Nutritional role of the 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 high variety of trophic elements that continuously supply nutrients to the farmed organisms. As in the natural ecosystems, these dietary components exhibit differing isotopic signatures at natural abundance levels and since these natural communities belong to different trophic positions and isotopic values of organisms tend to increase with trophic level, the isotopic differences can be measured and used to infer on the transfer of nutrients, hence allowing establishing relationships between consuming organisms and their diets. Isotopic values can be measured and integrated in mass-balance mixing models in order to quantify 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 the 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 consumers' tissues. The present review highlights the nutritional relevance that the natural productivity of semi-intensively managed ponds and the supplied formulated feed represent for farmed shrimp. A synthesis of results from studies applying isotopic techniques to determine the relative contribution of dietary carbon and nitrogen derived from different biota elements and formulated feeds to the growth of shrimp is presented.

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

Introduction

Shrimp farming through semi-intensive production methods has become predominant in the 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 the natural environments. Frequently, the natural populations established in the 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 the continuous foraging pressure by the farmed animals. The nutritional importance that the 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; Martinez-Córdova *et al.* 1998, 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 the natural communities of prey organisms. Nevertheless, efficient fertilization and rearing protocols and management strategies aimed to maintain the soil and water quality, allow fostering the presence of appropriate concentrations of individuals of the natural productivity over the whole culture cycle.

The natural productivity in aquaculture ponds

In the aquatic environment, micro- and macroalgae represent the link that converts solar energy in utilizable chemical energy, which is stored in the chemical bounds 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 the pond water is frequently associated with healthy microalgae populations, which in turn, guarantee the stability of the dissolved oxygen concentrations and also support the growth of zooplankton. In this context, it has been recently reported 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 presence of macroalgae is unwanted in aquaculture ponds because they compete for nutrients with the microalgae (and also because they interfere with the 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.

Nutrition wise, one of the most important components of the natural productivity is the zooplankton (Coman *et al.* 2003; Duffy *et al.* 2011), which is represented by a rich community of very diverse organisms such as mollusks, fish and crustacean larvae and adult forms of small species (copepods and nematodes). Most of these organisms directly forage on the phytoplankton and their populations sharply decrease when the latter is limited. The zoobenthos is composed by 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. Additionally, a diverse conglomerate of communities of the natural productivity, collectively receiving the term periphyton, is part of the benthic environment. The periphyton is frequently represented periphytic microalgae, cyanobacteria, micro-invertebrates and other organisms that constantly provide nutrients to the farmed shrimps. The periphyton not only acts as a nutrient source, but also as natural biological filter due to the activity of the biofilms, which are mostly conformed by heterotrophic microbes associated to these communities (Azim *et al*. 2003; Milstein *et al*. 2009). Given all these advantages, several methods have been developed to encourage the growth of periphyton by using different types of 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) on periphyton management and this information has ultimately focused in optimizing aquaculture production outputs through practices aimed to promote the onset, growth and monitoring of the periphyton.

For decades, the detrital material naturally found in the bottoms of aquaculture pond was classified as an unwanted characteristic associated to bad 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 aerated 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 to it. Up to 5 to 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 can easily represent a source of nutrients to the shrimps because after the cell walls have been digested, the available nutrients can be utilized (Hood & Meyers 1974), representing in turn, 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 the 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 remarkably evolved 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).

The 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 the current knowledge on the biological utilization and the nutritional suitability of natural and formulated feeds. Such information would allow optimizing 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 their 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 inert feed is its contribution to the primary productivity through the leaching of nutrients (Cam *et al*. 1991). In addition to ongoing efforts to substitute fish meal and oil in aquaculture diets with other ingredients, the fast lixiviation of nutrients and inappropriate formulation of practical aquaculture diets used for semi-intensive shrimp production still represent two of the problems that nutritionist have to conduct research on 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 of 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 to ultimately 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). Individuals belonging to the zooplankton and zoobenthos communities first appear in aquaculture ponds as they come through the effluents and other inputs. The natural productivity of a pond can be increased through careful management and constant monitoring of the variables associated to a specific culture (Boyd & Tucker, 1998). The pond bottom (soil-water interface) is considered as the "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 specific conditions. The majority of fertilization techniques yields better results when applied in conjunction with an efficient monitoring program focused on nutrient dynamics, available live feed and 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 population 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 behavior, the consumption rate of material attached to substrates drastically increases. Setting 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 to 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. Very 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, 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) in marine organisms having economic importance or deemed suitable for farming. Such studies have generated an important amount of information about the type of feed or preys that cultured animals choose under culture conditions. Some of the indicators applied to determine how animals select their trophic elements and how the latter contribute, are the following.

Stomach content evaluation

The 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 also complex and it 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, preys, artificial feed, detritus, minerals and semi-digested or unrecognizable material. Dissecting and isolating the consumed material allows conducting quantitative and qualitative analyses that provide information on the animal's 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 on 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 to this technique are represented by the need of skillful 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 estimating the real nutritional contribution of a specific dietary item to the somatic growth.

Chemical analyses of the ingested material and tissues of consuming organisms

The transference of nutrients to tissue can be inferred through the use of different techniques. Analyses of the fatty acid and amino acid profiles of dietary elements and consuming organisms allow inferring on 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 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 serologic estimations in order to estimate the residency time of the protein supplied by different types of consumed preys 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 biologic relevance have two or more stable isotopes (for example, ^{12}C and ^{13}C for carbon, ^{14}N and ^{15}N for nitrogen). The only difference between different isotopes of the same element is the number of neutrons, which do not affect the reactive properties of such isotopes. Frequently, one of these isotopes is present at a natural abundance level much higher 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. Isotopic values are 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 gaseous form before introduction into the mass spectrometer. The most commonly used IRMS approaches to analyze 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 analyzed, the isotopic values are expressed in delta

notation (δ) to indicate that reported values, in parts per thousand (‰), are based in an isotopic proportion that was compared with the respective isotopic proportion of a standard. The commercial availability of these analytical techniques, together with laboratory instrumentation having increasing accuracy levels, has allowed tracing nutrients in different organisms and ecosystems. The sampling techniques for a representative nutritional study using stable isotopes should consider all the possible sources of nutrients for the target organism. Considerations on analyzing whole animal carcass or specific tissues of the consuming organism are defined according to the aim of the study (*e.g.* muscle tissue to trace dietary nitrogen, whole bodies to trace dietary carbon). Sample 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 radio-isotopes, 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 consuming organism 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 estimating dietary contributions to infer about trophic relationships (DeNiro & Epstein 1978, 1981; Van der Zanden *et al*. 1999). As described in figure 1, this relationship has been previously used 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; Jomori *et al*. 2008; Gamboa-Delgado *et al*. 2008; Gamboa-Delgado & Le Vay 2009b).

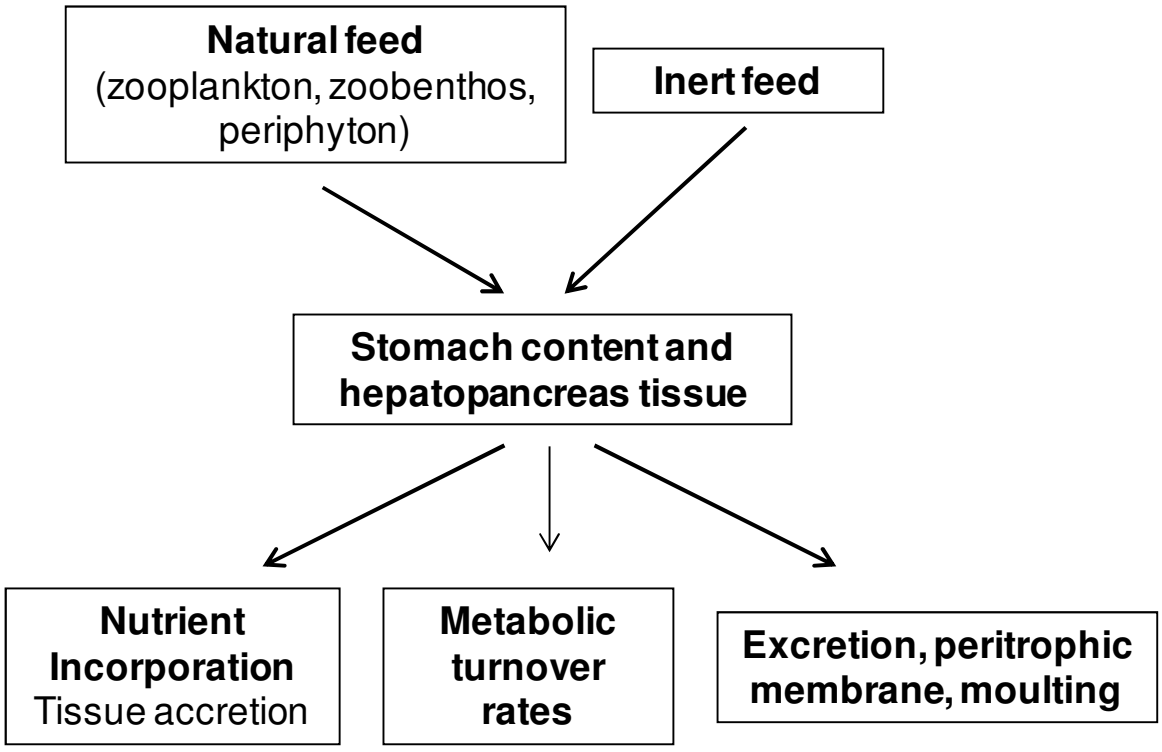

Fig. 1. Carbon and nitrogen flow in farmed shrimps under semi-intensive farming conditions. Bold arrows indicate origin and fate of components that can be isotopically analyzed. Metabolic turnover rates can be estimated using exponential models of isotopic change in tissue over time.

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 make them a very useful tool when assessing the nutritional physiology of larvae. The isotopic techniques have allowed estimating ingestion, assimilation efficiencies and elemental metabolic turnover rates in small zooplanktonic organisms by mean of direct methods instead of using indirect techniques (Verschoor *et al*. 2005). Gamboa-Delgado & Le Vay (2009b) applied carbon stable isotopes 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 the larval 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 incorporation using stable isotopes also presents several practical applications in the assessment of the nutritional performance of different aquaculture ingredients used to reduce or replace fish meal as protein and energy source in aquafeeds. For example Gamboa-Delgado & Le Vay (2009a) and Martinez-Rocha *et al*. (2012) formulated practical diets for shrimp *L. vannamei* using different proportions of soy protein isolate and pea meal,

respectively, to replace different levels of the dietary nitrogen supplied by fish meal. After isotopic analyses of ingredients, diets and animal tissue, results indicated that fish meal 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 pea meal in muscle tissue was similar to the levels established in four of five mixed experimental diets containing different proportions of fish meal and pea meal.

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 estimating 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 mean 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 the growth of shrimp?

1) Field studies

Different studies have shown that, even in the presence of supplied artificial feed, shrimps farmed under semi-intensive conditions derive most of their structural carbon and nitrogen from trophic elements supplied by the natural food (Table 1). For example, in a study conducted on Pacific white shrimp *L. vannamei*, the stomach content was quantitative and qualitatively analyzed at different shrimp sizes (from 2 to 10 g) over a semi-intensive culture cycle in Ecuador; results indicated that, when compared to formulated feed consumption, shrimps strongly select the available elements of the natural biota as the contribution of the latter to the stomach content was as high as 80-98 % (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 artificial feed and 84% by different elements of the natural biota. However, in this same study, results from stable isotope analysis of food items and shrimp tissue, indicated that at the end of the culture cycle, the formulated feed contributed with 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 the 30th day of culture to 67% at the 120th day of culture, hereby explaining that the nutritional importance of the formulated feed is higher as the shrimp biomass increases its grazing activity on the progressively diminishing communities of the pond biota. In a similar way, and 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 for the natural productivity in conjunction with the higher grazing activity exerted by the shrimps under these

Table 1. Proportional amount of live and inert 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
	Inert feed	Natural feed	Inert feed	Natural feed	
L. vannamei Ponds [a]	2-20 [a]	80-98	-	-	Gamboa-Delgado *et al.* 2003
P. japonicus Ponds [a]	4[a]	37-47	-	-	Reymond & Lagardare 1987
P. japonicus Ponds [a]	-	-	23-47	53-77	Anderson *et al*. 1987
P. subtilis Ponds [a]	16 [a]	84	25	75	Nunes *et al.* 1997
P. monodon Ponds [a]	22-29 [a]	71-88	-	-	Focken *et al.* 1998
F. chinensis Ponds [b]	-	-	93	7	Su *et al.* 2008
L. vannamei PL Lab - vials	50 [b]	50	27	73	Gamboa-Delgado & Le Vay 2009
P. esculentus Ponds [b]	-	-	47-61	39-53	Burford *et al.* 2004b
P. japonicus Ponds [a]	-	-	13-65	35-87	Cam *et al.* 1991
L. vannamei Tanks	49 [b]	51	20	80	Gamboa-Delgado *et al.* 2011

[a] Semi-intensively managed ponds.
[b] Intensively managed ponds.
[c] Intensively managed ponds. Pond biota mainly composed of microbial flocs.

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 postlarval shrimp *Penaeus esculentus.* Epiphytes significantly contributed to the carbon requirements of post-larval shrimp (39 - 53%), despite addition of formulated feed *ad libitum.* In a study conducted on other crustacean species, Duffy *et al.* (2011) analyzed the isotopic values of several elements of the natural productivity and the formulated feed consumed by common yabbies (*Cherax destructor*) reared in tubs. As 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 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) to eventually obtain average isotopic values that are incorporated into mixing models to infer on 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 highly increases the isotopic signal of a specific tracer (^{15}N-labelled protein) as it is incorporated by the phytoplankton, zooplankton, final consumer and even its excretion products (Burford *et al.* 2004b). However, measurements at natural abundance levels frequently generate enough relevant information on the trophic relationships occurring in a specific system, and the use of isotopically-labeled substrates is mainly applied 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.

2) Laboratory trials

Laboratory studies have had the main objective of evaluating the nutritional contribution of the formulated and natural feed. For example, in the larval culture phase, results from these experiments have shown that the 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, shrimps 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 the 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 carps (*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 (1) the nutritional sources have different isotopic values, (2) the elemental composition and assimilation efficiencies of nutritional sources are known, (3) isotopic equilibrium has been reached between diet and consumer and the isotopic discrimination factors (isotopic difference between consumer and diet/prey) are known (4) isotopically distinct dietary components are differentially allocated to different tissues (isotopic routing) (see review: Martínez 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 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 designing experiments aimed to determine 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 conducting 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 less trophic elements than those found in the 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 (fish meal, plant-derived protein, microbial protein) can be assessed after incorporating previously-analyzed ingredients into experimental practical diets. It is forecasted that in aquaculture nutrition, isotopic data in conjunction with production parameters will

provide a wider scheme on 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 fate of individual dietary fatty acids and amino-acids (see review: Le Vay and Gamboa-Delgado, 2011). It has been demonstrated that the isotopic values of the carbon and nitrogen found in amino acids of aquatic species consistently show a wide range of values of up to 20 ‰ (Fantle *et al.* 1999; McClelland & Montoya 2002; Chikaraishi *et al.* 2007; McCullagh *et al*. 2008). Isotopic differences are useful to avoid isotopic overlappings when intending to trace specific amino acids. CSIA for 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. Studies in insects have applied CSIA of individual amino acids to identify dietary requirements for amino acids (O'Brien *et al.* 2003, 2005) and results 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 high natural isotopic signature of carbon in heterotrophic microalgae *Schizochytrium* sp. to trace the transfer and conservation of fatty acids along a two-step food chain through rotifers (*Brachionus plicatilis*) and cod (*Gadus morhua*) larvae. In shrimp nutrition, 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. 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 on 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. . In addition to the natural ecological successions, there are strong fluctuations of 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 programs and frequent monitoring of the natural productivity are essential activities aimed to maintain a constant presence of natural feed

for the farmed shrimps. 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 in 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; 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 time periods on 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 to determine the dietary contributions 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 the isotopic values of live feed, represents an additional opportunity to increase the resolution and reach of nutritional studies applying isotopic techniques.

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