Chapter 11
The Use of Stable Isotopes Analysis in Wildlife Studies

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Abstract The application of stable isotopes analysis in wildlife studies has increased in recent decades due to the wide range of information that can be obtained with this methodology. This chapter aims to present the basic principles of the stable isotopes analysis and their potential applications in wildlife studies. The main topics presented are diet reconstruction, trophic level, animal movements, tissue turnover rates, and ecotoxicology.

11.1 Introduction

Stable isotopes analysis show a wide range of applications in biological, earth, and environmental sciences. However, in recent decades wildlife studies using this methodology increased exponentially promoting a major development in this area of knowledge (Gannes et al. 1997, 1998; Crawford et al. 2008; Layman et al. 2012). This is probably due to the large amount of information that can be obtained through this methodology to answer different types of questions related to wild animals.

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The use of stable isotopes is based on the fact that the isotopic compositions vary in a predictable way, as the element moves through the various ecosystem compartments (Martinelli et al. 2009). Many chemical processes result in isotopic fractionation, because of the mass difference between the light and heavy isotopes. Due to these characteristics, stable isotopes can be used as a biological tracer in ecological studies.

The analysis of stable isotopes provides a clear advantage in identifying differences in resources use at different scales (Dalerum and Angerbjörn 2005) allowing the assessment of long-term ecological trends, needed to management and conservation plans for wild species. Furthermore, new quantitative analytical approaches have emerged to elucidate various aspects of the biology of wild species based on the stable isotopes composition increasing its potential applicability (Layman et al. 2012).

The conservation biology deals with the causes and consequences of biodiversity loss. In this context, the development of both technological tools and conceptual basis is necessary to perceive, identify, and solve problems. This chapter has the purpose of showing how the stable isotopes tool can be used to answer ecological questions.

This chapter provides the presentation of main stable isotope analysis applications in wildlife studies comprising diet reconstruction, trophic level, animal movements, tissue turnover rates, and ecotoxicology. The themes breadth and the rapid growth of studies using this methodology make unfeasible a great depth and presentation of all published studies for each application. Therefore, our goal is to present the topics in a simple and concise form, wherever possible, providing examples of studies conducted in tropical environments. Our intention is that this work will serve as a guide for researchers wishing to get to know the applications of isotope methodology in wildlife studies.

Box 1: Stable Isotope Methodology

The study of applications of stable isotopes analysis in wildlife studies requires a brief review of some basic concepts discussed below. Isotopes are species of the same chemical element that have different atomic masses. This is due to variations in the neutrons number in the nucleus, for example, nitrogen isotope ^{14}N presents atomic mass equal to 14 (7 protons + 7 neutrons), and ^{15}N presents atomic mass equal to 15 (7 protons + 8 neutrons). In addition, the isotopes are considered stable when they do not undergo radioactive decay, thus maintaining the same mass over time.

Stable isotopes have different natural abundances being lighter isotopes (lower atomic mass) more abundant, while heavier isotopes (higher atomic mass) are less abundant. These differences in the isotopes concentrations can be measured using a mass spectrometer.

The stable isotope ratios (heavy/light, e.g., $^{13}C/^{12}C$, $^{15}N/^{14}N$ $^{2}H/^{1}H$) are usually expressed by the notation (δ) and are related to international standards (Fry 2006). The δ values are numerically small (of the order of 10^{-2}), so the results of these expressions are usually multiplied by 1,000,

being referred to as parts per mil (‰). These values can be either positive or negative, depending on the isotopes ratios. International standards have been defined for each of the elements. For example, the carbon standard is Peedee Belamite (PDB), a Belemnnitella fossil of the Peedee formation in the South Carolina (USA), nitrogen standard is air (N_2) and hydrogen standard is Vienna Standard Mean Ocean Water (VSMOW).

11.2 Uses in Wildlife Studies

11.2.1 Diet Reconstruction

Conventional methods of direct observation, stomach contents, and feces analysis have been traditionally used in ecological studies in order to understand individuals' diet (Litvaitis 2000). These methodologies provide important information about the recent diet; however, biases associated with different levels of items digestibility and accidental ingestion are common (Martinelli et al. 2009). In this context, the application of stable isotopes analysis to reconstruct the diet has gained more prominence in recent years (Boecklen et al. 2011).

The use of this methodology is based on the fact that the isotopic composition of animal tissues reflect the isotopic composition of their diet discounted the isotopic fractionation between diet–animals (DeNiro and Epstein 1978), so there is the possibility to track the diet assimilated by the animal (Ramos and González-Solís 2012). The stable isotopes analysis provide valuable ecological information in situations that conventional methods are disabled or as complementary to classical studies of stomach content analysis, for example, the investigation of the resources partition from individual to community level (Inguer and Bearhop 2008).

Some important aspects about isotopic analyzes should be highlighted before utilization. The isotopic composition of food sources must be distinct (e.g., C_3 or C_4 plants) and the animal tissue analyzed must be chosen appropriately taking into account the study objectives, the turnover rate and the isotopic fractionation (Gannes et al. 1998). When these aspects are attended, the isotopic compositions of consumers and their potential prey can be used for a qualitative or quantitative analysis of diet reconstruction (Layman et al. 2012).

Currently, there are many mixing models that yield relative contributions of several diet sources providing benefits in comparison with traditional methods for diet analysis (Phillips and Gregg 2003; Parnell et al. 2010, 2013; Boecklen et al. 2011; Erhardt and Bedrick 2013). The stable isotope analysis in R (SIAR), (Parnell et al. 2010) has been one of the most common models presently used by researchers in wildlife studies. This model utilizes linear equations and Bayesian statistical techniques to report ranges of proportional source pool contributions to consumers. Its advantage over previous models is incorporate uncertainty and variation in input parameters. Several examples of the isotope methodology application for diet studies can be found in the literature.

Oliveira (2006) studied seasonality of energy sources of tambaqui (*Colossoma macropomum*), fish of great economic importance in the floodplains of the Amazon region and that exhibit diverse feeding habitats, which can only be accessed by the combination of classical analysis of stomach contents and stable isotope techniques. The analysis of stomach contents showed that the relative importance of the food items varied with water level (rising, high, falling, and low). Fruits and seeds become available during periods of high water level when tambaqui have access to the flooded forest. However, lakes are disconnected from the rivers during the low water period, making the availability of food resources different. The $\delta^{13}C$ and $\delta^{15}N$ values of fish tissues also varied during the hydrological cycle. C_3 plant material (including fruits and seeds) was the main contributor to the tambaqui biomass with values between 55 and 95 % depending on the water level. C_4 plants have little importance in the tambaqui diet (maximum contribution = 26 %) probably because of its low nutritional value. Zooplankton played a role in supplying nitrogen to tambaqui.

Lara et al. (2012) studied the trophic relationship and primary carbon sources of diets between two sympatric freshwater turtles widely distributed in the Amazon basin, *Podocnemis unifilis* and *Podocnemis expansa*, using carbon and nitrogen stable isotopes analysis. No differences were found between the two species in relation to $\delta^{13}C$ (means *P. unifilis*: −26.2 ‰; *P. expansa*: −26.1 ‰), but *P. unifilis* had higher values of $\delta^{15}N$ than *P. expansa* (means *P. unifilis*: 7.6 ‰; *P. expansa*: 5.1 ‰), indicating a possible trophic change due to exploitation of different food resources. In addition, the values of $\delta^{13}C$ show the dependence of these species on C_3 plants, which represent their main source of basal energy. These two species of freshwater turtles have a diet based on aquatic plants, algae, seeds, leaves, fruits, flowers, roots, stems, and occasionally small animals (Pritchard and Trebbau 1984). However, migrations to small lakes made by *P. unifilis* during the flood season enables the exploitation of a broader range of food resources that *P. expansa*.

Marques et al. (2013) studied the intraspecific isotopic niche variation in Broad-snouted caiman (*Caiman latirostris*) in a silvicultural landscape in Brazil through the use of carbon and nitrogen stable isotopes. Discrete ontogenetic variations in the isotopic niche and sexual difference only for juveniles were identified analyzing claw samples collected from juveniles, adults, and hatchlings of *C. latirostris*. There is a progressive increase in stable isotope compositions values ($\delta^{15}N$ and $\delta^{13}C$) in relationships to animals' snout–vent length. These results may indicate differences in the exploitation of diet resources to decrease intraspecific competition. Crocodilians show a dramatic increase in body mass during ontogenetic development, which can result in diet shift from invertebrate to vertebrate items. Dietary studies using stomach contents suggest that the species can exploit a wide variety of prey such as insects, arachnids, crustaceans, snails, fish, amphibians, reptiles, birds, and mammals (Melo 2002; Borteiro et al. 2009).

Furthermore, the stable isotopes methodology has also been applied to the paleodiet reconstruction using fossil samples (Parkington 1991; Pate 1997; MacFadden 2000; Koch 2007; Clementz 2012). This methodology has opened a new perspective to become an important tool for paleontologists infer the diet of extinct animals because direct observation is not possible. The collagen (protein of bone tissue) removed from

bones and teeth are the material commonly used for these studies because remain preserved even with the passage of time (DeNiro 1987). The preservation quality of original isotopic information in this material can be evaluated by ratio of carbon to nitrogen (C:N) in samples (Ambrose 1990; van Klinken 1999). The C/N values should be between 2 and 3, so it can be sure that there was no contamination from exogenous sources (DeNiro 1985; Martinelli et al. 2009).

The power of isotopic tool in paleodiet reconstruction can be exemplified through the study of MacFadden et al. (1999) with six sympatric horses of 5 million years old (late Hemphillian) from fossil deposits of Florida. Traditional morphological studies of tooth crown height indicate that these animals with high-crowned teeth have fed on abrasive grasses, but enamel $\delta^{13}C$ values in combination with tooth microwear data indicate that these horses in this study were not exclusive C_4 grazers but also included mixed feeders and C_3 browsers. C_4 plants in this context include most grasses, while C_3 plants include most leafy, woody, and other soft plants (browse). Therefore, this study demonstrated that horses can partition their food resources from almost pure C_4 grazers to principally C_3 browsers, contrary to previous studies with others approaches.

11.2.2 Trophic Level

The stable isotopes analysis are extremely useful for studies about nutrients and energy transfer in food webs. The nitrogen stable isotopes are often used in trophic web studies due to the expected increase in ^{15}N over successive levels, according consumers tissues are enriched relative to its diet (Kelly 2000; Fry 2006). The consumer tissues have differents $\delta^{15}N$ values due to assimilation and excretion of nitrogen (Macko et al. 1986; Olive et al. 2003), with the excretion of lighter nitrogen (^{14}N) in the urine. This preferential removal of ^{14}N amine groups occurs by the enzymes responsible by desamination and transamination of aminoacids (Macko et al. 1986, 1987).

In this context, the trophic position of an animal can be estimated based on the $\delta^{15}N$ values of the food chain and on the ^{15}N enrichment values in each trophic level (Post 2002). The transfer of trophic level varies on average 2.5 ‰ (Fry 1991) to 3.4 ‰ for $\delta^{15}N$ (DeNiro and Epstein 1981; Minagawa and Wada 1984). However, these values can vary according to the number of trophic transfers. In general, 3.4 ‰ refers to calculations of trophic multiple paths (Post 2002), whereas values for a single transfer trophic may vary between 2 and 5 ‰ (Adams and Sterner 2000; McCutchan et al. 2003). Furthermore, trophic level of consumer can be estimated applying the formula adapted from Vander Zanden et al. (1997):

$$TP = \left(\frac{\delta^{15}N_{consumer} - \delta^{15}N_{baseline}}{\Delta\delta^{15}N} \right) + \lambda$$

where *TP* is the trophic position of the consumer, $\delta^{15}N_{consumer}$ is the nitrogen isotopic value of the consumer, $\delta^{15}N_{baseline}$ is the mean nitrogen isotopic value of the base of

the food chain assumed (i.e., primary producers), $\Delta\delta^{15}N$ is the "enrichment factor," and $\lambda =$ is the trophic position of the organism used to estimated $\delta^{15}N$ baseline.

This method is highly dependent on the generation of the suitable base isotopic representing the spatial and temporal variation of $\delta^{15}N$ within and between systems of interest (Post 2002). Therefore, it depends on a good estimate of the isotopic values on the lower trophic level of the system and the resources used by consumers. In addition, the use of this methodology depends on the estimation of discrimination factors ($\Delta^{15}N$) between tissues and diet (Caut et al. 2009). Discrimination factors show several sources of variation, like food type, physiological stress, lipid extraction, diet quality, taxa, and tissues (Hobson et al. 1993; McCutchan et al. 2003; Roth and Hobson 2000; Caut et al. 2009).

The body condition and consequent metabolic state also may affect the fractionation in the organisms. Animals in a starvation state show a progressive enrichment in $^{15}N/^{14}N$ rate, in a similar process to what happens along the trophic chain (Hobson et al. 1993). In this case, ^{14}N excreted is not replaced by the protein diet, so the animal becomes progressively enriched in ^{15}N as its hunger state increases. Therefore, the $\delta^{15}N$ can also be used as an indicator of changes in body condition (Hobson et al. 1993).

Manetta et al. (2003) used stomach contents and stable isotopes composition of nitrogen ($\delta^{15}N$) to verify the trophic position (TP) of the main species of fishes, of the Paraná River floodplain, Brazil. There was no difference between both methods and indicate that *Loricariichthys platymetopon* (TP by stomach contents: 2.0; TP by stable isotope: 2.1), *Schizodon borellii* (TP by stomach contents: 2.0; TP by stable isotope: 2.4), *Leporinus lacustris* (TP by stomach contents: 2.1; TP by stable isotope: 2.7), and *L. friderici* (TP by stomach contents: 2.0; TP by stable isotope: 2.3) are primary consumers and *Auchenipterus osteomystax* (TP by stomach contents: 3.5; TP by stable isotope: 3.8), *Iheringichthys labrosus* (TP by stomach contents: 3.0; TP by stable isotope: 3.6), and *Serrasalmus marginatus* (TP by stomach contents: 3.9; TP by stable isotope: 3.5) are secondary consumers. A great intraespecific variability of $\delta^{15}N$ was found in several fish species, for example, *I. labrosus* (omnivorous) possibly as a result of great diversity of food items in its diet, including higher plants, detritus, besides prey from different trophic levels. The high plasticity of food itens in fish species may mean that changes in the trophic hierarchy can occur depending on environmental conditions.

Estrada et al. (2003) estimated the trophic positions of the blue shark (*Prionace glauca*), shortfin mako (*Isurus oxyrinchus*), thresher shark (*Alopias vulpinus*), and basking shark (*Cetorhinus maximus*) from Atlantic Ocean near to Martha's Vineyard island, USA using stable isotope ratios of nitrogen ($\delta^{15}N$). Sharks are apex predators in the marine environment and their feeding ecology can affect the community structure. The basking shark had the lowest trophic positions (3.1) followed in crescent order by blue shark (3.8), shortfin mako (4.0), and thresher shark (4.5). Trophic position of sharks is closely related to the exploitation of food resources, for example, basking shark known to feed solely on zooplankton, comparisons with isotopic values of prey species suggest that blue shark and shortfin mako forage primarily on fish prey and thresher shark feed mainly on cephalopods.

11.2.3 Animal Movements

Traditional radiotelemetry techniques have been used to detect movement patterns in wild animals (Millspaugh and Marzluff 2001; Jacob and Rudran 2003). However, the high cost and possible adverse effects of transmitters on the individual's behavior can be considered as possible disadvantages of this methodology (Jacob and Rudran 2003). In such context, the stable isotopes technique applied to trace the origin and movement of animals has been gaining strength in animal ecology (Rubenstein and Hobson 2004; Hobson and Wassenaar 2008).

The isotopic composition of animal tissues reflects the values of their local food chain and can be used to trace movements between isotopically distinct food webs (McKechnie 2004). Several biogeochemical processes can cause spatial variation in isotopic composition of food webs (Hobson 2008). However, the choice of animal tissue to be analyzed is a key part in the research design about the origin and movements of animals using stable isotopes, because different tissues reflect different temporal scales (Dalerum and Angerbjörn 2005). Metabolically inert tissues (e.g., nail, hair, and feather) reflect the isotopic composition of where they are synthesized, whereas metabolically active tissues (e.g., muscle, skin, and blood plasma) reflect the integration of dietary sources in different sites depending on their turnover rate (Bearhop et al. 2002; Ethier et al. 2010).

Hydrogen stable isotopes compositions (δD or δ^2H) are used in many studies of animal migration (Bowen et al. 2005), because the δD values have large amplitude (~500 ‰) and variation among distinct environments in nature (e.g., terrestrial and marine) (Wassenaar 2008). In addition, δD values vary according to the latitude, altitude, distance from the sea and precipitation (climatic process) (Dansgaard 1964; Chamberlain et al. 1997; Hobson and Wassenaar 1997; Hobson 2005; Hobson et al. 2012). Therefore, analysis of different parts of inert tissues may reveal the origin of migratory animals (Chamberlain et al. 1997). The application of hydrogen stable isotopes for this purpose has been particularly successful in studies with birds based on feather analysis (e.g., González-Prieto et al. 2011; Greenwood and Dawson 2011; Marquiss et al. 2012).

Hobson et al. (2003) provide an interesting example of the application of δD in the study of animal movement. The authors investigated the potential for this approach by measuring isotopic compositions (δ^{13}C, δD, and δ^{15}N) in tail feathers of eight species of hummingbirds along an altitudinal gradient (300–3,290 m) in the Andes Mountains of Ecuador. Avifauna inhabiting montane regions can move and feed between in isotopically distinct regions. This study found a strong relationship between δ^{13}C, δD values of hummingbird feather, and elevation in the Ecuadorean Andes. In addition, the authors also discuss the possible origin of some species sampled in lower or higher elevation than their capture site.

Isotopic composition of other stable isotopes (δ^{13}C, δ^{15}N, and δ^{18}O) individually or in combination can also be used to infer about origin or movements of organisms (Hobson 1999), for example, movements between environments with a predominance of C_3 and C_4 plants (Chisholm et al. 1986; Alisauskas

et al. 1998); C_3 and crassulacean plants (Fleming et al. 1993) and marine and freshwater environments (Meyer-Rochow et al. 1992; Smith et al. 1996; Rosenblatt and Heithaus 2011).

Ogden et al. (2005) were able to quantify the proportional use of estuarine and terrestrial farmland resources by *Calidris alpina pacifica* (Dunlin) on the Fraser River Delta, British Columbia, using stable isotopes analysis ($\delta^{15}N$ and $\delta^{13}C$) of blood tissue. They found a great difference in intraspecific behavioral strategies, because the contribution of terrestrial farmland in diet ranging from 1 to 95 % between individuals. However, the proportion of diet attributed to terrestrial sources was 38 % when considering the mean isotopic values for Dunlin over four winters, 1997 through 2000. Juveniles showed higher terrestrial contribution to diet (43 %) than adults (35 %). Juveniles can forage more successfully in terrestrial farmland until gain experience to capture prey on the marine intertidal flats. In addition, Dunlin obtains most of its diet in these environments during periods of severe weather conditions. This study demonstrated that farmland terrestrial zone play an important role in the survival of Dunlin.

Maruyama et al. (2001) studied fluvial–lacustrine migrations of *Rhinogobius* sp. (landlocked goby: orange form) in the Lake Biwa water system, Japan, using stable isotope compositions (nitrogen and carbon isotope ratios). Previous reports showed that Lake Biwa has sediment and benthic animals with $\delta^{15}N$ values higher than those in the tributary rivers, then this isotope ratio could be used to trace fluvial–lacustrine migrations. This pattern of isotopic nitrogen distribution also occurred in *Rhinogobius* sp., and authors were able to detect that small individuals collected in the fluvial water body had spent their larval periods in the lake.

11.2.4 Tissue Turnover Rates

The application of stable isotopes analysis and correct interpretation of field data in wildlife studies rely on good estimates of tissue turnover rates. Isotopic turnover rate may be defined as the time that a tissue or whole consumer takes to reflect the isotopic composition of their diet (Tieszen et al. 1983; Gannes et al. 1998), in a process that occurs due to tissue growth and tissue replacement (MacAvoy et al. 2005).

The knowledge of differences in turnover rates is crucial to choose the appropriate tissue and to decide the sampling frequency in the individuals according to the objectives of a particular study, because the turnover rates varies between tissue types reflecting different timescales (Dalerum and Angerbjörn 2005; Rio and Carleton 2012). Tissues with a high turnover rate reflect the isotopic composition of food items consumed recently; on the other hand, tissues with low turnover rates reflect isotopic composition of food items consumed over a period of time (Hobson and Clark 1992, 1993).

The determination of turnover rates is also important for the interpretation of isotopic data, because accurate estimation of this parameter can improve interpretation of output isotope models (Phillips and Gregg 2001). Turnover rate may vary among individuals due to various factors, as growth rate, body size, and protein

turnover rate (Newsome et al. 2010). Therefore, lab-controlled studies with the highest number possible of taxons are needed to better understand the factors that influence the dynamics of isotopic incorporation into animal tissues.

Experiments under controlled conditions have been conducted in order to determine the turnover rates of several tissues (e.g., Voigt et al. 2003; Seminoff et al. 2007; Murray and Wolf 2012; Storm-Suke et al. 2012). In this case, tissues of interest are analyzed to verify the time required for them to reflect the new consumer's diet. Murray and Wolf (2012) conducted studies with the desert tortoise (*Gopherus agassizii*) and observed a mean turnover rate of 126.7 days for red blood cells and 32.9 days for the plasma when analyzed $\delta^{13}C$. Hobson and Clark (1992) in controlled experiments with quails (*Coturnix japonica*) measured the carbon half-life of 11.4 days for the whole blood, 12.4 days for the muscle *pectoralis,* and 173.3 days for the bones collagen.

Oliveira (2003) investigating the dynamics of incorporation of carbon and nitrogen in the tissues of tambaqui fingerlings (*C. macropomum*) observed that the replacement rate of these elements vary according to the quality of the food source and the tissue functionality. In individuals fed with a diet based on C_3 plants turnover rate for the $\delta^{13}C$ and $\delta^{15}N$ was 42.7 and 28.9 d for liver, 77.9 and 85.5 d for muscle, and 104.5 and 125.7 d for scale, respectively. The turnover in visceral fat tested only for $\delta^{13}C$ was 184.7 d. In individuals with a diet based only on C_4 plants, liver reached equilibrium with the diet for $\delta^{13}C$ in 85.2 d. However, the author had observed that carbon substitution is faster than nitrogen substitution in all tissues in the C_4 plant-based diet.

Rosenblatt and Heithaus (2013) conducted an experiment under controlled conditions to estimate turnover rates for three tissues (scutes, red blood cells, and plasma) in American alligators (*Alligator mississippiensis*). This study tries to fill the gap in our understanding about turnover rates for crocodilians. Juvenile American alligators were housed in an enclosed and fed with equal amounts of food two times per week. Diet of pellet was changed to diet of channel catfish, and the tissues were collected over time. The isotope turnover rates of American alligators found in this study were considerably slower than those of most other taxa studied. The estimated $\delta^{13}C$ turnover rates for blood plasma, red blood cells, and scutes were 252, 566, and 590 d, respectively, and the estimated $\delta^{15}N$ turnover rates were 249.6, 1,109.2, and 414 d, respectively.

11.2.5 Ecotoxicology

Stable isotopes analysis is a powerful tool in ecotoxicological studies to understand the dynamics of contaminants on individuals and food webs (Crawford et al. 2008). This analysis provides a considerable advance to the ecotoxicology field by linking wild animals to their diet and contaminant source (Jardine et al. 2006).

Understanding the diet of organisms has a key role in ecotoxicological studies because most contaminants (heavy metals, organochlorine compounds, and other

persistent contaminants) in animals are obtained by food consumption (Thomann and Connolly 1984; Hall et al. 1997). These contaminants pass through the process called bioaccumulation or biomagnification in the environment, in which concentrations of contaminants in consumers exceed those concentrations in diets (Gobas et al. 1993; Gobas and Morrison 2000). As the $\delta^{15}N$ also increases along the trophic chain as already seen, it is possible to relate it to the isotopic compositions values in food chains.

Jardine et al. (2006) considers three general categories of ecotoxicology studies that use stable isotopes analysis: qualitative linkages between dietary habits of animals and their contaminant concentrations, food web biomagnification studies, and quantitative assessments of habitat-specific foraging as a means of explaining biotic contaminant concentrations. In this context, the $\delta^{15}N$ enrichment in trophic webs helps to understand the contaminants paths along the food webs (Borga et al. 2004; Campbell et al. 2005), because there is a strong association between $\delta^{15}N$ enrichment and increasing concentrations of organochlorine and Hg contaminants (Broman et al. 1992; Kidd et al. 1995; Atwell et al. 1998; Campbell et al. 2005; Garcia and Carignan 2009). On the other hand, $\delta^{13}C$ values allow the traceability of foraging strategy and hence the specific sources of such contaminants (Crawford et al. 2008). There are several examples of studies adopting the approaches mentioned above (e.g., Atwell et al. 1998; Camusso et al. 1998; Thompson et al. 1998; Fox et al. 2002), however, we will address two papers in detail.

Di Beneditto et al. (2013) evaluate the trophic status and feeding ground of *Trichiurus lepturus* (ribbonfish) using total mercury concentration and stable isotope compositions ($\delta^{15}N$ and $\delta^{13}C$) during its ontogeny in the northern region of the State of Rio de Janeiro, south-eastern Brazil. Mercury is an environmental pollutant that bioaccumulates through the aquatic food chain and affects negatively human health. Mercury concentrations and $\delta^{15}N$ were different between sub-adult (planktivorous) and adult (carnivorous) specimens, indicating difference in trophic position of ontogenetic phases. However, the similarity of $\delta^{13}C$ values between sub-adults and adults suggest that both share the same feeding area (marine coastal waters). The mercury concentrations found in adults of *T. lepturus* are close to the tolerable limit for safe regular ingestion established by World Health Organization, so mercury levels in this fish species and environment should be monitored by public health authorities.

Das et al. (2004) studied trophic status, potential intraespecific segregation according to the source of prey and trace metals levels in harbor porpoise (*Phocoena phocoena relicta*) from the Black Sea. This environment has undergone an extensive human impact over the past decades which affected negatively wildlife populations. Harbor porpoises are at risk of disappearing and information on contaminant, their ecology and status are extremely important. The main result of this study was that differences in $\delta^{13}C$ between the sexes suggest that females use more the coastal environment (shallow waters) and males offshore habitats. The contaminant levels (hepatic Hg) in animals reflected the different exposure linked to coastal vs offshore feeding habitats.

11.3 What Next

Natural variations in abundance of stable isotopes provide an interesting tool for the study of energy flow systems. Currently, there is a growth in the use of stable isotopes analysis in animal ecology accompanying methodological development of the area (e.g. advances in analysis of stable isotope data and mass spectrometry). In this work, we addressed the main applications of isotopic analyses in wildlife studies emphasizing the ecological responses that can be achieved by this methodology. The topics were treated in a simple and concise form, and readers can deepen their knowledge in specific subjects in various articles and reviews available in the literature.

The stable isotope methodology has proven to be an interesting alternative in wildlife studies; however, some limitations need to be considered. Understanding the discrimination factors and routing processes in different tissues is needed to correct interpretation of isotopic data, beyond the knowledge of possible factors that may influence them (e.g., growth rate, age, and stress level). The call for controlled experiments to meet these goals has been performed by several authors (Gannes et al. 1997; Wolf et al. 2009) aiming to increase our ability to interpret values of stable isotopes.

Another limiting factor to be considered is the data resolution to distinguish different food sources and environments, for example, it is difficult to infer differences in diet contribution when food resources have similar isotopic composition. Technological developments in mass spectrometry, cost reduction, and concomitant analysis of a larger isotopes number can further improve the resolution studies. In this respect, technological development has enabled the use of compound-specific stable isotopes analysis of individual amino acids and fatty acids arouse great possibilities for studies in nutritional biochemistry of organisms.

The increased application of isotopic analysis in animal ecology also highlights the need to develop protocols for collecting and processing tissues. The method of tissues conservation, lipids extraction, and laboratory practices has significant effects on the isotopic compositions (Arrington and Winemiller 2002; Post et al. 2007).

Furthermore, there is a need to perform the isotopic monitoring trends over time. In the future, it is expected a major technological development and advances in the form of statistical analysis of isotopic data. The use of Bayesian inference in mixture models to estimate diet contribution incorporating uncertainty has provided more accurate estimates in recent years. The refinement of these types of analyzes might provide a better interpretation of isotopic patterns.

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