Naturally-occurring stable isotopes as direct measures of larval feeding efficiency, nutrient incorporation and turnover

Le Vay, Lewis [a,*] and Gamboa-Delgado, Julián [b]

[a] School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey, Wales, LL59 5AB, United Kingdom

[b] Programa Maricultura, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Cd. Universitaria Apdo. Postal F-56, San Nicolás de los Garza, Nuevo León 66450, Mexico

*corresponding author: Tel: +44 (0) 1248 351151; Fax: +44 (0) 1248 716367 l.levay@bangor.ac.uk

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Abstract

Stable isotopes are non-hazardous markers that have been widely-used in assessing energy flow within aquatic ecosystems. Hatchery systems are also highly amenable to this approach, as they represent controlled mesocosms with a limited number of food sources and short planktonic food chains with rapid and measurable bioaccumulation of the heavier stable isotopes of carbon and nitrogen at each trophic step. Differences in the natural isotopic composition of dietary components may be used to provide direct integrated measures of ingestion, nutrient incorporation and growth through development under normal feeding and environmental conditions, in either the laboratory or the hatchery. Simple isotopic mixing models allow estimation of relative utilisation of inert diets and live feeds, and individual components of compound feeds. Such experiments have investigated the effectiveness of co-feeding regimes, optimal timing of live food transitions (eg from rotifers to *Artemia*), presentation of inert diets, optimal size/age for weaning and incorporation of specific dietary components. Furthermore, time-series measurement of changes in tissue isotopic signature ($\delta^{15}N$, $\delta^{13}C$) enables modelling of growth dilution and tissue turnover components of isotopic change driven by nutritional sources. These measures need to take into account the difference in isotope values that is typically observed between the diet and consumer (isotopic discrimination factor, Δ). In marine larvae and early post-larvae, $\Delta^{13}C$ and $\Delta^{15}N$ have been found to range widely, from 0.4-4.1‰ and 0.1-5.3‰ respectively. The observation of such a high level of variation within species and life stages indicates a strong effect of diet quality on isotopic discrimination. Elucidating mechanisms underlying such observations, and much greater resolution in larval nutritional studies, can be achieved by application of rapidly-developing techniques for compound specific stable isotope analysis in tracing the transfer of dietary sources of carbon and nitrogen into tissue components. Fast growing aquatic larvae represent excellent model organisms exhibiting rapid transitions in isotopic composition in

response to diet, rapidly changing feeding behaviour and transitions in trophic level with ready ingestion of modifiable experimental diets in short and controlled food chains. Thus results of studies of the effects of diet composition, developmental stage, growth rates or environmental conditions on stable isotope incorporation will be of broad relevance not only in terms of larval nutrition but can also more broadly inform the design and interpretation of ecological studies.

Keywords: Stable isotopes, larval nutrition, assimilation, metabolic turnover

1. Introduction

Despite extensive research, the quantitative nutritional requirements of larvae of most marine species are not yet well understood and this has been mainly due to the difficulties in quantifying feed intake and assimilation. These are typically problematic to estimate in aquatic larval organisms due to size constraints, sample collection difficulties and rapid leaching of nutrients from micro-diets. Consequently, indirect indicators are commonly used to infer nutritional effects and measure performance of larval diets and feeding regimes, including comparison of diet and larval tissue composition, survival, rates of growth and development and responses to stress tests. More precise investigation of larval nutrition requires the use of tracers to follow the fate of specific dietary components. Radioactive and enriched stable isotopes have provided some of the most reliable tracers used in determination of ingestion rates, assimilation efficiencies and retention of nutrients (see recent review by Conceição et al., 2007). The use of radioactive isotopes (^{14}C, ^{3}H) as nutritional tracers was successfully applied in early studies of crustacean larval nutrition, to assess lipid incorporation and metabolism (Teshima and Kanasawa 1971; Teshima et al., 1976, 1986a,

1986b) and similarly radio-labelled compounds have been also applied to trace utilisation of nutrients in fish larvae (eg Koven et al., 1998; Rønnestad et al., 2001; Morais et al., 2005). However, the use of radiolabels is constrained by the need for appropriate safety management and their relatively rapid rate of dilution. Hence their application in larval nutrition research is typically restricted to short-term studies in small-scale, isolated, experimental culture systems. In contrast, stable isotopes are non-hazardous, non-invasive markers that can be used to determine the contribution of dietary sources to growth in individuals or at the population level. The stable isotope signature (frequently expressed in delta notation: δ) of a consumer organism reflects that of its diet, and hence represents a direct measure of nutrient incorporation and an integrated record of feeding over time (Peterson and Fry, 1987). Due to their natural abundance, the stable isotope ratios of carbon and nitrogen ($^{13}C/^{12}C$ and $^{15}N/^{14}N$, $\delta^{13}C$ and $\delta^{15}N$, hereafter in the text) are the most commonly used in ecological studies, identifying energy sources and trophic level, respectively, and have been a very effective tool in assessing energy flow within aquatic systems (Michener and Schell, 1994). In experimental studies of cultured aquatic species, isotopes of these elements are also the most commonly used, providing measures of energy transfer and protein utilization. In aquaculture pond systems, which represent semi-controlled aquatic mesocosms, both measurements of stable isotopes at natural abundance levels and isotopically-enriched nutritional substrates have been used to assess the sources and sinks for dietary carbon and nitrogen (Schroeder, 1983; Bombeo-Tuburan, et al*.,* 1993; Nunes et al., 1997; Epp et al., 2002; Burford et al., 2004a, 2004b). Such studies have determined, for example, the flow of nutrients from feeds into sediments (Yokoyama et al., 2006), from feeds to microbial flocs (Burford et al., 2002), and the relative contribution of formulated feeds and natural productivity to tissue growth (Parker et al., 1989). In laboratory studies, the use and application of stable isotopes allows the direct determination of ingestion and assimilation rates, with straightforward collection techniques

and rapid, accurate, sample analysis (Michener and Schell, 1994; Dittel et al., 1997; Verschoor et al., 2005). Adaptation of a similar approach to the scale of larval nutrition is attractive to circumvent some of the difficulties associated with assessment of ingestion and assimilation in such small and fast-changing life stages, with direct measurement of nutrient incorporation rather than use of indirect indices or added tracers. Hatchery systems are highly amenable to this approach, as they represent very controlled mesocosms with a limited number of food sources and short planktonic food chains with rapid and measurable bioaccumulation of the heavier stable isotopes of carbon and nitrogen at each trophic step. This paper reviews the current use of natural stable isotopes in larval nutrition research, compared to enriched stable isotope and radio-labeled tracers, and proposes a range of potentially valuable extensions of these applications in future studies.

2. Natural stable isotopes versus enriched stable isotope tracers

The use of larval diets, especially live feeds, enriched or labelled with very high levels of ^{13}C or ^{15}N has been applied as an alternative to radiolabels in a range of species. This is typically achieved by culturing algae in media containing the heavier isotope (for example, $NaH^{13}CO_3$ or $Na^{15}NO_3$) with rapid incorporation over a period of 12-24h, prior to feeding to live prey such as rotifers. In this way, the prey may accumulate heavier isotope concentrations of up to 18 atom% (Hino et al., 1997; Verschoor et al., 2005), providing a clearly distinguishable tracer signal in the consuming larva (Conceição et al., 2001). Very short term measurement of the incorporation (or depletion) of such labels, over less than the gut transit time, provides a measure of ingestion (or egestion) rates. In the case of ^{15}N, time series measurement of the ensuing changes in label concentrations in the free amino-acid pool and bound protein in larval tissue can be used as an alternative to single amino-acid radio-labels in flooding-dose studies to estimate protein synthesis and turnover rates (Carter et al. 1994; Houlihan et al.,

1995a, 1995b; Carter et al., 1998 ; Fraser et al., 1998; Conceição et al., 2001). However, in larvae such studies are typically run over a short timescale of 12-24 h and, as with most tracer methodologies, involve delivery of specific nutrient source under controlled or constrained conditions, providing a relatively instantaneous measure of physiological performance (Conceição et al., 2007). In contrast, studies that take advantage of the natural isotopic composition of dietary components may be designed to investigate integrated measures of ingestion, assimilation and growth over longer time periods under normal feeding and environmental conditions. To date, relatively few studies have adopted this approach, which is particularly useful in determining the sources and fate of nutrients (Schlechtriem et al., 2004; Jomori et al., 2005; Gamboa-Delgado et al., 2008) and in assessing tissue carbon and nitrogen turnover rates (Hesslein et al., 1993; Herzka et al., 2001; Gamboa-Delgado et al., 2008; Gamboa-Delgado and Le Vay, 2009b). Unlike the very high levels of heavy isotopes present in enriched feeds, natural abundance of carbon and nitrogen isotopes is very strongly biased toward the lighter ^{12}C and ^{14}N isotopes, and the differences in isotopic signature between dietary components is small. However, there is a sufficient range of values to allow design of useful contrasts between diets (Table 1) and these are easily measurable using widely-available isotope ratio measurement techniques developed for ecological samples, with dual stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) of animal tissue usually requiring very small sample sizes (800 to 1200 µg). In some cases, resolution of mixing models can be further improved by manipulation of the dietary isotopic composition, for example by feeding prey with C_3 and C_4 plant meals (Schlechtriem et al., 2004) or culturing algae with tank CO_2, but remaining within the normal range of values for natural samples.

Table 1. Examples of natural stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) and C:N ratios of different live and inert feeds frequently used in fish and crustacean larviculture.

Organism/feeding item	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C:N ratio	References
Phytoplankton				
Skeletonema costatum	-23.5 ± 1.1	-	-	Hinga et al., 1994
Phaeodactylum tricornutum[a]	-23.6 ± 1.1	-	-	Johnston and Raven, 1992
Chaetoceros muelleri[b]	-19.0 ± 0.2	0.0 ± 0.2	10.5	Gamboa-Delgado, unpublished
Chaetoceros gracilis[b]	-14.8 ± 0.0	-1.9 ± 0.1	7.8	Gamboa-Delgado, unpublished
Tetraselmis chuii[b]	-9.8 ± 0.1	5.7 ± 0.2	6.0	Gamboa-Delgado, unpublished
Rhinomonas reticulata[b]	-14.8 ± 0.1	6.4 ± 0.1	5.0	Gamboa-Delgado, unpublished
Isochrysis galbana[a]	-26.9 ± 0.9	-	-	Leboulanger et al., 1995
Isochrysis galbana[a](T-ISO)				
Air only	-21.0 ± 0.3	14.9 ± 0.4	7.1	Gamboa-Delgado et al., 2008
Air + injected CO_2	-43.3 ± 0.1	16.2 ± 0.1	7.7	
Zooplankton				
Copepods				
Temora longicornis	-19.1 ± 0.0	14.9 ± 0.1	-	Gentsch et al., 2009
Calanus finmarchicus	-23.4 ± 1.0	8.3 ± 1.1	-	Sato et al., 2002
Rotifers				
Brachionus calyciflorus[c]	-27.0 ± 3.0	8.5 ± 1.2	-	Yoshioka et al., 1994
Brachionus plicatilis				Gamboa-Delgado et al., 2008
Cultured on yeast	-23.9 ± 0.1	3.3 ± 0.2	4.2	
Enriched (T-ISO)	-22.2 ± 0.0	4.2 ± 0.0	3.9	
Artemia				
Vinh-Chau strain, Viet Nam	-16.0 ± 0.1	8.2 ± 0.0	5.3	Gamboa-Delgado et al., 2008
Posthatched nauplii	-18.5 ± 0.3	9.3 ± 0.2	4.2	
Enriched metanauplii (T-ISO)				
INVE-07332				Gamboa-Delgado, unpublished
Posthatched nauplii	-19.9 ± 0.1	11.7 ± 0.1	5.5	
Enriched metanauplii (T-ISO)	-23.3 ± 0.2	12.5 ± 0.1	4.7	
GSL, UTAH, USA (1178)[d]	-15.0 ± 0.3	5.4	-	Spero et al., 1993
San Francisco Bay, USA (1157)	-21.4 ± 0.3	4.8	-	Spero et al., 1993
Macau strain, Brazil (1128)	-13.6 ± 0.1	9.4	-	Spero et al., 1993
Aibi Lake strain, China (1198)	-18.1	12.8	-	Spero et al., 1993
Daphnia magna (inert feed)	-19.6 ± 0.5	13.6 ± 0.6	-	Power et al., 2003
Moina micrura	-30.1 ± 3.0	5.1 ± 1.0	-	Lindholm and Hessen, 2007
Nematodes				
Panagrellus redivivus[e]				Schlechtriem et al., 2004
(grown on corn meal)	-10.8	-	-	
(grown on wheat meal)	-22.9	-	-	Moens et al., 2005
Metachromadora remanei	-15.8	15.7	-	

Inert diets				
AgloNorse (EWOS)	-22.4 ± 0.4	8.5 ± 0.5	4.8	Gamboa-Delgado, unpublished
Frippak 2CD, 3CD (INVE)	-20.1 ± 0.2	9.4 ± 0.3	4.7	Gamboa-Delgado, unpublished
MeM (Bernaqua)	-21.4 ± 0.1	10.5 ± 0.2	3.9	Gamboa-Delgado, unpublished
Baker's yeast	-23.2 ± 0.4	-1.2 ± 0.5	5.9	Gamboa-Delgado, unpublished
Larval organisms				
Sciaenops ocellatus (fed rotifers, 18 d)	-26.1	-2.2	-	Herzka et al., 2001
Solea senegalensis[f]	-19.4 ± 0.0	14.2 ± 0.1	5.3	Gamboa-Delgado et al., 2008
Litopenaeus vannamei	-19.7 ± 0.1	12.6 ± 0.1	5.4	Gamboa-Delgado and Le Vay, 2009b

[a] Microalgae grown using a commercial liquid fertilizer (Cell-hi W, Varicon Aqua). [b] Microalgae produced on Guillard's F/2 medium. [c] Other zooplankton species sampled. [d] Artemia Reference Centre Number.
[e] Lipid-extracted. [f] Recently hatched.

3. Diet-consumer isotopic discrimination factors

Dietary components, or elements of a food web, may have naturally distinct stable isotope signatures, so that a "consumer–diet" relationship, particularly in terms of $\delta^{13}C$, can be used to identify those dietary sources contributing to growth, and mixing and mass balance models can be used to quantity the relative contribution of multiple carbon sources (Fry, 2006). The carbon and nitrogen isotopic signatures of animals typically reflect the isotopic signatures of their diets plus a discrimination factor (isotopic discrimination, $\Delta = \delta_{tissue}-\delta_{diet}$) caused by the different isotopes of the same element being incorporated into tissues at different rates, most probably through differential selection of the heavier isotope at each metabolic step (isotopic fractionation) (Martinez del Rio and Wolf, 2005; Martinez del Rio et al., 2009). The discrimination factor can vary according to tissue or element being studied, and also due to differences in tissue composition and physiology between species and individuals (Post 2002; McCutchan et al., 2003; Vanderklift and Ponsard, 2003). In ecological studies in aquatic systems, $\Delta^{13}C$ is assumed to be *circa* +1‰, reflecting only a slight increase in ^{13}C content

relative to diet (Michener and Schell, 1994; Fry and Sherr, 1984). Processes that may cause this discrimination are slight biases to loss of $^{12}CO_2$ during respiration and to uptake of ^{13}C compounds during digestion or the biosynthesis of different tissues (DeNiro and Epstein, 1981; Tiezen et al., 1983) while the generally larger $\Delta^{15}N$ values appear to result from the selective excretion of ^{15}N-depleted nitrogen (Minagawa and Wada, 1984; Tibbets et al., 2007). $\Delta^{15}N$ values have been used to estimate relative trophic level of organisms within a food web, with a mean difference of *circa* +3.2‰ normally assumed to represent one trophic level (DeNiro and Epstein, 1981, Minagawa and Wada, 1984; Kelly, 2000). Application of such generalised $\Delta^{15}N$ values has been accepted almost universally in determining trophic position in terrestrial and aquatic environments. (DeNiro and Epstein 1978, 1981; Fry and Sherr, 1984; Fry, 1991; Van der Zanden et al., 1999). However, the relationships between nitrogen isotopic discrimination and other factors such as dietary protein supply and quality remain poorly understood (Robbins et al., 2005; Martinez del Rio et al., 2009). Moreover, there is increasing evidence that isotopic discrimination factors are species- and tissue-specific (DeNiro and Epstein, 1981; Tiezen et al., 1983; Yokoyama et al., 2005; Stenroth et al., 2006) and the considerable variance in the reported values demonstrate that careful validation of assumptions about discrimination factors are critical for interpreting stable isotope data from experimental feeding studies (Cabana and Rasmussen, 1996; McCutchan et al., 2003; Crawley et al., 2007; Martinez del Rio et al., 2009). Caut et al. (2009) recently conducted a review of published studies applying stable isotope techniques, reporting that in more than half of the studies using isotopic mixing models for dietary reconstruction discrimination factors were not estimated, but were taken from published reviews. Available data for experimentally-determined discrimination factors across a range of aquatic larvae and post-larvae, measured under laboratory conditions, demonstrate a very considerable range in $\Delta^{13}C$ (0.4‰ – 4.1‰) and $\Delta^{15}N$ (0.1‰ – 5.3‰) (Table 2).

Table 2. Comparison of carbon and nitrogen isotopic discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) observed in controlled feeding experiments and average values and ranges reported from field studies.

Species/Stage/Tissue	Diet type	$\Delta^{13}C$	$\Delta^{15}N$	Reference
Average values between animal tissues and diet	-	0.5-1.0	3.2	Peterson and Fry, 1987; Fry and Sherr, 1984; Michener and Schell, 1994
Aquatic food webs	-	1.0	1.5-3.4	Van der Zanden and Rasmussen, 2001; McCutchan et al., 2003
Skeletonema costatum *Eucampia zodiacus* *Thalassionema nitzschioides*	CO_2 and HCO_3 ($\Delta^{13}C$ values relative to CO_2)	10-16	-	Trimborn, 2008
Brachionus plicatilis	Baker's yeast	0.2	5.1	Gamboa-Delgado, unpublished
Crassostrea gigas juvenile (adductor muscle)	*Chaetoceros neogracile*	-0.2	8.7	Yokoyama et al., 2008
Panulirus cygnus juvenile (abdominal muscle)	Mussel Sardine Coraline algae	3.3 3.6 2.9	2.8 1.8 2.8	Waddington and MacArthur, 2008
Penaeus esculentus postlarvae	*Artemia* nauplii Microbial mat Practical diet	1.6 4.0 3.5	0.1 3.5 5.3	Al-Maslamani et al., 2009 Al-Maslamani, 2006
Litopenaeus vannamei postlarvae	Zooplankton Detritus	0.4 7.0	2.7 0.4	Dittel et al. 1997
Litopenaeus vannamei postlarvae	*Artemia* nauplii Inert diet	1.3 4.1	0.9* 2.2*	Gamboa-Delgado and Le Vay, 2009b
Panulirus japonicus phyllosomata	*Artemia* metanauplii	-	2.5	Matsuda et al., 2009
Litopenaeus vannamei postlarvae	46% protein compound diet 100% N fish meal 100% N soy	 2.3 3.5	 0.8 3.6	Gamboa-Delgado and Le Vay, 2009a
juveniles	100% N fish meal 100% N soy	3.0 4.1	1.3 6.6	
Callinectes sapidus juveniles	Zooplankton *Artemia* Detritus	-0.1 1.0 -3.2	0.1 1.6 2.2	Fantle et al., 1999
Solea senegalensis postlarvae	*Artemia* nauplii Inert diet	0.8 2.3	1.7 1.5	Gamboa-Delgado et al., 2008

*Estimated values, full isotopic equilibrium was not reached.

There is also clearly considerable variation with diet. For example, Gamboa-Delgado and Le Vay (2009a) observed that protein quality can strongly affect $\Delta^{15}N$, with values of 0.8‰ and 3.6‰ observed in *Litopenaeus vannamei* fed iso-nitrogenous diets containing only fishmeal or soy as nitrogen sources, respectively. In another study, postlarvae of the same species reared through the mysis stages on *Artemia* or an inert diet exhibited $\Delta^{13}C$ values of 1.3‰ and 4.1‰, respectively (Gamboa-Delgado and Le Vay, 2009b). The occurrence of unusually high discrimination factors may

indicate an imbalance in dietary nutrients necessary for larval development. In addition, increased feeding rates as animals adapt to nutrient deficiencies may increase metabolic cycling of nonessential nutrients and cause greater isotopic fractionation (Martínez del Rio and Wolf, 2005). The very wide range of observed values in both $\Delta^{13}C$ and $\Delta^{15}N$ highlights the need for including experimental determination of discrimination factors into the design of experiments applying stable isotopes to larval nutrition. This may need to be repeated in each experimental study as isotopic discrimination may vary during ontogenesis of aquatic larvae due to changes in metabolic rate and in relation to the specific diets being studied (Hentschel, 1998; Rossi et al., 2004; Gamboa-Delgado and Le Vay, 2009b). In feeding experiments, the discrimination factor can be normally determined by waiting until a constant difference between diet and animal is achieved. For some larvae, for example those of tropical crustacean species, this can be difficult to accomplish due to their rapid metamorphic development and trophic changes, so that food types may only be suitable for short developmental stages during which larvae may not reach equilibrium with its diet (Schlechtriem et al., 2004; Comtet and Riera, 2006). Nevertheless, larvae and postlarvae of most decapod crustaceans, develop sufficiently fast to provide a window of opportunity for feeding experiments aiming to establish isotopic equilibrium values as part of the design. For example**,** Schwamborn et al. (2002) reported short isotopic equilibrium periods for larvae of

two decapod species, *Sesarma rectum* and *Petrolisthes armatus* (6-9 d), which is similar to the time (5 d) required for *L. vannamei* mysis larvae to reach isotopic equilibrium with *Artemia* and inert diets (Gamboa-Delgado and Le Vay, 2009b). In early-stage postlarval shrimp, Al-Maslamani (2006) detected carbon and nitrogen isotopic equilibriums between *Penaeus semisulcatus* and their diets after 15 d of growth. Fry and Arnold (1982) also observed that fast-growing postlarval *Farfantepenaeus aztecus* needed to gain a 4-fold increase in biomass to achieve carbon isotopic equilibrium with their diets. Such weight increases are typical of rapid growth during larval development, although in some species ontogenetic changes may prevent use of consistent diets over longer periods of time than those reported above. Similar transitions in diet may be required in marine fish larvae, though results in *Solea senegalensis* show that $\Delta^{13}C$ equilibrium may be attained sequentially in both the rotifer and *Artemia*-fed stages (Gamboa-Delgado et al. 2008). However, in fish larvae there may be differences in the period required for larvae to reach equilibrium with their diet in terms, depending on the isotope being studied. For example, Jomori et al. (2008) found that *Piaractus mesopotamicus* larvae fed *Artemia* nauplii took only 9 d from first feeding to achieve consumer-diet equilibrium in terms $\Delta^{15}N$, but up to 18 d in terms of $\Delta^{13}C$, most likely reflecting the longer time taken to utilise maternally- transferred carbon in lipid reserves.

4. Rate of isotope incorporation: growth and turnover

Stable isotopes can be used to estimate the tissue turnover rate of elements and, in the case of nitrogen, can be used as a reliable indicator of protein turnover, especially in muscle tissue. Protein, as a macronutrient, may limit the growth of larvae and is also the most expensive ingredient in aquaculture formulated diets; therefore, the metabolism of proteins has been widely studied as a mean to understand and improve the growth process in aquatic animals

(Carter et al., 1994, 1998; Beltran et al., 2008) and the rate of protein turnover has been determined in several fish and crustaceans species (see reviews by Houlihan et al., 1995a; Waterlow, 2006; Fraser and Rogers, 2007). Protein turnover rates have been frequently estimated by the flooding dose method (Garlick et al., 1980; Houlihan et al., 1988) using radioactive isotopes (^{14}C-labelled lysine or ^{3}H-labelled phenylalanine) that are incorporated through injection or constant infusion as metabolic tracers into the free amino-acid pool (Waterlow, 2006). The metabolism of proteins has also been evaluated using stable isotope tracers as an alternative to radioactive isotopes. Protein synthesis studies in trout (*Oncorhynchus mykiss*) have shown that results obtained using enriched stable isotopes are similar to those obtained using radio-labelled amino-acids (Houlihan et al., 1995a). Carter et al. (1994, 1998) used stable isotopes in trout (*O. mykiss*) and flounder (*Pleuronectes flesus*) in order to assess protein synthesis, protein turnover rates and to construct nitrogen budgets. Conceição et al. (2001) extended this approach to larval turbot (*Psetta maxima*) using ^{15}N-labelled rotifers to demonstrate that exposure to an immunostimulant increased the fractional rates of protein synthesis.

The rate of incorporation of a nutrient into specific tissues or whole bodies can also be estimated directly by measuring natural stable isotope changes over longer time periods, after a dietary shift has been applied to the consumer (Pearson et al., 2003) and provide a further indicator of diet performance because tissues of fast growing animals exhibit shorter half-times (t_{50}) for carbon and nitrogen than slow growing animals (MacAvoy et al., 2005). Short tissue half times are common for carbon and nitrogen in early life stages of fish (2.8-5.2 d) (Van der Zanden et al., 1998; Herzka and Holt, 2000; Bosley et al., 2002; Gamboa-Delgado et al., 2008) and crustaceans (1.2-4.9 d) (Fry and Arnold, 1982; Al-Maslamani, 2006; Gamboa-Delgado and Le Vay, 2009b). This is due to the very fast growth rates characteristic of early

life stages, so that observed carbon and nitrogen isotopic changes in larvae are thus mainly due to tissue accretion and not to tissue metabolic turnover, the converse of typical observations in adult organisms (Martinez de Rio et al., 2009). Exponential models applied to associate isotopic changes with time (or biomass increase) can also be used to assess elemental turnover rates (Fry and Arnold, 1982; Hesslein et al., 1993). As is also the case for isotopic mixing models (see following section), the resolution of such models in the estimation of elemental turnover rates and elemental t_{50} is improved, with better fit to predicted values and lower variability, when there is a clear contrast between the initial isotopic signature of the consumer and the diet. The model first applied by Hesslein et al. (1993) to tissue changes in larval whitefish (*Coregonus nasus*) and later by Gamboa-Delgado et al (2008) to larval *S. senegalensis* and by Gamboa-Delgado & Le Vay (2009a, 2009b) to *L. vannamei* has the advantage of distinguishing between isotopic change due to metabolic turnover (m) and that due to isotopic dilution through growth (k). The latter value can be derived from the exponential growth equation, while the former can be calculated using iterative nonlinear least squares regression once the initial and final isotope values in the consumer (after a dietary shift) and k have been integrated into an exponential equation. Similarly, Herzka et al. (2001) applied a model proposed by Fry and Arnold (1982) to estimate the relative influence of growth dilution and metabolic turnover components of isotopic tissue changes in larvae of red drum, *Sciaenops ocellatus*, resulting from habitat changes at settlement. Table 3 presents examples of estimated carbon and nitrogen turnover rates and metabolic elemental half times in tissue using stable isotopes at natural abundance levels in larval and post-larval fish and crustaceans. Turnover rates are greatly influenced, among some other factors, by water temperature, metamorphosis stage and dietary conditions. Thus, assessment of nutrient elemental turnover rates in larval tissue can provide an additional

indicator of nutritional performance of a specific diet or feeding regime under specific conditions.

Table 3. Growth rates (*k*), carbon and nitrogen turnover rates (*m*) and estimated elemental half times in tissue (t_{50}) of different aquatic organisms as indicated by natural stable isotope changes integrated in exponential models.

Species/Stage	**Weight**	**Isotope**	***k* (d^{-1}) and *m* (d^{-1})**	**t_{50} (d)**	**Reference**
Solea senegalensis postlarvae	481-924 µg dw	$\delta^{13}C$	*k* 0.022-0.122 *m* 0.145-0.218	3.1-5.2	Gamboa Delgado unpublished
Sciaenops ocellatus larvae	0.02-0.89 mg dw	$\delta^{15}N$	*k*+*m* 0.25*	2.8	Herzka and Holt, 2000
Pseudopleuronectes americanus postlarvae	1.0-1.4 mg dw	$\delta^{15}N$	*k*+*m* 0.18-0.22*	3.1-3.9	Bosley et al., 2002
Oreochromis niloticus fingerlings	3.5 g dw	$\delta^{13}C$	*k*+*m* 0.020-0.053	13-33	Zuanon et al., 2007
Micropterus dolomieui larvae	<1.0 mg dw	$\delta^{15}N$	*k*+*m* 0.14-0.23*	3-5	Van der Zanden et al., 1998
Penaeus semisulcatus postlarvae	3.8 mg	$\delta^{13}C$	*K* 0.093 *m* 0.016	-	Al-Maslamani, 2006
	18 mg ww		*k* 0.096 *m* 0.048	4.9	
Farfantepenaeus aztecus Postlarvae	38 mg dw	$\delta^{13}C$	-	4.0	Fry and Arnold, 1982
Litopenaeus vannamei early postlarvae	241 µg dw	$\delta^{13}C$	*k* 0.204-0.239 *m* 0.239-0.381	1.2-1.6	Gamboa-Delgado and Le Vay, 2009b
Litopenaeus vannamei early postlarvae	360 µg dw	$\delta^{15}N$	*k* 0.139-0.178 *m* 0.002-0.117	2.8-4.0	Gamboa-Delgado and Le Vay, 2009a

* Data recalculated by McIntyre and Flecker (2006) after applying an exponential model to original published data.

5. Identification of nutrient sources

By applying mixing and mass balance models, the relative contribution of nutrients derived from different food sources and retained in the consumer organism can be calculated (Phillips and Gregg, 2001, 2003; Fry, 2006). Thus, in larval studies, the relative contribution of elements provided in co-feeding regimes may be investigated, as well as the relative utilisation of dietary sources (eg protein) within compound feeds. The application of isotopic mixing models usually requires certain assumptions and conditions to be met in the experimental design (see review by Martinez del Rio et al., 2009). Not least of these is that larvae should be in isotopic equilibrium with their diet. This may require time series sampling, where changes of diet occur, or sufficient baseline data to determine the minimum time required for the species being studied to attain equilibrium with a particular diet (see previous section on rates of isotope incorporation). In addition, food sources should have similar elemental composition (eg to be iso-nitrogenous), although correction factors can be applied if this assumption is not precisely met (Fry, 2006). Isotopic discrimination factors for each isotope need to be quantified, in relation to each of the dietary treatments being investigated. The systematic estimation of discrimination factors provides positive and negative control values in experiments where two (or more) nutrient sources are co-fed to a consumer in varying proportions. The measured discrimination factors are then used to provide correction factors, increasing the resolution of the mixing model. Similarly, assimilation efficiencies for each element should be estimated to allow correction of the model for differences between diet types in terms of actual uptake of nutrients from the gut. The potential for isotopic routing, for example the transfer of carbon from dietary carbohydrate into tissue protein (eg biosynthesis of dispensable amino-acids from intermediate metabolites produced in the glycolysis or the citric acid cycle), prevents that application of mixing models to carbon isotope data comparing whole diets with isolated

consumer tissues, but in the case of larvae this is commonly avoided by use of entire animals for tissue analysis. Similarly, when using mixing models to determine overall sources of dietary carbon, lipid extraction of diet and larval samples should be avoided due to the often-encountered difficulties in differentiating between carbon derived from lipid, carbohydrate or protein fractions. However, where dietary lipids can be selected with isotope signatures that are sufficiently different from other diet components, stable isotope analysis of complete and lipid-extracted whole larval samples may usefully complement the traditional use of C:N ratios to investigate utilisation of dietary or maternally-transferred lipids. In studies concentrating only on nitrogen isotopes, the situation is simpler as all nitrogen is usually assumed to be in the amino-acid and protein pool.

Isotopic mixing models generate higher output resolution when the different feeding sources have contrasting isotopic values, allowing estimation of carbon and nitrogen contributions from different dietary ingredients into a target organism (Schlechtriem et al., 2004; Beltran et al., 2008). In larviculture rearing systems, different approaches can be taken to manipulate the isotopic values of prey items in order to avoid overlapping isotopic values, but due to its simplicity, the use of different culture media for phytoplankton and the option to inject tank CO_2 or only air into algal culture vessels provide simple and effective means to modify the nitrogen and carbon isotopic values of the primary producers (Table 1), hence simplifying further isotopic manipulations up in the larval trophic chain (rotifers, copepods, *Artemia*, larval and postlarval organisms). Estimation of the relative contribution of nutrients using mixing models is not necessarily limited to only two sources. Some models can integrate additional sources, with the concentration of the element being studied in each source also taken into account in assessing relative nutritional contributions. For example, Phillips and Gregg (2003) proposed a method (IsoSource, www.epa.gov/wed/pages/models.htm) in which

Table 4. Estimated mean proportions of carbon or nitrogen contributed from different nutritional sources and incorporated in tissue of fish and crustacean, as indicated by isotope mixing models using foods with at natural stable isotope abundance levels.

Species/ Developmental stage	Dietary items/ Isotope	Estimated relative contributions to growth	References
Solea senegalensis postlarvae	*Artemia* nauplii and inert diet (70:30 dry weight), $\delta^{13}C$	88 % *Artemia* 12 % inert diet	Gamboa-Delgado et al., 2008
Piaractus mesopotamicus larvae	*Artemia* nauplii and inert diet, $\delta^{15}N$	60% *Artemia* 40% inert diet (36 DAH)* 15% *Artemia* 85% inert diet (42 DAH)	Jomori et al., 2008
Cyprinus carpio larvae	Lipid-free matter and lipids in nematodes, $\delta^{13}C$	35-45 % of initial offered lipid free matter was assimilated**	Schlechtriem et al., 2005
Awaous guamensis larvae	Stream leaf litter, stream algae and marine sources, Dual: $\delta^{13}C$ and $\delta^{15}N$	50% marine sources 16% stream leaf litter 34% stream algae	Hobson et al., 2006
Penaeus esculentus postlarvae	Inert feed and epiphytes growing on different artificial substrates, $\delta^{13}C$	47-61% inert feed 39-53% periphyton	Burford et al., 2004b
Litopenaeus vannamei postlarvae	*Artemia* nauplii and inert diet (50:50 C), $\delta^{13}C$	77 % *Artemia* 23 % inert diet	Gamboa-Delgado and Le Vay, 2009b
Panulirus japonicus phyllosomata	*Artemia* metanauplii and mussel gonad, $\delta^{15}N$	66% *Artemia* 34% mussel gonad	Matsuda et al., 2009
Litopenaeus vannamei postlarvae	Practical diet (50:50 N from fish meal and soy protein), $\delta^{15}N$	69% fishmeal 31% soy protein isolate	Gamboa-Delgado and Le Vay, 2009a

* Days after hatching
** 6 days after feeding. Assumed discrimination factor = 2.

all possible combinations of each dietary source contribution (0–100%) are examined in small increments. In order to demonstrate its application they used several published data sets (*e.g.* Ben-David et al., 1997) to perform dietary analysis using two isotopes ($\delta^{13}C$ and $\delta^{15}N$) to assess nutritional contributions of up to seven dietary sources.

Isotopic composition of larval tissue provides an integrated measure of diet history, but does not in itself differentiate mechanisms for differential retention of nutrients between dietary components. However, it provides a versatile approach to investigation of specific aspects of feeding efficiency under realistic hatchery conditions, with appropriate experimental design depending on the objectives of the study. Thus experiments may potentially address topics such as the effectiveness of larval diets and co-feeding regimes, optimal timing of live food transitions (eg from rotifers to *Artemia*), mechanical presentation of inert feeds (frequency, particle size, tank design), optimal size/age for weaning and digestibility of different protein sources. To date, the application of isotopic mixing models has been applied in several nutritional studies on larval and postlarval aquatic organisms (Table 4). For example, Schlechtriem et al. (2005) manipulated the $\delta^{13}C$ signatures of the nematode *Panagrellus redivivus* by pre-feeding with C_3 and C_4 plant meals. The nematodes were then fed to common carp (*Cyprinus carpio*) larvae to differentially determine the assimilation of lipids and lipid-free matter. More recently Gamboa-Delgado et al. (2008) and Gamboa-Delgado and Le Vay (2009b) assessed the $\delta^{13}C$ values of different live feeds and applied isotopic mixing models in order to estimate nutritional contributions from co-fed *Artemia* and inert diets to the growth of larval and postlarval Senegalese sole (*S. senegalensis*) and Pacific white shrimp (*L. vannamei*), respectively. In a similar study, Matsuda et al. (2009) estimated the relative nutritional contributions of *Artemia* and mussel gonad to the growth of *Panulirus japonicus* larvae. The technique has also been applied to identify periods when larval fish are physiologically better adapted to incorporate specific dietary nutrients from live and inert diets (Jomori et al., 2008) and it has also been recently extended to estimation of nutritional contributions from individual ingredients within compound diets to tissue growth of *L. vannamei* (Gamboa-Delgado and Le Vay, 2009a). However, the full interpretation of the

results of the latter study will require more detailed investigation of the transfer of dietary nitrogen and carbon into consumer amino-acids using compound specific isotope analysis.

6. Compound specific stable isotope analysis

Separation of sub-units of complex organic molecules prior to stable isotope analysis (compound specific isotope analysis, CSIA) has been used to trace sources and fate of individual dietary fatty acids and amino-acids, as well as their synthesis from labeled precursors. In the case of fatty acids, this approach has used isotopically-enriched lipids in pulse-chase experimental designs. For example, Bell et al. (2007) demonstrated the limited e ability of filter-feeding marine copepods to synthesise long chain PUFA from deuterium-labeled 18:3n-3 delivered in liposome suspensions, with subsequent derivatisation of fatty acids extracted from tissue samples to pentafluorobenzyl esters and separation and quantification of label incorporation into individual fatty acids by GC-MS. In a longer term study, Parrish et al. (2007) used the relatively high natural $\delta^{13}C$ signature typical of mass-produced *Schizochytrium* sp. to trace the transfer and conservation of n-6 docosapentaenoic acid along a two-step food chain through rotifers (*Brachionus plicatilis*) and cod (*Gadus morhua*) larvae, with results suggesting a previously unreported potential dietary requirement for this fatty acid in early stage cod larvae. CSIA for individual amino-acids has been applied in a range of marine species and studies that are most directly relevant to larval nutrition research in the laboratory include ecological studies of juvenile crabs, *Callinectes sapidus* (Fantle et al. 1999) and rotifers, *Brachionus plicatilis* (McClelland and Montoya, 2002), in which laboratory experiments were used to interpret field observations of wild zooplankton. More generally, results for marine species demonstrate consistently wide ranges of up to 20‰ for both $\delta^{13}C$ and $\delta^{15}N$ values in amino-acids in rotifers, crustaceans, gastropods and fish (Fantle et al., 1999; McClelland and Montoya, 2002; Schmidt et al., 2004; Chikaraishi et al.,

2007; McCullagh et al., 2008). In the case of carbon stable isotopes, this variation appears to reflect the very limited scope for isotopic discrimination in the indispensable amino-acids, with the transfer of intact carbon skeletons between diet and consumer at any trophic step (Fantle et al., 1999). In contrast, dispensable amino-acids in consumer tissue may include carbon from a range of dietary components, with isotopic discrimination at each of the steps in the metabolic pathways involved in amino-acid synthesis, resulting in high variability in carbon isotopic composition, especially at higher trophic levels. While there is also wide variation in nitrogen stable isotope signatures between amino-acids, there is no clear distinction between behaviour of dispensable and non-dispensable amino acids, and the underlying mechanisms determining $\Delta^{15}N$ values may include the degree of conservation of particular amino-acids and the scope for isotopic fractionation during transamination and deamination (McClelland and Montoya, 2002). Carbon and nitrogen discrimination factors between specific amino acids and consumer tissue, as opposed to bulk values, potentially provide a better understanding of trophic relationships in experimental ecological studies. For example, experiments on zooplankton suggest that amino-acids showing large $\Delta^{15}N$ between diet to consumer may be better indicators of trophic level than bulk isotopic values, while those which are more conservative may provide insight into dietary sources (McClelland and Montoya, 2002). Similarly, $\Delta^{13}C$ values for specific amino-acids can provide insight into the transfer of carbon from dietary sources into animal tissue protein (Berthold et al., 1991; Fantle et al., 1999; O'Brien et al., 2003; McCullagh et al., 2008**).** Those amino acids that are conserved (ie low $\Delta^{13}C$) are indicative of direct use of intact dietary amino-acids, including essential amino-acids, while non-conservative behaviour (ie high $\Delta^{13}C$) indicates *de novo* synthesis of amino-acids using carbon derived from other compounds. In designing experiments investigating marine larval nutrition, useful lessons can be drawn from work on terrestrial species. For example, some very informative studies in insects demonstrate that

$\Delta^{13}C$ can be used to indicate dietary sources of carbon and to identify dietary requirements for amino-acids, with $\delta^{13}C$ of essential amino-acids in adults remaining close to values for plant proteins consumed by larvae, while $\delta^{13}C$ for non-essential amino-acids reflecting carbohydrates consumed by adults (O'Brien et al. 2003, 2005). Similarly, low $\Delta^{13}C$ values for essential amino-acids have been observed in poultry, where use of uniformly ^{13}C-labelled dietary amino-acids confirmed conservation of essential amino-acids, but with evidence of some endogenous synthesis of methionine, while proline behaved isotopically as an essential amino-acid (Berthold et al., 1991). CSIA for amino acids has until recently used coupled gas chromatography/isotope ratio mass spectrometry (GC-IRMS) separation and analysis of esterified derivatives of amino acids (Preston, 1992; Preston et al., 1994). When studying naturally-occurring isotopic ratios, the introduction of a derivatisation step introduces systematic errors due to addition of reagent carbon and fractionation during derivatisation, that can be corrected for in data interpretation. A similar approach can be adopted using isotopically-enriched tracers, but these do not measure differences in diet-consumer discrimination between specific amino-acids, as the isotope tracer signal is several orders of magnitude greater. A good example of this approach is given by Saavedra et al. (2007), who used ^{15}N-labelled rotifers to investigate relative utilisation of dietary amino acids by *Diplodus puntazzo* larvae. More recently, the development of liquid chromatography/isotope ratio mass spectrometry (LC-IRMS, Krummen et al., 2004) enables measurement of natural isotopic signatures of individual amino-acids without need for derivatisation and offers the potential for the higher throughput analysis that is required for large numbers of small samples typically generated by larval nutritional studies. While considerable fundamental research on diet-consumer relationships for natural stable isotopes in amino-acids in marine larvae and their behaviour under varying nutritional conditions is still required, examples of potential practical applications include investigation of the relative contribution of dietary components,

either within compound feeds or in co-feeding regimes, to larval growth and energy requirements (ie sources and fate of dietary carbon), development of improved dietary protein:energy ratios, as well as investigation of dietary amino-acid requirements and their utilisation with life stage and growth rate.

7. Summary and conclusions

The experimental studies in fish and crustacean larvae reviewed here demonstrate the effectiveness of carbon and nitrogen stable isotope analysis in larval nutrition research. Measurement of bulk stable isotope signatures in larvae represents an integrated and direct measure of nutrient uptake resulting from the combined effects of diet ingestion, incorporation and turnover without the use of added tracers or labels. Thus experiments can be conducted under realistic hatchery rearing conditions using typical feeds, in larvae exhibiting normal growth and development. With appropriate experimental design, studies can address specific research questions on particular aspects of larval nutrition and culture systems. For example, co-feeding regimes might be evaluated in a series of treatments with varying degrees of live food replacement, or diet-switching experiments may be used to investigate rates of tissue incorporation and turnover. However, in all such studies care must be taken to ensure that underlying assumptions are met, and appropriate corrections may need to be made for digestibility and diet-consumer isotopic discrimination. Evidence from a range of species and diets demonstrate that discrimination factors may be highly variable and the importance of inclusion of measurement of actual values within most larval studies. The wider adoption of compound specific analysis, particularly for amino-acids, represents an opportunity to greatly improve our understanding of nutrient utilisation during the larval development of different species. Experience from studies in zooplankton and terrestrial species demonstrates the potential for use of compound specific stable isotope analysis in

well-designed studies to trace the transfer of dietary carbon from different components of the diet into multiple amino-acids, simultaneously, under normal feeding and husbandry conditions. It is also important to recognise that the implications of such research reach beyond the practical goals of improved larviculture. There is a long history of application of carbon and nitrogen stable isotope analysis in trophic ecology (see recent review by Martínez del Rio et al., 2009). However, it is only in recent years that the generalised isotopic discrimination factors between consumers and their diets that are typically applied in such work have begun to be more fully underpinned by experimental data. Fast growing aquatic larvae represent excellent model organisms to investigate factors influencing diet-consumer isotopic relationships, exhibiting rapidly-attained isotopic equilibria with changes in diet, rapidly-changing feeding behaviour, transitions in trophic level and ready ingestion of modifiable experimental diets in short and controlled food chains. Thus results of studies into the effects of diet composition, developmental stage, growth rates and environmental conditions on stable isotope incorporation will be of broad relevance not only in terms of larval nutrition but can also inform the design and interpretation of ecological studies.

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