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# Nutritional role of natural productivity and formulated feed in semi-intensive shrimp farming as indicated by natural stable isotopes

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## Abstract

The natural productivity of semi-intensively managed shrimp ponds is frequently represented by a diverse trophic structure that forms part of the diet of farmed organisms. As in natural ecosystems, these dietary components exhibit differing isotopic signatures that vary with diet and trophic level. These isotopic differences can be used to infer the transfer of nutrients, as the isotopic values of prey items and consumers can be integrated in mass-balance mixing models, allowing the quantification of the relative contribution of multiple nutritional sources to the growth of a specific organism. By applying such methodology, it has been possible to estimate the relative dietary contribution of several elements that belong either to the biota of the farming environment or that are part of formulated diets. Careful sampling methods and isotopic analysis of these samples provide valuable information, not only in terms of what the consumer organism has selected, captured and ingested, but also in terms of the proportions of assimilated nutrients in the consumer's tissues. Results from several studies indicate that the natural productivity found in semi-intensively managed ponds frequently supplies higher proportions of dietary carbon and nitrogen to shrimp growth than the formulated feed, emphasizing the nutritional relevance of the former. A synthesis of field and laboratory studies applying isotopic techniques to determine the relative contribution of nutrients derived from different biota elements and formulated feeds to the growth of farmed shrimp is presented.

**Key words:** dietary carbon, dietary nitrogen, *Litopenaeus vannamei*, natural productivity, nutritional contributions, stable isotopes.

## Introduction

Shrimp farming through semi-intensive production methods has become predominant in tropical areas of the western hemisphere (Moles & Bunge 2002; CONAPESCA 2010; Stern & Sonnenholzner 2011). Semi-intensive production methods are typically characterized by the periodic application of fertilizers to stimulate the natural productivity of the ponds and by the addition of supplementary, formulated feed. Aquaculture ponds managed extensively and semi-intensively are very similar to small ecosystems and, as such, they show many of the processes that are observed in natural environments. Frequently, natural populations established in shrimp ponds are represented by diverse communities of organisms undergoing temporal variations, as a consequence of natural successions, but more importantly, as a result of continuous foraging pressure by farmed animals. The nutritional importance that natural productivity of ponds represents for farmed shrimp in terms of production parameters has been demonstrated in numerous studies, under both laboratory and field conditions (Hunter *et al.* 1987; Martínez-Córdova *et al.* 1998; Martinez-Córdova *et al.* 2002; Gamboa-Delgado *et al.* 2011; Porchas-Cornejo *et al.* 2011). Shrimps are constant feeders and such behaviour is only suppressed at the pre- and post-moulting stages. Therefore, as the culture cycle progresses, their cumulative feeding activity exerts a strong influence on natural communities of prey organisms. Nevertheless, efficient fertilization and rearing protocols and management strategies aimed at maintaining the soil and

water quality, promote the presence of appropriate concentrations of individuals of natural productivity over the whole culture cycle.

## Natural productivity in aquaculture ponds

In the aquatic environment, micro- and macroalgae represent the link that converts solar energy into utilizable chemical energy, which is stored in the chemical bonds of the organic compounds of different plant tissues. This first link comprises an essential source of nutrients for organisms belonging to upper trophic levels. The phytoplankton communities rapidly respond to optimal variables such as temperature, salinity and nutrient concentration. An appropriate turbidity in pond water is frequently associated with healthy microalgae populations, which in turn, guarantee the stability of dissolved oxygen concentrations and also support the growth of zooplankton. In this context, it has been reported recently that Pacific white shrimp (*Litopenaeus vannamei*) juveniles can filter, ingest and digest suspended microalgae. The filtering action occurs at the third pair of maxillipeds, which have net-like setae and could potentially select for microorganisms as small as 10 μm in size (Kent *et al.* 2011), therefore, it might be possible that Penaeid shrimp ingest other microorganisms suspended in the water column.

The term phytobenthos is employed to refer to the micro- and macroalgal communities attached onto different substrates such as rocks, pebbles, sediment and submerged plant material. Although the presence of macroalgae is unwanted in aquaculture ponds because they compete for nutrients with the microalgae (and also because they interfere with harvesting activities), there are examples of pilot assays in which the presence of macroalgae of certain species is encouraged with the aim of providing feed and substrate to the cultured shrimps (Cruz-Suárez *et al.* 2010). The macroalgal biomass is harvested at the end of the culture cycle because it represents an extra economic asset when used as forage or as a source of fine chemicals having biological activities ranging from immunostimulants to components for culture media for microorganisms.

Nutritionally, one of the most important components of natural productivity for farmed animals is the zooplankton (Coman *et al.* 2003; Duffy *et al.* 2011), which is represented by a rich community of very diverse organisms such as mollusc, fish and crustacean larvae and adult forms of small species (copepods and nematodes). Most of these organisms forage directly on phytoplankton and their populations sharply decrease when the latter is limited. The zoobenthos is composed of animals living on or in sedimentary environments and among these organisms, some of the most important nutritional sources for shrimps can be found, for example, polychaete worms, harpacticoid copepods and sessile rotifers. An important additional part of the natural productivity is a diverse benthic community termed the periphyton. The periphyton is composed of periphytic microalgae, cyanobacteria, micro-invertebrates and other organisms that constantly provide nutrients to farmed shrimps. The periphyton not only acts as a nutrient source, but also as a natural biological filter due to the activity of heterotrophic microbes associated with these communities (Azim *et al.* 2003; Milstein *et al.* 2009). Given these benefits, several methods have been developed to encourage the growth of periphyton using different substrates that multiply the available substrate area for both the periphyton and the cultured shrimps. There are extensive studies and revisions (e.g. Azim *et al.* 2005 and references therein) of periphyton management and this information has ultimately focused on optimizing aquaculture production outputs through practices that promote the onset, growth and monitoring of periphyton.

For decades, the detrital material naturally found in the bottom of aquaculture ponds was classified as an unwanted characteristic associated with poor water (and/or soil) quality, and although excessive detrital material accumulated in the pond bottom can lead to anaerobic conditions, the detrital material may be an important source of nutrients under management promoting aeration and mixing conditions. For example, Nunes *et al.* (1997) and Gamboa-Delgado *et al.* (2003) confirmed that shrimps reared under semi-intensive conditions select and ingest important amounts of detritus. Such studies have based their findings on stomach content analysis, which in turn have shown that the detrital material is one of the nutritional sources most frequently found in the stomach contents of farmed shrimps. The digestibility of detritus is high and this material supplies important amounts of protein from the bacterial communities associated with it. Up to 5–10% of the weight of the detrital mass is constituted by microorganisms (Moriarty 1997) and it has been suggested that the microbial component of the detritus has an important nutritive value to shrimp (Fenchel 1970). Bacteria could be an important nutrient source for shrimp as once the cell walls have been digested, the available nutrients can be utilized (Hood & Meyers 1974), representing a significant source of vitamins and digestive enzymes (Ceccaldi 1997). Hunter *et al.* (1987) evaluated the chemical composition of the biota found in aquaculture ponds and determined that the composition of the detrital material is (on a dry weight basis) 14.8% protein, 1.6% lipids and 1.1% carbohydrates. Interestingly, this study reported that the protein: energy ratio was highest for the detritus than for any other component of the natural productivity in ponds. Digestibility trials conducted on shrimp *Metapenaeus monoceros* have indicated that the digestibility of the detrital material can be as high as 93%; however, its energy content is low

(458 cal $g^{-1}$ on a wet weight basis). The management of detritus has evolved markedly into microbial-based intensive shrimp rearing systems in which the growth of microbial aggregates is promoted as an important source of nutrients for shrimps, while also having an influence on the maintenance of good water quality conditions (Burford *et al.* 2004c; Ju *et al.* 2008; Ray *et al.* 2011; Emerenciano *et al.* 2012).

## Dietary role of formulated feed in semi-intensive ponds

The addition of formulated feed and fertilizers is one the main characteristics of semi-intensively managed shrimp ponds. Although formulated feeds are physically and nutritionally designed for a particular species and size/age of shrimp, scarce information exists on shrimp dietary nutrient requirements under semi-intensive farming conditions (Venero *et al.* 2007) as the majority of studies on nutritional requirements have been performed under controlled, indoor laboratory conditions (Tacon 1995). Additional studies are needed to improve current knowledge on the biological utilization and the nutritional suitability of natural and formulated feeds. Such information would allow optimizing the feeding protocols and dietary formulations for shrimps farmed under specific conditions. Previous studies conducted on semi-intensively farmed shrimp have indicated that from 2% to 20% of the shrimp stomach contents is represented by ingested formulated feed (Nunes *et al.* 1997; Gamboa-Delgado *et al.* 2003); however, as described below, the nutritional contribution of the formulated feed to the growth of shrimps is higher than the proportions that have been found in the stomachs. Formulated feeds may also contribute vitamins, minerals, pigments and other micronutrients that are absent or become progressively scarce in the pond biota as the culture cycle progresses. A secondary, but important, role of the formulated feed is its contribution to the primary productivity through the leaching of nutrients (Cam *et al.* 1991). In addition to ongoing efforts to substitute fishmeal and oil in aquaculture diets with other ingredients, the rapid leaching of nutrients and inappropriate formulation of practical aquaculture diets used for semi-intensive shrimp production still represent two areas of much needed research in order to formulate cost-effective, nutritionally suitable diets and to identify possible synergistic effects of the natural productivity and the formulated feeds on the farmed organisms.

## Growth of the natural biota in aquaculture ponds

The natural productivity of an aquaculture pond is generally established in an ecological succession supported by the available nutrients for the primary producers. For example, the composition of phytoplankton growing in a rich nutrient media is 45–50% carbon and 8–10% nitrogen (Edwards 1982), hence, microalgal cells are entirely dependent on the biological supply of these primary elements to thrive and nutrients should not become a limiting factor. The basic principle of any fertilization scheme in aquaculture is to boost the production of natural feed sources available in the pond ultimately to supply the farmed animals with various nutrient sources. In general, a controlled addition of chemical fertilizers is encouraged to feed the autotrophic organisms (phytoplankton, benthic macroalgae and vascular plants), while the use of organic manure can be a resource to increase the growth of heterotrophic organisms (zooplankton, zoobenthos and farmed animals); however, food safety regulations restrict the use of untreated organic fertilizers. Individuals belonging to the zooplankton and zoobenthos communities first appear in aquaculture ponds as they are flushed into the aquaculture ponds through the water inputs. The natural productivity of a pond can be increased through careful management and constant monitoring of the variables associated with a specific culture (Boyd & Tucker 1998). The pond bottom (soil–water interface) is considered as a 'reservoir of primary nutrients' of the pond ecosystem and as such, plays an important role in maintaining the natural productivity (FAO 1989). There are numerous fertilization techniques and methods developed to maximize the natural productivity of aquaculture ponds under a variety of different conditions. The majority of fertilization techniques yield better results when applied in conjunction with an efficient monitoring programme focused on nutrient dynamics, available live feed and the biological performance of the cultured organisms in each individual pond.

## Natural productivity in shrimp nursery phase

The tanks or raceways used for the shrimp nursery phase are frequently managed so as to encourage small blooms of specific species of microalgae: however, the relatively short residency time of the cultured organisms, the high animal densities and the different water management techniques in this phase do not allow the establishment and full development of the trophic chains observed in aquaculture ponds (Burford *et al.* 2004b). Many shrimp hatcheries and nurseries have chosen to foster the production of benthic microalgae such as *Navicula* and *Amphora* with the objective of providing the postlarval shrimps with natural feed before their final transfer to grow out. The availability of natural feed under these particular conditions is thus very limited as the high ingestion rates characteristic of the early life stages of shrimp do not allow further settlement of additional live feed. Once the animals have adopted benthic behaviour, the consumption rate of material attached to

substrates drastically increases. Placing different types of artificial substrates into the tanks or ponds is a frequent strategy aimed to increase the available surface for both the densely packed organisms and the natural biota associated with substrates. Under these conditions, the constant supply of nutritionally balanced formulated feeds is thus critical to maintain healthy shrimp postlarvae exhibiting high growth and survival rates. Often, the nursery phase represents the last stage of the production cycle in which a higher degree of control can be exerted over variables such as water quality and feed management. After this stage and once the animals have been transferred to grow out ponds, the diverse culture system variables are more difficult to control.

## Methods applied to assess nutritional contributions to shrimps

Different methodologies have been applied to nutritionally evaluate the performance of natural and formulated diets (and the ingredients used to manufacture them) for marine organisms that have economic importance or are deemed suitable for farming. Such studies have generated an important amount of information about the type of feed or prey that cultured animals select under culture conditions. Some of the indicators applied to determine the dietary composition of the culture species are described below.

### Stomach content evaluation

Digestion implies a complex process that includes the mechanical breakdown of feed, enzyme secretion and different mechanisms for nutrient mobility. In the particular case of crustaceans, the ingestion process is complex and is initiated by an array of appendages that grind and separate fine feed particles, however, most of the mechanical breakdown of feed actually occurs within the stomach (Ceccaldi 1997). Analysis of the stomach content has the advantage of allowing identification of the material that was selected, captured and ingested by shrimp. Generally, this ingested material has been classified into the following categories: plant material, prey, artificial feed, detritus, minerals and semi-digested or unrecognizable material. Dissecting and isolating the consumed material allows quantitative and qualitative analyses that provide information on the animal's dietary preferences. These observations can be used in conjunction with data related to the size of the animals, moulting cycle and the trophic conditions of the culture system to infer the effect of combined variables on the biological performance of the target species (Nunes *et al.* 1997; Focken *et al.* 1998; Gamboa-Delgado *et al.* 2003). Some of the drawbacks associated with this technique are represented by the need for skilled personnel to conduct the dissections and the identification of ingested material. The short residency time of the feed in the digestive tract of the shrimp causes a fast degradation of the soft material, rendering it unrecognizable. In addition to the laborious aspects of the technique, there is the difficulty of distinguishing the artificial feed from the detritus. Nevertheless, it is possible to apply diverse dying techniques that stain the starch granules naturally present in the artificial feed, thereby distinguishing the latter from the detrital material. The natural fluorescence of the pigments found in the artificial feed has also been used to estimate ingestion (Kelly *et al.* 2000). Although the analysis of the stomach content provides a very good indicator of the dietary preferences of a particular consumer, it does not allow estimation of the real nutritional contribution of a specific dietary item to somatic growth.

### Chemical analyses of ingested material and tissues of consuming organisms

The transference of nutrients to tissue can be inferred through the use of different techniques. Analysis of the fatty acid and amino acid profiles of dietary elements and consumer permit assessment of the intake and metabolic fate of specific nutrients. For example, Gonzalez-Felix *et al.* (2009) evaluated the effects of different levels of dietary fatty acids on the growth and fatty acid composition of hepatopancreas and muscle tissue of Pacific white shrimp *L. vannamei*, while Forster *et al.* (2011) recently reported that the fatty acid profiles of shrimps reflected those of their respective diets when evaluating the physiological capacity of this shrimp species to utilize soy oil as a replacement for fish oil.

Immunological assays have also been applied to determine the presence of specific dietary particles in the digestive tract (Feller 1991). Alternative techniques have had the final objective of applying serological estimations in order to estimate the residency time of the protein supplied by different types of consumed prey and also to estimate amounts of ingested feed (Hoyt *et al.* 2000).

### Isotopic analysis of animal tissue and diets

One of the most reliable methods applied to determine assimilation efficiencies is by means of isotopic assessments. Most of the elements having biological relevance have two or more stable isotopes (for example, $^{12}C$ and $^{13}C$ for carbon, $^{14}N$ and $^{15}N$ for nitrogen). The difference between isotopes of the same element is the number of neutrons, which changes the weight of the atom but does not affect its reactive properties. Frequently, the 'lighter' isotope is present at a much higher natural abundance level than the 'heavy' isotope (Ehleringer & Rundel 1989); however, all participate in biochemical reactions. Animals

have a tendency to accumulate the heavier isotopes due to a discriminating effect of the different enzymatic pathways preferentially incorporating the heavier isotopes, while the lighter isotopes are excreted (Martínez del Rio & Wolf 2005). This physiological effect confers specific isotopic values to organisms belonging to different trophic levels in the aquatic and terrestrial ecosystems and thus, isotopic values can be used as natural biomarkers. The ratio between the heavy and light isotopes ($^{13}C/^{12}C$) is measured by means of isotope ratio mass spectrometry (IRMS). Under this technique, the target compound must be first combusted in order to convert the elements of interest to the gaseous form before introduction into the mass spectrometer. The most commonly used IRMS approaches for analysing carbon and nitrogen stable isotopes involve gas purification to introduce carbon as $CO_2$ and nitrogen as $N_2$. The purified compounds are then transferred to a source where gases are ionized before flowing through a flight tube where the paths of different isotopic species (e.g. $^{12}CO_2$ or $^{13}CO_2$) are magnetically deflected before colliding with a detection system. Once analysed, the isotopic values are expressed in delta notation ($\delta$) to indicate how the reported values, in parts per thousand (‰), vary with respect to the isotopic proportion of an international standard. The commercial availability of these analytical techniques, together with increasing accuracy levels of laboratory instrumentation, has allowed the tracing of nutrients in different organisms and ecosystems. Sampling techniques for a representative nutritional study using stable isotopes should consider all the possible sources of nutrients for the target organism. The decision to analyse the whole animal carcass or specific tissues of the consumer is defined according to the aim of the study (e.g. muscle tissue to trace dietary nitrogen, whole bodies to trace dietary carbon). Pretreatment of solid samples includes drying, grinding and lipid extraction (for samples containing high lipid levels, as lipids are isotopically depleted in $^{13}C$) and/or acidification to remove inorganic carbon. The isotopes are thus an integral part of the organic tissues but compounds having heavy isotopes can also be added to a specific substrate in order to label it or enrich ('spike') it. In contrast to the radioisotopes, the stable isotopes can be measured at natural abundance levels and are not dangerous, they are not invasive and several estimations can be done on a population, individual or specific tissue. Additional applications of the isotopic techniques include their use as pollution biomarkers (e.g. aquaculture waste tracing, Felsing *et al.* 2006) and their application in estimating metabolic turnover rates (MacAvoy *et al.* 2006), authenticating production methods (e.g. wild vs. farmed fish, Serrano *et al.* 2007), tracing animal migrations (Fry *et al.* 2003) and reconstructing palaeodiets (Richards *et al.* 2006).

The application of stable isotopes as nutritional tracers presents a powerful tool to estimate energy and nutrient flows in aquatic systems (Michener & Schell 1994). This is possible because the isotopic signature of a consumer mirrors the isotopic value of the assimilated material and thus provides information on the feeding habit over a period of time (Peterson & Fry 1987) and allows the estimation of dietary contribution and hence provides information on trophic relationships (DeNiro & Epstein 1978, 1981; Van der Zanden *et al.* 1999). As described in Figure 1, this relationship has been used previously in aquaculture nutrition to identify the origin and fate of different dietary components contributing to the growth or metabolic turnover of farmed animals in both grow out ponds (Schroeder 1983; Nunes *et al.* 1997; Burford *et al.* 2004a) and larval rearing systems (Schlechtriem *et al.* 2004; Gamboa-Delgado *et al.* 2008; Jomori *et al.* 2008; Gamboa-Delgado & Le Vay 2009b). Studies on larval nutrition are frequently constrained by the small larval size and the high sample size required for other analytical techniques. In contrast, stable isotope analysis requires samples of dry tissue of less than 1 mg, which makes it a very useful tool when assessing the nutritional physiology of larvae. Isotopic techniques have allowed the estimation of ingestion, assimilation efficiencies and elemental metabolic turnover rates in small zooplanktonic organisms by using direct methods as opposed to normal indirect techniques (Verschoor *et al.* 2005). Gamboa-Delgado and Le Vay (2009b) applied carbon stable isotope analysis to estimate the nutritional contribution of the dietary carbon supplied by live *Artemia* nauplii and formulated larval feeds (both offered in co-feeding regimes) to the growth of larvae and postlarvae of *L. vannamei*. The contribution of dietary carbon from *Artemia* to larval

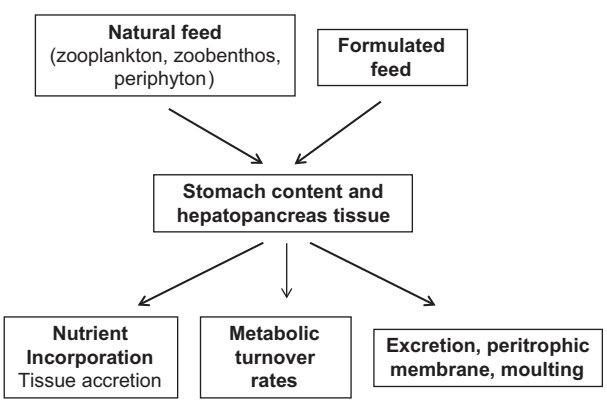


**Figure 1** Carbon and nitrogen flow in farmed shrimp under semi-intensive farming conditions. Bold arrows indicate origin and fate of components that can be isotopically analysed. Metabolic turnover rates can be estimated using exponential models of isotopic change in tissue over time.

growth was significantly higher than contributions supplied by formulated feeds. The sensitivity of the isotopic technique is such that in the latter study, the authors were able to detect carbon isotopic changes occurring from fertilized shrimp eggs and throughout all the shrimp larval stages. These isotopic shifts were correlated to the physiological utilization of endogenous and exogenous nutrient sources. In a similar approach, Matsuda *et al.* (2009) measured the nitrogen stable isotope values in diets and larval consumers and also reported that *Artemia* seem to be a more important food item for lobster (*Panulirus japonicus*) larvae than mussel gonad, as the respective, relative proportions of dietary nitrogen contributed to growth were 66% and 34%.

The estimation of nutrient assimilation using stable isotopes also presents several practical applications in the assessment of the nutritional performance of different aquaculture ingredients used to reduce or replace fishmeal as protein and energy source in aquafeeds. For example, Gamboa-Delgado and Le Vay (2009a) and Martinez-Rocha *et al.* (2013) formulated practical diets for shrimp *L. vannamei* using different proportions of soy protein isolate and peameal, respectively, to replace different levels of dietary nitrogen supplied by fishmeal. After isotopic analyses of the ingredients, diets and animal tissue, results from the former study indicated that fishmeal contributes significantly higher amounts of dietary nitrogen to the muscle tissue that the soy protein isolate, possibly due to a restriction of essential amino acids (methionine and lysine) in the latter. In contrast, incorporation of the dietary nitrogen from peameal in muscle tissue was similar to the levels established in four of five mixed experimental diets containing different proportions of fishmeal and peameal.

Different dietary components found in semi-intensively managed ponds may show naturally contrasting isotopic signatures, therefore, it is possible to establish a relationship between animals and their known diets. These isotopic values can be integrated in simple, mass-balance, isotopic mixing models (e.g. Phillips & Gregg 2001, 2003; Fry 2006) with the objective of quantifying the relative contribution of two or more nutrient sources to growth. Hence, nutritional studies have been conducted by using the natural isotopic variations specific to each trophic level and population. The availability of isotopic data obtained under controlled conditions has allowed the estimation of the nutritional contribution of several elements found in the natural environment or incorporated into specific experimental feeds and feeding regimes (Le Vay & Gamboa-Delgado 2011). The relative utilization of different dietary sources (protein, lipids) in live and formulated feeds can also be quantified by means of isotopic techniques (Schlechtriem *et al.* 2004; Beltran *et al.* 2008).

## Isotopic assessments: what are the nutritional contributions of the natural and formulated feed to shrimp growth?

### Field studies

Different studies have shown that, even in the presence of supplied artificial feed, shrimp farmed under semi-intensive conditions derive most of their structural carbon and nitrogen from the natural pond productivity (Table 1). For example, when the stomach content of different sized (2–10 g) Pacific white shrimp *L. vannamei* was quantitatively and qualitatively analysed over a semi-intensive cul-

**Table 1** Proportional amount of live and formulated feed found either in shrimp stomach contents (a) or supplied as experimental dietary regimes (b) and their actual contributions to the somatic growth of Penaeid shrimp as indicated by stable isotope analysis

| Species/environment | Stomach content (a) or Dietary proportion (b) (%) | | Actual contribution to tissue growth (%) | | Reference |
|---|---|---|---|---|---|
| | Formulated feed | Natural feed | Formulated feed | Natural feed | |
| *L. vannamei* Ponds† | 2–20[a] | 80–98 | – | – | Gamboa-Delgado *et al.* 2003; |
| *P. japonicus* Ponds† | 4[a] | 37–47 | – | – | Reymond & Lagardere 1990; |
| *P. japonicus* Ponds† | – | – | 23–47 | 53–77 | Anderson *et al.* 1987; |
| *P. subtilis* Ponds | 16[a] | 84 | 25 | 75 | Nunes *et al.* 1997; |
| *P. monodon* Ponds† | 22–29[a] | 71–88 | – | – | Focken *et al.* 1998; |
| *F. chinensis* Ponds‡ | – | – | 93 | 7 | Su *et al.* 2008; |
| *L. vannamei* Ponds§ | – | – | 71–82 | 18–29 | Burford *et al.* 2004c |
| *L. vannamei* Laboratory – culture vials | 50[b] | 50 | 27 | 73 | Gamboa-Delgado & Le Vay 2009b |
| *P. esculentus* Ponds‡ | – | – | 47–61 | 39–53 | Burford *et al.* 2004b |
| *P. japonicus* Ponds† | – | – | 13–65 | 35–87 | Cam *et al.* 1991 |
| *L. vannamei* Tanks | 49[b] | 51 | 20 | 80 | Gamboa-Delgado *et al.* 2011 |

†Semi-intensively managed ponds.

‡Intensively managed ponds.

§Intensively managed ponds. Pond biota mainly composed of microbial flocs.

ture cycle in Ecuador, the results indicated that shrimp strongly selected the available elements of the natural biota over the formulated feed as it was shown that 80–98% of stomach contents were derived from the natural biota (Gamboa-Delgado *et al.* 2003). In another trial carried out on *Penaeus subtilis* in Brazil, stomach content analysis indicated that 16% of the ingested material was represented by formulated feed and 84% by different elements of the natural biota. However, in this same study, the results from stable isotope analysis of food items and shrimp tissue, indicated that at the end of the culture cycle, the formulated feed contributed 25% of the dietary carbon incorporated as somatic growth, while 75% was attributed to the pond's natural productivity (Nunes *et al.* 1997). Cam *et al.* (1991) observed in semi-intensively farmed *Penaeus japonicus* that the contribution of the dietary carbon supplied by the formulated feed increased from 13% at day 30 of culture to 67% at day 120 of culture, indicating the increasing nutritional importance of the formulated feed as pond biota is diminished by the grazing pressure of expanding shrimp biomass. Similarly, as shrimp production methods intensify, the contribution of formulated feed to growth tends to increase (Table 1) due to the restriction of nutrients and substrate available to the natural production in conjunction with the higher grazing activity exerted by shrimp under these culture conditions. For example, Burford *et al.* (2004b) applied stable isotope analysis to determine the contribution of epiphytes growing on artificial substrates in ponds having different high densities of post-larval shrimp *Penaeus esculentus*. Epiphytes significantly contributed to the carbon requirements of post-larval shrimp (39–53%), despite the addition of formulated feed *ad libitum*. In a study conducted on other crustacean species, Duffy *et al.* (2011) analysed the isotopic values of several elements of natural productivity and the formulated feed consumed by common yabbies (*Cherax destructor*) reared in tubs. As the results indicated that zooplankton and microphytobenthos were the main contributors to growth and that animals consumed the diverse elements of the natural productivity even in the presence of pelleted feed, the authors recommended the use of formulated diets having a protein content lower than 19% for *Ch. destructor* reared under natural biota availability. As the natural communities of the pond biota can be very diverse, sampling techniques for isotopic determinations usually require pooling organisms that are representative of each trophic level (e.g. phytoplankton, phytobenthos, microzooplankton) eventually to obtain average isotopic values that are incorporated into mixing models to infer the nutritional contributions supplied by each group. Another approximation is to add an isotopically enriched substrate (e.g. $^{15}NH_4Cl$) into the pond or raceway, which greatly increases the isotopic signal of a specific tracer ($^{15}N$-labelled protein) as it is incorporated by the phytoplankton, zooplankton, final consumer and even in its excretion products (Burford *et al.* 2004b). However, measurements at natural abundance levels frequently generate enough relevant information on trophic relationships occurring in a specific system, and the use of isotopically labelled substrates is applied mainly to identify metabolic precursors and to label primary producers. Enriched substrates are also applied to increase the resolution of studies facing overlapping of the isotopic values of the dietary elements, which prevents the use of isotopic mixing models.

### Laboratory trials

Laboratory studies have had the main objective of evaluating the nutritional contribution of formulated and natural feed. For example, in the larval culture phase, results from these experiments have shown that larval and postlarval stages of marine fish and shrimp acquire significantly higher amounts of dietary carbon from live preys (*Artemia* and rotifers) than from formulated feed supplied at similar dietary proportions (Gamboa-Delgado *et al.* 2008; Gamboa-Delgado & Le Vay 2009b). Likewise, it has been determined that juvenile shrimps co-fed with formulated feed and live biomass of macroalgae *Ulva clathrata*, incorporate significantly higher amounts of dietary carbon and nitrogen from the latter. However, the high amount of dietary carbon and nitrogen supplied by the live macroalgae biomass in co-feeding regimes supplying more than 50% of macroalgae was not reflected in a fast increase of somatic growth due to the restriction of other nutrients in this macroalgae species (low lipid and energy content). Interestingly, shrimp under a co-feeding regime supplying 75% of formulated feed and 25% of live macroalgae biomass (on a dry weight basis) showed higher growth rates than animals reared only on commercial formulated feed, although the difference was not statistically significant (Gamboa-Delgado *et al.* 2011).

In studies conducted on other species, Schlechtriem *et al.* (2004) manipulated the isotopic values of nematodes by feeding them on meals from plants having different photosynthetic pathways (C3 and C4), which imprints differing isotopic values. The grown nematodes were in turn offered as live feed to carp (*Cyprinus carpio*) with the aim of estimating lipid and lipid-free matter assimilation. The use of isotopic mixing models to estimate nutritional contributions requires some assumptions and conditions to be met and these are more easily verified and fulfilled under laboratory conditions than in field studies. For example, important assumptions take into account that (i) the nutritional sources have different isotopic values, (ii) the elemental composition and assimilation efficiencies of nutritional sources are known, (iii) isotopic equilibrium has been reached between diet and consumer and the isotopic dis-

crimination factors (isotopic difference between consumer and diet/prey) are known, (iv) isotopically distinct dietary components are differentially allocated to different tissues (isotopic routing) (see review: Martinez del Rio *et al.* 2009). As one of these assumptions indicates that the consuming organism should be in isotopic equilibrium (or isotopic steady state) with their respective diet, preliminary laboratory experiments are frequently required to verify that the consuming organism's tissues reflect the isotopic values of a previously fed diet and to estimate the isotopic discrimination factors in order to introduce correction factors into the isotopic mixing models. The isotopic equilibrium can be reached through tissue accretion, metabolic turnover or both, and the amount of time necessary for an animal to reach isotopic equilibrium with its diet depends on the growth rate, metabolic rates, size/age of the individual and dietary quality. In the dynamics of isotopic transfers, there is a physiological effect termed 'isotopic routing' (Gannes *et al.* 1997) in which the different dietary elements (and their isotopes) are not evenly mixed and directed to all tissues, but are selectively metabolized and incorporated. This effect has to be taken into consideration when selecting either specific tissues or whole animals for a particular study. In the particular case of larval nutrition, the isotopic routing is commonly avoided because, due to the small larval size, whole animals are used for analysis and the ensuing data interpretation (Le Vay & Gamboa-Delgado 2011). Alternatively, it is possible to trace a specific dietary element (e.g. nitrogen) to a specific tissue-reservoir (e.g. muscle). The isotopic values of carbon and nitrogen present at natural abundance levels in different organisms are frequently very contrasting and thus allow experiments to be designed aimed at determining nutrient incorporation. Additionally, the isotopic values of primary producers and filter-feeding organisms can be easily manipulated through the use of specific culture media and dietary substrates (Gamboa-Delgado *et al.* 2008, 2011). This allows experiments using nutritional sources having contrasting isotopic values, which improves the resolution of mixing models and exponential models of isotopic change when estimating nutritional contributions to growth and metabolic turnover rates, respectively.

## Future studies

Since aquaculture systems have the advantage of being composed of relatively fewer trophic elements than those found in natural ecosystems, nutritional studies using isotopic techniques require less intensive sampling. Nevertheless, the trophic relationships and the flow of energy and nutrients in semi-intensive aquaculture ponds can be complex. These processes can be systematically disentangled through isotopic analysis, careful sampling and appropriate experimental designs. For example, the nutritional components of the pond biota that are thought to contribute to shrimp nutrition can be transferred to experimental units in laboratory conditions to be offered to farmed animals in order to assess the nutritional contributions of specific elements to growth by means of isotopic assays. The use of isotopic mixing models to estimate nutritional contributions is not limited to the assessment of two dietary sources (e.g. live and formulated feed). In ecology studies, dietary contributions to animal growth have been estimated for up to seven nutritional sources, although an underlying condition is that the sources must have different isotopic values (Ben-David *et al.* 1997; Phillips & Gregg 2003). Under this approach, the nutritional contribution of multiple individual ingredients having contrasting isotopic values (fishmeal, plant-derived protein, microbial protein) can be assessed after incorporating previously analysed ingredients into experimental practical diets. It is forecast that in aquaculture nutrition, isotopic data in conjunction with production parameters will provide a wider scheme for the physiological utilization of different ingredients delivered through new dietary formulations for larval and juvenile stages. At a finer level of analytical detail, chromatographic 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 the fate of individual dietary fatty acids and amino acids (see review Le Vay & Gamboa-Delgado 2011). It has been demonstrated that the isotopic values of carbon and nitrogen found in amino acids of aquatic species consistently show a wide range of values of up to 20 units (Fantle *et al.* 1999; McClelland & Montoya 2002; Chikaraishi *et al.* 2007; McCullagh *et al.* 2008). Isotopic differences are useful to avoid isotopic overlap when tracing specific amino acids. Compound specific isotope analysis of individual amino acids has been applied in studies on juvenile crabs *Callinectes sapidus* (Fantle *et al.* 1999) in laboratory experiments aimed to interpret field observations related to the transfer of essential and non-essential amino acids. The application of CSIA to individual amino acids to identify dietary requirements of insects for amino acids (O'Brien *et al.* 2003, 2005) have shown that the carbon isotopic signature of essential amino acids in adult insects remains close to the values of the amino acids found in the plant proteins consumed by larvae, while the isotopic signature of carbon for non-essential amino acids reflects carbohydrates consumed by adults. In the case of fatty acids, Parrish *et al.* (2007) used the relatively heavy natural isotopic signature of carbon in heterotrophic microalgae *Schizochytrium* sp. to trace the transfer and conservation of the essential fatty acid, ω6 docosapentaenoic acid, through a two-step food chain through rotifers (*Brachionus plicatilis*) and into cod (*Gadus morhua*) larvae. In shrimp nutrition, there exists a

constant interest in delivering appropriate amino acid profiles through formulated feeds, and although formulated feeds experience moderate to high leaching of nutrients, it seems that the elements of the natural productivity frequently compensate for these losses and promote high growth and survival rates. The CSIA of amino acids may have the potential to elucidate how and which of the different amino acids are primarily transferred from the live or formulated feeds into the shrimp tissues. The growing adoption of CSIA in many fields of biology holds great potential and will further increase the current knowledge of the dietary roles and biological utilization of specific nutrients available in different aquaculture systems. The commercial availability of a variety of isotopically enriched substrates (amino acids, fatty acids, cholesterol, vitamins) labelled with up to three heavy isotopes ($^{13}C$, $^{15}N$, $^{2}H$), extends the range of applications in studies focusing on the nutritional physiology of aquatic species.

## Conclusion

Results from studies applying stable isotope techniques indicate that the different elements of the natural productivity found in semi-intensively managed shrimp ponds frequently represent the main source of dietary carbon and nitrogen for the farmed animals. Penaeid shrimps are very efficient at utilizing this pond biota, which provides highly digestible macronutrients for shrimps, as well as vitamins and minerals. The natural productivity rapidly responds to foraging pressure, nutrient availability and to diverse environmental conditions causing natural ecological successions. Additionally, there are strong fluctuations in the natural populations caused by shrimp predation and foraging, and while some communities such as the zooplankton rapidly decrease over the first few weeks of culture (Coman *et al.* 2003), other organisms may show a tendency to recover, as in the case of organisms finding temporary shelter in the pond substrate (Nunes & Parsons 2000). Although each semi-intensively managed pond and its natural populations have very particular characteristics, effective fertilization programmes and frequent monitoring of the natural productivity are essential activities aimed at maintaining a constant presence of natural feed for the farmed shrimp. Previous studies have applied stable isotopes as tracers and have indicated that at the end of the farming cycle (larval or grow out), the majority of macronutrients are derived from the natural feed. Although the formulated feed contributes relatively lower proportions to the stomach content, its contribution to growth in terms of dietary carbon and nitrogen is comparatively larger than the proportions observed in the stomach. This can be explained by the high digestibility coefficients and high protein content of formulated feeds. Therefore, the constant availability of formulated feed represents an excellent nutritional supplement in both the shrimp nursery stage and semi-intensive grow out operations. The isotopic values present at natural abundance levels in shrimps and their natural diets can provide relevant information to elucidate the flow and incorporation of nutrients contributing to growth, by also defining the time periods at which the animals are physiologically better prepared to ingest and assimilate nutrients. Nutritional evaluations conducted by the application of stable isotopes provide a very useful analytical technique to interpret the digestive physiology of aquatic organisms, being of particular assistance in nutritional studies aimed at determining the dietary contribution that the different trophic elements provide to the consuming organisms under farming conditions. The feasibility of manipulating the isotopic profiles of dietary ingredients, formulated diets and even live feeds, represents an additional opportunity to increase the resolution and reach of nutritional studies applying isotopic techniques.

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