Temperature was preset at 17°C and the light:dark regime to 12:12 h. The aquaria were provided with a 2 cm sand layer in which the gobies could hide, and a fine meshed gauze cover to prevent their escape. During the translocation fish were anesthetized (MS-222) and subsequently marked individually with visible implant elastomers (VIE), measured to the nearest mm (standard length, SL) and weighed (fresh weight) to assess subsequent individual growth. Only fish within a certain length range, corresponding to the average (\pm SD) length of the population in the Scheldt estuary at that time, were considered for the experiment.

The aquaria were then randomly assigned to a pellet (PEL) and a starvation (STARV) treatment (Table 1). The PEL group consisted of 18 aquaria and received a pellet diet based on fishmeal (producer: N.V. Joosen-Luyckx, Art 10120). The $\delta^{13}\text{C}$ value of this food differs by approximately 6‰ from that of the initial goby muscle (see Table 3); this resembles the isotopic difference between the marine and oligohaline zone of the Scheldt estuary (Guelinckx et al. 2006). Feeding was twice a day, similar to the natural feeding frequency of

sand gobies (Healey 1972). Rations were supplied in such a way that the food was clearly visible, and corresponded to portions that were approximately 3% of fish body weight. During the experiment, the rations were adapted to the increasing fish biomass. The pellet feed was initially selected from a range of feeds based on their isotopic composition and sand goby feeding preference, which was *a priori* tested in a pilot study. The STARV group consisted of 3 aquaria in which the fish were deprived of food for 20 d.

The actual experiment (feeding) started the day after allocation to the aquaria, namely August 28, which was designated Day 0, and lasted 90 d. At specific time intervals all the fish in 3 randomly chosen aquaria were killed (Table 1) to document changes in isotopic composition. In the STARV group only 2 fish per aquarium were killed on Day 10; all others were killed on Day 20. Fish were measured and weighed again before storage at -20°C. Every 2 d feces and the remaining food were siphoned away. Dead and sick fish were immediately removed and replaced by marked fish from a reserve stock in order to maintain the same density in each aquarium. These specimens were not considered for further analysis. Aquarium temperature was monitored 3 times a week and pH, oxygen and salinity twice a week. NO_2^- and $\text{NH}_3\text{-NH}_4^+$ were monitored occasionally. All variables were found to be stable (Table 2).

Stable isotope analysis. To reduce ecological variability (Gearing 1991), dorsal muscle, liver and heart tissue of 2 specimens from each aquarium were used for stable isotope measurements. Consequently, tissues from 6 individuals from each sampling event were analysed resulting in 3 replicas. Those fish with the largest increase in biomass per aquarium were selected for analysis because we assumed they were the best acclimatized during the experiment. Within the STARV group, fish were chosen randomly for isotope analyses.

All tissue samples were dried for 2 d (55°C) to constant weight and ground with a mortar and pestle. An aliquot (0.5 to 0.6 mg) was subsequently packed in tin containers for isotope analysis (for heart tissue this often meant the entire sample). Lipids were not removed from our samples prior to analysis to avoid dispersion of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ (Pinnegar & Polunin 1999). Stable isotope measurements were done at the Laboratory for Analytical and Environmental Chemistry at the Vrije Universiteit Brussel (Belgium) on a Flash series 1112 elemental analyzer interfaced to a Delta^{plus} XL Thermo Finnigan IRMS. The working standards were high-purity N_2 and CO_2 , while IAEA-C-6 and IAEA-N2 were used as reference materials. Stable isotopic compositions are expressed in the conventional δ -notation:

$$\delta X (\text{‰}) = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 10^3$$

Table 1. *Pomatoschistus minutus*. Overview of experimental design with mean (\pm SD) initial biomass per aquarium and fish length for the 2 treatments (PEL: pellet food; STARV: starvation). There was no significant difference between treatments for biomass or fish length (Student's *t*-test, $p > 0.05$). Biochemical composition and energy content of dried pellets are also shown. Energy content values (\pm SD) are based on 3 measurements

Parameter	PEL	STARV
No. of aquaria	18	3
No. of fish per aquarium	6	6
Initial biomass per aquarium (g)	5.85 \pm 0.44	6.30 \pm 0.75
Mean initial standard length (mm)	42.8 \pm 1.4	43.5 \pm 0.9
Sacrificed on Day	10, 20, 30, 45, 60, 90	10, 20
% ash	7.8	
% crude protein	61.7	
% carbohydrate	14.2	
% lipid	13.6	
Energy content (kcal g ⁻¹)	4.62 \pm 0.05	

Table 2. Mean (\pm SD) abiotic conditions of experiment and of the Eastern Scheldt

	T (°C)	Salinity (psu)	Oxygen saturation (%)	pH	NH ₃ -NH ₄ ⁺ (mg l ⁻¹)	NO ₂ ⁻ (mg l ⁻¹)
Aquaria	16.9 \pm 0.5	31.8 \pm 0.1	92.86 \pm 4.05	7.9 \pm 0.1	0.46 \pm 0.28	0.06 \pm 0.05
Eastern Scheldt	14.2 \pm 4.2	31.7 \pm 0.3	91.67 \pm 13.4	8.1 \pm 0.4	0.25	/

where X is ¹³C or ¹⁵N and R is ¹³C/¹²C or ¹⁵N/¹⁴N. $\delta^{13}\text{C}$ values are expressed relative to the VPDB (Vienna PeeDee Belemnite) standard, while $\delta^{15}\text{N}$ values are expressed relative to atmospheric N₂. Reproducibility for different aliquots of the reference materials was generally better than 0.3‰ for $\delta^{13}\text{C}$ and better than 0.2‰ for $\delta^{15}\text{N}$.

Differences in isotopic composition among tissues before the diet switch and within the same tissue among sampling events in the STARV group were tested using 1-way ANOVA. When the assumptions were not fulfilled the non-parametric Kruskal-Wallis test was applied. Consequently, post hoc Tukey's HSD or Mann-Whitney *U* tests were used to identify the significantly differing groups (Statistica 6.0, StatSoft).

Turnover modeling. Data processing was done with the mean values (2 fish) of each aquarium (3 replicates), resulting in 3 values per sampling event for each tissue. Single-pool models as a function of time and growth were used to describe the shift in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ within each tissue.

Model as a function of time: The following exponential model describes the change in isotopic composition as a function of time (Tieszen et al. 1983):

$$\delta_t = \delta_f + (\delta_i - \delta_f)\exp(vt) \quad (1)$$

where δ_t is the stable isotopic composition of a tissue at the time of fish collection from the aquaria, δ_i is the initial value before the diet switch, δ_f is the final isotopic composition equilibrated to the new diet, *t* is the time that fish were in the experiment (d), *v* is a measure of the turnover rate and has units of time⁻¹. δ_f and *v* were determined by fitting (least squares method) the model to the data using Sigma Plot 2000 6.0 (SPSS). During the model fitting procedure, the value of δ_i was fixed to the mean value of the 7 individuals sampled from the Eastern Scheldt.

A more conventional way to express the isotopic turnover rate is by the half-life value (*t*_{1/2}), i.e. the amount of time required to reach the midpoint value of the initial (δ_i) and final values (δ_f). By transforming Eq. (1) the half-life value for each tissue was calculated as follows (Tieszen et al. 1983):

$$t_{1/2} = \ln(0.5)/v \quad (2)$$

Model as a function of growth: The relative contributions of growth and metabolic turnover to the

observed changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were examined using the following equation (Fry & Arnold 1982, Herzka et al. 2001):

$$\delta_t = \delta_f + (\delta_i - \delta_f)(W_t/W_i)^c \quad (3)$$

where δ_t , δ_i and δ_f have the same meaning as in the time model, *W_i* and *W_t* indicate the fresh weight of an individual before the experiment and at the moment of collection respectively, *c* is the exponent of metabolic decay and is a measure of the relative contribution of growth and metabolic activity to the isotopic turnover rate. When *c* = -1, the rate of change in isotopic composition is mediated by growth alone and the dilution model prevails. When *c* < -1, metabolic replacement increases the rate of isotopic change (Fry & Arnold 1982, Herzka et al. 2001). δ_f and *c* were estimated by the Levenburg-Marquardt iterative, non-linear, least squares fitting algorithm using Statistica 6.0 (StatSoft). Again, for the curve-fitting procedure, δ_i was set to the mean initial value.

RESULTS

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ before the diet switch

The mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (δ_i) of the 3 tissues before the diet switch (August 24) are presented in Table 3. Liver (L) was significantly depleted in ¹³C relative to muscle (M) ($\Delta\delta^{13}\text{C}_{\text{L-M}} = 4.92$ ‰) and heart (H) tissues ($\Delta\delta^{13}\text{C}_{\text{L-H}} = 4.76$ ‰) (Tukey's HSD test, *p* < 0.001), while muscle and heart tissue had very similar $\delta^{13}\text{C}$ -values. For $\delta^{15}\text{N}$, the 3 tissues differed significantly from each other, although these differences were small. Muscle tissue was more enriched in ¹⁵N than was heart ($\Delta\delta^{15}\text{N}_{\text{M-H}} = 1.75$ ‰) and liver ($\Delta\delta^{15}\text{N}_{\text{M-L}} = 2.93$ ‰) tissue (Tukey's HSD test, *p* < 0.001) while heart tissue was also slightly more enriched than liver tissue ($\Delta\delta^{15}\text{N}_{\text{H-L}} = 0.64$ ‰) (Tukey's HSD test, *p* < 0.01). Table 3 also shows the mean isotopic composition of the pellet diet and the expected isotopic composition of the tissues when equilibrated to the pellet diet. Pellet samples were taken every 2 wk and confirmed that there was no change in diet isotopic composition during the experiment. The expected muscle values were predicted from the mean pellet values by assuming a trophic enrichment factor of +1.0‰ for $\delta^{13}\text{C}$ and +3.4‰

Table 3. *Pomatoschistus minutus*. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (mean \pm SD) of pellet diet, sand goby muscle, liver and heart tissue in the Eastern Scheldt and values expected for the 3 tissues when in equilibrium with their pellet diet. Fish were sampled on July 20, 2004 (preliminary study) and August 24, 2004 (start of experiment). For muscle tissue, expected values were calculated by adding +1.0 and 3.4‰ to pellet $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively; for liver and heart tissue, isotopic differences from muscle tissue were taken into account

Tissue	$\delta^{13}\text{C}$ (‰)			$\delta^{15}\text{N}$ (‰)		
	July 20	August 24	Expected	July 20	August 24	Expected
Muscle	-16.07 ± 0.13	-16.23 ± 0.56	-22.41	15.84 ± 0.39	16.13 ± 0.37	12.54
Liver	-20.91 ± 0.46	-21.15 ± 0.49	-27.33	13.87 ± 0.26	13.74 ± 0.15	10.14
Heart	-15.96 ± 0.65	-16.39 ± 0.80	-22.57	14.35 ± 0.18	14.38 ± 0.21	10.49
Pellet diet		-23.41 ± 0.38			9.14 ± 0.22	

for $\delta^{15}\text{N}$ (DeNiro & Epstein 1978, Minagawa & Wada 1984, Owens 1987). For liver and heart tissue the deviation of these tissues from muscle values before the experiment was additionally taken into account. The expected final values differed by about 6.2‰ for $\delta^{13}\text{C}$ and 3.6‰ for $\delta^{15}\text{N}$ from sand goby values before the diet switch.

Turnover modeling as a function of time

During the 3 mo of the experiment the goby tissues shifted towards a new equilibrium as a result of the

pellet diet (Fig. 1). All estimates and models were statistically significant ($p < 0.05$) and explained between 81 and 95% of the variation. The rate of isotopic change differed among tissues and between C and N. Muscle tissue had the slowest turnover with similar half-lives of 24.7 and 27.8 d for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. For $\delta^{13}\text{C}$ the shortest half-life was in heart tissue (6.1 d), while liver had an intermediate half-life of 9.1 d. For $\delta^{15}\text{N}$, the highest isotopic turnover rate was recorded in liver tissue (2.8 d). Surprisingly, heart $\delta^{15}\text{N}$ had a similar turnover rate (27.6 d) to muscle $\delta^{15}\text{N}$. Our data approached the expected values in 4 of 6 tissue–isotope combina-

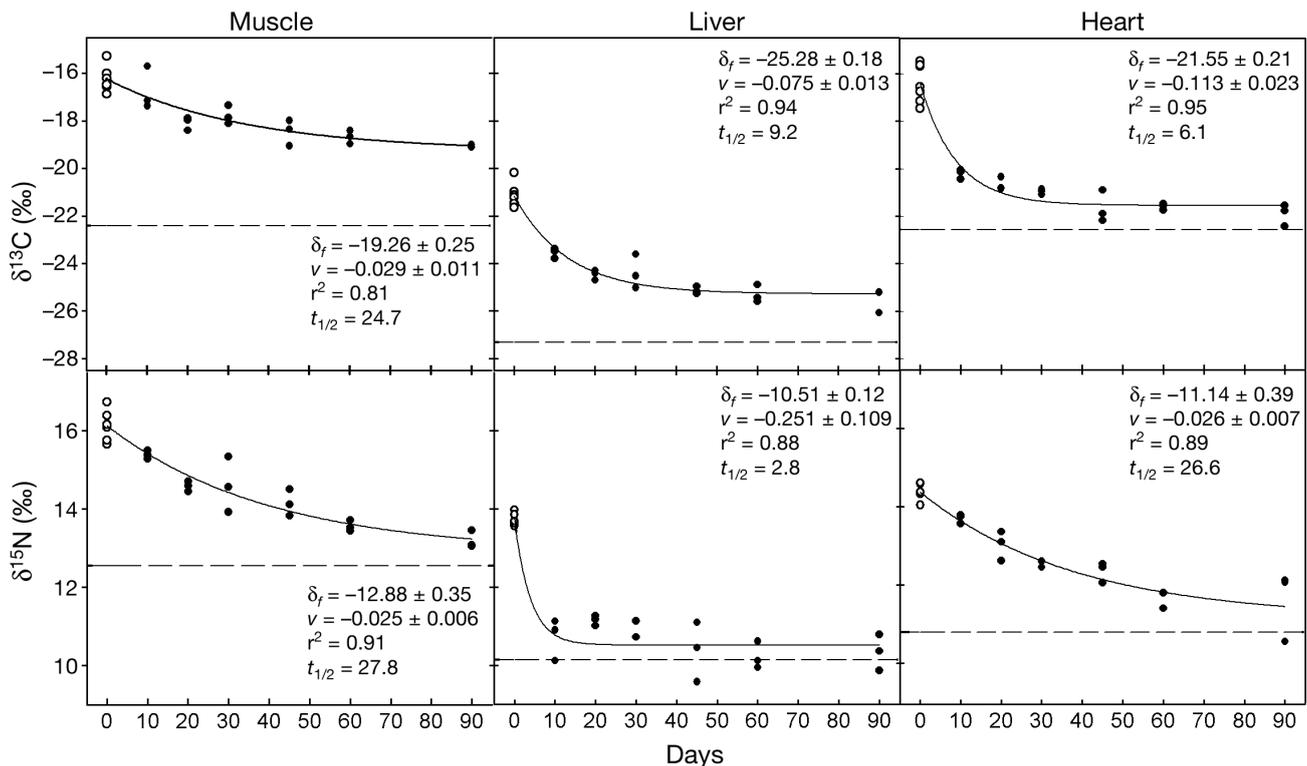


Fig. 1. *Pomatoschistus minutus*. Changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as a function of time (t) for muscle, liver and heart tissue in the PEL group. (●) δ_t , mean value of 2 fish (1 aquarium); (○) δ_i (1 individual). Continuous lines: best fit through data following $\delta_t = \delta_f + (\delta_i - \delta_f)\exp(-vt)$; δ_f (‰) and v were estimated by the model (\pm SE); r^2 and $t_{1/2}$ (d) are also shown. Dashed lines: expected final values when in equilibrium with pellet diet (cf. Table 3)

tions, with the exception of $\delta^{13}\text{C}$ in muscle and liver. There was a discrepancy of 3.15‰ for muscle and 2.05‰ for liver between the expected value and δ_f estimated by the model.

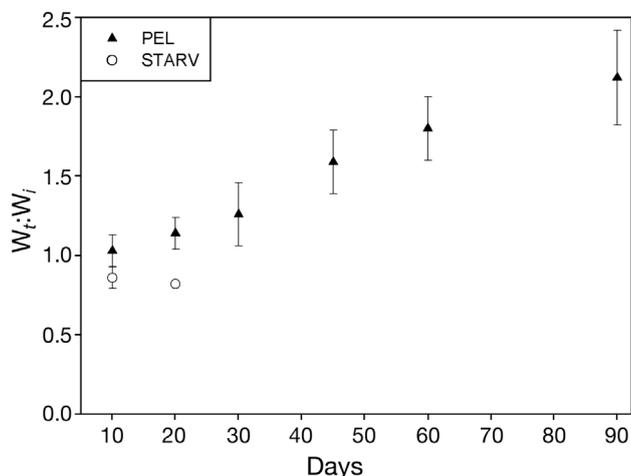


Fig. 2. *Pomatoschistus minutus*. Average (\pm SD) change in biomass, expressed as $W_t:W_i$, for fish in the pellet-fed (PEL) and starved (STARV) groups. Data are based on all surviving individuals from Day 0

Turnover modeling as a function of growth

On average, fish in the PEL group had doubled their biomass by the end of the experiment (Fig. 2). Throughout the experiment the mean growth rate k ($= \log_e[W_t/W_i] \times t^{-1}$) in the PEL group was 0.012 d^{-1} . The change in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in the 3 tissues was also described as a function of biomass increase (Fig. 3). Again, all estimates were statistically significant and the models explained between 79 and 90% of the variation. The exponent of metabolic decay c was not statistically significantly different from -1 for $\delta^{15}\text{N}$ in muscle and heart tissue. However, metabolic activity seemed to become increasingly more important in muscle $\delta^{13}\text{C}$, liver $\delta^{13}\text{C}$, heart $\delta^{13}\text{C}$ and liver $\delta^{15}\text{N}$, in which the lowest c value was observed (-15.9). The dotted lines in Fig. 3 represent simple dilution models, where c assumes a value of -1 .

Effect of short-term fasting

Fish in the STARV group had decreased in biomass by approximately 14% after 10 d and 20% after 20 d (Fig. 2). No change in muscle $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ or in liver

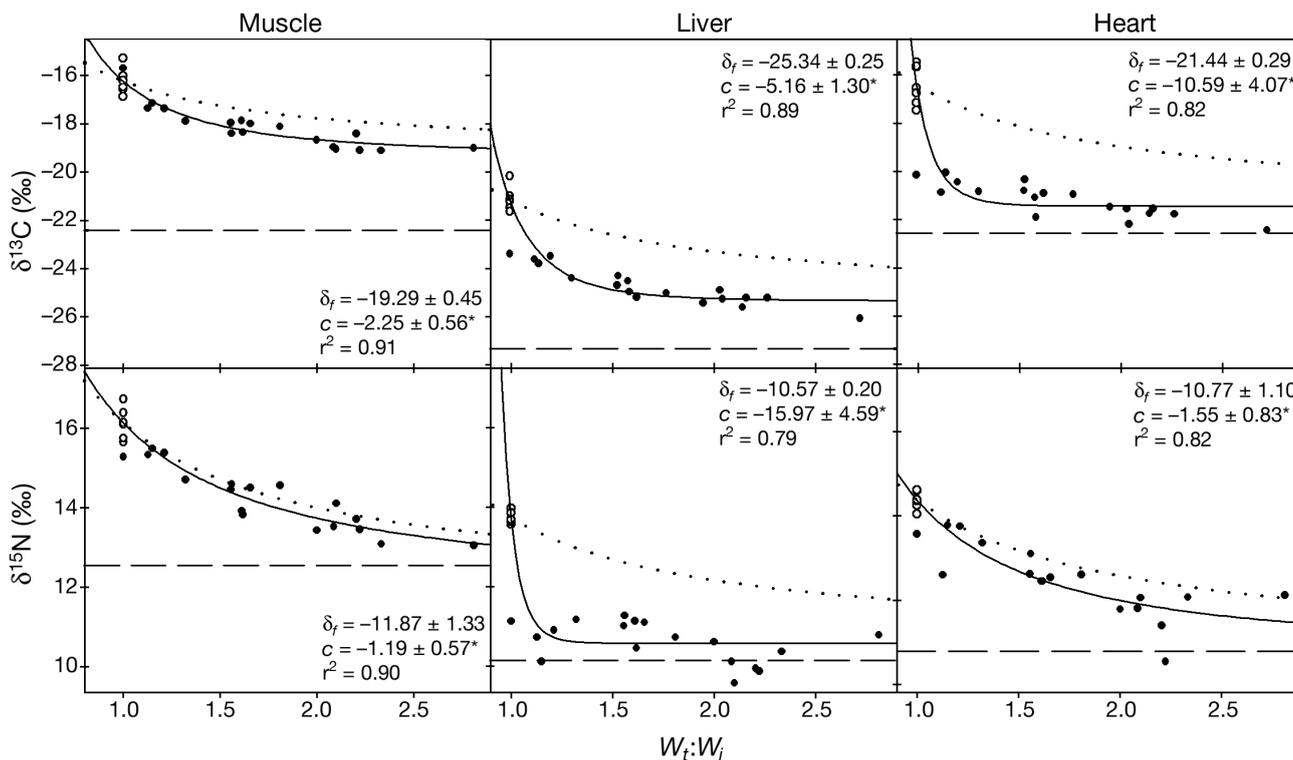


Fig. 3. *Pomatoschistus minutus*. Changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as a function of fish biomass for muscle, liver and heart tissue in the PEL group. (●) δ_f , mean value of 2 individuals (1 aquarium); (○) δ_i (1 individual). Continuous lines: best fit through data following $\delta_t = \delta_f + (\delta_i - \delta_f)(W_t/W_i)^c$, where δ_f (‰) and c were estimated by the model (\pm SE); *: indicates when c is statistically significantly different from -1 ; dotted lines: change in isotopic composition due to dilution only ($c = -1$); dashed lines: expected signatures when in equilibrium with pellet diet (cf. Table 3)

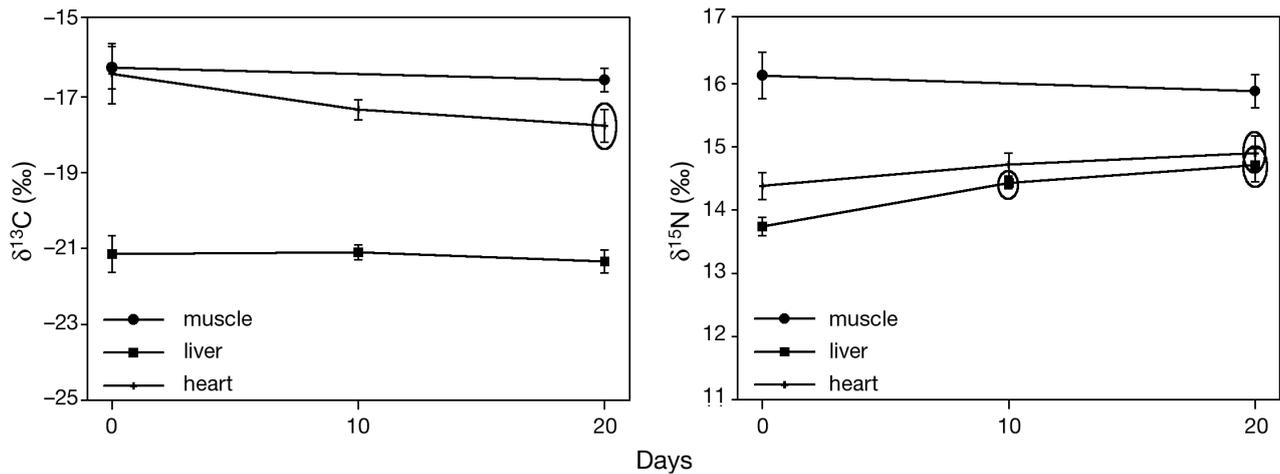


Fig. 4. *Pomatoschistus minutus*. STARV group: effect of food deprivation on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of muscle, liver and heart tissue (mean value \pm SD) after 10 and 20 d. δ -values significantly different ($p < 0.05$) from those before diet switch are encircled

$\delta^{13}\text{C}$ was observed after 20 d of fasting (Fig. 4). However, liver $\delta^{15}\text{N}$ and heart $\delta^{15}\text{N}$ increased while heart $\delta^{13}\text{C}$ decreased as a result of food deprivation. For liver $\delta^{15}\text{N}$, the effect occurred after 10 d; for heart $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ the effect of starvation was only detectable on Day 20.

DISCUSSION

Mortality and growth

Fish mortality was highest during the first 2 wk, during which several fish died. Mortality was the same in both treatments and never exceeded 20%. It could not be attributed to one specific cause. We observed that some fish did not forage on the pellet diet and became weak and died. Some suffered visibly from bacterial and fungal infections. Marine gobies are highly susceptible to mycosis and bacterial infections and this causes a high mortality at sea. Such infections probably affected some of our freshly caught gobies, and were enhanced by external stress occasioned by capture and captivity (M. Fonds pers. comm.). The remaining fish were probably resistant to the infections and their average growth rate (PEL group) equalled that in their natural habitat for the same period (J. Guelinckx unpubl. results), indicating that the experimental conditions were satisfactory for *Pomatoschistus minutus* and that the experimental results can be applied to field data.

Tissue-specific $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

The initial $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were tightly grouped in each tissue and corresponded strongly (Mann-Whitney U tests, $p > 0.05$) to values for sand gobies sampled

1 mo earlier (July 20, 2004) in the Eastern Scheldt during a preliminary study. These results are also shown in Table 3 for comparison. The consistency in isotopic composition of both sampling events strongly suggests that the tissues were in equilibrium with the local marine food web on Day 0. The statistically significant differences among the tissues indicate tissue specific trophic shifts. The same ranking in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for muscle, liver and heart tissue was also found in other studies, e.g. for rainbow trout *Oncorhynchus mykiss* (Pinnegar & Polunin 1999). These isotopic variations result partly from differences in biochemical composition. The lipid content in particular seems to be important for $\delta^{13}\text{C}$, with lipids being depleted in ^{13}C relative to the other biochemical components (DeNiro & Epstein 1977, Tieszen et al. 1983, Gearing 1991). Liver, which has a high lipid content, generally has lower $\delta^{13}\text{C}$ values (Focken & Becker 1998, Pinnegar & Polunin 1999, Lorrain et al. 2002). Hesslein et al. (1993) found for broad whitefish *Coregonus nasus* that liver $\delta^{13}\text{C}$ was on average $4.1 \pm 0.5\text{‰}$ more depleted than muscle tissue, which is highly consistent with our results. However, they also found that lipid removal from liver tissue could only account for a small portion ($0.7 \pm 0.3\text{‰}$) of the difference between liver and muscle. This, together with an increased variability in isotopic composition after lipid removal (Pinnegar & Polunin 1999), made us decide not to extract lipids from our samples.

The tissues are also distinct in terms of $\delta^{15}\text{N}$, probably due to the relative abundance of different amino acids in the various tissues. The isotopic composition of essential amino acids exhibits little change during assimilation. However, amino acids that are wholly synthesized or at least partly modified, may undergo shifts in $\delta^{15}\text{N}$ of varying magnitude depending on the

biosynthetic pathway (Pinnegar & Polunin 1999). Liver protein is known to have a greater proportion of essential amino acids, which might explain the lower $\delta^{15}\text{N}$ value observed for liver (Pinnegar & Polunin 1999, Kurle & Worthy 2002, McClelland & Montoya 2002). Thus, in addition to the different biochemical composition of tissues, secondary fractionation during physiological processes contributes to the isotopic variation among tissues. Also, lipid synthesized from dietary carbohydrate is relatively enriched in ^{12}C (DeNiro & Epstein 1977) in comparison to fat deposited directly from dietary lipid. Due to metabolic activity and/or biochemical components these secondary fractionation effects governing nitrogen and carbon assimilation are tissue specific (Kurle & Worthy 2002).

Furthermore, differences in isotopic composition among organs could also reflect the phenomenon of isotopic routing, which means that dietary nutrient components are allocated differentially to specific tissues and tissue components. Consequently, a tissue often reflects the isotopic composition of the nutrient component of the diet from which it was synthesized, and not the isotopic composition of the whole diet (Gannes et al. 1997).

We suspect that the nutrient components of the formulated pellets were not homogeneous in C isotopic composition, and that differential assimilation and routing of these components led to the observed discrepancy between the model-predicted $\delta^{13}\text{C}$ value (δ_r) and the expected $\delta^{13}\text{C}$ value in muscle and liver tissue (Figs. 1 & 3). The other models, especially those for $\delta^{15}\text{N}$, predicted a final value, δ_r , close to expected values, supporting the applied classical trophic enrichment factors and the observed differences among the tissues before the diet switch.

The carbon trophic enrichment factor in marine vertebrates is generally very small (Kurle & Worthy 2002). Post (2002) reviewed the trophic fractionation within aquatic organisms and found an average increase of $0.4 \pm 1.3\text{‰}$ ($n = 107$) for $\delta^{13}\text{C}$. There was, however, a relatively large variation around this value from approximately -3 to $+4\text{‰}$. Peterson & Fry (1987) and Vander Zanden & Rasmussen (2001) reported similar values. High trophic enrichment factors for $\delta^{13}\text{C}$ have been reported for muscle tissue of brook trout *Salvelinus fontinalis* ($+3.3 \pm 0.29\text{‰}$) (McCutchan et al. 2003) and of juvenile mado *Atypichthys strigatus* ($+3.7\text{‰}$) reared on a diet of commercial flake for 12 mo (Gaston & Suthers 2004). It remains unclear as to what the most important underlying biochemical and physiological mechanisms are that control the isotopic differences among tissues. It is obvious that more field and laboratory investigations are required to elucidate the sources of variation in tissue isotopic composition and specific trophic shifts. This is all the more important

since isotopic variability among organs may reflect their different metabolic activity as well as their isotopic turnover rates (Gannes et al. 1997, 1998, Lorrain et al. 2002).

Change in isotopic composition following dietary change

Isotopic turnover rates vary widely among different tissues and organs in endothermic vertebrates (e.g. Tieszen et al. 1983, Hobson & Clark 1992, 1993, MacAvoy et al. 2005), but this has not yet been unambiguously established for poikilothermic fish. C, N and S turnover rates for liver and muscle tissue were similar in broad whitefish, suggesting that the large differences in turnover rates among tissues of endotherms might not occur in fishes (Hesslein et al. 1993). This was (among others) supported by Herzka & Holt (2000), who stated that the high basal metabolism of endotherms, that probably results in tissue specific turnover rates does not occur in ectotherms, explaining the negligible effect of metabolic replacement to isotopic change often found for ectotherms. The half-life periods found in the present study, however, clearly show that different fish organ tissues can vary in isotopic turnover rate. Different $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic turnover rates were found for goby muscle, liver and heart tissue, with muscle having the slowest turnover rate (Fig. 1). Moreover, our results indicate that metabolic activity is not always negligible in fish tissue and can play a dominant role in isotopic change for tissues other than muscle (Fig. 3). Similar results were recently reported for whitefish *Coregonus lavaratus* (Perga & Gerdeaux 2005), Japanese temperate bass *Lateolabrax japonicus* (Suzuki et al. 2005) and juvenile mummichogs *Fundulus heteroclitus* (Logan et al. 2006).

The exponent of metabolic decay, c allows interpretation of the isotopic turnover rate in terms of growth and metabolic replacement. A decrease in c corresponds to faster isotopic turnover rates in our results (Figs. 1 & 3) and this implies an increase in the relative contribution of metabolic replacement to isotopic change. c was statistically significantly different from -1 for $\delta^{15}\text{N}$ in liver and for $\delta^{13}\text{C}$ in muscle, liver and heart tissue, demonstrating that metabolic replacement accelerates the rate of isotopic change and causes tissue specific isotopic turnover rates. The liver is a regulatory tissue with a continuous protein turnover which exceeds that in muscle tissue (de la Higuera et al. 1999). This explains the shorter half-life of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in liver compared to muscle. Muscle is the tissue most representative of growth when protein synthesis and deposition are considered, but at the

same time it has a relatively low protein turnover in fish (de la Higuera et al. 1999). The $\delta^{13}\text{C}$ in goby heart tissue had a relatively high turnover rate. To some extent, this could have been caused by adhering blood plasma fractions, which have a short $\delta^{13}\text{C}$ half-life period (Hobson & Clark 1993).

Metabolic activity was found not to be important for nitrogen isotopic change in goby muscle, with the estimated c not significantly different from -1 (95% CI: $-2.37, -0.01$). For carbon, however, -1 fell just outside the estimated 95% CI for c ($-3.45, -1.07$). Our data support the general view that growth is mainly responsible for isotopic change in fish muscle tissue. Nevertheless, a refinement to the model regarding the role of metabolic replacement seems necessary. The effect of metabolic activity on the isotopic turnover rate is most likely negligible in early life stages or fast growing fish, and a simple dilution model is adequate to describe the change in isotopic composition of the whole body (Hesslein et al. 1993, Herzka & Holt 2000, MacAvoy et al. 2001, Bosley et al. 2002). However, metabolic replacement cannot be ignored in muscle tissue of almost full-grown fish such as the gobies in this study. The exponent c for muscle $\delta^{13}\text{C}$ had a relatively low value (-2.26) compared to other published metabolic decay values for larvae and juvenile fish (Herzka & Holt 2000, Herzka et al. 2001, Bosley et al. 2002) and was indeed different from -1 , indicating a statistically significant contribution of metabolic replacement to isotopic change. Thus, in tissues of moderate- to slow-growing fish, both metabolic turnover and growth are likely to play an important role in isotopic changes (Suzuki et al. 2005, Logan et al. 2006, Miller 2006) as already hypothesized by Fry & Arnold (1982). Sakano et al. (2005) showed for sockeye salmon *Oncorhynchus nerka* that the degree of metabolic contribution became increasingly more important with age, as growth rate decreased.

Isotopic composition during starvation

Fasting animals tend to have stable isotope ratios that are distinct from those fed ad libitum. The effects of food deprivation are especially expected to be manifested in metabolically more active tissues (Hobson et al. 1993) or tissues for which a low exponent of metabolic decay is detected. Indeed, in this study, no effects were found for muscle tissue, but statistically significant changes occurred in liver $\delta^{15}\text{N}$ and heart $\delta^{13}\text{C}$, consistent with their low exponent of metabolic decay, c . Liver $\delta^{13}\text{C}$, however, exhibited no change although its c was rather low, and the ^{15}N enrichment in the heart was unexpected based on its c close to -1 (Figs. 3 & 4).

The tissues of starving animals often show a progressive increase in their $^{15}\text{N}/^{14}\text{N}$ ratio as body mass decreases (Hobson et al. 1993, Oelbermann & Scheu 2002, Olive et al. 2003). The mechanisms by which their tissues become enriched in ^{15}N are partly the same as those causing trophic fractionation. The catabolized and excreted lighter ^{14}N is not replaced by dietary protein; therefore the animal becomes progressively more ^{15}N -enriched during starvation (Gannes et al. 1997). The rate of increase in the animal's ^{15}N concentration happens faster when the ratio of the excretion rate of ^{14}N to ^{15}N is high and N turnover is large (Ponsard & Averbuch 1999). An alternative explanation for the ^{15}N enrichment involves possible changes in amino acid composition (Hobson & Clark 1992).

The decrease in $\delta^{13}\text{C}$ noted in heart tissue is quite remarkable, since most other starvation studies do not report a $\delta^{13}\text{C}$ change and, where they do, then the change is toward ^{13}C enrichment (Frazer et al. 1997, Gorokhova & Hansson 1999, Oelbermann & Scheu 2002, Olive et al. 2003, Tominaga et al. 2003). A ^{13}C enrichment can be explained by the preferential loss of ^{12}C during oxidation of acetyl groups derived from catabolism of lipids, proteins and carbohydrates (Hobson et al. 1993). Depleted $\delta^{13}\text{C}$ values as a result of food deprivation have to our knowledge, not yet been reported. One explanation for the lower values in heart tissue could be that blood components 'contaminating' the heart tissue contained catabolized products enriched in ^{12}C . Another explanation could be that ^{13}C -depleted lipids are used as fuel by heart muscle tissue. It is evident that more research is needed for complete understanding of the change in isotopic composition and the origin and fate of mobilized biochemical components during catabolism.

Comments on experimental design

The experiment was carefully designed to resemble natural conditions as much as possible. The start of the experiment coincided with the start of sand goby migration into the Scheldt estuary. Consequently, sampled individuals were of approximately the same age and length as those from the estuarine population under study. In addition, during transfer of the fish to aquaria, all specimens not corresponding to the average length \pm SD of the estuarine population at that time were excluded from the experiment. Temperature and photoperiod corresponded to average conditions for the Scheldt estuary during the study period. Despite the fact that experimental conditions mimicked natural conditions, care should be taken when applying the experimental results to field data, especially having regard to the high estimated trophic shifts for $\delta^{13}\text{C}$ in

muscle and liver. It would have been better to prolong the experiment until all tissues reached isotopic equilibrium with certainty and to use a natural diet. This was however not possible in our study. A shorter sampling interval during the first 20 d would also have improved our experimental design. Nevertheless, our study has provided a useful first insight into stable isotopic turnover rates in juvenile sand goby tissues. However further research is required to achieve a better understanding of how variations in metabolic activity (e.g. due to temperature, body size, stress, etc.) influence isotopic turnover rate in tissues.

Finally, we highly recommend the use of a control treatment to detect shifts in isotopic composition not caused by a new diet. A control treatment based on mussel meat *Mytilus edulis* was *a posteriori* rejected from our experiment because, after accounting for trophic enrichment, mussel $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values deviated from those of goby muscle before the diet switch, a result in contrast to that of a preliminary study. In addition, from Day 45 onwards there was a difference in growth rate between fish fed the pellet and those fed the mussel diet. When selecting diets for an isotopic turnover study care should be taken to not only consider the isotopic composition, but also to confirm that the nutritional composition and the food quality of the respective diets are equal and adequately represent the quality of the natural food sources of the species under study.

Conclusions

The results of this study encourage the use of stable C and N isotopes for analyzing sand goby migrations in terms of several days to several weeks. The controlled laboratory conditions resulted in clear changes in the isotopic composition of sand goby muscle, liver and heart tissue. Observed isotopic turnover rates were specific for tissue type and element. Muscle tissue was identified as having a temporal resolution ($t_{1/2}$ = ca. 26 d) appropriate for determining the residence times of individuals caught on a monthly basis in the upper Scheldt estuary. Moreover, muscle is to be preferred since it is not influenced by short-term fasting, in contrast to the metabolically more active liver and heart tissue. The isotopic turnover rate in muscle is predominantly controlled by somatic growth, although we found a statistically significant contribution of metabolic replacement for $\delta^{13}\text{C}$. The relative contribution of basal metabolism is likely to increase in older, slow growing (sub)adult fish and/or during warm periods. The isotopic turnover rate for liver and heart tissue was quite high and can probably be explained by the important role of metabolic replacement in these tis-

sues. This feature is generally accepted for endotherms but not for ectotherms like fish.

The observed fractionation factors for $\delta^{13}\text{C}$ in muscle and liver emphasize the need for further research on the physiological and biochemical mechanisms that control the isotopic shift between an organism and its diet.

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