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Diet estimation of *Paralichthys orbignyanus* in a coastal lagoon via quantitative fatty acid signature analysis

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ABSTRACT

Quantitative fatty acid analysis (QFASA) is a statistical model designed to quantitatively estimate predator diets using fatty acid (FA) signatures among the predator and its potential prey. QFASA estimated the diet of a migratory flatfish *Paralichthys orbignyanus* over its fattening stage in the Rocha lagoon (a semi-closed estuary) where all prey available to this top predator species are well known. A 20-week controlled feeding trial obtained calibration coefficients (CC) for *P. orbignyanus* fed two types of prey (silverside and menhaden). Several subsets of FA were tested in order to elucidate which is the most suitable for applying QFASA to this species. QFASA was applied to all CC and FA subsets to validate the model. The model predicts better the consumed diet with silverside CC than with menhaden CC. The subset which best adjusts the diet over the validation process, includes approximately 34% of total FA, containing mainly dietary FA. The diet estimation in nature for *P. orbignyanus* varied according to whether the model is applied with or without CC. When the diet was estimated without CC, results were similar to those based on stomach content analysis (reported in previous studies); it fed mainly on silverside (~88%), but also some minor soft-body species that are only evident using this kind of methodology (QFASA). When the diet was estimated with silverside CC, a higher presence of silverside (~97%) was observed. These results seem to indicate a tendency to overestimate the presence of the item used as prey for CC calculations.

1. Introduction

Continental shelves and their associated estuaries are among the most productive ecosystems in the world (Day et al., 1981), where major fishery resources, including flatfish (Munroe, 2005) are located. Estuaries constitute areas used by several species as permanent or transitory habitats for reproduction, migration, feeding and nursery (Elliott and Hemingway, 2002). Establishing and quantifying trophic relationships between the species of an ecosystem is of primary importance to understand the ecosystem functioning (Connan et al., 2007).

Paralichthyidae flatfishes constitute important commercial and recreational fisheries throughout the Atlantic, from the deep Arctic to the coasts of southern Africa and South America (Díaz de Astarloa, 2002). They are the most productive demersal fisheries in the world from the commercial point of view and Paralichthyidae flatfishes are by far the most valuable fish per unit weight landed. (Díaz de Astarloa and Munroe, 1998). In Uruguay, there are three species of flatfishes, but only one (*Paralichthys orbignyanus*) inhabits estuaries. This flatfish occurs from Río de Janeiro, Brazil, to San Matías Gulf, Argentina (Fabré and Díaz de Astarloa, 1996). It is categorized as a eurihaline and euritherm species (López Cazorla, 2005) and in summer it is captured mainly in coastal areas (Lopez Cazorla, 1987). *P. orbignyanus*, like other North Atlantic flatfishes, is a catadromous fish spawning in marine water, but its juveniles migrate towards coastal lagoons (Bergman et al., 1988; Koutsikopoulos and Lacroix, 1992; Whitfield, 1998) and fatten there (Robaldo, 2003).

Across the Atlantic shoreline of Uruguay there are several coastal lagoons and stream mouths where *P. orbignyanus* is found from juvenile to adult stages throughout the year (Rivera Prisco et al., 2001). The Rocha Lagoon is a sand flat coastal lagoon that, as an estuarine environment, serves as a nursery and sheltering area for migrating birds and fish (Mianzan et al., 2001). In recent years, increasing eutrophication of the lagoon has been observed (Aubriot et al., 2005) related to the main activities of the land use: extensive cattle raising and agriculture. Today, this ecosystem belongs to a conservation area where

Abbreviations: CC, calibration coefficients; DHA, docosahexaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid; FA, fatty acids; FAMEs, fatty acid methyl esters; HIS, hepatosomatic index; HUFA, high unsaturated fatty acids; KL, Kullback–Liebler; MUFA, monounsaturated fatty acids; PER, predator energy reservoir; PUFA, polyunsaturated fatty acids; QFASA, quantitative fatty acid signature analysis; SAFA, saturated fatty acids.

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P. orbignyanus – which has been reported as a top predator (Norbis and Galli, 2004; Rodriguez-Graña et al., 2008) – represents a high proportion of the captures by local fishermen.

Top predators play an important role in determining the structure and functioning of ecosystems (Bowen, 1997). The dynamics of predator–prey relationships, the structure of food webs, and the foraging behavior of individuals are key factors to understand the functioning of these types of areas (Pimm et al., 1991; Schoener, 1971; Sih et al., 1998), which is crucial for their management (King et al., 1995).

The common way to address the study of trophic relationships is by producing accurate estimates of the diet of predators. Currently, the diet of *P. orbignyanus* has been estimated using the classic method of stomach content analysis (Norbis and Galli, 2004). Although often used for determining diets, such estimates can be biased since soft-bodied prey are rapidly digested whereas prey with hard parts can be overestimated (Bowen, 2000). In addition, this estimate provides only a snapshot of the last meal of an animal. For these reasons, methods to assess the feeding habits based on fatty acid (FA) signatures seem to be a promising alternative. They can provide new insight into the long-term diet of species taking advantage of FA as trophic markers. Moreover, the detection of soft-bodied prey can be improved and the sampling process can be undertaken while keeping the predator alive.

Fatty acids have been extensively used in qualitative studies about trophic relationships in food webs (Dalsgaard et al., 2003) based on the demonstrated influence of dietary FA on predator fat stores (Colby et al., 1993; Kanazawa et al., 1979; Kirsch et al., 1998; Raclot et al., 1998; Rouvinen and Kiiskinen, 1989). Specifically the concept of individual lipid biomarkers has been focused on mainly in the linkage between organisms at lower levels of the food webs (Falk-Petersen et al., 2002; John and Lund, 1996; Leveill et al., 1997; Mansour et al., 1999). Recently, Iverson et al. (2004) have developed a new method to quantitatively estimate top predators' long-term diet using fatty acid signatures (quantitative fatty acid signature analysis, QFASA). The technique involves the use of a statistical model to determine the combination of prey FA signatures that most closely resembles the predator FA stores to infer its diet. The predator differential metabolism of FA is taken into account by introducing calibration coefficients (CC) in the model, which are obtained from controlled feeding experiments. These experiments not only provide correction factors that allow a more accurate quantitative estimation, but they also provide a rigorous validation of the method. The determination of how long these experiments have to last to truly reflect the diet in the predator fat storage tissue is critical (Budge et al., 2006). Several studies have been conducted to determine calibration coefficients for birds and mammals (Iverson et al., 2007; Nordstrom et al., 2008; Rosen and Tollit, 2012; Wang et al., 2010; Williams et al., 2009) but, in regard to fish, only Atlantic salmon has been studied (Budge et al., 2011, 2012). A careful selection of the predator fat store tissue to use in QFASA has been shown to be of crucial importance (Budge et al., 2006; Iverson, 2009). The adipose tissue is usually selected in vertebrates as it should experience a rapid turnover in response to dietary lipid intake. Fish, despite being vertebrates, have their lipid stores in muscle with skin, viscera or liver, and it is well known that the fatty acid composition of these tissues in fish largely resembles the fatty acid composition of the diet (Ackman, 1980; Jobling, 1993; Shearer, 1994).

Although qualitative FA techniques have been used to infer foraging ecology in fish (Elsdon, 2010; Stowasser et al., 2009; Young et al., 2010), to this date, to our knowledge, quantitative analysis has not been performed or validated in this group of vertebrates. The QFASA method was designed and assessed for upper trophic level endothermic vertebrates (Iverson et al., 2007; Nordstrom et al., 2008; Thiemann et al., 2008; Tucker et al., 2009; Wang et al., 2010), but it has not yet been applied to lower vertebrates (Iverson, 2009).

The aim of this work was to obtain a quantitative estimation of the diet of a flatfish (*P. orbignyanus*) in an estuarine coastal lagoon by applying the QFASA. Additionally, we aimed to determine calibration coefficients and validate the model for this species under controlled experimental conditions.

2. Materials and methods

2.1. Sampling site and sample database

Field sampling for wild prey and predator fish was conducted in Rocha Lagoon, Uruguay (Fig. 1). Over the validation process of the model, fish were housed and managed in the Experimental Institute of Marine Aquaculture of DINARA (Department of Rocha, Uruguay).

2.2. Site

Rocha lagoon is a brackish, shallow, and microtidal coastal lagoon (mean depth = 0.6 m, area = 72 km^2) located on the Atlantic coast of South America (34° 38′ S, 54°17′W) (Sommaruga and Conde, 1990), included in a protected area of MaB-UNESCO. At irregular intervals of time, a connection with the ocean opens through a restricted inlet in the southernmost region of the lagoon, allowing the migration of many species, including *P. orbignyanus*, and producing a north–south salinity gradient (Conde et al., 2000).

2.3. Wild P. orbignyanus

A total of 33 adult *P. orbignyanus* (23 females and 10 males) obtained at Rocha Lagoon with the help of local fishermen from April 2008 to October 2010, were measured and weighed (44.0 ± 8.1 cm and 1.2 ± 0.5 kg) and sampled for lipid and fatty acid analysis. Samples were obtained from gonads, liver and a piece of upper dorsal muscle with skin (sampled together to include subdermal lipids) and stored at −20 °C. The livers of the fish were also weighed to obtain the hepatosomatic index (HSI), calculated as: [liver weight (g) / fish weight (g)] × 100.

2.4. Potential prey

Based on available information about the items cited as prey for *P. orbignyanus* according to Rivera Prisco et al. (2001), Norbis and Galli (2004), López Cazorla (2005) and Rodriguez-Graña et al. (2008), a comprehensive sampling of the cited prey and non-cited potential prey of *P. orbignyanus* was carried out. Prey samples were collected from April 2008 to October 2010 at Rocha lagoon using gill nets, seine nets, corer samples, dredge samples and manual collections. A total of 17 dietary items were collected and identified to the lowest possible taxonomic level.

Prey, with the exception of *Heteromastus similis*, were counted, measured and weighed (total length with 1 mm precision and wet weight with 0.0001 g precision). As *P. orbignyanus* displays cannibalism, juveniles of this species could be considered as a prey item. However, this option was not considered in order to avoid artificial noise in the diet estimation, due to the resemblance between this prey and the predator FA profile.

2.5. Lipids and FA analysis

All samples for biochemical procedures were stored at −20 °C until analysis. Lipid extraction and quantification was made in duplicate according to Folch et al. (1957). To generate the predator profile, lipids were extracted from freeze-dried and homogenized dorsal muscle with skin. In the case of potential prey, whole organisms were freeze-dried and homogenized prior to lipid extraction. FA methyl esters (FAMEs) of total lipids of all samples were methylated by transesterification with H_2SO_4 in methanol solution (Christie, 1982). FAMEs were separated using gas chromatography (Hewlett Packard 5890) equipped with a flame ionization detector, a Supelcowax fused silica capillary column (30 m 0.32 mm ID, Supelco, USA) and nitrogen as a carrier gas. Samples

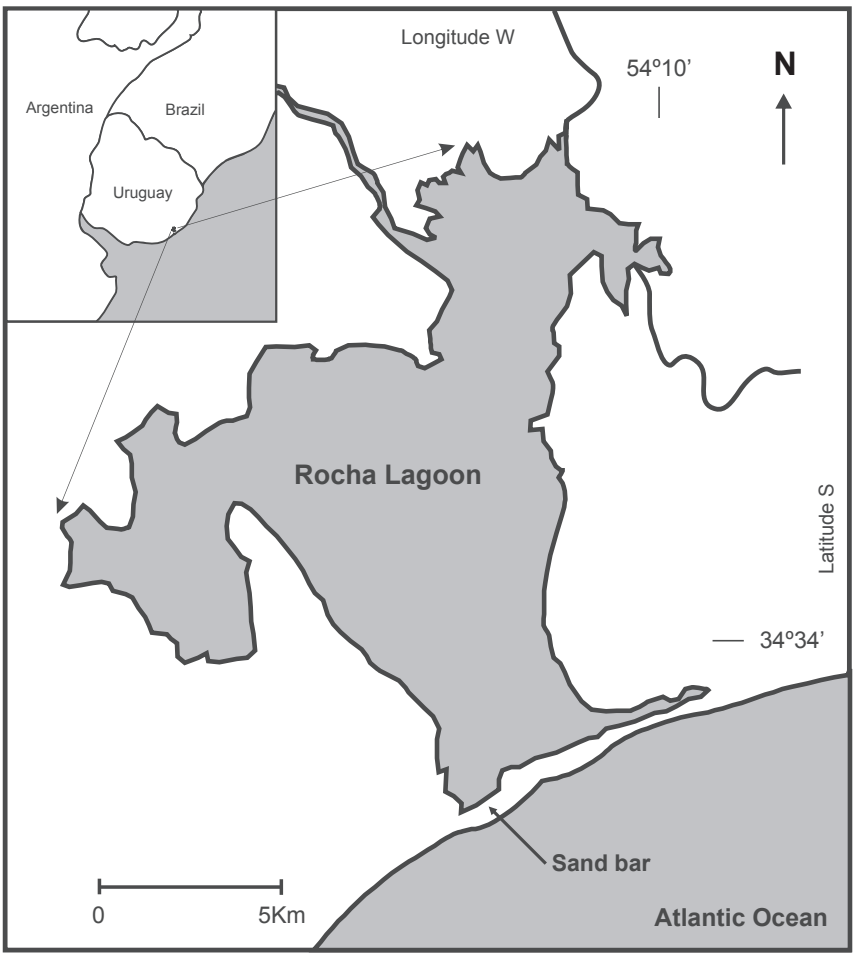

Fig. 1. Study area of *Paralichthys orbignyanus* for the quantitative fatty acid signature analysis of diet estimations in nature.

were injected in split mode at 250 °C. FAs were identified by comparing retention times of methyl esters standards (Supelco) and by reference to a well characterized fish oil (Salhi and Bessonart, 2013). FA data were expressed as the mass percentage of total FA ± sd.

2.6. Predator database and prey database

2.6.1. Predator database

Since over the reproductive period (December to March) *P. orbignyanus* migrates towards marine water for spawning, only the FA signature of flounders from April to November was included in the predator database. This period corresponds to the fattening stage prior to the fish breeding season out at sea.

2.6.2. Prey database

To capture the variability in the potential prey FA profiles, 150 fatty acid signatures were gathered belonging to 17 prey items. In the case of large prey (such as fish) some were analyzed as individual or small groups of fish. Small prey were usually grouped to be biochemically analyzed (at least 3 samples per prey item were analyzed).

2.7. Quantitative fatty acid statistical analysis method

The estimate of predator diet was obtained by considering a weighted combination of the fatty acid signatures of the prey and then determining the weighting coefficients that best explain the fatty acid signature of the predator. This was implemented by minimizing the statistical distance between the combination of fatty acid signatures and that of the predator.

Let y be a vector, whose elements y_j describe the proportion of each fatty acid j of the predator. Following Iverson et al. (2004), the model can be stated as estimating y as a weighted sum $\hat{y}$ of the fatty acids of the prey,

$$\hat{y} = \sum_k p_k \hat{x}_k$$

where $\hat{x}_k$ is a vector, whose elements $\hat{x}_{kj}$ are the mean of each fatty acid j of the prey type k and P_k is a weighting coefficient that corresponds to the estimated proportion of the prey type k in the predator diet. The goal of the analysis is to choose the $\hat{P}_k$ values such that the model estimation $\hat{y}$ is as close as possible to the real profile y. Since y and $\hat{y}$ are distributions over the fatty acids, the Kullback–Liebler (KL) distance is used to compare fatty acid profiles where,

$$\mathrm{KL} = \sum_j \left(y_j - \hat{y}_j\right) \log\left(\frac{y_j}{\hat{y}_j}\right).$$

In addition, the p_k svalues are constrained to be positive and sum to 1. Therefore, the estimation can be formulated as an optimization problem with constraints,

$$\min \quad KL = \sum_j \left(y_j - \hat{y}_j\right) \log\left(\frac{y_j}{\hat{y}_j}\right).$$
$$\text{subject to} \quad \sum_k p_k = 1, \quad p_k \geq 0.$$

The optimization was carried out in Matlab using its optimization toolbox. As in Iverson et al. (2004), no prior bias was assumed for any

prey, so the optimization was initialized with equal values for all p_k coefficients. To account for variations in the key signature of a prey type due to differences in the fatty acid profile of the individuals in the sample, a bootstrap procedure was carried out in which the optimization was repeated. In each iteration, for a total of 100 repetitions, the sample of each prey type was randomly selected with replacement from the database. This allowed the computation of standard deviation for the experiments.

2.8. Captive feeding trials, calibration coefficients (CC) and validation of the model

Additional methodological issues have to be taken into account to estimate the diet based on the described method. Due to the fact that the fatty acid profiles of the prey are not transferred exactly to the predator because of the effect of its lipid metabolism, calibration coefficients (CC) must be determined by feeding individuals in captivity with a known diet. Additionally, since not all fatty acids provide equal information about diet due to predator metabolism, subsets of FA considered to better reflect the diet were selected (instead of using all the identified fatty acids). Finally, the contribution from each prey in the diet must be adjusted to account for the differences in fat content of the different prey items.

2.8.1. Captive feeding trials

In order to determine the quantity of the FA signature of prey that resembles the FA deposition in *P. orbignyanus*, and to obtain calibration coefficients, a 136 day feeding trial with two groups of flounders fed with two types of prey, was designed. The duration of the experiment was set up to 20 weeks, based on studies reporting that after 2 to 12 weeks of being fed a diet, the fatty acid profile of the storage tissue of fish is stabilized (Budge et al., 2011; Copeman et al., 2013; Kirsch et al., 1998; Skonberg et al., 1994).

For this experiment, 23 *P. orbignyanus* were captured at Rocha lagoon. Six of them were used to obtain the initial FA profile of the feeding trials. The others were housed at the Experimental Station for Marine and Aquaculture Research where they were acclimated for three months until they were domesticated to be fed in captivity. The captive fish were separated into two groups, one group (n = 9, min. 482 g, max. 1777 g) was fed with headless and gutless *Odonthestes argentinensis*, and the other group (n = 8, min. 427 g, max. 1597 g), was fed with filets of *Brevoortia aurea*. The animals were fed daily at sunset to satiation.

To minimize the variation of the fatty acid profile of the items offered as prey during the feeding trial, all the fish used as food during the whole experiment were caught from the same batch, at the same time in the Rocha lagoon on April 2010 (*O. argentinensis* 53.5 ± 14.1 g and *B. aurea* 45.0 ± 9.9 g). Samples of twelve individuals of *B. aurea* and *O. argentinesis* were analyzed for FA to calculate the CC.

Formulated dry food was not used for these trials, because flounders of this species caught as adults in the wild do not accept artificial food once domesticated, but only dead prey.

2.8.2. Live biopsy technique

At weeks 5, 12 and 20 a live biopsy technique was applied to the flounders of the feeding trials. At first, only 3 individuals of each treatment were biopsied; at week 12, all individuals of each group were biopsied; and at week 20 all individuals were biopsied and 6 of them per feeding group were euthanized to obtain samples of liver tissue. The live biopsy allows sampling the same individual each time in order to see the temporal evolution in the FA profile. To prevent opportunistic infections, the day before the biopsy, the flounders were kept in oxitetracycline 0.02 g/l. After 5 min of benzocaine 0.001 g/l anesthetization, a cube of muscle with skin from the upper dorsal part of the fish body was taken (approximately 7 mm by side), followed by cauterization and propolis treatment of the area. The following seven days, the flounders were treated with oxitetracycline 0.02 g/l. In the euthanized individuals, the liver was also sampled for FA analysis and a larger piece of muscle with skin was collected in order to determine if the smaller pieces taken in previous biopsies were representative of the FA profile.

2.9. Calibration coefficients (CC)

The CC for *P. orbignyanus* were calculated according to Iverson et al. (2004) by dividing FA levels of dorsal muscle with skin or liver tissue of flounders of the feeding trials, by FA levels of the prey item offered (*O. argentinensis* or *B. aurea*). Fig. 2 shows the experimental design of feeding trials and the number of sets of calibration coefficients calculated in each biopsy (at weeks 5, 12 and 20). All sets of CC were calculated as a 10% trimmed mean.

2.10. Validation of the QFASA model

To determine the optimum CC to be applied in the model, and also to validate the use of this approximation in our model fish, we ran the QFASA model using not only data of feeding prey offered during the feeding trials, but also data of all prey items sampled in the Rocha lagoon. Additionally, a crossvalidation between all FA subsets and both tissues sampled (muscle with skin and liver) with and without CC, was tested. The optimum CC and the subset of FA that best resembled the prey offered during feeding trials were evaluated.

2.11. Diet determination using QFASA

2.11.1. Qualitative analysis of diet items

Hierarchical cluster analysis was used in the prey database, prior to the application of QFASA to assess how well the FA profile separated potential prey. To minimize the noise around the diet estimates, species or group of species too close in the analysis were grouped. The distance among clusters was computed by an agglomerative method using average linkage between groups and KL distance measured using Matlab 7.0 software.

2.11.2. FA subset selection and model evaluation

Seven subsets of FA were selected to evaluate the performance of the model. The number of FA included in each subset varied from 36 to 10 FA, ranging from 98.7 ± 1.3% to 12.6 ± 4.3% of total FA (Table 1) and all subsets were normalized to 100% before each evaluation. The criteria utilized to select the FA included in each subset, were based on groups of FA selected according to their essentiality for a marine fish.

3. Results

3.1. Sample collection

Sample size and size of potential prey collected at Rocha lagoon are shown in Table 2. Lipid content and selected fatty acids from the FA profile of the predator and prey used to construct the prey-database, are presented in Table 3.

The five species of fish sampled included individuals of a wide range of size, from around 2.5 cm to 20 cm. Their lipid content (% WW) varied from 1.7 ± 0.4 for *Jenynsia multidentata* to 2.6 ± 0.8 for *Oligosarcus* sp. The other three species had a similar content of lipids (around 1.8% WW). 12 items of invertebrates were collected consisting of 10 single species, one group of Amphipoda and one group of Isopoda. Three groups can be distinguished regarding their lipid content: Amphipoda, *Heleobia australis* and *Erodona mactroides* had the lowest lipid level (around 0.4% WW), Isopoda and *Penaeus paulensis* had the highest (around 2.5% WW) and the rest were intermediate (from around 0.6 to 1.4% WW).

3.2. Feeding trial

During the feeding trial, flounders accepted in equal manner the food offered in both groups (data not shown). After 20 weeks of the feeding trial, total weight gained by the fish did not represent more than 10.0 or 14.0% of the initial weight (silverside and menhaden groups respectively). We assumed that the change in fatty acid profile of their muscles was mostly due to turnover of their FA, rather than the result of a dilution process (Jobling, 1993).

Although menhaden filets contained a higher level of lipids, no significant differences were found ($P > 0.05$) in the lipid content of muscle with skin between the flounders undergoing the different diets (Table 4). Samples of menhaden filets showed a high variation in their lipid (relative standard deviation = 46.8%).

To gain confidence on how representative the samples of muscle with skin were, different parts of this tissue of seven wild flatfish were sampled before the feeding trials. FA profiles obtained were similar, confirming that the smaller portion of biopsied tissue was representative of the FA profile of muscle with skin (data not shown).

The fatty acid profile of prey (silverside and menhaden) and flounders of the feeding trial at the beginning of the experiment (initial) and at weeks 5, 12 and 20 are presented in Fig. 3.

The characteristic FA profile of the captive flounders, changed over time as a result of the two different diets (silverside or menhaden) supplied (Fig. 3). For the initial biopsy, both groups of flatfish (fed with silverside and fed with menhaden) showed a similar FA profile, where saturated (SAFA) represented 53.7 and 55.1%, monounsaturated (MUFA) 35.3 and 32.1% and polyunsaturated (PUFA) 11.1 and 11.8% respectively, differing strongly from initial FA profile.

After 20 weeks, the FA profile of silverside and menhaden flatfish groups changed: SAFA represented 34.8 and 43.1%, MUFA 25.2 and 22.7% and PUFA 40.1 and 34.2%, respectively, resembling the supplied diet in each case. It could be noted that the major differences found in PUFA relate to n3−HUFA content, which was higher in silverside (24.9 and 11.0% in menhaden).

Silverside contained higher proportions of 18:1n−9, 20:4n−6, 20:5n-3, 22:5n−3 and 22:6n−3 than menhaden, that was richer in SAFA and MUFA such as 14:0, 16:0, 16:1n−7, 18:0 and 20:1. Relative amounts of 18:2n−6 and 18:3n−3 were similar in both prey. In general, FA profile of *P. orbignyanus* varied according to the FA profile of the prey supplied during the feeding trial. In the case of 14:0, 16:0, 16:1n−7 and 18:0, higher in menhaden than in silverside, levels found in *P. orbignyanus* fed silverside reached higher levels on weeks 5 or 12 than in flounders fed menhaden; but at week 20 proportions were higher in *P. orbignyanus* fed menhaden. In spite of a higher level of 20:1 in silverside, at week 20, almost no difference was found in the proportion of this FA in flounders fed the different diets. Regarding 18:1n−9, 20:5n−3, 22:5n−3 and 22:6n−3, higher in silverside than in menhaden, levels in *P. orbignyanus* fed the different diets were similar up to week 20 when they became higher in flounders fed silverside, especially for PUFA which proportionally was more than 38% higher in these flounders compared to those fed menhaden. However, in the case of 20:4n−6, although its proportion in prey was four times higher in silverside than in menhaden, relative amounts found in flounders at week 20 were similar regardless the prey fed.

4. Calibration coefficients (CC)

The CC obtained during feeding trials (5, 12 and 20 weeks) for silverside and menhaden are shown in Fig. 4.

Several CC, such as those obtained for 12:0, 16:4n−1, 18:4n−1, 20:1, 20:4n−6, 22:1, 22:4n−9, 22:4n−6, 22:5n−3 and 22:6n−3, had still not stabilized by feeding week 20. Others, such as 16:1, 16:3n−4, 17:1, 18:3n−3, 18:4n−3, 20:2n−6, 20:3n−9 and 20:3n−6, were mainly constant after 5 weeks of feeding. In the group of flounders fed on menhaden, the highest CC were obtained for 16:4n−1, 18:4n−1, 20:1, 20:4n−6, 22:1, 22:4n−9, 22:4n−6 and 22:6n−3. Some of them (16:4n−1, 18:4n−1, 22:4n−9) appeared in low proportion (generally at 0.5% of total FA in flounders and prey) and because of the high relative standard deviation in minor or trace

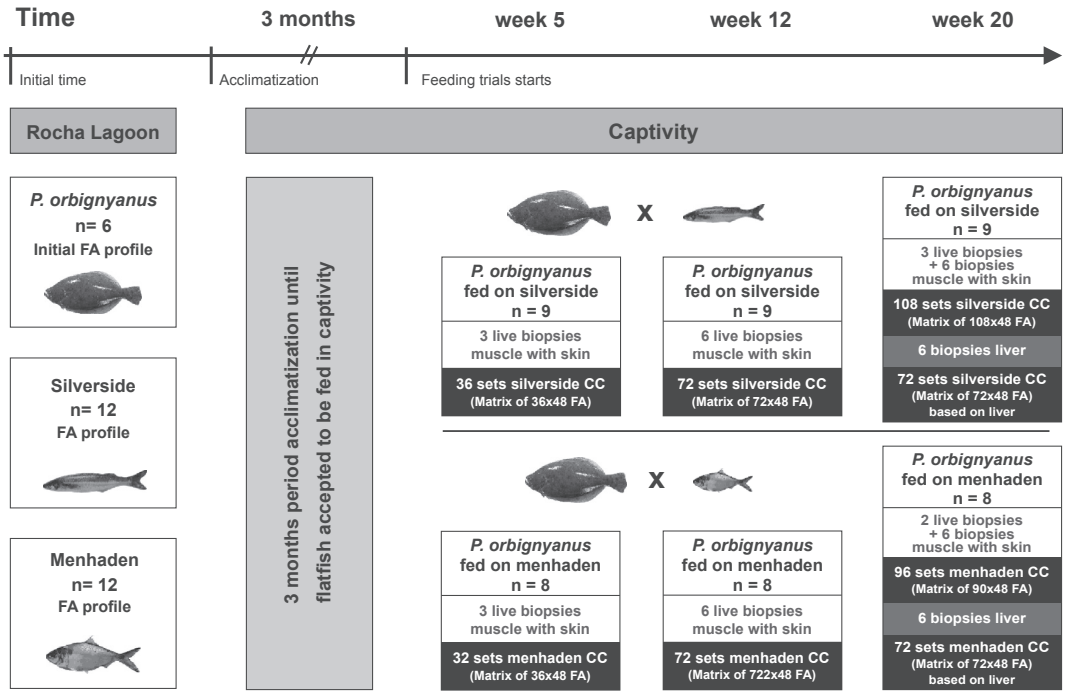

Fig. 2. Experimental design of feeding trials and calibration coefficients for both groups of *Paralichthys orbignyanus* fed with silverside and menhaden.

Table 1
FA subsets tested for *Paralichthys orbignyanus* over the validation process.

FA	12:0	14:0	14:1	15:0	16:iso	16:0	16:1n−7	16:1n−5	16:2	17:0	16:3n−4	17:1	16:4n−3	18:0	18:1n−9	18:1n−7	18:2n−6	18:3n−6	18:3n−3
Subset A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Subset B									X		X		X				X	X	X
Subset C									X		X		X				X	X	X
Subset D									X		X		X				X	X	X
Subset E		X				X	X		X		X		X	X	X	X	X	X	X
Subset F											X		X				X	X	X
Subset G		X				X	X							X	X	X			
Subset H		X				X	X							X	X	X			

Table 1 (continued)

FA	18:4n−3	18:4n−1	20:0	20:1	20:2n−9	20:2n−6	20:3n−6	20:4n−6	20:3n−3	20:4n−3	20:5n−3	22:1	22:3n−6	22:4n−6	22:5n−6	22:5n−3	22:6n−3	#of FA	% FA
Subset A	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	36	97.8 ± 1.3
Subset B	X	X		X		X	X	X	X	X	X	X	X	X	X	X	X	21	34.0 ± 10.6
Subset C	X	X		X		X	X	X				X	X	X	X			16	17.93 ± 5.6
Subset D	X	X		X								X						10	12.6 ± 4.3
Subset E	X	X		X								X						16	71.0 ± 10.4
Subset F	X					X	X	X	X	X	X		X	X	X	X	X	17	32.1 ± 11.8
Subset G						X	X	X	X	X			X	X	X	X	X	17	82.7 ± 5.3
Subset H						X	X	X	X	X			X	X	X	X	X	16	80.8 ± 5.0

Table 2
Sample size and size of potential prey items sampled at Rocha lagoon. x = mean, sd = standard deviation. Min: minimum, Max: maximum.

Items	Number of individuals	Number of samples analyzed for lipids and FA[a]	Length (x ± sd) or min–max (cm)	Weight (x ± sd) or min–max (g)
Fishes				
Odontesthes bonaerensis	13	13	15.05 ± 2.47	24.63 ± 10.49
Micropogonias furnieri	12	6	3.90–19.30	0.60–64.00
Brevoortia aurea	14	14	5.10–20.40	10.50–20.40
Jenynsia multidentata	36	7	3.01 ± 0.42	0.30 ± 0.13
Oligosarcus sp.	3	3	6.50 ± 0.50	5.50 ± 0.70
Invertebrates				
Callinectes sapidus	24	6	1.74 ± 0.43	0.43 ± 0.31
Chasmagnatus granulatus	51	17	2.51 ± 0.21	9.24 ± 1.97
Cyptograpsus angulatus	48	18	1.99 ± 0.57	3.87 ± 2.47
Palaemonetes argentinus	18	12	2.17 ± 0.85	2.15 ± 0.99
Penaeus paulensis	25	5	10.80–18.00	7.30–31.00
Neomysis americana	400	7	1.35 ± 0.16	0.01 ± 0.00
Laeonereis acuta	258	5	3.29 ± 0.81	0.00 ± 0.00
Heteromastus similis	31	3	–	–
Amphipoda	585	8	0.60 ± 0.20	0.01 ± 0.00
Isopoda	63	7	0.99 ± 0.34	0.02 ± 0.01
Heleobia australis	263	8	5.90 ± 1.26	0.02 ± 0.01
Erodona mactroides	88	11	12.00 ± 3.70	0.50 ± 0.25

[a] In cases where the number of individuals does not match the number of analyses, the latter were analyzed as a pooled sample for analytical purposes.

FA, those resulted in high CC. As this might have considerable effects on estimates, CC for these FA were removed from modeling subsets.

4.1. Validation of the model

Estimations of the diet of flounders fed with silverside and menhaden during the feeding trial at week 20 are shown in Table 5. This table represents the estimation made using muscle with skin and liver as predator storage tissue. Seven subsets of FA were proposed, and for each group the validation was checked using four sets of CC (silverside, menhaden, the mean CC between silverside and menhaden and without CC), 102 results of estimations were obtained (Table 5).

When considering the best tissue as the predator energy reservoir (PER), no statistical differences ($P < 0.05$) were found between muscle with skin and liver. This held true regardless of the FA subsets when CC applied corresponded with the diet offered.

When liver was used as PER, both groups showed the same trend (low percentages of estimation), in the case of applying silverside CC to estimate menhaden diet, or applying menhaden CC to estimate silverside diet (0.5 to 44%).

The silverside CC obtained resulted in the best predictors of the diet for both groups (fed silverside and menhaden) when muscle with skin was used as PER. However, when liver was used as PER, silverside CC was good at estimating the silverside diet, but did not work well at estimating the menhaden diet.

When the model was run with menhaden CC to predict the diet of flatfish fed menhaden, good estimations were obtained (54.6 ± 19.1% to 88.5 ± 13.2%, Table 5), but never as good as when using silverside CC (87.5 ± 10.7% to 98.7 ± 2.5%, Table 5).

Silverside CC when applied, for the estimation of the diet of flatfish fed silverside, explained more than 88% of the diet regardless the FA subset. Estimations obtained using menhaden CC always explained less of the diet than silverside CC.

When the average of the CC from menhaden and silverside was applied, both groups (muscle with skin and liver) showed a wide range of levels of diet estimation (from 23 to 80%), in most cases representing more than 40% of the correctly identified consumed prey.

Regarding the FA subsets, subset B was on average, always the best in all groups (except in the case of menhaden group without applying CC). Subsets F and G showed on average the worst results identifying consumed prey.

Given the previous results – of verification if the model consistently predicts what the flatfish have eaten – the silverside CC was the best (Table 5) with high percentages of success (averaging all FA subsets 93.0 ± 4.7%). For this reason, subsequent application of the QFASA model on the wild, it will utilize this CC obtained at week 20 of the feeding trial. The high percentage of success with silverside CC, in particular using subset B (98.7 ± 2.5%), together with the fact that the diet of *P. orbignyanus* in Laguna de Rocha is known through analysis of stomach contents (Norbis and Galli, 2004), encouraged us to apply this model during the period in which this flatfish is feeding in the same location of the previous study.

4.2. Diet determination using QFASA

As the clustering analysis did not show relevant similarity between the mean FA profile of the prey, it was not necessary to group any of the items to form the prey matrix. Thus, the 17 potential dietary items were reliably identified on the basis of their fatty acid patterns (Fig. 5) and the model was run with all these 17 groups.

The different items proposed as prey were grouped as expected with regard to their FA profile (Table 3). Crustaceans were grouped together, with the exception of *Neomysis americana* as this species showed a ratio of DHA/EPA > 1, different from that found in crustaceans as already reported by Richoux et al. (2005). Fish were also grouped together, with the minimum distance between the pair *Odonthestes argentinensis* and *Oligosarcus* sp. in this subcluster. *H. australis and L. acuta* were grouped together probably because of their similar feeding habits.

The relative contributions of each prey item in the diet of *P. orbignyanus*, tested over 4 subsets of FA proposed (A–D) are shown in Fig. 6. FA subsets E–G are not included since they did not improve results obtained with the previous subsets.

The main prey item seemed to be *O. argentinensis*, even when CC were not used. The use of CC (obtained from muscle with skin of *P. orbignyanus* fed on silverside) resulted in similar percentage of *O. argentinensis* eaten regardless of the FA subset used. The use of CC implied the exclusion of prey items, such as *J. multidentata*, *Micropogonias furnieri* and *Oligosarcus* sp., that otherwise appeared as prey items for *P. orbignyanus*. Using CC, a very small percentage of *N. americana*, *B. aurea* and *H. australis* appeared as prey items of *P. orbignyanus*.

5. Discussion

5.1. Meeting requirements to QFASA application

In this work we applied QFASA to determine the diet of a top predator in a coastal lagoon in a conservation area. Ecological and methodological reasons led us to conduct this study in this site. From the

Table 3
Main fatty acids composition (% of total FA) of the predator and the prey. Values are expressed as mean ± standard deviation.

	Paralichthys orbignyanus adults	*Odontesthes bonaerensis*	*Micropogonias furnieri*	*Brevoortia aurea*	*Jenynsia multidentata*	*Oligosarcus sp.*	*Calllinectes sapidus*	*Chasmagnatus granulatus*	*Cyptograpsus angulatus*
Lip % DW	4.1 ± 1.5	7.2 ± 1.7	8.6 ± 5.1	9.8 ± 2.4	7.2 ± 1.4	10.7 ± 9.9	3.9 ± 1.6	2.7 ± 1.2	3.3 ± 0.8
Lip % WW	0.8 ± 0.3	1.8 ± 0.3	1.8 ± 1.1	2.1 ± 1.2	1.7 ± 0.4	2.6 ± 0.8	0.9 ± 0.5	0.6 ± 0.3	0.8 ± 0.3
FA % area									
14:0	2.3 ± 0.9	3.5 ± 1.7	1.7 ± 0.8	8.6 ± 1.9	3.0 ± 0.9	2.7 ± 0.2	1.8 ± 1.0	1.8 ± 0.6	1.8 ± 0.3
16:0	22.7 ± 4.1	23.5 ± 3.2	21.4 ± 2.8	26.1 ± 5.5	27.1 ± 3.5	29.4 ± 1.6	15.0 ± 2.2	17.1 ± 3.3	19.1 ± 1.8
16:2	1.0 ± 0.2	0.6 ± 0.2	1.0 ± 0.4	1.7 ± 0.4	0.6 ± 0.3	0.7 ± 0.3	1.1 ± 0.6	0.4 ± 0.4	0.2 ± 0.1
18:0	7.4 ± 1.3	6.2 ± 2.1	11.3 ± 2.2	6.2 ± 2.3	11.6 ± 1.7	5.8 ± 0.7	7.7 ± 1.1	6.7 ± 1.4	6.5 ± 1.0
18:1n−9	13.2 ± 3.1	16.0 ± 4.8	11.5 ± 0.5	14.0 ± 3.4	13.2 ± 2.3	5.7 ± 1.1	6.1 ± 0.7	11.2 ± 2.0	9.6 ± 2.9
18:2n−6	1.6 ± 0.6	1.2 ± 0.7	1.2 ± 0.3	1.3 ± 0.3	3.7 ± 1.4	0.8 ± 0.4	3.6 ± 2.9	5.1 ± 1.4	2.6 ± 1.0
18:3n−3	0.9 ± 0.4	1.1 ± 0.1	0.7 ± 0.6	1.0 ± 0.7	1.0 ± 0.3	1.1 ± 0.0	1.7 ± 0.7	3.2 ± 1.8	2.1 ± 0.7
20:1	1.1 ± 0.7	1.6 ± 0.6	2.7 ± 2.5	3.4 ± 0.7	1.5 ± 0.3	1.3 ± 1.2	3.2 ± 1.9	1.6 ± 0.4	2.7 ± 1.0
20:2n−6	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.2 ± 0.2	1.2 ± 0.4	0.8 ± 0.2	0.9 ± 0.2
20:4n−6	3.9 ± 1.4	2.6 ± 1.2	3.2 ± 1.4	0.7 ± 0.3	3.1 ± 0.9	2.2 ± 0.6	4.8 ± 2.3	6.1 ± 2.3	5.6 ± 0.9
20:5n−3	3.5 ± 1.3	4.9 ± 2.0	4.8 ± 1.3	4.1 ± 2.7	0.9 ± 0.4	6.4 ± 1.5	17.1 ± 2.1	11.8 ± 4.1	13.3 ± 3.1
22:1	0.4 ± 0.2	0.6 ± 0.4	1.0 ± 0.3	0.7 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	1.0 ± 1.0	0.6 ± 0.4	0.8 ± 0.3
22:5n−3	3.2 ± 1.1	3.4 ± 1.4	2.4 ± 0.6	1.1 ± 0.6	1.5 ± 0.6	2.0 ± 0.0	2.3 ± 0.5	0.7 ± 0.3	1.1 ± 0.3
22:6n−3	17.4 ± 7.3	13.1 ± 3.5	12.9 ± 8.8	5.9 ± 3.3	3.9 ± 1.7	22.0 ± 5.7	7.4 ± 4.1	5.0 ± 2.4	5.3 ± 1.1
Saturated	38.1 ± 7.7	36.6 ± 7.7	37.4 ± 4.6	45.0 ± 8.0	50.0 ± 7.6	34.9 ± 6.7	27.0 ± 2.8	30.4 ± 4.0	31.5 ± 2.7
Monounsaturated	25.5 ± 6.5	30.9 ± 4.1	26.9 ± 7.8	31.7 ± 4.4	31.1 ± 2.2	20.5 ± 7.8	24.1 ± 5.7	28.4 ± 5.8	29.3 ± 3.8
Polyunsaturated	35.6 ± ###	32.5 ± 6.9	29.5 ± 4.0	23.3 ± 8.9	18.9 ± 6.8	44.6 ± 1.1	32.4 ± 9.8	41.2 ± 9.1	39.2 ± 5.4
n−9	15.4 ± 3.4	18.2 ± 4.9	14.7 ± 3.2	18.0 ± 3.9	16.0 ± 2.6	9.1 ± 2.0	8.5 ± 3.5	13.6 ± 2.4	12.8 ± 3.4
n−6	7.4 ± 1.8	5.3 ± 1.6	6.7 ± 1.8	3.9 ± 0.7	7.9 ± 2.6	4.3 ± 0.0	11.6 ± 4.8	13.2 ± 2.4	10.3 ± 1.8
n−3	26.9 ± 8.7	24.3 ± 5.3	22.5 ± 8.6	14.9 ± 7.7	9.0 ± 3.1	35.1 ± 3.9	30.9 ± 4.7	24.2 ± 7.1	25.3 ± 4.4
n−3 HUFA	25.0 ± 8.7	22.0 ± 5.5	20.6 ± 9.6	12.1 ± 6.8	6.8 ± 2.7	32.5 ± 4.8	27.6 ± 5.3	18.2 ± 6.6	20.5 ± 3.7

	Palaemonetes argentinus	*Penaeus paulensis*	*Neomysis americana*	*Laeonereis acuta*	*Heteromastus similis*	Amphipoda	Isopoda	*Heleobia australis*	*Erodona mactroides*
Lip % DW	5.4 ± 0.6	7.1 ± 2.2	9.2 ± 0.4	6.9 ± 1.8	–	3.8 ± 0.9	–	0.9 ± 0.5	0.7 ± 0.0
Lip % WW	0.9 ± 0.1	2.1 ± 0.2	0.7 ± 0.0	0.9 ± 0.1	1.4 ± 0.1	0.5 ± 0.1	2.5 ± 0.5	0.5 ± 0.3	0.40 ± 0.0
FA % area									
14:0	1.6 ± 0.3	1.8 ± 0.5	1.8 ± 0.2	5.0 ± 1.5	5.3 ± 0.4	2.3 ± 0.0	4.0 ± 0.2	4.7 ± 1.2	2.7 ± 0.4
16:0	14.0 ± 0.8	15.7 ± 3.3	23.1 ± 0.8	24.8 ± 2.1	13.4 ± 1.3	18.8 ± 1.4	20.2 ± 2.0	25.9 ± 4.4	22.6 ± 4.2
16:2	0.4 ± 0.3	0.5 ± 0.1	0.3 ± 0.0	1.0 ± 0.2	0.8 ± 0.5	0.4 ± 0.1	1.7 ± 0.3	2.0 ± 0.8	1.3 ± 0.7
18:0	7.2 ± 0.3	7.9 ± 1.8	4.3 ± 0.3	8.0 ± 0.8	3.6 ± 1.2	4.7 ± 1.5	3.4 ± 1.1	15.8 ± 2.7	5.4 ± 1.5
18:1n−9	12.5 ± 0.8	7.8 ± 1.1	7.7 ± 0.5	4.0 ± 0.4	3.0 ± 0.3	11.8 ± 0.3	8.5 ± 0.5	2.3 ± 0.9	2.9 ± 1.0
18:2n−6	3.4 ± 1.3	3.0 ± 1.5	1.6 ± 0.0	0.7 ± 0.0	2.3 ± 0.3	3.6 ± 1.9	1.6 ± 0.2	1.8 ± 1.2	0.9 ± 0.1
18:3n−3	1.8 ± 0.2	1.3 ± 0.6	1.2 ± 0.0	0.7 ± 0.0	1.7 ± 0.5	3.5 ± 1.3	1.1 ± 0.2	1.8 ± 0.6	2.3 ± 0.4
20:1	1.6 ± 0.3	4.0 ± 1.0	1.5 ± 0.1	5.0 ± 0.7	4.9 ± 0.0	2.5 ± 0.3	1.2 ± 0.0	4.9 ± 1.6	4.7 ± 1.5
20:2n−6	0.7 ± 0.2	1.0 ± 0.2	0.0 ± 0.0	0.5 ± 0.1	1.2 ± 0.0	0.7 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.9 ± 0.0
20:4n−6	5.3 ± 1.2	5.0 ± 0.4	2.4 ± 0.0	1.1 ± 0.3	3.6 ± 0.3	4.8 ± 1.7	3.1 ± 0.2	2.7 ± 1.8	2.2 ± 0.1
20:5n−3	17.1 ± 0.4	14.2 ± 3.4	16.5 ± 1.3	5.7 ± 2.1	8.5 ± 0.2	12.8 ± 0.5	14.2 ± 1.1	2.5 ± 2.0	7.2 ± 3.7
22:1	0.4 ± 0.0	0.7 ± 0.3	0.5 ± 0.0	1.5 ± 0.6	1.1 ± 0.3	0.9 ± 0.2	0.8 ± 0.5	4.0 ± 1.8	1.1 ± 0.3
22:5n−3	1.0 ± 0.1	3.4 ± 0.8	0.9 ± 0.1	1.8 ± 0.6	3.2 ± 0.4	1.2 ± 0.7	0.5 ± 0.0	0.5 ± 0.4	2.0 ± 0.8
22:6n−3	7.8 ± 0.4	5.6 ± 1.4	21.1 ± 2.0	0.9 ± 0.5	1.6 ± 0.2	4.2 ± 2.0	1.9 ± 0.1	2.7 ± 1.5	7.0 ± 3.3
Saturated	25.9 ± 0.5	29.4 ± 2.3	32.8 ± 1.3	44.6 ± 3.0	27.2 ± 7.3	29.7 ± 1.1	29.5 ± 3.0	52.3 ± 5.9	35.2 ± 8.4
Monoinsaturated	29.2 ± 1.0	27.2 ± 2.9	16.1 ± 1.1	33.5 ± 1.4	32.4 ± 3.1	30.3 ± 1.2	36.3 ± 2.5	25.6 ± 0.9	25.7 ± 1.8
Polyunsaturated	44.9 ± 0.5	31.3 ± 6.1	51.1 ± 2.4	21.9 ± 3.7	31.9 ± 7.8	40.0 ± 0.1	24.9 ± ##	22.2 ± 5.0	39.1 ± 7.9
n−9	14.7 ± 0.7	10.6 ± 4.0	10.4 ± 0.4	10.2 ± 1.9	8.1 ± 5.6	14.6 ± 0.2	9.9 ± 1.0	8.8 ± 1.7	9.2 ± 0.6
n−6	10.2 ± 0.3	11.8 ± 1.5	5.4 ± 0.1	3.0 ± 0.4	10.4 ± 2.2	10.5 ± 0.2	6.2 ± 1.1	5.7 ± 3.6	6.2 ± 0.7
n−3	32.1 ± 0.3	26.7 ± 3.5	41.5 ± 3.2	15.5 ± 3.2	19.8 ± 3.2	26.6 ± 0.2	20.8 ± 2.3	10.8 ± 2.2	27.5 ± 7.8
n−3 HUFA	26.8 ± 0.2	24.0 ± 4.8	40.1 ± 3.2	8.7 ± 3.1	13.9 ± 0.9	19.8 ± 1.6	17.3 ± 1.3	6.5 ± 2.0	17.4 ± 7.7

ecological point of view, it is well known that accurate diet estimations of predators are a key factor to understanding the functioning of ecosystems, especially those belonging to protected areas where trophic relationships constitute a very powerful tool to develop effective management strategies (Sodhi and Ehrlich, 2010). From the methodological point of view, this well-studied semi-closed estuary (that remains disconnected from the sea for most of the year) constitutes an ideal place to apply QFASA to determine the diet of fish in a semi-closed system, where all potential prey available to this predator are well known (Conde et al., 2002; Giménez et al., 2006; Norbis and Galli, 2004; Rodriguez-Graña et al., 2008).

In the present study, all the necessary requirements to successfully apply QFASA were considered namely: a comprehensive database matrix representing the FA signature of a representative sample of any possible prey for a given predator; a FA profile of the predator obtained from the tissues where energy is stored as lipids; an optimization model developed to minimize the distance between predators and prey FA profiles and calibration coefficients to account for the metabolism of the predator (Budge et al., 2006; Iverson et al., 2004).

Table 4
Percentage of lipids in the diets (silverside and menhaden) and in two groups of flounders in the feeding trial (WW = wet weight; mean ± standard deviation).

	Lipids % WW
Odontesthes argentinensis	2.9 ± 0.7
Brevortia aurea	9.4 ± 4.4
Paralichthys orbignyanus fed with silverside	0.74 ± 0.30
Paralichthys orbignyanus fed with menhaden	0.80 ± 0.32

5.2. Sample collection and matrix of FA construction

The matrix of the prey database used in this study, which included 17 items, largely reflects the prey available in the Rocha lagoon for the

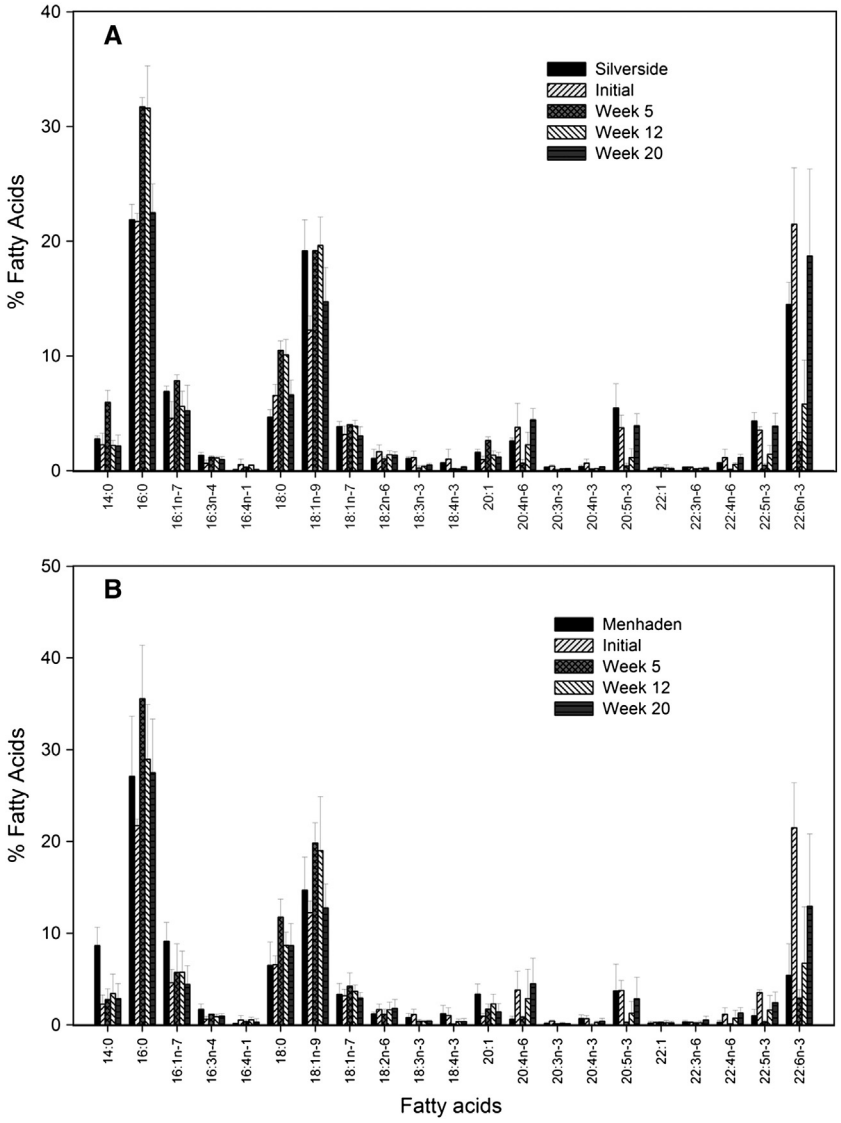

Fig. 3. Percentage of FA of prey and muscle with skin of *Paralichthys orbignyanus* fed with silverside (A) and menhaden (B) at weeks 0, 5, 12 and 20 of the captive feeding trial.

size range of the predator. Moreover, the proposed items (Table 2) covered all trophic interactions on the lagoon food web, between macro fauna and fish, excluding seabirds at the top of the web and plankton and macrophytes at the base (Rodriguez-Graña et al., 2008). The robustness of the matrix of prey was explored in order to ensure that the differences between the profiles of prey included in the database were big enough to effectively distinguish species based on their FA signature.

5.3. Energy reservoir identification

The next key factor to take into account in applying QFASA is the FA profile of the predator obtained from the main energy storage reservoir (Iverson, 2009). Fish can store their energy as lipids in different parts of their body, such as belly flaps, liver or lipid reservoirs between skin and muscle (sub-dermal lipids, usually sampled as muscle with skin) (Ackman, 1980; Brown, 1957). Since *P. orbignyanus* has no belly flaps and its fattening strategy was not clear, both muscle with skin and liver fatty acids were included to perform the validation of QFASA. The hepatosomatic index (HSI) variation in this study was not relevant (0.7–2.1) regardless of the fish size compared to other species that store lipids in the liver, such as *Gadus morhua* whose HSI varies between 2 and 9 (Yaragina and Marshall, 2000). On the other hand, when we considered the lipid content of muscle with skin, significant differences ($p < 0.05$) were found between pre spawning season (0.79 ± 0.28% WW) and post spawning season (0.47 ± 0.13% WW), pointing out that this flounder stores its lipid reserves in subdermal tissue. Furthermore, we found that both, data obtained from muscle with skin and liver, gave good estimations of the real diet over the validation process, considering the results with and without calibration coefficients (98.7 and 77.3% in muscle and 99.0 and 88.2% in liver). However, considering the small monthly variation of the HSI during the pre-spawning season, we have a reasonable doubt about the role of the liver in *P. orbignyanus* as energy reservoir. In this case, the liver could be reflecting the short term metabolism, instead of the long term assimilated diet. Moreover, the fact that subdermal tissue of *P. orbignyanus* seems to be the metabolically active lipid reservoir, led us to decide to focus our analysis of estimations of diet in wild populations based on muscle with skin, although the results of the validation estimations in the liver were somewhat higher than in the muscle, depending on the selected subset of FA (Table 5). Since FA of polar lipids are best preserved than those of neutral lipids that better reflect dietary FA (Henderson and Tocher, 1987), the most accurate way to face this matter would be to use FA from muscle with skin neutral lipids to estimate the diet.

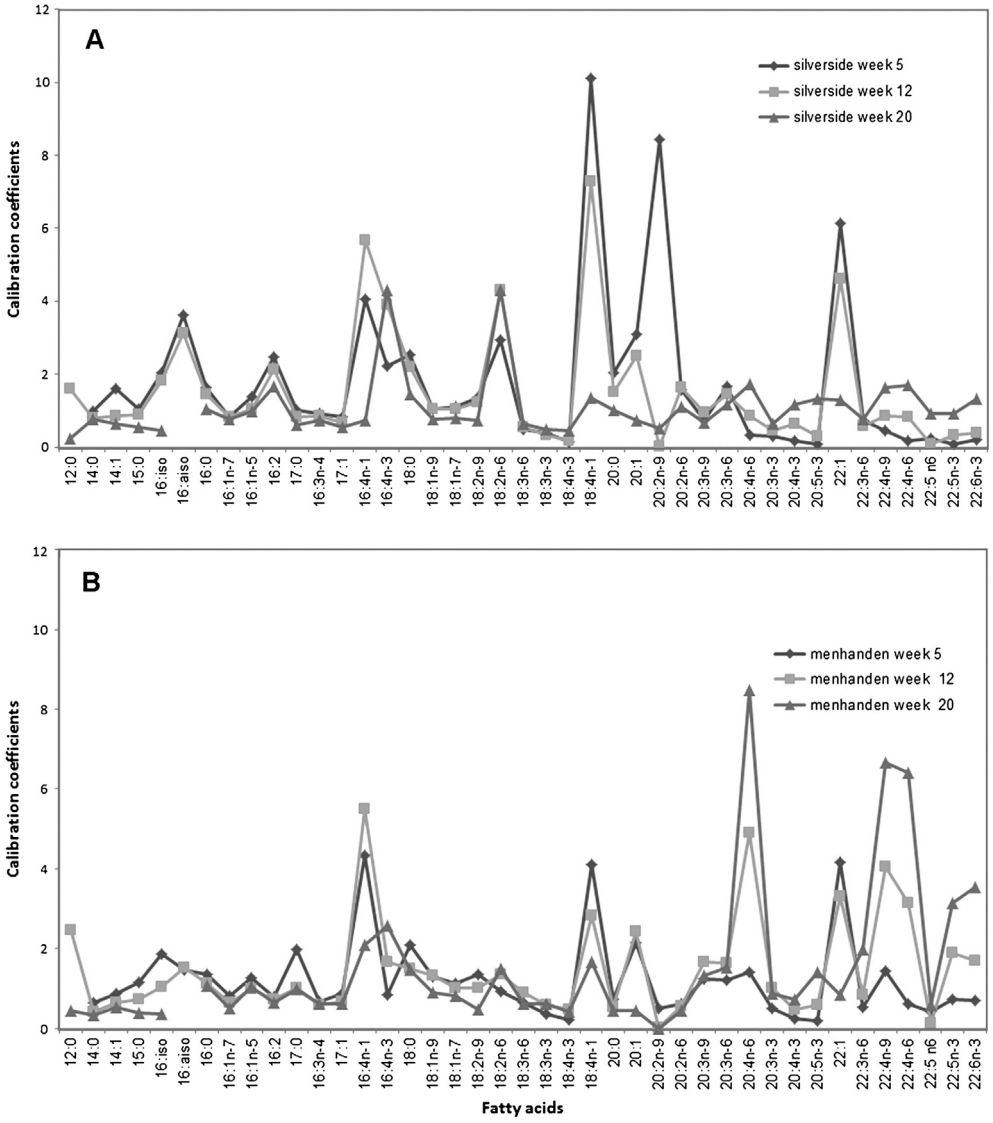

Fig. 4. Temporal evolution of calibration coefficients for *Paralichthys orbignyanus* obtained from dorsal muscle with skin at 5, 12 and 20 weeks of feeding on silverside (A) and menhaden (B).

Table 5
Estimation (% ± sd) of the real diet (silverside or menhaden) supplied in the feeding trial (week 20) over the validation process, using different predator tissues (muscle with skin or liver) and different FA subsets.

Dorsal muscle with skin		FA subset A	FA subset B	FA subset C	FA subset D	FA subset E	FA subset F	FA subset G
Flatfish fed with silverside	No CC	49.0 ± 5.4	77.3 ± 12.3	29.9 ± 13.8	0.0 ± 0.0	34.4 ± 5.9	56.1 ± 7.1	52.7 ± 7.4
(FA profile of week 20)	Silverside CC	94.9 ± 2.9	98.7 ± 2.5	94.7 ± 5.8	87.5 ± 10.7	97.8 ± 2.2	88.8 ± 3.5	88.3 ± 3.7
	Menhaden CC	5.2 ± 7.1	42.6 ± 8.0	4.0 ± 5.7	42.2 ± 10.1	19.5 ± 14.1	5.6 ± 5.9	4.3 ± 5.4
	Mean CC	45.6 ± 5.6	80.8 ± 6.5	57.7 ± 5.1	74.2 ± 11.5	84.4 ± 4.5	46.5 ± 5.6	46.5 ± 5.8
Flatfish fed with menhaden	No CC	1.2 ± 1.4	–	–	13.1 ± 6.0	2.3 ± 1.2	0.3 ± 0.9	0.3 ± 0.9
(FA profile of week 20)	Silverside CC	73.7 ± 17.4	81.1 ± 10.2	89.7 ± 12.9	73.6 ± 18.9	75.6 ± 13.9	54.5 ± 20.7	58.7 ± 19.1
	Menhaden CC	64.5 ± 15.5	80.5 ± 4.6	88.5 ± 13.2	73.8 ± 19.3	78.8 ± 13.5	57.7 ± 19.7	54.6 ± 19.1
	Mean CC	41.8 ± 10.1	38.6 ± 4.9	55.4 ± 4.6	50.5 ± 13.9	23.4 ± 7.1	39.0 ± 8.3	39.5 ± 9.2
Liver		FA subset A	FA subset F	FA subset C	FA subset D	FA subset E	FA subset G	FA subset H
Flatfish fed with silverside	No CC	64.6 ± 4.4	88.2 ± 14.0	5.9 ± 8.7	0.2 ± 1.3	79.5 ± 10.6	59.0 ± 4.2	56.8 ± 4.5
(FA profile of week 20)	Silverside CC	96.1 ± 2.6	99.0 ± 2.2	94.3 ± 6.0	87.5 ± 8.8	97.6 ± 3.3	89.5 ± 2.9	88.4 ± 3.3
	Menhaden CC	2.4 ± 4.9	30.3 ± 12.1	–	–	44.8 ± 11.1	0.5 ± 1.8	1.2 ± 3.6
	Mean CC	42.1 ± 5.9	75.7 ± 5.8	46.9 ± 7.8	32.3 ± 12.7	83.7 ± 4.3	30.6 ± 5.9	34.1 ± 5.7
Flatfish fed with menhaden	No CC	20.0 ± 2.2	10.0 ± 2.1	48.4 ± 10.0	70.6 ± 20.5	25.4 ± 5.9	17.4 ± 2.6	18.2 ± 3.2
(FA profile of week 20)	Silverside CC	4.4 ± 1.9	9.2 ± 1.9	21.7 ± 6.6	17.4 ± 18.4	10.7 ± 4.0	0.4 ± 0.5	1.1 ± 0.8
	Menhaden CC	79.6 ± 9.7	70.4 ± 16.9	86.5 ± 10.6	80.1 ± 13.8	78.6 ± 11.2	70.7 ± 14.4	71.9 ± 14.2
	Mean CC	38.9 ± 5.7	31.6 ± 10.6	60.3 ± 6.6	54.1 ± 19.0	30.2 ± 3.8	28.3 ± 4.9	26.2 ± 3.8

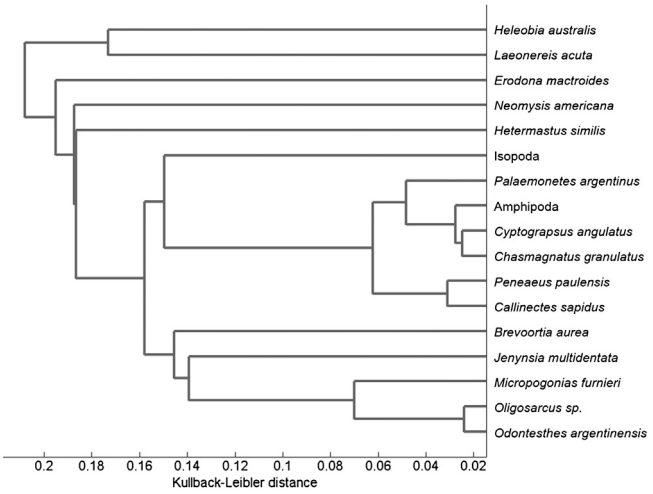

Fig. 5. Hierarchical cluster analysis based on FA profile over the 17 species recognized as potential prey for *Paralichthys orbignyanus* at Rocha lagoon.

5.4. Feeding trial and calibration coefficients

As fish used in the feeding trials were subadults and adults in prespawning season, variations or interferences due to the influence of physiological processes associated with starvation, spawning, maturity, migration, etc. on lipid metabolism (Sargent and Henderson, 1995) were avoided. Moreover, taking into account that this flatfish is a migratory species that spends about four months spawning in the sea and the rest of the year feeding in the coastal lagoons, our dietary studies were focused on the period when these fish remain feeding in the lagoon.

There were many similar patterns between the CC obtained during the feeding trials with silverside and menhaden (Fig. 4), suggesting that the underlying metabolic processes were common among animals and diets. It could be noted that there is a generalized trend towards reaching a value of 1 in CC over time, although the CC of flounders fed silverside showed a strong tendency towards 1. Despite this fact, over the whole period of the feeding trials, the FA profile of flounders fed both menhaden and silverside did not clearly reach a constant value of CC 1 for all FAs.

Since many FAs did not stabilize within 20 weeks of feeding trial, this could be suggesting that QFASA is a model that fits better in those species consuming constant diets for long periods, rather than migratory species like *P. orbignyanus*.

Turchini et al. (2009) stated that high lipid diets may suppress de novo synthesis or increase β-oxidation of FAs in tissues of captive fish, and to account for predator metabolisms, feeding the experimental fish with a similar percentage of lipids of their metabolically active energy storage. In the present study, both items offered in the feeding trials had higher lipid content than the flatfish itself, especially menhaden (Table 4), even though the prey offered was, in the case of silverside the real prey in wild populations (Norbis and Galli, 2004), and in the case of menhaden, a potential prey. The elevated lipid content in menhaden and also its high intraspecific variation most likely makes this prey item not suitable for this type of feeding trial, although in both cases (silverside and menhaden) prey was collected as a single batch from the same site where the diet estimation was conducted. The slight adjustment of menhaden feeding trials would be related to the high variability of FA profiles shown by this species. Furthermore,

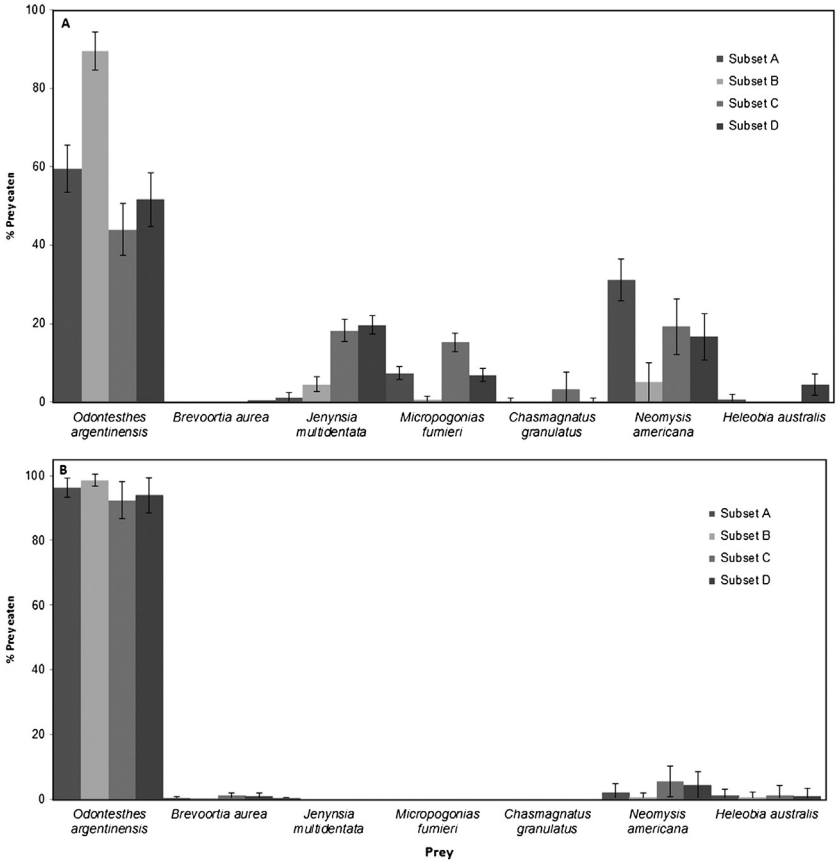

Fig. 6. QFASA diet estimations of *Paralichthys orbignyanus*, using the prey matrix of Rocha lagoon. Results are expressed as a percentage of eaten items, with four different subsets of FA (A–D). A. Results without calibration coefficients. B. Results with calibration coefficients obtained from flatfish muscle with skin at week 20 of feeding on silverside.

when menhaden CC were applied, the model correctly identified 80.5 ± 4.6% over the validation process, conversely, with results obtained with silverside feeding trials, without CC there was only a small resemblance of menhaden in real diet estimation, suggesting again that this species is not suitable for this kind of experiment.

In accord with previous studies (Budge et al., 2012; Rosen and Tollit, 2012), overestimating the presence of the item used as prey for CC calculations was also observed. Without questioning the necessity of using CC to compensate for lipid metabolism of the predator, it is important to highlight that the incorrect construction or improper use of CC can lead to poorer estimations. One way to attenuate the bias produced by the application of CC in the diet estimation, could be the application of CC on the prey FA profile, instead of on the predator one. From the practical point of view, this implies the development of CC for each potential prey and their application for the correction of each prey FA profile prior to running QFASA.

5.5. Subsets of FA performed in the model

The accuracy of the diet estimation is highly influenced by the FA subset selected to run QFASA, hence special attention should be paid in this regard (Budge et al., 2006). The construction of FA subsets includes endogenous FA, which could be present by de novo biosynthesis and dietary or exogenous FA, which content is mostly affected by dietary FA (Dalsgaard et al., 2003). The latter ones are the most informative about diet consumed, and hence the candidates to be included beforehand in the design of the FA subsets. Dietary fatty acid (DFA) are those that better resemble diet because they are not synthesized de novo by vertebrates and are those belongings to n − 3 and n − 6 series. Particularly, docosahexaenoic acid (22:6n − 3, DHA), eicosapentaenoic acid (20:5n − 3, EPA) (Cowey, 1988) and arachidonic acid (20:4n − 6, AA) (Bessonart et al., 1999), all constitute essential FA (EFA) for marine fish, because they lack the ability to elongate and/or to desaturate their shorter chain precursors: linoleic (18:2n − 6) and linolenic (18:3n − 3) acids, which several freshwater fish can do (Sargent et al., 2002). Selecting the FA subsets to run QFASA, the best option is to include the dietary FA, despite the higher percentage of all FA are the endogenous. QFASA model takes into account the relative amount of FA and for this reason it was designed to choose a distance as KL, which weighted rare or minor FA. Recently, Budge et al. (2012) observed that working with controlled feeding trials with fish (*Salmo salar*), the subsets which best resemble diets, were those which incorporate the dietary FA combined with other endogenous FA (such as 16:0, 16:1n − 7, 18:1n − 9, 18:0, etc.) which increase from approximately 39% to 90% of total FA. Thus, these authors proposed that a good strategy to establish the best subset would be to incorporate FA according to their concentration. Taking into account these observations, in this work the subsets tested contained different combinations of these FA comprising from 12.6 to 97.8% of total FA. However, the one which best resembled the diet in the feeding trials was subset B, which comprised only 34.0 ± 10.6% of total FA and included almost exclusively DFA.

During the comparison of performance of subset B (that includes only DFA) with subset A (which includes almost all but the trace FA), we noted that subset B showed a better resemblance to the diet. This was in accord with our expectations when we removed all non-dietary FA from subset A to create subset B (Table 1). Due to EFA could be involved in the selective retention processes associated with its essentiality (Henderson and Tocher, 1987), in subsets C and D different EFAs were removed in order to improve the resemblance of the diet to the subset. However no subset showed better performance than B, indicating that in this case the essential features of the FA did not affect its incorporation in reservoir tissues. Therefore the inclusion of the EFA into the FA subsets can improve the diet estimation, but according to other authors we cannot assume this to be always true (Budge et al., 2012). Moreover, even the inclusion of major FA in the subsets did not improve our estimations. Subset selection seems to be a promising area for research, where physiological and maybe environmental considerations should be included in the design of the right subset selection for diet estimation of a given fish.

5.6. Diet determination using QFASA

Paralichthyidae flatfishes are one of the most studied flatfish groups in the world, due to their large size, they are the target of very important fisheries and aquaculture developments worldwide (Howell and Yamashita, 2005). Flatfish usually present two major feeding strategies: predators in benthic communities or piscivores.

Within the Paralichthyidae family there are species belonging to both groups (Link et al., 2005). Among piscivores, some species of the genus *Paralichthys* are proposed to be apex predators (Lee et al., 2010) and within the benthic predator group, several species of this family play an important role in energy conversion from macrobenthic fauna to superior levels, mainly feeding on polychaetes and small benthic crustaceans (Link et al., 2005).

In the case of *P. orbignyanus* previous studies on feeding habits of the species based on stomach content, reported that adult fish fed on both fish (Norbis and Galli, 2004) and benthic crustaceans (Carnikián, 2006), depending on the area and prey availability. It is known that some flatfishes can feed on small prey when the abundance of this kind of prey increases (Link et al., 2005).

Traditionally, studies on feeding habits of fish are based on qualitative and quantitative analysis of stomach content. These methods usually reflect the last food intake, several times inferring the diet from the identification of parts of hard tissues or remainders of skeletons and the occurrence of empty stomachs is common (Hyslop, 1980; Rindfort and Lewy, 2004). Alternatively the robustness of QFASA is associated with its capacity to detect small prey even reflecting the food intake from the previous weeks. According to that, determining a diet applying QFASA we found a greater number of prey than previous studies based on stomach content analysis (Carnikián, 2006; Norbis and Galli, 2004). The main difference was given by the presence in the diet of two groups of invertebrates (gastropoda and mysidacea).

According to Norbis and Galli (2004) the diet estimation by stomach content shows that *P. orbignyanus* feeds exclusively on fish (88% silverside, and 12% other fish) in the Rocha Lagoon. Using QFASA with CC we found that fish constitute around 97% of *P. orbignyanus* diet but when the model was applied without CC, fish presence in the diet varies from 68 to 95% depending on the FA subset. Interestingly, without calibration coefficients those prey hard to identify with the classic method increased both in number and amount, opening an interesting discussion about the suitability of the CC utilization, as other authors mentioned (Budge et al., 2012).

Considering the exclusion of CC when running QFASA to estimate the diet of *P. orbignyanus* and choosing subset B as the best subset, it could be observed that during the study period when this species entered the estuaries to fatten, particularly in Rocha lagoon, this flounder fed mainly on *O. argentinensis* with a minor percentage of other fish and crustacean (*N. americana*, at 5.1 ± 2.4%). This result was largely in agreement with the feeding habits previously reported for this species in the same study area (Norbis and Galli, 2004). Although minor differences could be highlighted, especially those referred to as soft-bodied prey (*N. americana*), that enhances the robustness of this type of model in detecting those species hard to identify with conventional methodologies due to its elevated rates of digestion. However, these results raise the additional question if this tiny crustacean (*N. americana*) could be a real prey for this large sit-and-wait top predator. The possible explanation concerns the feeding strategy of the main prey of *P. orbignyanus*, the silverside (*O. argentinensis*), that it is known to feed on *N. americana* when they are in the filter feeding stage (Sagretti and Bistoni, 2001), in which case the model could be reflecting the stomach content of the silverside.

6. Conclusions

QFASA was initially developed for upper trophic level endothermic vertebrates, and to the best of our knowledge this is the first study to quantify the diet of lower vertebrate species (*P. orbignyanus*). This flatfish was selected for having well characterized feeding habits from previous researches in the study area. Application of QFASA without calibration coefficients resulted in a good approximation of the natural diet of this fish, in line with results from previous studies, but detecting soft-bodied prey. It would be interesting to focus future work in the development of prey calibration coefficients for a given predator instead of predator CC for any potential prey.

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