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Effect of digestion on the $\delta^{13}C$ and $\delta^{15}N$ of fish-gut contents

J. Guelinckx*†, F. Dehairs‡ and F. Ollevier*

*Laboratory of Aquatic Ecology, Katholieke Universiteit Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium and ‡Department of Analytical Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

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Gut contents of sand goby *Pomatoschistus minutus* showed higher C and N isotope values than the food before consumption. This enrichment was more pronounced in the hindgut than in the foregut, probably because of preferential assimilation of ^{12}C and ^{14}N along the gastro-intestinal tract. The results indicated that the shift towards higher values in the alimentary canal occurs in the first 2 h after feeding.

Key words: assimilation; carbon and nitrogen stable isotopes; *Pomatoschistus minutus*; stomach content analysis; trophic enrichment.

Stable isotope techniques are routinely used in the study of trophic relationships and animal migrations in aquatic and terrestrial ecosystems (Gannes *et al.*, 1998; Hobson, 1999; Thompson *et al.*, 2005). Use of stable C and N isotopes to elucidate trophodynamics requires *a priori* estimates of the alteration in $\delta^{15}N$ and $\delta^{13}C$ values between prey and predator (known as trophic fractionation). The stable isotopic composition of a consumer was commonly accepted to be enriched in ^{13}C by *c.* 0–1‰ and in ^{15}N by *c.* 3·4 ± 1·1‰ (mean ± S.D.) relative to its diet (DeNiro & Epstein, 1978; Minagawa & Wada, 1984; Owens, 1987). Considerable variation in the trophic shift for $\delta^{13}C$ and $\delta^{15}N$, however, has been observed between and within trophic groups or even among conspecifics. Multiple potential sources for this variation have been recognized, such as taxon identity (Minagawa & Wada, 1984), main biochemical form of the nitrogenous waste (Vanderklift & Ponsard, 2003), dietary differences (Adams & Sterner, 2000; Vander Zanden & Rasmussen, 2001; McCutchan *et al.*, 2003; Robbins *et al.*, 2005), ration size, temperature (Barnes *et al.*, 2007), variable tissue composition (Focken & Becker, 1998; Pinnegar & Polunin, 1999), isotopic routing (Gannes *et al.*, 1997) and physiological stresses such as starvation (Hobson *et al.*, 1993). Nevertheless, the real underlying physiological and biochemical mechanisms

†Author to whom correspondence should be addressed. Tel.: +32 16 324572; fax: +32 16 324575; email: jef.guelinckx@bio.kuleuven.be

responsible for the change in isotopic ratios between food source and consumer remain poorly understood (Gannes *et al.*, 1997; Sponheimer *et al.*, 2003).

Ponsard & Averbuch (1999) proposed that the isotopic composition of a whole organism is the result of a dynamic equilibrium between isotopic discrimination during food digestion and incorporation on the one hand and isotopic discrimination associated with the discharge of excretion products on the other hand. The ratio of fractionation during assimilation (including digestion and absorption) to fractionation during excretion thus determines the direction and magnitude of the trophic shift (Ponsard & Averbuch, 1999; Olive *et al.*, 2003). The lighter isotope is preferentially used in biochemical reactions, due to its weaker bonds. Consequently, the consumer's body should become isotopically lighter relative to the diet during food assimilation, while the unabsorbed food in the gut becomes more enriched in the heavier isotope (Fry *et al.*, 1984). Catabolic reactions, however, also favour the lighter isotope (DeNiro & Epstein, 1978; Minagawa & Wada, 1984) and the consumer must become enriched in the heavier isotope as excretory products are eliminated (Olive *et al.*, 2003).

Stable isotope analysis (SIA) on stomach or gut contents may be a powerful tool in food web and animal migration studies. SIA on gut contents can provide direct information about the diet of consumers and subsequently also about the food sources of their prey (Grey *et al.*, 2002). This way some of the uncertainty associated with trophic fractionation can be circumvented. Additionally, it may contribute to establish an appropriate isotopic base line (Post, 2002) in food web studies. In order to study animal movement patterns using stable isotopes, it is necessary that the locations between which migration occurs are isotopically different (Hobson, 1999; Herzka, 2005). As migrating animals gradually adapt their isotopic composition to that of the new feeding ground (Hobson, 1999), their tissues cannot be used to test this prerequisite, especially if the timing of arrival and the isotopic turnover rate of the tissues is unknown. Gastro-intestinal contents can then provide a solution to establish isotopic differences between target locations and to predict completely adapted isotope values of migrants at specific locations (Guelinckx *et al.*, 2006).

Using SIA on gut contents may avoid additional sampling efforts towards prey items and eliminate the problem of investigating and identifying the food sources in their contributing proportions (Peterson, 1999). This is all the more important when the species under study forages on one or a few specialized diet items within the available prey spectrum (Jardine *et al.*, 2005).

Although Tieszen *et al.* (1983) and Peterson (1999) suggested performing SIA on stomach or gut contents, only a few studies actually have done so (Fry *et al.*, 1984; Fry, 1988; Peterson *et al.*, 1993; Grey *et al.*, 2002; Page & Lastra, 2003; Yatsuya & Nakahara, 2004; Jardine *et al.*, 2005; Guelinckx *et al.*, 2006). Only two studies reported possible effects on the stability of stable isotope ratios of ingested food due to digestion or fractionation during absorption and excretion (Fry *et al.*, 1984; Grey *et al.*, 2002). These effects may complicate the interpretation of isotope measurements on ingested food items.

This study is part of a research project that uses stable C and N isotopes to investigate recruitment of the marine sand goby *Pomatoschistus minutus* (Pallas) to the Scheldt Estuary (Belgium). Following Guelinckx *et al.* (2006), gut contents are analysed to determine the C and N isotopic gradient between

the marine and the brackish-water food web and to predict estuarine end member signatures for fish muscle tissue. A major concern here is the degree and timing of the change in the isotopic composition of the ingested food due to digestive and assimilation processes. In addition, isotope measurements of gut contents can help to understand the mechanisms controlling trophic fractionation. Thus, the aims of the present study were twofold: to test for any change in diet $\delta^{13}C$ and $\delta^{15}N$ in the gastro-intestinal tract of the sand goby as a function of time and to assess the isotopic differences among foregut content, hindgut content and the food prior to ingestion. It was expected that the gut content would become enriched in the heavy isotope over time and that this effect would be stronger in the hindgut than in the foregut.

A feeding experiment was conducted in which gut contents were collected for SIA at regular time intervals after foraging. The experimental design was constrained by the limited number of available fish in a laboratory stock of a larger experiment (Guelinckx *et al.*, 2007). Fifteen specimens were distributed in equal numbers over three 20 l aquaria, which were installed in a continuous flow-through system. At least 20% of the water volume was changed on a daily basis. Temperature was preset on 17° C and the photoperiod regime was 12L:12D. Twice a day, fish were fed *ad libitum* a formulated pellet food (Table I) and were observed to forage well after 4 days. On the fifth day, the fish were fed in excess for 30 min. The remaining food was then removed and 1, 2, 4, 6 and 8 h later one fish from each aquarium was killed by severing the CNS and immediately stored at −20° C. This resulted in three replicates for each sampling event. The experimental design did not include any measurement after 8 h, since calculations of the depletion rate of gastro-intestinal content, based on the model of Andersen (1984), predicted that only 9% of the ingested food remained 9 h after feeding. For additional details on aquarium set up and maintenance see Guelinckx *et al.* (2007).

Whenever possible, food samples from the foregut (oesophagus and proximal region of the intestines) as well as from the hindgut (distal region of the intestines without the rectum) were collected from the same fish for isotope ratio measurements. Surprisingly, several gastro-intestinal tracts were empty even though the fish were observed to forage on the pellets. In order to obtain more samples, the whole experiment was immediately repeated with an additional nine fish. They were killed 2, 4 and 6 h after foraging on the pellet food. Results of both experiments were treated together for statistical analysis. Dorsal muscle tissue of one randomly chosen fish from each aquarium was also collected for analysis ($n = 6$).

Gut content and muscle tissue samples were dried at 55° C to constant mass, homogenized using a mortar and pestle, and aliquots were weighed into tin containers. Stable C and N isotope ratio measurements were performed at the Laboratory for Analytical and Environmental Chemistry (ANCH) at the Vrije Universiteit Brussel (Belgium) on a Flash series 1112 elemental analyser interfaced to a DeltaPlus XL Thermo Finnigan IRMS. The working standards were high-purity N_2 and CO_2, while IAEA-C-6 (mean ± S.D. −10·4 ± 0·1‰) and IAEA-N_2 (20·41 ± 0·12‰) were used as reference materials. Stable isotopic compositions are expressed in the conventional δ-notation: $\delta X = [(R_{sample} - R_{standard})R^{-1}_{standard}] \times 10^3$, where X is ^{13}C or ^{15}N and R is ^{13}C:^{12}C or ^{15}N:^{14}N. $\delta^{13}C$ values are expressed relative to the VPDB (Vienna PeeDee Belemnite)

TABLE I. Mean ± S.D. $\delta^{13}C$ and $\delta^{15}N$ of the pellet feed, muscle tissue, foregut and hindgut content samples. Mann–Whitney *U*-tests were used to test for differences between the isotopic composition of pellets and gut content samples (Fgut, foregut; Hgut, hindgut) and Wilcoxon matched pairs tests for differences between foregut and hindgut samples

	Pellet	Muscle	Foregut	Hindgut	△ Fgut-pel	△ Hgut-pel
$\delta^{13}C$ (‰)	−23·25 ± 0·55 ($n = 7$)	−16·59 ± 0·79 ($n = 6$)	−22·81 ± 0·43 ($n = 12$)	−22·04 ± 0·36 ($n = 9$)	0·44	1·20
			$n = 9$, $P < 0·05$		$P = 0·17$	$P < 0·01$
$\delta^{15}N$ (‰)	9·14 ± 0·22 ($n = 7$)	15·74 ± 0·55 ($n = 6$)	10·45 ± 0·36 ($n = 12$)	11·02 ± 0·47 ($n = 9$)	1·31	1·89
			$n = 9$, $P < 0·01$		$P < 0·001$	$P < 0·001$

standard, while $\delta^{15}N$ values are expressed to atmospheric N_2. Reproducibility for different aliquots of the reference materials was generally better than 0·3‰ for $\delta^{13}C$ and 0·2‰ for $\delta^{15}N$.

Linear regressions were used to examine temporal trends in the $\delta^{13}C$ and $\delta^{15}N$ data of gut contents. Subsequently, the Mann–Whitney *U*-test was applied to test for isotopic differences between the diet and gut contents, while the Wilcoxon matched pairs test was used to evaluate whether significant differences occurred between foregut and hindgut contents (STATISTICA 6.0, StatSoft Inc., Tulsa, OK, U.S.A.).

Assuming a preferential assimilation of ^{12}C and ^{14}N, it was expected that gastric $\delta^{13}C$ and $\delta^{15}N$ values would become enriched relative to the pellet diet (Olive *et al.*, 2003). The results seem to confirm this, though quite a lot of variation was observed and interpretation should be done with caution. Mean ± S.D. $\delta^{13}C$ and $\delta^{15}N$ values for the pellets, dorsal muscle tissue, foregut and hindgut contents are summarized in Table I. For $\delta^{15}N$, both foregut and hindgut contents differed significantly from the pellets (+1·31 and +1·89‰, respectively), and for $\delta^{13}C$ there was an enrichment of +1·20‰ in the hindgut content, but no significant difference was detected in the foregut compared to the pellets. Although this enrichment was probably caused by differential absorption of the lighter isotope, mixing of the undigested food with digestive tract material such as intestinal cells, digestive enzymes and mucus cannot be ruled out completely (Ponsard & Averbuch, 1999; Sponheimer *et al.*, 2003; Jardine *et al.*, 2005). Sand goby muscle tissue was *c.* 6·6‰ enriched in ^{13}C and ^{15}N relative to the pellet food (Table I). If only contamination with endogenous material was responsible for this enrichment between pellets and gut contents, however, equal shifts for $\delta^{13}C$ and $\delta^{15}N$ would occur, assuming a similar C:N stochiometry. Yet, the isotopic shift appeared to be higher for $\delta^{15}N$ than for $\delta^{13}C$ in foregut and hindgut. The significantly higher $\delta^{13}C$ and $\delta^{15}N$ values of the hindgut samples relative to the foregut samples also suggest a preferential digestion and uptake of ^{12}C and ^{14}N along the digestive tract.

As a result of discrimination against heavy isotopes during assimilation, the consumer's body is expected to become depleted in heavy isotopes relative to the diet. This contradicts the fact that consumers are generally enriched compared to their diet (DeNiro & Epstein, 1978; Minagawa & Wada, 1984; Vander Zanden & Rasmussen, 2001; Vanderklift & Ponsard, 2003). Consequently, the fractionation against heavy isotopes must be larger during processes leading to excretion than during the assimilation processes in order to account for trophic enrichment of animals (Ponsard & Averbuch, 1999; Olive *et al.*, 2003).

The higher values for $\delta^{13}C$ and $\delta^{15}N$ in the hindgut and for $\delta^{15}N$ in the foregut contrast with results reported by Grey *et al.* (2002), who observed that the $\delta^{13}C$ and $\delta^{15}N$ composition of crustacean zooplankton and Arctic charr *Salvelinus alpinus* (L.), both derived from fish-gut samples, were within the inherent natural variability exhibited by local specimens. They concluded that the effects of digestive processes on $\delta^{13}C$ and $\delta^{15}N$ of ingested food were negligible, yet it might well be that differences between food sources in the natural environment and the gut contents remained undetected due to a higher natural variability. Fry (1981) reported no influence of digestion on $\delta^{13}C$ of food recovered from the stomachs of benthic crustaceans (Fry *et al.*, 1984). This is in agreement with

the foregut $\delta^{13}C$ data of sand goby. Fry *et al.* (1984) on the other hand showed that midgut and faecal samples of benthic shrimps became actually depleted in ^{13}C relative to foregut samples during assimilation. In contrast to the latter study, there are many reports showing isotopically enriched faeces (DeNiro & Epstein, 1978; Steele & Daniel, 1978; Sponheimer *et al.*, 2003) confirming the observed enrichment in sand goby guts. The present study is, as far as is known, the first to present data on the rate of this enrichment. The shift towards higher $\delta^{13}C$ and $\delta^{15}N$ values for ingested food seems to occur within the first 2 h of feeding (Fig. 1), as $\delta^{13}C$ and $\delta^{15}N$ of foregut and hindgut did

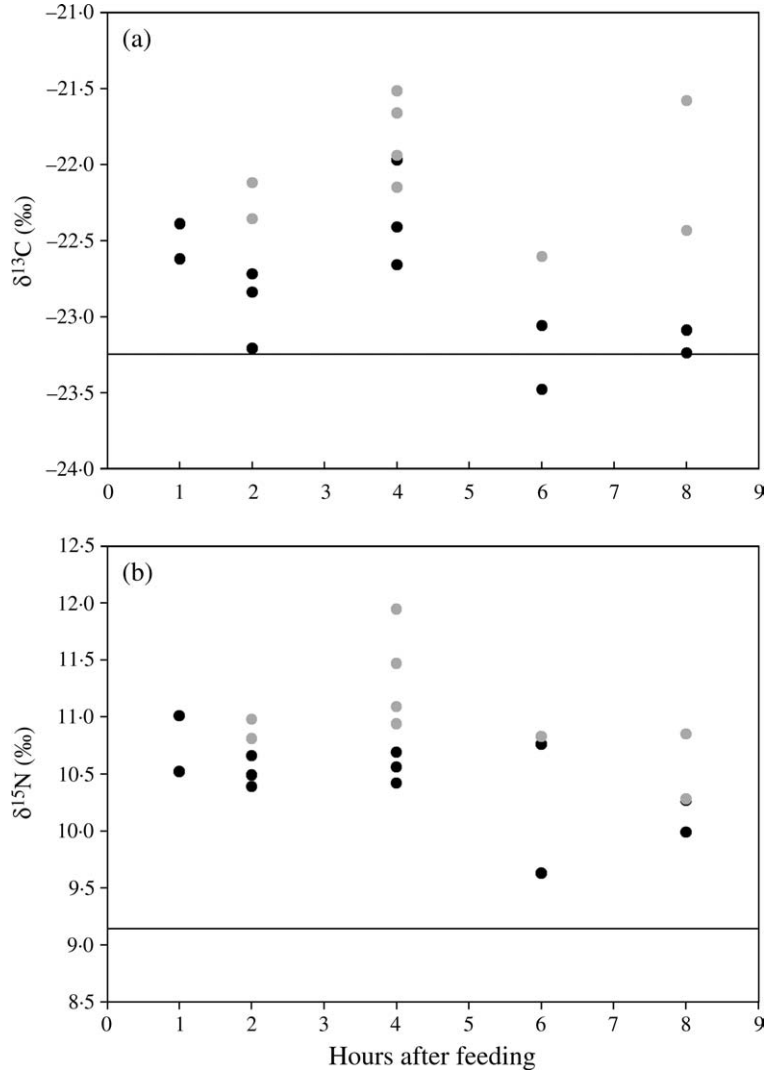

FIG. 1. (a) $\delta^{13}C$ and (b) $\delta^{15}N$ of foregut (●) and hindgut contents (●) sampled 1, 2, 4, 6 and 8 h after feeding. ——, the mean value of the formulated pellet feed. There were no samples for hindgut 1 h after feeding.

not show an increasing trend between 1 and 8 h after foraging. On the contrary, the $\delta^{13}C$ and $\delta^{15}N$ data suggested decreasing trends over time, although none of slopes were significantly different ($P > 0{\cdot}05$) from zero (Fig. 1). The relatively fast isotopic shift might be explained by high initial absorption rates (Goldstein & Elwood, 1971; Andersen, 1984) and the preferential digestion and uptake of the lighter isotope. The absence of a persisting $\delta^{13}C$ and $\delta^{15}N$ increase over time probably results from the decreasing overall absorption rate of nutrients and a relatively greater uptake of the heavier isotope which is expected to become more available as digestion proceeds.

Although the experiment was limited in its design and the results showed considerable variation, this study provides insight in processes leading to trophic fractionation. The results seem to support the model proposed by Ponsard & Averbuch (1999), stating that trophic fractionation derives from a combination of isotopic fractionation during assimilation and synthesis, as well as during catabolic processes and excretion. Additionally, with respect to applications of SIA on gut contents, this study suggests that the moment of sampling after feeding time is relatively unimportant and does not confound isotopic values. This confirms that gut contents can be applied to investigate isotopic gradients in the environment, *i.e.* the relative differences in isotopic composition between habitats. Nevertheless, care should be taken in determining absolute isotopic signatures for prey items or trophic fractionation based on gut contents. Differences in isotopic composition between food and gut contents were observed, especially for the hindgut. More elaborate studies are needed to identify the influencing factors such as temperature, diet quality, food ration and consumer's physiological status, and ultimately the underlying physiological mechanisms behind diet and tissue fractionation. Efforts should hereby be made to perform (compound specific) SIA on excretion products and on gut contents free of adhering digestive tract fragments, in order to assess the degree of isotopic fractionation during catabolic and assimilation processes, respectively.

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