UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS DO MAR E DO AMBIENTE

TROPHIC ECOLOGY OF THE ATLANTO-IBERIAN SARDINE (*Sardina pilchardus*)

(Tese para a obtenção do grau de doutor no ramo de Ciências do Mar, especialidade de Ecologia Marinha)

CARLA SUSANA FERREIRA GARRIDO COELHO

Orientador: Professora Doutora Maria Alexandra Anica Teodósio Chícharo

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Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

Ricardo Reis *1933*

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DATA: 7 de Fevereiro de 2007

TÍTULO DA TESE: Ecologia trófica da sardinha Atlanto-Ibérica (*Sardina pilchardus*)

RESUMO

Esta tese tem por objectivo estudar a ecologia trófica da sardinha Atlanto-Ibérica (*Sardina pilchardus*). Em laboratório, estudou-se o comportamento alimentar e a dieta de sardinhas recolhidas na costa oeste (W) e sul (S) de Portugal através da análise das presas e dos ácidos gordos (AG). Comparou-se dados de clorofila de satélite com a dieta e a ingestão de *Pseudo-nitzschia* com a acumulação de ácido domóico. Estudou-se a influência da dieta nos AG do músculo e o efeito maternal na composição de AG dos oócitos. As sardinhas usam filtração ou predação activa dependendo do tamanho das presas, sendo as mais importantes crustáceos (principalmente ovos e copépodes), ovos de peixe, dinoflagelados e diatomáceas. Os dados de satélite correlacionaram-se com a intensidade alimentar, superior na costa W. As sardinhas podem actuar como vectores de ácido domóico para os predadores mas os riscos para o Homem são mínimos. Os AG da dieta da sardinha reflectiram a dominância de fitoplâncton no W e de carnivoria no S. As diferenças espaciais da dieta reflectiram-se no músculo. A sazonalidade de AG do músculo é condicionada pela desova. As reservas das fêmeas influenciaram a composição de AG dos oócitos, podendo ter implicações na sua qualidade e no recrutamento.

Palavras-chave: *Sardina pilchardus*, comportamento alimentar, dieta, ácido domóico, ácidos gordos biomarcadores e efeitos maternais

TROPHIC ECOLOGY OF THE ATLANTO-IBERIAN SARDINE (*Sardina pilchardus*)

ABSTRACT

This dissertation aims at describing the trophic ecology of the Atlanto-Iberian sardine (*Sardina pilchardus*). Laboratory experiments studied the feeding behaviour of adult sardines while diet in the west (W) and south (S) Portuguese coast was examined through stomach and fatty acid (FA) analysis. Remote-sensed chlorophyll was compared to sardine diet. The ingestion of *Pseudo-nitzschia* was compared to the accumulation of domoic acid. The influence of diet on muscle composition and the maternal effects on the FA composition of the oocytes were studied. Sardines are highly omnivorous, using filter- and particulate-feeding depending of prey size and the most important prey were crustaceans (mainly eggs and copepods), fish eggs, dinoflagellates and diatoms. Feeding intensity matched satellite data being higher for the W. Sardines are vectors of domoic acid for predators, although no implications for human consumption are expected. The FA of sardine diet matched the dominance of phytoplankton in the diet for the W and carnivory for the S. Spatial differences of diet were reflected in the muscle, with higher EPA, EPA/DHA and (n-3)/(n-6) for the W. Seasonality of muscle FA composition was mainly driven by spawning. Female reserves influenced the FA composition of the oocytes, which might have implications for egg quality and ultimately, recruitment.

Key-words: *Sardina pilchardus*, feeding behaviour, diet composition, domoic acid, fatty acid biomarkers and maternal effects

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LIST OF ABBREVIATIONS

AA – Arachidonic acid

ANOVA – Analysis of variance

ASP- Amnesic Shellfish Poisoning

BRANCH – Branched fatty acids

% C – Percentage contribution to dietary carbon

CF – Condition factor

DA – Domoic acid

DAD – Diode array detector

DHA – Docosahexaenoic acid

DSP – Diarrhetic Shellfish Poisoning

DW – Dry weight

ED – Energy density

EPA – Eicosapentaenoic acid

F – Clearance rate

F_{max} – Maximum clearance rate

FA – Fatty acid

FI – Feeding intensity

FID – Flame ionization detector

g – Instantaneous clearance rate

GLM – Generalized linear model

GSI – Gonadosomatic index

HAB – Harmful Algae Blooms

HPLC – High Performance Liquid Chromatography

HUFA – Highly unsaturated fatty acids

ICES - International Council for the Exploration of the Sea

IMAT – Immature sardines

IPIMAR – Instituto de Investigação das Pescas e do Mar

Mg – Mouth gape

mIRI – Modified index of relative importance of prey

MAT – mature sardines

MUFA – Monounsaturated fatty acids

NRC – National Research Council of Canada

%O – Frequency of occurrence

PSN - *Pseudo-nitzschia*

PUFA – Polyunsaturated fatty acids

r – resting stage of reproduction

S – South coast of Portugal

SAT – Saturated fatty acids

SD – Standard deviation

SEM – Scanning electron microscopy

SFA - Saturated fatty acids

sp – spawning sardines

SSE – Sum of squares due to error

SST – Sea Surface Temperature

SW – Swimming speed

TL – Total length

Tukey HSD – Tukey Honestly Significantly Different

W – West coast of Portugal

CHAPTER 1

INTRODUCTION

1.1 - European sardine (*Sardina pilchardus*) and the Atlanto-Iberian stock

The European sardine (*Sardina pilchardus* Walbaum, 1792) is a small pelagic fish distributed in the upwelling system of the Northeast Atlantic, from the North Sea to Mauritania (Culley 1971). It is also distributed in the Mediterranean and adjacent waters, and off the Madeira, Azores and Canary Archipelagos (Andreu 1969, Suau 1959).

Sardine abundance is especially high off the Moroccan Atlantic coast, with average catches of 600 000 tons in recent years (Kifani 1998) contributing 70% of fish landings from that area (Amenzoui 2006). The second area of high sardine abundance is off the Iberian Peninsula, where an average of 100 000 tons are caught annually by the purse-seine fisheries of Portugal and Spain (ICES 2006).

The Atlanto-Iberian sardine stock is distributed at the northern limit of the east central Atlantic coastal upwelling system and extends from the Gulf of Cadiz to the Spanish/French border, and is considered by the *International Council for the Exploration of the Sea* (ICES) to be a consistent stock.

For Portugal, sardines represent approximately half of the annual fish landings, being an important resource for the country, both economically and socially (Pestana 1989). More than 50% of the Portuguese commercial catches of sardines are taken off the western coast, while the south coast represents approximately 15% of the total catches of the Atlanto- Iberian stock in recent years (Fig. 1.1).

Historical data (ICES 2006) reveal that the Atlanto-Iberian sardine stock has shown high fluctuations in abundance and high inter-annual variability in recruitment. Abundance decreased significantly from 1996 to 1999, but strong recruitment in 2000 resulted in an important recovery that year and an increasing trend in population size was observed until 2003, while 2004 and 2005 showed a decrease in abundance and 2006 showed similar numbers to 2005.

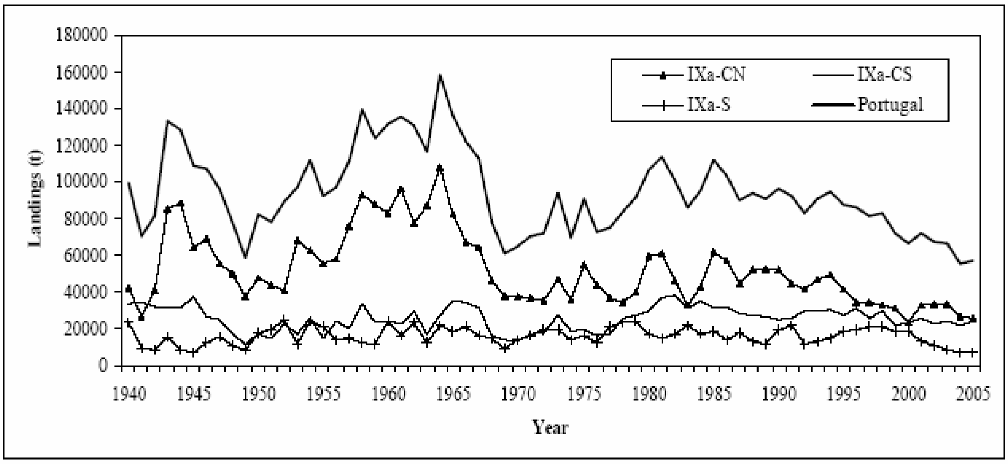

Figure 1.1 – Annual landings of sardine in ICES sub-areas IXa-CN (northwestern Portugal), IXa-CS (southwestern Portugal), IXa-S (South Portugal), and total landings off Portugal (figure from ICES WGMHSA Report 2006).

The most important recruitment area for sardines off Portugal is off the northwestern coast, mainly during winter, followed by southern waters and the Cantabrian Sea, mainly during the spring (Ré et al. 1990, ICES 2006). As a result, 0-group catches are mostly concentrated in northwestern Portuguese waters, while older fish (age groups 5 and 6+) concentrate in the Bay of Biscay/Cantabrian area and also off southwest Portugal. The 2000 year class that recruited off northwest Portugal made a large contribution to the increase in sardine abundance off western Iberian and northern Galicia but apparently had a limited impact in eastern Cantabria and southern Portugal, where catches showed a declining trend (ICES 2006).

Other contrasting signals for the Atlanto-Iberian sardine stock have occurred within its area of distribution. During the last two decades sardine length at maturity decreased and their spawning period extended off western Iberia, concomitant with an increase in fish condition and fat reserves during the summer, suggesting that the variation of the reproductive season for sardines might have been caused by changes in food availability (Silva et al. 2006). Sardine become mature at a smaller size off the south coast of Portugal (similar to that observed for fish in the Gulf of Cadiz and the southwestern Mediterranean) than off the west coast, which is probably related to distinct prevailing environmental conditions in those two areas (Silva et al. 2006). Morphometric studies have also shown that the shape (mainly the head-to-body ratio) of sardines from southern Portugal is similar to that of fish from Morocco, but different from fish distributed further north (Silva 2003).

Several hypotheses have been suggested to explain variations in the abundance of small pelagic fish, mostly related to the variations in the spawning fraction and to environmental factors. For planktivorous fish like sardines feeding is strongly dependent on environmental conditions, and might be a determining factor explaining variations in abundance and distribution (e.g. Parrish et al. 1989, Mathisen et al. 1978, Schwartzlose et al. 1999).

1.2 - Hydrological features off Portugal and productivity

If wind causes the movement of surface water away from the coast, then water from a deeper layer will move upward at the coast to take its place, a process known as coastal upwelling. Since deep water is characteristically rich in nutrients, upwelling has the effect of stimulating planktonic primary production and the food web based on it, and the five eastern currents of the world where this phenomenon occurs generate about 20% of the world fish production (Mann 2000).

The west coast of Portugal experiences upwelling-favourable winds from March until October (summer), with the months from July to September being the period of strongest upwelling (Fiúza et al. 1982). Upwelling results from strong northerly winds associated with the northern displacement of the Azores high pressure cell and the weakening of the Iceland low pressure cell (Relvas & Barton 2002). Although the wind regime favours coastal downwelling during winter time, upwelling events are also observed during that season (Ribeiro et al. 2005), and these seem to have increased in frequency and intensity over recent decades (Borges et al. 2003).

Occasionally, water upwelled from the west coast extends eastwards along the Algarve shelf off the southern Portuguese coast (Fiúza 1983), and the south coast of Portugal is also directly affected by upwelling events under favourable westerly winds. These are usually weaker and more intermittent than off the west coast and occur mainly during late spring/summer (Relvas & Barton 2002).

A decadal study of remotely-sensed chlorophyll data off Portugal (Peliz & Fiúza 1999) demonstrated that seasonal and interannual fluctuations in phytoplankton concentrations, as well as variations related mainly to upwelling events, river-runoff and topography. On the shelf, where sardines are distributed, the phytopigment maxima occurs in December-January (winter) off the northwestern Portuguese coast and is related to the cycle of riverine freshwater input that occurs in that area. Off the south coast the phytopigment maxima occurs during March and is associated with the discharge of the Guadalquivir River, although the influence of the Guadiana River fresh-water outflow is also important for the south shelf (Sanchez et al. 2006). Minimum phytopigment concentrations occur before August off the northwestern coast and during September off the south coast. During the summer (June-August) months upwelling events enhance productivity and the upwelled water mass extends productivity offshore, and productivity in autumn is generally low for both the west and south coasts (Peliz & Fiúza 1999). A two year study carried out off the central Algarve coast (off Ria Formosa), on the other hand, described a bimodal cycle of phytoplankton with maximum phytoplankton biomass during the spring months and at the end of summer or autumn, and minimal concentrations during the summer and winter (Barbosa 2006).

Studies carried out during the 1980's confirmed that phytoplankton biomass maxima off the Portuguese coast are generally found during all seasons off the northwestern coast (Moita 2001). The enhanced productivity of this area is related to upwelling events, but also to the fact that the shelf is larger and flatter and has higher thermo-haline stratification than in the south, which causes north-to-south differences in plankton composition along the Portuguese coast, although inshore-offshore variations in phytoplankton composition appear to be higher than latitudinal variations (Cunha 1993).

At low spawning stock sizes, sardines are preferentially located in zones with major river plumes (i.e. the Douro-Aveiro in northwestern Portugal, and the Adour and surrounding Basque rivers in the Cantabrian Sea), and continental features such as river-runoff may on occasion be more important for sardine recruitment than upwelling or other oceanographic regimes (Carrera & Porteiro 2003).

The distinct oceanographic regimes that occur within the area of distribution of Atlanto-Iberian sardines might explain the geographical differences that occur in morphometric characteristics, age structure, population dynamics and maturation (Carrera & Porteiro 2003, Silva 2003, ICES 2006, Silva et al. 2006) that have been previously described, namely through the influence on temporal and geographical variations in food availability.

1.3 - Trophic ecology of Atlanto-Iberian sardines

An ecological analysis of feeding must answer basic questions including “what is eaten, when is it eaten, where is it eaten and how much is eaten?” (Wootton 1998). Several aspects of the feeding ecology of fish, particularly of planktivorous clupeids as sardines will be focused in this section; the physiology of their feeding apparatus, the diet composition inferred mostly through stomach content analysis and the feeding behaviour used by planktivorous clupeids. Also, the role of planktivorous fish as vectors for toxic algae will be addressed and finally we will focus on the use of fatty acid trophic markers in the pelagic marine food web and the role of fatty acids in fish nutrition and reproduction.

1.3.1 – Feeding apparatus

Teleostean fishes evolved from generalized macrophagus predators towards feeding on smaller prey like the plankton. The development of microphagy led to the development of specialized structures such as the replacement of teeth by elaborate gill rakers, and a lengthening of the intestine in order to permit the processing of larger amounts of food without the necessity for a digestive pause (Lazzaro 1987). Gill-raker shape and abundance are related to the diet, and in fish that feed on small phyto and zooplankton prey gill-rakers probably sieve the particles from the respiratory current, although the detailed mechanism of the sieving action is not fully understood (Wooton 1998).

The filtering apparatus of juvenile and adult *S. pilchardus* is composed of five pairs of branchial arches, each one of which supports one series of gill-rakers covered with denticles on the anterior side, and branchiae on the posterior side of the first four branchial arches (Andreu 1969). Gill-rakers develop along the supporting basihyal bones and project forwards from the inner margins of the gill arches, and in the case of the first arch also project over the sides of the tongue, thus forming an efficient filter for food particles. The number of gill rakers and the separation between them is different in sardines from the Atlantic compared to those from the Mediterranean Sea, which have fewer gill rakers that are more widely separated. This difference was explained as an adaptation to the higher plankton abundance in the Atlantic by which sardine would benefit using filter-feeding, while Mediterranean sardines would preferentially capture individual prey via particulate-feeding (Andreu 1969).

1.3.2 – Diet composition

Only a small fraction of the ocean's productivity produced by phytoplankton is available to the higher trophic levels while a large fraction is available to plankton feeders (Lazzaro 1987), hence planktivorous fish live in areas where planktonic resources are dominant. Small pelagic clupeiformes are in general microphagous planktivores, and their high abundance levels in upwelling systems in particular was attributed to their ability to feed directly on phytoplankton prey (Ryther 1969).

Studies on the feeding ecology of European sardines using stomach content analysis suggest that its diet comprises phyto- and zooplankton prey in different proportions according to sampling area. An early report of a study carried out off England showed that numerically, European sardine diet was primarily composed of copepods, decapods, amphipods, euphausids, diatoms, peridinians, mollusca and crustacean eggs (Hickling 1945). Stomach contents of sardine from the Cantabrian Sea were numerically dominated by phytoplankton during the summer (Navarro & Navaz 1946), although other studies reported a numerical predominance (>50%) of zooplankton prey in sardines from Galician and Cantabrian waters in spring, summer and winter (Olivier 1951, Olivier & Navarro 1952, Varela et al. 1988, 1990). In the Mediterranean Sea sardine diet was dominated by zooplankton (Massutí & Olivier 1948) while a study carried out in the Adriatic (Vučetic 1963) described sardine as feeding almost exclusively on zooplankton, mainly copepods and decapod larvae.

In the studies carried out off the Portuguese coast phytoplankton, both diatoms and dinoflagellates, dominate sardine diet in terms of number of prey. Silva (1954) considered copepods as the most important prey of sardines off the west coast of Portugal due to their high frequency of occurrence in stomachs all year round, although a high diversity of phytoplankton prey were also present in large numbers. In recent studies, copepods and dinoflagellates showed the highest frequency of occurrence, while diatoms dominated in terms of number of prey, in sardine stomachs collected during spring, although copepods were the most important prey in terms of biovolume (Garrido 2003, Cunha et al. 2005).

From stable isotopes studies it appears that sardines obtain most of their muscle protein from zooplankton while phytoplankton would provide reserve materials (Bode et al. 2004). Those studies also point to an increased consumption of plankton of a lower trophic position in the food web as sardines grow and their filtering apparatus develops, with phytoplankton becoming increasingly important for older sardines (Bode et al. 2003).

From these studies it can be seen that sardine is omnivorous, with a highly diverse diet that varies both geographically and seasonally. Sardines have probably developed adaptations to predominant local conditions that are different within their area of distribution, and knowledge of seasonal variability in diet at different locations will permit a better understanding of the influence of the environment on the dynamics of this species, namely growth and reproduction.

The impact of sardine feeding on plankton communities also raises important questions regarding bottom-up mechanisms, one of them being the impact of cannibalism or predation of eggs and larvae of other fish species. More than 80% of the stomachs of sardines collected off the South African coast contained anchovy eggs, and it was estimated that 56% of the anchovy egg mortality was due to predation by sardines (Szeinfeld 1991). Fish eggs that may have been sardine eggs have also been found in stomach contents of sardine off Iberia (Silva 1954, Varela et al. 1988), and this may also represent an important source of mortality.

Extracting stomach contents and using several methods (frequency of occurrence, numeric, volumetric and gravimetric) to infer the relative importance of prey after their identification are essential in order to recognize patterns of seasonal and geographical variation in sardine diet. However, some food categories are quickly digested and very difficult to detect whilst others, even after ingestion, do not contribute to the diet because they are not successfully digested (Hyslop 1980). Studies involving stable isotopes and fatty acid trophic biomarkers constitute possible complementary techniques to improve the knowledge of marine fish diet. Furthermore, to fully understand the relationship between plankton availability and sardine feeding and be able to identify preferred food items, one must comprehend the mechanisms of prey recognition and preference, i.e. the feeding behaviour of the fish in the presence of different food items.

1.3.3 – Feeding behaviour

The diet of a fish reflects what is available in the environment, but is also dependent on the mechanisms of prey selection that the fish might possess. Selectivity can either be examined using indices that compare the proportion of prey in the stomachs to that available in the water in samples taken at the same time as fish samples were collected or through experiments in controlled conditions in the laboratory. The problem with the first method is that it assumes that stomach and habitat samples accurately reflect the relative abundance of the prey items in the environment, which for most cases is probably not true.

The quantitative and qualitative characteristics of the set of prey ingested by a predator per unit time are the product of the different success rates (one for each event) for each prey type corrected for the time used to detect, pursue, capture and retain a prey (Lazzaro 1987). All of these factors vary from species to species and for different environments. For example, the morphology of the feeding apparatus will determine the minimum size of prey the fish is able to retain, while the prey conspicuousness (which is a function of its size, shape, pigmentation and behaviour) will interact to determine the differential selection of prey in a fish diet. Environmental variables such as ambient light and other optical characteristics as well as predator visual ability will also influence how well a prey will be seen.

Most microphagous clupeoids possess two feeding modes and switch between the two depending on the feeding conditions, generally filter-feeding on smaller food particles and particulate-feeding on larger food particles. The ability to switch between these feeding modes makes these species highly opportunistic and flexible foragers which are able to maximise their energy intake employing the feeding mode most appropriate to a particular food environment.

Particulate-feeding is characterized by predators visually detecting and selecting individual prey items from the water column. Filter-feeding is characterized by surrounding prey items with the mouths held full agape while swimming rapidly (also recognized as tow-net filter feeding) or using rhythmic suctions to capture prey while remaining stationary or moving slowly (pump filter feeders). An intermediate feeding mode named “gulping” is similar to pump filter-feeding but is a visual feeding mode since fish were able to size-select their prey while using it was described for the alewife *Alosa pseudoharengus* and the cisco *Coregonus artedii* (Janssen 1976, 1978). The switch between filter- and particulate-feeding occurs as a consequence of different factors. It can depend on fish age, for example *Brevoortia tyranus* particulate feeds as larvae and filter feeds as adults (Durbin & Durbin 1975). Other fish retain the ability to change from one to the other feeding mode as adults, including *Engraulis mordax* (Leong & O'Connell 1969), *Scomber japonicus* (O'Connell & Zweifel 1972), *Alosa pseudoharengus* and *Coregonus artedii* (Janssen 1976), *Dorossoma petenense* (Holanov & Tash 1978), *Clupea harengus* (Gibson & Ezzi 1985), *Engraulis encrausicolus* (James & Findlay 1989), and *Sardinops sagax* (van der Lingen 1994). These fish alter between the two feeding modes depending of the concentration and/or size of the available prey, probably using the most efficient feeding mode in terms of energy intake in relation to the prey available, which varies according to fish species. Benguela sardines (*Sardinops sagax*) only use particulate-feeding if large prey are present in low concentrations. Calculations of respiration rate and swimming speed suggest that filter-feeding is the energetically less expensive feeding mode (van der Lingen 1995). Benguela anchovies (*Engraulis encrausicolus,* former *E. capensis*), on the other hand, start using particulate-feeding at lower prey sizes than sardines, and independently of prey concentration, and this feeding mode is the energetically cheapest for anchovy (James & Probyn 1989).

In the case of European sardines, the correspondence found between stomach contents and prey availability suggests that sardines are essentially filter feeders whose diet reflects plankton composition (Varela et al. 1990, Bode et al. 2003). However, Varela et al. (1988) found at some stations sampled off northern Spain that zooplankton items were far more abundant in sardine stomachs than in the water, where phytoplankton dominated, which suggests that particulate-feeding might also be used.

1.4 – Planktivorous fish as vectors of toxic microalgae, with special emphasis on the genus *Pseudo-nitzschia* spp.

Almost 80 phytoplankton species are considered to be producers of toxins (Sournia 1995), and depending on the combined action of several environmental factors can rapidly develop high population numbers and attain massive concentrations designated as harmful algae blooms (HAB's). Toxin accumulation in filter-feeding organisms can have devastating effects on higher-level predators, such as humans, sea birds, and marine mammals (Lefebvre et al. 2002). Domoic acid is one such toxin, and is naturally produced by some species of chain forming diatoms of the genus *Pseudo-nitzschia*. Domoic acid is a secondary amino acid that acts as an agonist to the main neurotransmitter, glutamate, and functions as a powerful neuroexcitor leading to neural damage (Debonel et al. 1989). This toxin is responsible for the neurotoxic illness called amnesic shellfish poisoning (ASP). Several ASP events have been reported from Canada, the USA, and New Zealand (e.g. Smith et al. 1990, Rhodes et al. 1996, Lefebvre et al. 1999), and domoic acid-producing species have also been detected in the coastal waters of Japan, Scotland, Denmark, Spain, Portugal, France and Italy (e.g. Míguez et al. 1996, Vale & Sampayo 2001, Cerino et al. 2005, Quiroga 2006). Bivalves are used as indicator species for the risk of human intoxication by domoic acid, but in at least two mass mortality events in the Monterey Bay, California, planktivorous fish were implicated as vectors of this neurotoxin (Fritz et al. 1992, Lefebvre et al. 1999, Scholin et al. 2000). In addition, anchovies kept in laboratory conditions were found to accumulate and suffer neurotoxic symptoms and mortality after ingestion of domoic acid (Lefebvre et al. 2001).

Domoic acid is detected in bivalves off the Portuguese coast several times during the year and high levels of toxicity have previously been registered for sardines collected off the southwestern Portuguese coast (Vale & Sampayo 2001). The occurrence of this ASP-causing toxin has been monitored by IPIMAR (Portuguese Institute for Sea and Fisheries Research) since 1992. The diatom that produces the toxin, *Pseudo-nitzschia* spp., frequently forms blooms off the western Portuguese coast as a result of enhanced productivity related to upwelling events that occur mainly during spring and summer months (Abrantes & Moita 1999) although it can also be a consequence of riverine inputs (Smith et al. 1990). The mechanisms of accumulation and excretion of domoic acid are well studied for bivalves, but not for planktivorous fish such as sardines whose diet includes phytoplankton, that might also be affected by the accumulation of this toxin as well as serving as vectors for the transmission to higher trophic levels.

1.5 – Fatty acids as trophic biomarkers in the marine pelagic foodweb

Animal lipids fulfil two broad general functions, the major role being the provision of energy but also being involved in the maintenance of the structural integrity of a wide variety of biological membranes (Cowey & Sargent 1972).

Fatty acids have carboxyl and methyl ends, and their chains are lengthened by the addition of two carbon atoms at a time to the carboxyl end. The designation of fatty acids is given according to the number of carbon atoms in the chain, the number of double bonds, and the inclusive number of carbon atoms from the terminal methyl to the carbon atom of the only or first double bond from the methyl end, also called the omega number. Therefore, 14:0 designates a fatty acid with 14 carbon atoms and no double bonds, while 18:1(n-9) designates a fatty acid with 18 carbon atoms, 1 double bond and

9 carbon atoms from the methyl end of the molecule. Saturated fatty acids (SATs) have no double bounds, monounsaturated fatty acids (MUFAs) have one double bond and polyunsaturated fatty acids (PUFAs) have two or more double bonds.

The interplay between dietary fatty acids and biosynthetic activities is especially important in considering the essential fatty acids required by fish. Marine fish with diets rich in lipids are not likely to biosynthesize fatty acids *de novo* to any significant extent, if they do so at all. This means that the lipid depots that fish accumulate are largely, if not exclusively, derived from dietary lipids (Tocher 2003). Every organism is capable of synthesizing SATs but all MUFAs are probably derived preformed from the diet. Fish cannot synthesize the PUFAs 18:2(n-6) or 18:3(n-3), but can convert these fatty acids from dietary sources into the longer chain fatty acids they require such as arachidonic acid (AA, 20:4(n-6)), eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n-3)). These three fatty acids are considered to be dietary essential fatty acids (Tocher 2003); DHA is a vital fatty acid for the neural and visual function of fish and AA is the chief precursor of the eicosanoids, which are hormone-like compounds produced by cells as a response to a variety of situations, namely blood clotting, immune and inflammatory response, renal function, neural function and reproduction. EPA competitively interferes with eicosanoid production, and therefore the ratio AA:EPA determines their action (Cowey & Sargent 1972). In contrast to fresh water species, it is likely that no marine fish species can convert any substantial amount of 18:3(n-3) to EPA and DHA (Cowey & Sargent 1972).

The fatty acid composition of several fish species is strongly and rapidly influenced by the composition of the diet (e.g. Linko et al. 1985, Iverson et al. 2002); e.g. the fatty acid composition of muscle of the Japanese sardine (*Sardinops melanostictus*) was influenced by spatial and seasonal variation in plankton composition (Shirai et al.

2002). In fact, fatty acids are in many circumstances incorporated into consumers in a conservative manner, permitting the fatty acid signatures to be used to identify trophic relationships in the marine environment. Contrary to gut content analysis, fatty acids provide information on dietary intake and food constituents over a long period of time, and are able to validate the importance attributed to prey through stomach content analysis (Dalsgaard et al. 2003). Furthermore, problems of differential digestion of prey that represent a bias in stomach content analysis are overcome by the fatty acid analysis. Two main problems arise from using fatty acids as trophic biomarkers to infer predator-prey relationships. The first is that no single fatty acid can be assigned uniquely to a prey species and secondly, the fatty acid composition of the predator can be altered by its metabolism.

Although individual fatty acids cannot be used as taxonomic indicators, combinations of fatty acids and fatty acid ratios give important insights to the diet of the fish (Daalsgard et al. 2003). Baccilariophyte fatty acid biomarkers include palmitoleic acid (16:1(n-7)), C16 and C20 fatty acids, C16 PUFA, EPA, and the ratios 16:1(n-7)/16:0 and EPA:DHA, and these are usually negatively correlated with C18 fatty acids. Dinophytes (together with Prymnesiophytes) are separated from the other algal classes by positive anomalies of stearic acid 18:0, oleic acid 18:1(n-9), C22 PUFA, DHA, and the ratio EPA:DHA. Strictly herbivorous copepods biosynthesise large amounts of 20:1(n-9) and 22:1(n-11) and high concentrations of C20 and C22 MUFA accumulate as the copepod grows, and these are used as tracers of herbivorous calanoid copepods in secondary and higher order consumers. Omnivorous and carnivorous copepods also biosynthesise fatty acids and accumulate large amounts of saturated fatty acids, but long chain MUFAs are not biosynthesized. Oleic acid 18:1(n-9) and the ratio 18:1(n-7)/18:1(n-9) are used to determine the degree of carnivory of the diet, while the ratio PUFA/SAT is considered an index to detect a change from omnivorous-carnivorous to herbivorous diet. The eggs and larvae of calanoid copepods, and the eggs of fish, are rich in DHA and EPA, while they are relatively deficient in long-chain MUFAs (Sargent & Falk-Peterson 1988).

Starvation, the production of large numbers of gametes in particular eggs, and migration, are examples of situations of high energy requirements which change fish fatty acid composition; for example, the energy requirements of the swimming red muscle are largely provided by fatty acids (Tocher 2003). The metabolism of fatty acids also depends on the physiological and behavioural traits of the fish, such as size and state of gonad maturity. In fish undergoing ovarian development, fatty acids mobilized from the diet are used for the biosynthesis of vitellogenin, which is transferred to the ovary (Sargent et al. 1989). The mobilization of particular fatty acids during this time depends on whether the fatty acids are used for the provision of metabolic energy or destined for gonad development; DHA, for example, is generally selectively transferred to the gonads due to its important role in neural and visual tissues of the larvae, while 22:1(n-11) and 20:1(n-9) are generally retained in the muscle (Wiegand 1996). Hence the fatty acid composition of fish muscle also depends on variations in the differential metabolism of fatty acids, which can be different during the spawning seasonal cycle and amongst sexes.

Some environmental factors can cause changes in the general composition of fatty acids in fish. For instance, lower temperatures are associated with an increase in the degree of unsaturation of fish lipids which itself is associated with changes in biomembrane fluidity at different temperatures (Cowey & Sargent 1972). However, the largest single factor in the environment that affects fish fatty acid composition is diet (Cowey & Sargent 1972).

The FA composition of fish is not only important for the well-being of the fish and particularly its successful reproduction, but also for human consumption (Sargent et al. 1989). Marine lipids have long been documented to be the major source of polyunsaturated fatty acids (PUFAs), especially DHA and EPA, that have a proven role in human health and nutrition, namely by preventing cardiovascular diseases, improving visual acuity and neural function and reducing inflammatory processes (reviews in Calder 2004, Fleith & Clandinin 2005, Wang et al. 2006). A higher ratio of (n-3)/(n-6) for human diet is also desirable, as prevention for many chronic diseases (Simopoulos 2002).

1.6 – Lipid nutrition and reproduction

The indeterminate relationship between the total biomass of spawners and the number of offspring produced, observed in many fish stocks has puzzled population dynamicists and impeded fisheries management (Marshall et al. 1999), but there is increasing awareness that the reproductive potential of individual fish affects recruitment. In Atlantic cod (*Gadus morhua*), the production of oocytes varies among years and during the spawning season and is related to maternal characteristics, with potential fecundity dependent on body weight (Kjesbu et al. 1998) and egg size dependent on fish age (Kjesbu et al. 1996).

The relationship between eggs and recruitment has been mainly viewed from the quantitative aspect, i.e., the number of eggs produced, and seldom from a qualitative aspect, i.e., egg quality (Schartzlose et al. 1999). However, a relationship between ovary mass and female lipid (and also the fatty acid EPA) content in the muscle was found for the Japanese sardine, *Sardinops melanostictus* (Morimoto 1991, 1998), indicating that the accumulation of reserves prior to the spawning season influences the quantity and quality of eggs produced, which is likely to have an effect on their survival. Similarly, two thirds of the energy required for spawning by northern anchovy (*Engraulis mordax*) over one spawning season comes from fat reserves stored almost a year previously during the annual spring bloom, and the remaining third is taken up during the current spawning season (Hunter & Leong 1981).

Fatty acids are not only the major source of metabolic energy in fish for growth from the egg to the adult, but also the major source of metabolic energy for reproduction (Sargent et al. 2002). Stored fat must support not only the immediate energy requirements of the parent fish but also the future requirements of the progeny.

Hence spawner nutrition is vital to producing eggs and larvae that have the optimal fatty acid composition to give the embryo the best chance of success (Sargent et al. 2002). Fatty acid composition has been related to egg quality in a large number of fish species (e.g. Fernández-Palacios et al. 1995, Bell et al. 1997, Almansa et al. 1999). For Iberian sardines, larval viability depends on the quality of the eggs produced (Riveiro et al. 2000), which is probably determined by the nutritional condition of females during or prior to the spawning season. The dietary fat of *Sardinops caerulea* (Lasker & Theilacker 1962) in the wild was shown to derive to a large extent from *Calanus helgolandicus*, being stored largely unaltered in the muscle, and gonad lipids were found to be identical.

Egg quality has been related to increased levels of HUFAs of the (n-3) series, particularly DHA and EPA, and also to increased levels of AA. Besides having a clearly established role in membrane structure and functioning, the HUFAs can serve as important energy sources during embryonic and early larval development of fish, especially marine species (Tocher 2003). Lipid utilization in marine fish generally occurs mainly after hatching, reflecting the greater energy demand of the free-swimming yolk-sac larvae compared to the egg (Sargent et al. 2002).

The spawning season of sardines off western and southern Portugal occurs practically year-round, although spawning is more frequent from September/October to March/April (Figueiredo & Santos 1989). This extended spawning season must result in a very high energetic requirement for reproduction, and probably causes the strong seasonal cycle in fat content that sardines experience off the Portuguese coast (Bandarra et al. 1997). Hence it seems likely that the availability of food for adult sardines will have a marked impact on their reproductive success, and be linked to the amount of reserves they are able to accumulate to sustain the spawning season and produce high quality eggs.

The survival, growth and reproduction of a fish depend on their energy and nutrient incomes generated from their feeding activities. Although fish have a considerable capacity to resist starvation and many fishes even cease to feed at times, their capacity to survive depends on being able to mobilize their accumulated reserves whenever needed (Wooton 1998). The size of those reserves will reflect their feeding success.

1.7 – Aims and Structure of this Dissertation

The aim of this dissertation is to describe the feeding ecology of the sardine *Sardina pilchardus* (Walbaum 1792) off Portugal and relate it to condition and reproduction.

A combined laboratory and field approach was employed to fulfil the above aim, with the following studies being conducted:

1) A laboratory examination of the feeding behaviour of sardine, and how feeding behaviour is affected by prey type, size and concentration;

2) A field investigation on temporal and geographical variation in the diet of sardines off the Portuguese coast, in which the diets of fish from two areas (the south and west coasts) characterized by different hydrological regimes related to the strength of seasonal upwelling events were examined and compared. In addition, the potential of using remotely-sensed chlorophyll data for each area to proxy sardine feeding intensity was evaluated. The two fishing ports selected in this study from which sardine samples were collected were Peniche, located along the central west coast, and Portimão on the south coast of Portugal. Given the differences in the main oceanographic features and associated productivity of these areas described above (see previous section on 'Hydrological features off Portugal and productivity') it was hypothesized that the two locations provide different feeding conditions for sardines as a result of different food web structure and seasonality;

3) A field study of the relationship between ingestion by sardines of the chain-forming diatom *Pseudo-nitzschia* spp. and the accumulation of the toxin domoic acid in their tissue;

4) An investigation of the temporal and geographical variation in the fatty acid composition of sardine diet, in which fatty acid biomarkers in sardine stomach contents were compared with muscle fatty acid composition and their effect on sardine condition assessed, and;

5) A study of the effects of female condition on the fatty acid concentration and composition of the oocytes produced.

The present dissertation is organized in 7 Chapters. This chapter provides a general introduction, and Chapters 2 to 6 present each of the above objectives; each of those chapters is in the form of a peer-reviewed scientific paper and contains a brief introduction, details of the particular methods used, a description of the results obtained, and discussion about the implications of those results. The last chapter summarizes the conclusions drawn from this work and presents future perspectives.

The research carried out in this thesis was developed within the programme PELAGICOS funded by the Portuguese Foundation for Science and Technology, that aims at describing and recovering data concerning the biology and ecology of, and fisheries for, small pelagic fish off Portugal. Specifically, this thesis work contributed to the task "Spatio-temporal variation in prey abundance available to recruits and adult fish". The research described in this thesis has contributed to knowledge of the trophic ecology of sardines, particularly off the Portuguese continental coast, and will be useful in future studies examining the relationship between sardine feeding ecology and population dynamics.

1.8 - References

Abrantes F & Moita MT (1999) Water column and recent sediment data on diatoms and cocolithophorids, off Portugal, confirm sediment record upwelling events. Oceanol Acta 22: 319-336

Almansa E, Perez MJ, Cejas JR, Badia P, Villamandos JE, Lorenzo A (1999) Influence of broodstock gilthead seabream (*Sparus aurata* L.) dietary fatty acids on egg quality and egg fatty acid composition throughout the spawning season. Aquaculture 170 (3-4): 323-336

Amenzoui K, Ferhan-Tachinante F, Yahyaoui A, Kifani S, Mesfioui AH (2006) Analysis of the cycle of reproduction of *Sardina pilchardus* (Walbaum, 1792) off the Moroccan Atlantic coast. Comptes Rendus Biologies 329 (11): 892-901

Andreu B (1969) Las branquispinas en la caracterización de las poblaciones de *Sardina pilchardus* (Walb.). Inv Pesq 33: 425-607

Bandarra NM, Batista I, Nunes ML, Empis JM, Christie WW (1997) Seasonal changes in lipid composition of sardine (*Sardina pilchardus*). J Food Sci 62 (1): 40-42

Barbosa A (2006) Estrutura e dinâmica da teia alimentar microbiana na Ria Formosa. PhD Thesis, University of the Algarve, Algarve

Bell JG, Farndale BM, Bruce MP, Navas JM, Carillo M (1997) Effects of broodstock dietary lipid on fatty acid compositions of eggs from sea bass (*Dicentrarchus labrax*). Aquaculture 149 (1-2): 107-119

Bode A, Alvarez-Ossorio MT, Carrera P, Lorenzo J (2004) Reconstruction of trophic pathways between plankton and the North Iberian sardine (*Sardina pilchardus*) using stable isotopes. Sci Mar 68: 165-178

Bode A, Carrera P, Lens S (2003) The pelagic foodweb in the upwelling ecosystem of Galicia (NW Spain) during Spring: natural abundance of stable carbon and nitrogen isotopes. ICES J Mar Sci 60: 11-22

Borges MF, Santos AMP, Crato N, Mendes H, Mota B (2003) Sardine regime shifts off Portugal: a time series analysis of catches and wind conditions. Sci Mar 67: 235-244 Suppl 1

Calder PC (2004) n-3 fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clinical Sci 107 (1): 1-11

Carrera P & Porteiro C (2003) Stock dynamic of the Iberian sardine (*Sardina pilchardus*, W.) and its implication on the fishery off Galicia (NW Spain). Sci Mar 67: 245-258 Suppl 1

Cerino F, Orsini L, Sarno D, Dell'Aversano C, Tartaglione L, Zingone A (2005) The alternation of different morphotypes in the seasonal cycle of toxic diatom *Pseudo-nitzschia galaxiae*. Harmful Algae 4:33-48

Cowey CB & Sargent JR (1972) Fish nutrition. Adv Mar Biol 10: 383-492

Culley M (1971) The Pilchard: Biology and Exploitation. Pergamon Press, Oxford & NY 1st ed. 241pp

Cunha ME (1993) Spatial variation of the zooplankton biomass in relation to the hydrographyc conditions off the Portuguese coast. ICES CM 1993/L:63, 9 pp

Cunha ME, Garrido S, Pissarra J (2005) The use of stomach fullness and colour indices to assess *Sardina pilchardus* feeding. J Mar Biol Ass U.K. 85: 425-431

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003) Fatty acid trophic markers in the pelagic marine environment. Adv Mar Biol 46: 225-340

Debonnel G, Beauchesne L, de Montigny C (1989) Domoic acid, the alleged "mussel toxin", might produce its neurotoxic effect through kainite receptor activation:

an electrophysiological study in the rat dorsal hippocampus. Can J Physiol Pharmac 67: 29-33

Durbin AG & Durbin EG (1975) Grazing rates of Atlantic menhaden *Brevoortia tyrannus* as a function of particle size and concentration. Mar Biol 33 (3): 265-277

Fernández-Palacios H, Izquierdo MS, Robaina L, Valencia A, Salhi M, Vergara JM (1995) Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L). Aquaculture 132 (3-4): 325-337

Figueiredo, I. & Santos, A.M. (1989) Reproductive biology of *Sardina pilchardus* (Walb.): Seasonal maturity evolution (1986 to 1988) ICES CM 1989/H:40

Fiúza AFG (1983) Upweling patterns off Portugal. I: Coastal upwelling Pt A. Ed by Erwin Suess and Jorn Thiede, Plenium Publish. Corporation

Fiúza AFD, DeMacedo ME, Guerreiro MR (1982) Climatological space and time-variation of the portuguese coastal upwelling. Oceanol Acta 5 (1): 31-40

Fleith M & Clandinin MT (2005) Dietary PUFA for preterm and term infants: Review of clinical studies. Critical Rev in food Sci and Nutrition 45 (3): 205-229

Fritz L, Quilliam MA, Wright JLC, Beale A, Work TM (1992) An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudonitzschia australis*. J Phycol 28: 439-442

Garrido S (2003) Alimentação de *Sardina pilchardus* (Walbaum, 1792) ao largo da costa continental portuguesa e implicações da condição nutricional das fêmeas na qualidade dos oócitos. MS thesis, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Porto

Gibson RN & Ezzi IA (1985) Effect of particle concentration on filter-feeding and particulate-feeding in the herring *Clupea harengus*. Mar Biol 88 (2): 109-116

Hickling C (1945) The seasonal cycle in the cornish pilchard, *Sardina pilchardus* (Walb). J Mar Biol Assoc UK 26: 115–138

Holanov SH & Tash JC (1978) Particulate and filter feeding in threadfin shad, *Dorosoma petenense*, at different light intensities. J Fish Biol13 (5): 619-625

Hunter JR & Leong R (1981) The spawning energetics of female northern anchovy, *Engraulis mordax*. Fish Bull 79 (2): 215-230

Hyslop EJ (1980) Stomach content analysis - a review of methods and their application. J Fish Biol 17: 411-429

ICES (2006) Report of the working group on the assessment of mackerel, horse mackerel, sardine and anchovy. International Council for the Exploration of the Sea (CM Papers and Reports) CM 2006/ ACFM 08, 631pp

Iverson SJ, Frost KJ, Lang SLC (2002) Fat content and fatty acid composition of forage fish and invertebrates in Prince William Sound, Alaska: factors contributing to among and within species variability. Mar Ecol Progr Ser 241: 161-181

James AG & Findlay KP (1989) Effect of particle size and concentration on feeding-behaviour, selectivity and rates of food ingestion by the Cape anchovy *Engraulis capensis*. Mar Ecol Progr Ser 50 (3): 275-294

James AG & Probyn T (1989) The relationship between respiration rate, swimming speed and feeding behaviour in Cape anchovy *Engraulis capensis* Gilchrist. J Exp Mar Biol Ecol 131: 81-100

Janssen J (1976) Feeding modes and prey size selection in alewife (*Alosa pseudoharengus*). J Fish Res Bd of Canada 33 (9): 1972-1975

Janssen J (1978) Feeding-behaviour repertoire of alewife, *Alosa pseudoharengus*, and *Ciscoes coregonus hoyi* and *Ciscoes artedii*. J Fish Res Bd Canada 35 (2): 249-253

Kifani S (1998) Climate dependent fluctuations of the Moroccan sardine and their impact on fisheries. In: Global versus local changes in upwelling systems. Eds. MH Durand, P Curry, R Mendelssohn, C Roy, A Bakun, D Pauly. Edition de l'Ostrom. Institut Français de Reserche Scientific pour le Development en Cooperation

Kjesbu OS, Solemdal P, Bratland P, Fonn M (1996) Variation in annual egg production in individual captive Atlantic cod (*Gadus morhua*). Can J of Fish and Aquat Sci 53 (3): 610-620

Kjesbu OS, Witthames PR, Solemdal P, Walker MG (1998) Temporal variations in the fecundity of Arcto-Norwegian cod (*Gadus morhua*) in response to natural changes in food and temperature. J of Sea Res 40 (3-4): 303-321

Lasker R & Theilacker GH (1962) Fatty acid composition of the lipids of some Pacific sardine tissues in relation to ovarian maturation and diet. J of Lipid Res 3 (1): 60-64

Lazzaro X (1987) A review of planktivorous fishes - their evolution, feeding behaviours, selectivities, and impacts. Hydrobiol 146 (2): 97-167

Lefebvre KA, Dovel SL, Silver MW (2001) Tissue distribution and neurotoxic effects of domoic acid in a prominent vector species, the northern anchovy *Engraulis mordax*. Mar Biol 138 (4): 693-700

Lefebvre KA, Powell CL, Busman M, Doucette GJ, Moeller PDR, Silver JB, Miller PE, Hughes MP, Singaram S, Silver MW, Tjeerdema RS (1999) Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. Nat Toxins 7:85-92

Lefebvre KA, Silver MW, Coale SL, Tjeerdema RS (2002) Domoic acid in planktivorous fish in relation to toxic *Pseudo-nitzchia* cell densities. Mar Biol 140: 625-631

Leong RJH & O'Connell CP (1969) A Laboratory study of particulate and filter feeding of northern anchovy (*Engraulis mordax*). J Fish Res Bd Canada 26 (3): 557-582

Linko RR, Kaitaranta JK, Vuorela R (1985) Comparison of the fatty acids in Baltic herring and available plankton feed. Comp Biochem and Physiol B-Biochem. & Molecular Biol. 82 (4): 699-705

Mann KH (2000) Ecology of coastal waters, with implications for management. Blackwell Publishing Professional; 2 ed., Malden USA

Marshall CT, Yaragina NA, Lambert Y, Kjesbu OS (1999) Total lipid energy as a proxy for total egg production by fish stocks. Nature 402 (6759): 288-290

Massuti M & Oliver M (1948) Estudio de la biometría y biología de la sardina de Mahón (Baleares), especialmente de su alimentación. Bol Inst Esp Oceanogr 3: 1-15

Mathisen OA, Thorne RE, Trumble RJ, Blackburn M (1978) Food consumption of pelagic fish in a upwelling area. In Upwelling Ecosystems Ed. R. Boje and M. Tomczak, Springer-Verlag, NY: 111-123

Míguez Á, Fernández ML, Fraga S (1996) First detection of domoic acid in Galicia (NW of Spain) In: Yasumoto T, Oshima Y, Fukuyo T (Eds). Harmful and Toxic Algal Blooms. IOC, UNESCO, Paris, pp 143-145

Moita MT (2001) Estrutura, variabilidade e dinâmica do fitoplâncton na costa de Portugal continental. PhD Thesis, University of Lisbon, Lisbon

Morimoto H (1991) Relationship between reproductive ability and nutritional body condition in Japanese sardine. ICES CM 1991/H:19

Morimoto H (1998) Relations between batch fecundity and egg size in Japanese sardine *Sardinops melanostictus* in Tosa Bay and off Kii Channel, southwestern Japan from 1990 to 1993. Fish Sci 64 (2): 220-227

Navarro FP & Navaz JM (1946) Apuntes para la biología y biometría de la sardina, anchoa, boga y chicharro de las costas vascas. Inst. Esp. Oceanogr., Notas y Resúmenes, 134

O'Connell CP & Zweifel JR (1972) Laboratory study of particulate and filter feeding of pacific mackerel, *Scomber japonicus*. Fish Bull (Wash DC) 70 (3): 973-981

Oliver M (1951) La sardina de la costa noroeste española en 1948 y 1949 (Estudio biométrico y biológico). Bol Inst Esp Oceanogr, 42: 1-22

Oliver M & Navarro FP (1952) Nuevos datos sobre la sardina de Vigo. Bol Inst Esp Oceanogr 56: 25-39

Parrish RH, Serra R, Grant WS (1989) The monotypic sardines, *Sardina* and *Sardinops*: their taxonomy, distribution, stock structure, and zoogeography. Can J Fish Aquat Sci 46: 2019-2036

Peliz AJ & Fiúza AFG (1999) Temporal and spatial variability of CZCS-derived phytoplankton pigment concentrations off the western Iberian Peninsula. Intern J of Remote Sensing 20 (7): 1363-1403

Pestana G (1989) Nota histórica sobre a avaliação do recurso de sardinha e seu desenvolvimento programático em Portugal (1950-1989). Prova complementar para as provas de acesso à categoria de Investigador auxiliar no IPIMAR

Quiroga I (2006) *Pseudo-nitzschia* blooms in the Bay of Banyuls-sur-Mer, northwestern Mediterranean Sea. Diatom Res 21 (1): 91-104

Ré P, Cabral e Silva R, Cunha E, Farinha A, Menezes I, Moita T (1990) Sardine spawning off Portugal. Boletim do Instituto Nacional de Investigação das Pescas, 15: 31-44

Relvas P & Barton ED (2002) Mesoscale patterns in the Cape São Vicente (Iberian Peninsula) upwelling region. J of Geophys Res 107(C10) 3164 doi:10.1029/2000JC00 0456

Ribeiro AC, Peliz A, Santos AMP (2005) A study of the response of chlorophyll-a biomass to a winter upwelling event off Western Iberia using SeaWiFS and in situ data. J of Mar Syst 53 (1-4): 87-107

Riveiro I, Guisande C, Lloves M, Maneiro I, Cabanas JM (2000) Importance of parental effects on larval survival in *Sardina pilchardus*. Mar Ecol Progr Ser 205: 249-258

Rhodes LL, White D, Syhre M, Atkinson M (1996) *Pseudo-nitzschia* species isolated from New Zealand coastal waters: domoic acid production in vitro and links with shellfish toxicity. In: Yasumoto T, Oshima Y, Fukuyo T (Eds). Harmful and Toxic Algal Blooms. IOC, UNESCO, Paris, pp 155-158

Ryther JH (1969) Relationship of photosynthesis to fish production in the sea. Science 166: 72-76

Sanchez RF, Mason E, Relvas P, da Silva AJ, Peliz A (2006) On the inner-shelf circulation in the northern Gulf of Cadiz, southern Portuguese shelf. Deep-Sea Res II(11-13): 1198-1218

Sargent JR & Falk-Peterson S (1988) The Lipid Biochemistry of calanoid copepods. Hydrobiol 167: 101-114

Sargent JR, Henderson RJ, Tocher DR (1989) The Lipids. In Fish Nutrition (ed JR Halver): 154-219. San Diego: Academic Press

Sargent JR, Henderson RJ, Tocher DR (2002) The Lipids. In Fish Nutrition (ed. JR Halver) pp 154-219. San Diego: Academic Press

Schwartzlose RA, Alheit J, Bakun A, Baumgartner TR, Cloete R, Crawford RJM, Fletcher WJ, Green-Ruiz Y, Hagen E, Kawasaki T, Lluch-Belda D, Lluch-Cota SE, MacCall AD, Matsuura Y, Nevárez-Martínez MO, Parrish RH, Roy C, Serra R, Shust KV, Ward MN, Zuzunaga JZ (1999) Worldwide large-scale fluctuations of sardine and anchovy populations. S Afr J Mar Sci 21: 289-347

Scholin CA, Gulland F, Doucette GJ, BensonS, Busman M, Chavez FP, Cordaro J, DeLong R, De Vogelaere A, Harvey J, Haulena M, Lefebvre K, Lipscomb T, Loscutoff S, Lowenstine LJ, Martin RI, Miller PE, McLellan WA, Moeller PDR, Powell CL, Rowles T, Silvagnl P, Silver M, Spraker T, Trainer V, Van Dolah FM, (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403:80-84

Shirai N, Terayama M, Takeda H (2002) Effect of season on the fatty acid composition and free amino acid content of the sardine *Sardinops melanostictus*. Comp Biochem Physiol B 131: 387-393

Silva A (2003) Morphometric variation among sardine (*Sardina pilchardus*) populations from the northeastern Atlantic and the western Mediterranean. ICES J Mar Sci 60 (6): 1352-1360

Silva A, Santos MB, Caneco B, Pestana G, Porteiro C, Carrera P, Stratoudakis Y (2006) Temporal and geographic variability of sardine maturity at length in the northeastern Atlantic and the western Mediterranean. ICES J of Mar Sci 63 (4): 663-676

Silva E (1954) Some notes on the food of the pilchard *Sardina pilchardus* (Walb.), of the Portuguese coasts. Rev Fac Cienc Lisboa, 2ª Série vol 4 fasc. 2º:281-294

Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & Pharmacotherapy 56 (8): 365-379

Smith JC, Cormier R, Worms J, Bird CJ, Quilliam MA, Pocklington R, Angus R, Hanic L (1990) Toxic blooms of the domoic acid containing diatom *Nitzschia pungens* in Cardigan river, Prince Edward Island, in 1988. In: Granéli E, Sundström B, Edler L, Anderson DM (Eds). Toxic marine phytoplankton. Elsevier, New York, pp 227-232

Sournia A (1995) Red tide and toxic marine phytoplankton of the world ocean: An inquiry into biodiversity. In: Lassus P, Arzul G, Erad E, Gentien P, Marcaillou C (Eds). Harmful Marine Algal Blooms. Lavoisier, Paris, pp 103-112

Suau P (1959) Contribution a la connaissance des stocks de sardines de la Méditerranée espagnole. Proc Gen Fish Coun Medit 5: 453-458

Szeinfeld EV (1991) Cannibalism and intraguild predation in clupeoids. Mar Ecol Prog Ser (79): 17-26

Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci 11 (2): 107-184

Vale P & Sampayo MAM (2001) Domoic acid in Portuguese shellfish and fish. Toxicon 39:893-904

van der Lingen CD (1994) Effect of particle size and concentration on the feeding behaviour of adult sardine *Sardinops sagax*. Mar Ecol Prog Ser 109:1-13

van der Lingen CD (1995) Respiration rate of adult pilchard *Sardinops sagax* in relation to temperature, voluntary swimming speed and feeding behaviour. Mar Ecol Progr Ser 129: 41-54

Varela M, Alvarez-Ossorio MT, Valdés L (1990) Método para el estudio cuantitativo del contenido estomacal de la sardina. Resultados preliminares. Bol Inst Esp Oceanogr 6: 117-126

Varela M, Larrañaga A, Costas E, Rodríguez B (1988) Contenido estomacal de la sardina (*Sardina pilchardus*, Walbaum) durante la campaña Saracus 871 en las plataformas Cantábrica y de Galicia en Febrero de 1987. Bol Inst Esp Oceanogr 5: 17-28

Vučetic T (1963) Sur la nutrition de la sardine adulte (*Sardina pilchardus* Walb) dans la partie moyenne de l'Adriatique orientale. Acta Adriatica 2: 1-47

Wang CC, Harris WS , Chung M, Lichtenstein AH, Balk EM, Kupelnick B, Jordan HS, Lau J (2006) n-3 fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. American J of Clinical Nutrition 84 (1): 5-17

Wiegand MD (1996) Composition, accumulation and utilization of yolk lipids in teleost fish. Rev Fish Biol Fish 6 (3): 259-286

Wootton RJ (1998) Ecology of Teleost Fishes. 2nd ed. Kluwer Academic Publishers, Fish and Fisheries Series 24, Dordrecht, The Netherlands, 386 pp

CHAPTER 2

LABORATORY INVESTIGATIONS ON THE EFFECT OF PREY SIZE AND CONCENTRATION ON THE FEEDING BEHAVIOUR OF SARDINE (*Sardina pilchardus*)[*]

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[*] Garrido S, Marçalo A, Zwolinski J, van der Lingen CD (2007) Laboratory investigations on the effect of prey size and concentration on the feeding behaviour of *Sardina pilchardus*. Mar Ecol Progr Ser 330: 189-199

2.1 – Abstract

Laboratory experiments were conducted to study the effects of different prey types and concentrations on the feeding behaviour of Iberian sardine (*Sardina pilchardus*). Experiments consisted of providing known concentrations of different prey types (both single prey type and a mixture of prey types were used) to a group of sardines acclimated to laboratory conditions and observing their feeding behaviour. Data on feeding mode choice, feeding selectivity, and filtration efficiency were collected, and clearance rates for different prey types and sizes were estimated. Sardines use two feeding modes and switch between the two depending on prey size; filter-feeding was used to capture prey ≤724 µm and particulate feeding to capture prey ≥780 µm, therefore the feeding mode switch occurs within these limits. Sardine are able to feed on nanoplankton and can retain prey items as small as 4 µm, and filtration efficiency increases from 20% at this prey size to close to maximum for prey >200 µm. Sardines show selective feeding, preferentially ingesting fish eggs compared to other prey types (even larger fish larvae) when fed cultured, mixed prey assemblages, and selecting copepods and decapods over other zooplankton prey when fed wild-collected, mixed prey assemblages. Clearance rates were generally low compared to other clupeids, arising from the smaller mouth gape and lower swimming speed of this species. Results obtained from this study suggest that filter-feeding is the dominant feeding mode of sardine and that it is able to efficiently utilize microplankton prey, and corroborate previous dietary studies indicating that small zooplankton and chain-forming diatoms dominated stomach contents.

2.2 – Introduction

The sardine (*Sardina pilchardus*, Walbaum 1792) is the main target of the purse-seine fishery off Portugal and Spain, with average annual landings of 100 000 tons during the last decade (ICES 2006). High fluctuations in both population size and catches off the Iberian coast have stimulated several studies of this commercially important resource (*e.g.* Borges et al. 2003, Stratoudakis et al. 2003, Guisande et al. 2004). Studies of the trophic ecology of sardine (Bode et al. 2003, 2004, Costa & Garrido 2004) have followed the assumption that the feeding ecology of small pelagic fishes is a key factor in regulating their abundance and distribution (*e.g.* Mathisen et al. 1978, Parrish et al. 1981, Schwartzlose et al. 1999).

Previous studies based on stomach content (Varela et al. 1988, 1990, Garrido 2003, Cunha et al. 2005) and stable isotope analyses (Bode et al. 2003, 2004) have shown that sardines have a highly diverse diet, dominated in terms of biovolume by micro and mesozooplankton prey. High numbers of phytoplankton cells, mainly chain-forming diatoms and dinoflagellates, usually occur in sardine stomach contents, but these generally represent <10% of total prey biovolume (Cunha et al. 2005). The correspondence between the ambient food environment and stomach contents suggest that sardines are essentially filter-feeders whose diet reflects plankton composition (Varela et al. 1990, Bode et al. 2003). However, although stomach content analysis is an essential tool to study seasonal and geographical variability in the diet of planktivorous fish, it is insufficient to address important aspects related to feeding behaviour, which may clarify the relationship between fish population dynamics and food availability.

Knowledge of the feeding behaviour of planktivorous fish allows the identification and recognition of favourable feeding conditions/environments for the fish, the potential niche separation between different co-habiting fish species, and the estimation of their impacts on plankton communities. Several laboratory experiments with planktivorous small pelagic fish species have shown that feeding behaviour and ingestion rates depend primarily on prey size and concentration (e.g. Leong & O'Connell 1969, Janssen 1976, Friedland et al. 1984, James & Findlay 1989, Gibson & Ezzi 1990, van der Lingen 1994). Planktivorous fish generally feed through filter-feeding, particulate-feeding or both (Lazzaro 1987), and it is assumed that if an individual alters its feeding behaviour in response to changing prey conditions, that change will result in a greater net energy gain (Gibson & Ezzi 1992).

The objective of this work was to study the feeding behaviour of *Sardina pilchardus*, specifically to characterize its feeding mode, swimming velocity and ingestion rates for different phyto, zoo and ichthyoplankton prey types and concentrations. To meet this objective several laboratory experiments were performed that closely followed the protocol used by James & Findlay (1989) and van der Lingen (1994) for Cape anchovy (*Engraulis encrasicolus;* formerly *E. capensis*; Grant & Bowen 1998) and sardine (*Sardinops sagax*), respectively. Experiments aimed at identifying and/or clarifying aspects of sardine feeding behaviour, including (1) a determination of whether *S. pilchardus* show preferential feeding modes that are adapted to specific environmental feeding conditions, (2) quantification of the filtering efficiency (the efficiency of the filtering apparatus in retaining particles, expressed as a % of the estimated maximum clearance rate), particularly for small sized prey, which will allow definition of prey length spectra available to sardines, (3) estimation of the feeding rates for different prey types, an essential input term for future bioenergetics studies, and (4) whether *S. pilchardus* exhibits feeding selectivity, which is crucial for estimating the impact of this species on plankton communities.

2.3 – Material and methods

2.3.1 – Acclimatization

Iberian sardines were captured in June 2003 by a commercial purse-seiner operating from the port of Olhão in southern Portugal. Details regarding capture and transfer techniques used are given by Peleteiro et al. (2004). The fish were transported to the Aquaculture Station of Olhão (IPIMAR) in 300 l plastic covered tanks, and there were transferred into 1500 l circular tanks supplied with a continuous flow of 100 µm sand-filtered sea water at ambient temperature (17.9 ± 0.6º C) and salinity (37). Sardines were acclimated to laboratory conditions for 4 months prior to the onset of experimentation, and were fed twice-daily with dry pellets (1mm diameter "Aquasoja" pellets) at an estimated daily ration of 1 to 2% total fish mass.

2.3.2 – Food types

Both cultured and wild plankton were used in a total of 24 experiments, 18 of which used single prey types and 6 of which used combinations of different prey types. Cultured zooplankton included *Brachionus plicatilis* and *Artemia salina* (nauplii to adults), and cultured phytoplankton included the diatom *Skeletonema costatum* (single cells only) and the chlorophyte *Tetraselmis suecica*. Additionally, eggs and larvae of seabream *Sparus aurata* and sole *Solea senegalensis* cultured at the Aquaculture Station were fed to the sardines. Wild plankton was collected off the port of Olhão using 53 and 20 µm WP2 drift nets towed for 30 minutes to 1 hour; collected plankton was then transported to the laboratory and immediately used in experiments.

To determine the prey length at which sardines changed from filter- to particulate-feeding, a set of six experiments using the same initial density of either *Brachionus* *plicatilis* or *Artemia salina* of successively increasing length were performed, assuming that the potential transition would occur within the range of 200 to 1000 µm. In order to study the effect of prey density on sardine feeding behaviour and ingestion rates, and to assess whether sardines filter-feed on high densities of large prey items, fish eggs were added to the experimental tank at 3 different initial concentrations. Experiments were done to 1) examine whether sardines are able to feed selectively when offered a mixed assemblage of prey, and 2) compare ingestion rates of sardine on particular prey items offered as the only prey type and as part of a mixed prey assemblage. To achieve these objectives experiments were conducted using mixed assemblages of both cultured (Experiments 19 – 22, Table 2.2) and wild (Experiments 23 – 24, Table 2.3) plankton. These assemblages comprised both small (assumed to elicit a filter-feeding response) and large (assumed to elicit a particulate-feeding response) prey items.

2.3.3 – Experimental set up

We studied the feeding behaviour of a small group of sardines following the introduction of known concentrations of different food types into the tank (van der Lingen 1994). Experiments were recorded using a digital Sony VX 1000 digital video camera mounted 3 m above the tank. Changes in food concentration during an experiment were determined through sampling at regular intervals. Groups of 14 sardines of the same length class (19.3 ± 1.9 cm total length) were transferred to a 530 l fibreglass experimental tank and acclimated there for 2 weeks, including a 4 day period before each set of experiments during which fish were not fed. With the 4 day starvation period we tried to standardize the hunger state (without compromising their condition) since sardines have been used to feeding on dry pellets for 4 months. The tank had a flat acrylic bottom marked with grid lines spaced 10 cm apart that were used as a reference for video recording (see below). In order to avoid habituation, each school was only used for 10 consecutive experiments before being replaced (van der Lingen 1994).

At the start of each experiment the water supply to the tank was switched off (experiments were performed with no water flow) and the fish were filmed for 15 minutes to determine non-feeding behaviour and swimming speeds. A known concentration of food contained in 5 l of water was then added to the tank, while a porous air tube placed around the inner tank perimeter was activated for 3 minutes to promote a homogeneous distribution of prey within the tank. Immediately after mixing, water samples were collected to determine prey density at the beginning of the experiment ($t = 0$), and samples were subsequently collected every 10 minutes during the first hour and every 30 minutes thereafter until the end of the experiment.

The concentration of zoo- and ichthyoplankton prey in the tank was measured at each sampling time by taking 5 replicate samples using a PVC sampling tube that fitted into a 200 µm mesh-bottomed cup and sampled a volume of 0.589 l. Phytoplankton prey concentration in the tank was measured by collecting 3 replicates of 250 ml. Prey items in each replicate sample were counted under a stereoscope (x63; for zoo- and ichthyoplankton), an inversion microscope (x400; for natural phytoplankton) or a Coulter Counter EPICS XL (for cultured phytoplankton). Food items were either counted immediately or were preserved in 4% buffered formalin for later examination. Thirty individual food particles were measured (major and minor axis) for each experiment to determine average particle size and to estimate mean prey biovolume by assuming the closest geometric shape.

Analyses of video recordings taken during experiments were performed to determine feeding mode (whether filter- or particulate-feeding) used by sardines and fish swimming speed. A second video camera positioned close to the tank was used to determine the fish mouth gape and feeding bout duration during 2 experiments of each feeding mode (Experiments 6, 8, 15 and 17; see Table 2.1) in a total of 78 feeding act durations measured, and it was assumed that the values obtained were representative of each feeding mode. The average feeding bout duration was calculated by counting the number of frames sardines spent with their mouths open in the course of the experiments, and the time spent with the mouth closed between two successive feeding acts was also determined. Swimming speed was determined by counting the number of frames an individual sardine took to completely cross a grid line, and was recorded as body lengths per second (bl s^{-1}). Sardines whose path deviated more than 20º during a swimming speed measurement were excluded. We made 30 measurements of swimming speed for each time interval between successive samplings of the food concentration, excluding the first 3 minutes after subsampling since this could have interfered with feeding behaviour and stressed the sardines.

The duration of the experiments was pre-determined as 1.5h for experiments using zoo- and ichthyoplankton prey and 4.5h for experiments using phytoplankton prey, unless sardines had completely removed prey and ceased feeding before this time.

2.3.4 – Data analysis

Food concentration was tested for an exponential decrease with time using generalized linear models (GLM; Venables & Ripley 2002), as $s(concentration) = \beta 0 + \beta 1(time)$ where s is the logarithm link function and concentration follows either a Poisson or Negative Binomial distribution. Initial prey concentration was obtained as the exponential of the model intercept $\beta 0$. The estimated slope parameter $\beta 1$, is then equivalent to the instantaneous grazing rate, g, defined by Durbin & Durbin (1975). Testing for differences in g according to food types and experiments was done by deviance analysis at a significance level of $p < 0.05$. Statistical analysis was performed using the open source software R 1.9 (R Development Core Team 2005).

The clearance rate F or volume swept clear (Harvey 1937) is calculated from the equation: $F = Vg/N$ (l fish^{-1}min^{-1}), where V = *water volume in the tank* and N = *number of fish in the tank*. The clearance rate represents the volume of water from which prey would have to have been removed to account for the measured decline in food concentration. The clearance rate for each experiment in which sardines used filter-feeding was compared to the maximum clearance rate in order to assess filtration efficiency. Maximum clearance rate was calculated from: $F_{max} = Sw\ Mg\ Mo$, where Sw = *swimming speed* (cm s^{-1}), Mg = *mouth gape* (cm^2) and Mo = *percentage of time spent with their mouths open* (%). The comparison of F with F_{max} is a measure of the retention ability for the food particles, and when $F = F_{max}$ it is assumed that all the particles in the water passing through the gill rakers are retained and that filtering efficiency is 100%. Initial prey biovolume was calculated by multiplying the initial prey concentration by the average prey biovolume.

2.4 – Results

2.4.1 – Feeding modes

Before the introduction of food to the experimental tank sardines swam around continuously in a loose shoal, with a mean (±SD) swimming speed of 0.93 ± 0.23 bl s^{-1}, equivalent to 18.1 ± 4.5 cm s^{-1} (Fig. 2.1). The introduction of food items in all experiments elicited a feeding response, characterized by an increase in swimming speed and the initiation of filter- or particulate-feeding, depending on prey size (Table 2.1). Mg while feeding was determined as 0.5 (0.1) cm^2.

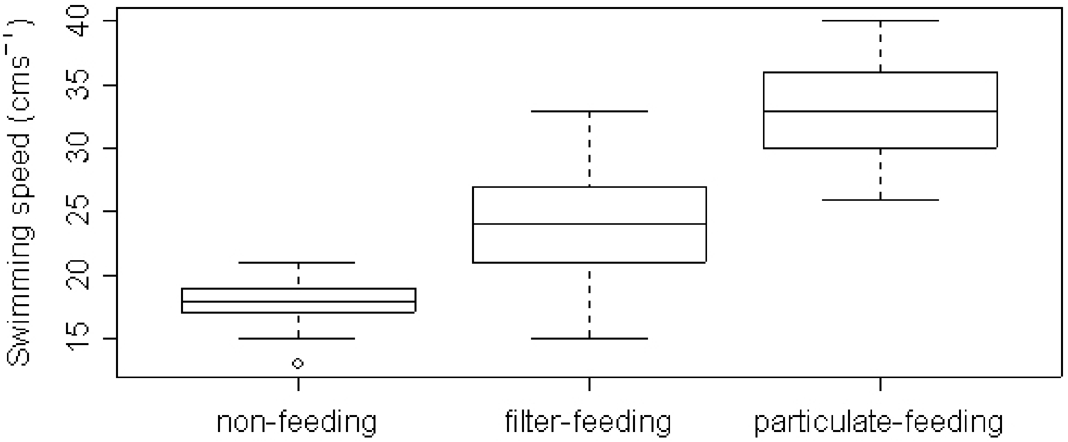

Figure 2.1 – Swimming speeds of *Sardina pilchardus* during non-feeding, filter-feeding and particulate-feeding; the median, 1st and 3rd quartile, and range of observed values for each activity type are shown.

Sardines used filter-feeding when single phytoplankton cells and zooplankton of a size <780 µm were introduced into the tank. During this feeding mode fish engulf of a volume of water containing food organisms and do not appear to visually select prey. Swimming speed when filter-feeding remained constant during the entire experiment and had an average value of 23.0 ± 7.6 cm s^{-1}. In addition to swimming faster than non-feeding fish, filter-feeding sardine schooled tightly and swam with their mouths held agape and opercula widely flared. Average feeding act duration when filter-feeding was 0.500 (0.258) s, and fish kept their mouths open for 52% on average of the whole experimental duration.

Table 2.1 – Average prey length (µm), experiment number (Exp), initial concentration (ic; number of prey l^{-1}), dominant feeding mode (dfm; f indicates filter-feeding and p indicates particulate-feeding), instantaneous clearance rate (g; number of prey l^{-1} s^{-1}), clearance rate (F; as volume swept clear in l min^{-1} ind^{-1}) and average swimming speed (sw; cm s^{-1}) for feeding experiments on *Sardina pilchardus* in which single prey types were used. Where filter-feeding was the feeding mode, the filtering efficiency (% Fmax) is presented. Values in brackets represent the standard deviation. Wild phytoplankton species were given in a single experiment (nº18).

Prey	**Mean length**	**Exp**	***ic***	***dfm***	***g***	***F***	***sw***	***% Fmax***
Cultured Phytoplankton								
Skeletonema costatum	4 (1)	1	1.1E+08	f	0.0022	0.08	23.5	23
		2	2.3E+08	f	0.0022	0.08	23.1	23
Tetraselmis suecica	12 (1)	3	1.7E+07	f	0.0022	0.08	19.1	28
		4	8.2E+07	f	0.0022	0.08	17.2	31
Cultured Zooplankton								
Brachionus plicatilis	190 (42)	5	631	f	0.0096	0.36	28.2	83
		6	942	f	0.0096	0.36	29.4	79
Artemia salina	592 (61)	7	667	f	0.0096	0.36	26.7	87
	722 (85)	8	942	f	0.0096	0.36	26.6	88
	724 (29)	9	942	f	0.0096	0.36	26.1	89
	780 (136)	10	616	p	0.0175	0.66	32.7 (10)	-
	828 (84)	11	852	p	0.0268	1.02	35.8 (11)	-
	833 (74)	12	381	p	0.0175	0.66	33.1 (10)	-
Ichthyoplankton								
Solea senegalensis eggs	1045 (43)	13	52	p	0.1340	5.07	32.0 (9)	-
		14	73	p	0.0300	1.14	33.3 (10)	-
Sparus aurata eggs	1003 (40)	15	140	p	0.0110	0.42	30.6 (10)	-
S. senegalensis larvae	2470 (250)	16	40	p	0	0	24.8 (6)	-
S. aurata larvae	3030 (230)	17	255	p	0.0639	2.43	33.7 (7)	-
Wild phytoplankton								
Dinoflagellates								
Scripsiella spp.	24 (4)	18	607		0	0		
Dinoflagellates n.i	24 (4)							
Protoperidinium sp.	48 (9)			f			21.9 (6)	-
Diatoms								
Thalassionema sp	48		262		0	0		
Chaetoceros sp.	11							

Sardines used particulate-feeding in every experiment where prey >780 µm were offered, which included large *Artemia salina*, cultured fish eggs and larvae, and wild zooplankton assemblages. This feeding mode was characterized by the visual detection and attack of single prey items, and a higher swimming speed after the introduction of food compared to filter-feeding fish (Fig. 2.1). There was also higher between-experiment variability in swimming speed while filter-feeding than while particulate-feeding. Swimming speed by particulate-feeding fish decreased slightly in the course of the experiments and had an average value of 30.0 (9.0) cm s^{-1}. When particulate-feeding, sardines dispersed from the shoal and aligned themselves towards prey items, with frequent turns and changes of direction being observed. In contrast to filter-feeding the opercula were only slightly flared, and feeding act duration was shorter than during filter feeding with an average value of 0.135 ± 0.055 s. Additionally, the period between particulate-feeding acts was more variable than for filter-feeding, depending on the selection of other prey items. Fish all used the same feeding mode during a given experiment, and the transition between filter and particulate-feeding was not gradual (see below).

2.4.2 – Filtration efficiency and clearance rates

Sardines were able to retain particles as small as 4 to15 µm (corresponding to single-cells of the diatom *Skeletonema costatum* and the Chlorophyta *Tetraselmis suecica*) at the same clearance rate (0.08 l $fish^{-1}$ min^{-1}; see Fig. 2.2). However, swimming speed was slightly higher when feeding on *S. costatum* compared with *T. suecica*, corresponding to a filtering efficiency of 23% for *S. costatum* and 28% for *T. suecica*.

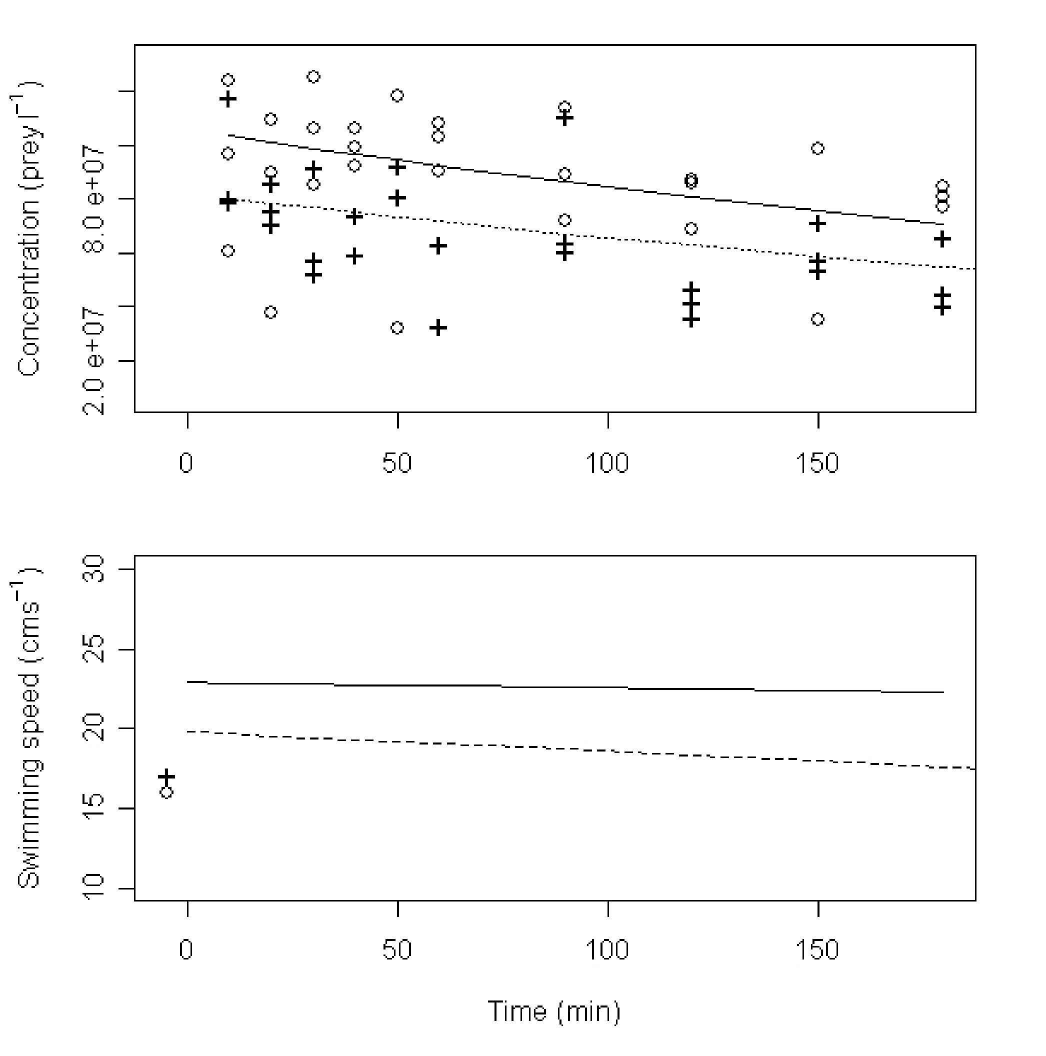

Figure 2.2 – Change in food concentration (upper graph) and swimming speed (lower graph) with time for two experiments where *Sardina pilchardus* filter-fed on cultured phytoplankton: *Tetraselmis suecica* (+ and dashed line) and *Skeletonema costatum* (o and straight line). Food concentration decrease was modelled by GLM; the slope of the fitted line is the instantaneous grazing rate. Swimming speed across time was fitted by linear regression, and the symbols (+ and o) represent the non-feeding average swimming speed prior to the addition of food.

Results of the experiment using wild phytoplankton (dominated by small dinoflagellates and diatom chains and having an average size of 30 μm) showed no significant decrease in prey density with time (when using either the total number of phytoplankton prey or for separate phytoplankton classes), most likely due to the very low initial phytoplankton concentration (800 cells l^{-1}). The initial swimming speed after the introduction of food (t = 10 min) was slightly higher (1.38 ± 0.5 bl s^{-1}) than in experiments using cultured phytoplankton, although in this instance and in contrast to other experiments in which fish filter-fed, swimming speed decreased during the course of the experiment.

Clearance rates of sardines filter-feeding on cultured *Brachionus plicatilis* (≈ 190 µm) and small *Artemia salina* (≤724 µm) were the same at 0.36 l fish^{-1} min^{-1}, higher than values obtained for fish feeding on phytoplankton. However, because the swimming speed of sardines feeding on *B. plicatilis* was higher than when feeding on *A. salina* (Fig. 2.3), this results in filtering efficiency of 80% for *B. plicatilis* and 90% for small *A. salina*. Overall, sardine filtering efficiency increased from the smallest prey used in these experiments (4 to12 µm) up to ≈ 230 µm where it reaches a plateau around 90%, indicating that sardines are highly efficient in retaining particles above this size (Fig. 2.4).

Sardines particulate-fed on large zoo- and ichthyoplankton prey, which included cultured *Artemia* of 780 µm and larger, indicating that the size at which sardine switches from filter- to particulate-feeding lies in the range 724 to 780 µm. Both the clearance rate (0.66 l fish^{-1} min^{-1}) and swimming speed (average 32.7-35.8 cm s^{-1}) of sardines particulate-feeding on large *Artemia* were higher than fish filter-feeding on small *Artemia* (Fig. 2.3).

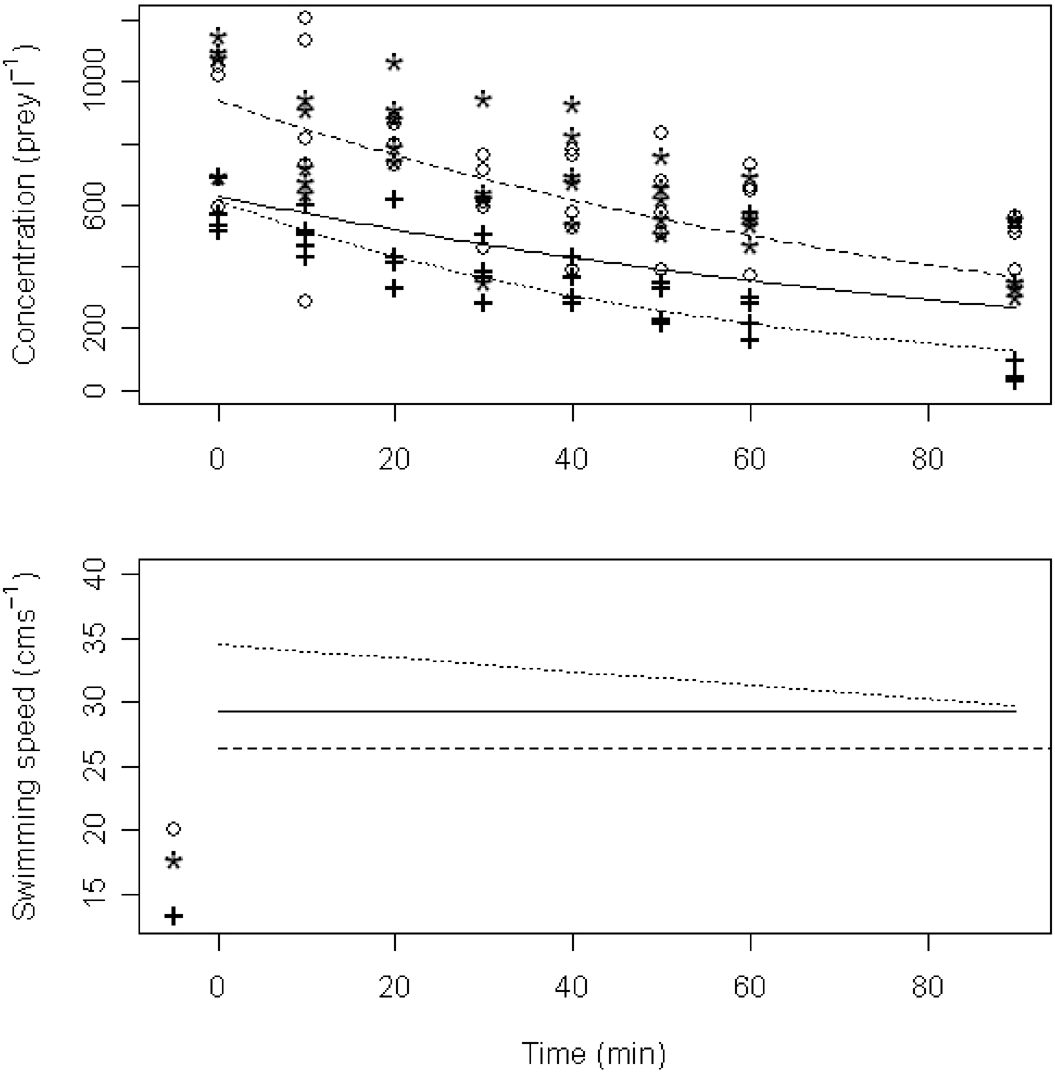

Figure 2.3 – Change in food concentration (upper graph) and swimming speed (lower graph) with time for three experiments where *Sardina pilchardus* fed on cultured zooplankton. Sardines filter-fed on *Brachionus* (* and dashed line) and *Artemia* <780 µm (o and straight line), and particulate fed on *Artemia* >780 µm (+ and dotted line). Food concentration decrease was modelled by GLM; the slope of the fitted line is the instantaneous grazing rate. Swimming speed across time was fitted by linear regression, and symbols (*, o, +) represent the non-feeding swimming speed prior to the addition of food.

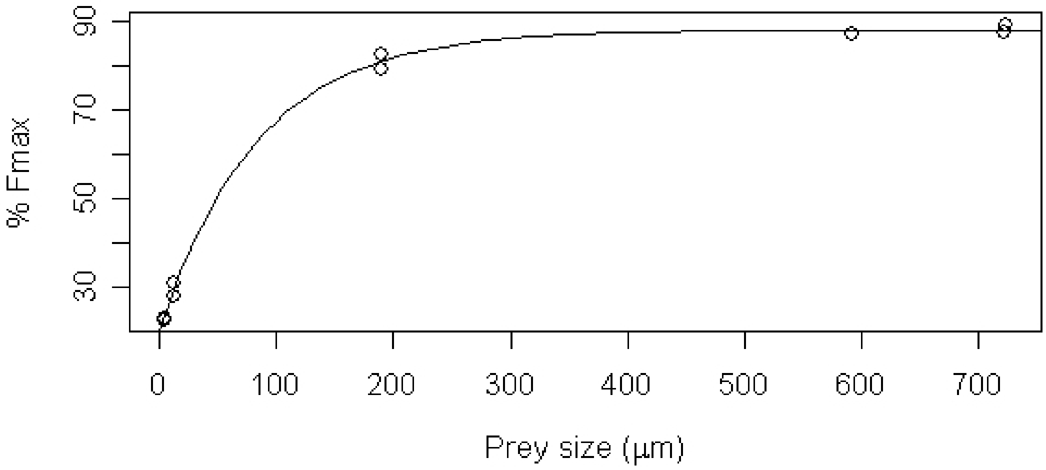

Figure 2.4 – Filtration efficiency as a function of particle size for filter-feeding *Sardina pilchardus*. The fitted model is %F$_{\max}$ = $20.02 + 67.93 * \left(1 - \exp\left(-\frac{\Pr ey\, size}{83.37}\right)\right)$.

The highest clearance rate and swimming speed was recorded for sardines particulate-feeding on fish eggs. Sardines particulate-fed in experiments where three different initial concentrations of fish eggs were used, with the highest clearance rate (5.07 l fish^{-1} min^{-1}) being observed in the experiment that had the lowest initial concentration (Fig. 2.5). Additionally, swimming speed after the introduction of food was also higher at the beginning of that experiment and decreased sharply as food concentration decreased**,** whereas for the other two experiments where initial egg density was higher initial swimming speed was lower, and swimming speed did not change with time. Sardines feeding on *Sparus aurata* larvae, which distribute themselves in midwater and are able to actively swim, showed a high clearance rate (2.43 l fish^{-1} min^{-1}) and high swimming speed (33.7 ± 7 cm s^{-1}), similar to the estimates obtained for experiment in which *Solea senegalensis* eggs were given in low concentrations. Conversely, when feeding on *S. senegalensis* larvae which float at the surface, sardine showed only a small increase in fish swimming speed from non-feeding levels (24.8 ± 6 cm s^{-1}), and neither filter nor particulate-feeding was observed during this experiment. No decrease in the concentration of these fish larvae through the experiment was detected by the model, indicating inefficient or unsuccessful feeding.

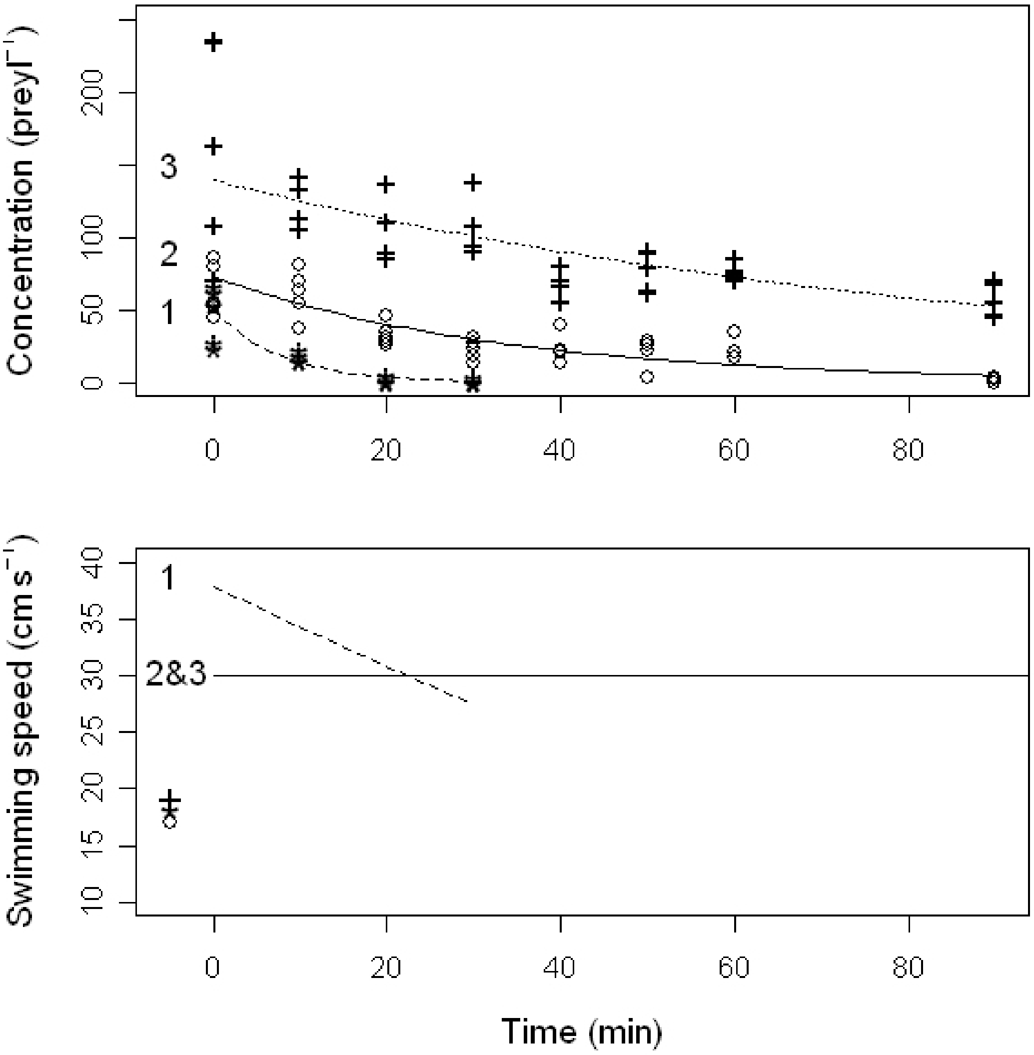

Figure 2.5 – Change in food concentration (upper graph) and swimming speed (lower graph) with time for three experiments where *Sardina pilchardus* particulate-fed on fish eggs at different initial concentrations - numbers 1 (+), 2(o) of *Solea senegalensis* and 3 (*) of *Sparus aurata*. Food concentration decrease was modelled by GLM; the slope of the fitted line is the instantaneous grazing rate. Swimming speed across time was fitted by linear regression, and the symbols (*, o, +) represent the non-feeding average swimming speed prior to the addition of food.

In general, the clearance rates and swimming speeds of particulate-feeding sardines were higher than for fish engaged in filter-feeding (Figs. 2.1 & 2.6, Table 2.1). Since the feeding mode used by sardines is determined by prey size, both ingestion rates and initial swimming speeds of sardines feeding on large prey (>780 µm) were higher than when feeding on prey smaller than <724 µm (Fig. 2.6). The clearance rate of filter-feeding sardines was related to prey size as a result of the increase in the filtering efficiency with increasing prey length (Fig. 2.2). However, fish swimming speed was not correlated with prey length but rather to the prey type, being lower while filter-feeding on phytoplankton and higher while filter-feeding on zooplankton (Table 2.1). Clearance rates and swimming speeds of filter-feeding sardines (above a certain concentration threshold) were not correlated to initial prey density. While particulate-feeding, clearance rates and swimming speeds of sardines were not related to prey size but to prey type (Table 2.1). The highest clearance rate and swimming speed were obtained with sardines preying on fish eggs, but both variables decreased when higher densities of this prey were used in the experiments (Fig. 2.5).

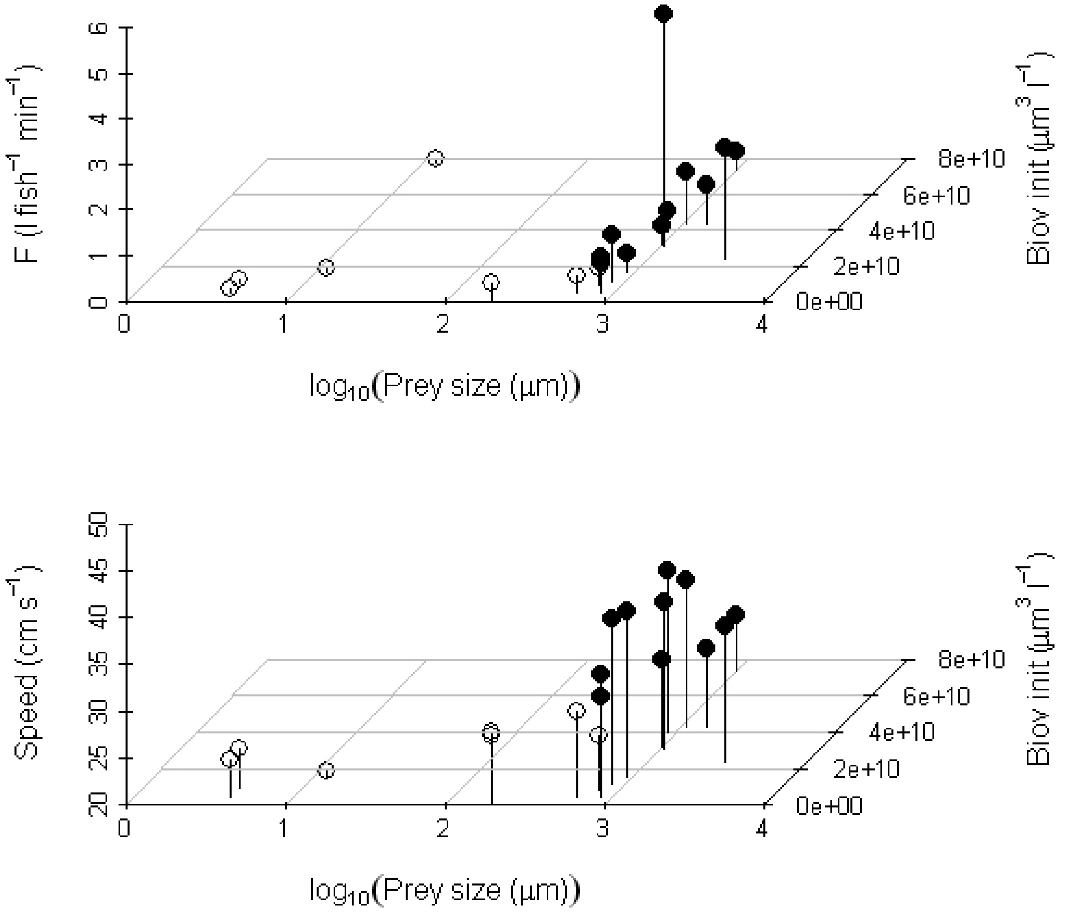

Figure 2.6 – Relationships between prey size (shown as the $\log_{10}$ of prey length), initial prey biovolume (biov init) and clearance rate (F in l fish^{-1}min^{-1} ; upper graphic) and prey size, initial prey biovolume and swimming speed (Speed; lower graphic) for *Sardina pilchardus* filter-feeding (white circles) and particulate-feeding (black circles) on cultured prey used in feeding experiments.

2.4.3 – Mixed experiments: Selectivity

In all the experiments conducted to examine feeding selectivity (Tables 2.2 & 2.3) sardines particulate-fed only and no filter-feeding was observed. Even at high prey densities (Experiments 20-22, Table 2.2), sardines particulate-fed selectively, with fish eggs being depleted faster. Clearance rates of sardines preying on fish eggs ranged from 0.64 to 3.01 l fish^{-1} min^{-1}, higher than the clearance rates for fish larvae (0.23 - 0.53l fish^{-1} min^{-1}) and large *Artemia* (0.36 - 0.4l fish^{-1} min^{-1}) which indicates that sardine feeding selectivity is toward specific prey types and not the larger prey available. Comparison of the clearance rates obtained in single prey type experiments and those obtained in mixed assemblages reveals that fish eggs were cleared at rates within the range of those obtained in single experiments. Similarly, both *Artemia* and *Sparus aurata* larvae were cleared at comparable rates in single and mixed prey experiments. On the other hand, *Solea senegalensis* larvae, which were apparently ignored in experiments using a single food type, had a clearance rate of 0.23 l fish^{-1} min^{-1} when given in a mixture with sole eggs, probably as a result of incidental capture. Feeding selectivity was also observed in experiments using mixed assemblages of wild plankton where the initial food concentration was low (Table 2.3). The higher clearance rates observed for copepods and decapods (0.73 to 0.86 l fish^{-1} min^{-1}) indicate that these items were preferred to gelatinous plankton (F = 0.35 to 0.40 l fish^{-1} min^{-1}). This again indicates that prey size is not the only determinant of prey selection since the gelatinous plankton were larger than the copepods. Sardines fed on dinoflagellates, diatoms and crustacean eggs with similar clearance rates (0.011 l fish^{-1} min^{-1}).

Table 2.2 – *Sardina pilchardus*. Average ± standard deviation prey length (µm) and initial concentration (ic; prey l^{-1}) of mixed cultured prey types used in feeding experiments. The instantaneous clearance rate (g) corresponds to the slope of the generalized linear model fitted to the decrease of prey concentration *vs* time; F is the clearance rate (l $fish^{-1}$ min^{-1}); and sw is swimming speed (cm s^{-1}; values in brackets indicate the standard deviation).

Prey	**Average length**	***ic***	***g***	***F***	***sw***
19					
***Solea senegalensis* eggs**	1045±43	77	0.0703	2.66	26.9 (6)
***Solea senegalensis* larvae**	2470±249	29	0.0060	0.23	
20					
Artemia salina	886±60	453	0.0108	0.41	29.7 (8)
***Sparus aurata* eggs**	1003±40	53	0.0170	0.64	
21					
Tetraselmis suecica	12 ± 1	2.3E+07	0.0039	0.15	27.5 (9)
Artemia salina	910 ± 80	627	0.0108	0.41	
***Sparus aurata* eggs**	1003 ± 40	25	0.0482	1.82	
22					
Artemia salina	923 ± 86	973	0.0096	0.36	28 (8)
***Solea senegalensis* eggs**	1045±43	24	0.0795	3.01	
***Sparus aurata* larvae**	3029 ± 225	66	0.0140	0.53	

Table 2.3 – *Sardina pilchardus*. Average ± standard deviation prey length (µm) and initial concentration (ic; prey l^{-1}) of mixed wild plankton used in feeding experiments. The instantaneous clearance rate (g) corresponds to the slope of the generalized linear model fitted to the decrease of prey concentration *vs* time; F is the clearance rate (l $fish^{-1}$ min^{-1}); and sw is swimming speed (cm s^{-1}; values in brackets indicate the standard deviation).

Prey	**Average prey length**	***ic***	***g***	***F***	***sw***
23					
Crustacean eggs	250	90	0.0060	0.23	30.2 (9)
Copepods	1070	43	0.0227	0.86	
Decapods	2230	2	0.0227	0.86	
Gelatinous plankton	1398	10	0.0092	0.35	
24					
Dinoflagellates	30	560	0.0107	0.40	24.3 (6)
Diatoms	80	346	0.0107	0.40	
Crustacean eggs	115	42	0.0107	0.40	
Calanoids	884	12	0.0192	0.73	
Doliolids	993	8	0.0107	0.40	

2.5 – Discussion

This study has shown that adult *Sardina pilchardus* use two different feeding modes: filter-feeding on small (≤724 µm) prey and particulate-feeding for large (≥ 780 µm) prey or mixed assemblages of both small and large prey. The feeding mode selection depends exclusively on prey size and is not related to prey density. The ability to use these two feeding modes has been described for several clupeids, among which Iberian sardines appear similar to the Cape anchovy (*Engraulis encrasicolus*), which also switches to particulate feeding at a relatively small prey size of around 720 µm, irrespective of prey density (James & Findlay 1989). This is in contrast to southern Benguela sardine (*Sardinops sagax*), a species that changes from filter- to particulate-feeding at larger prey sizes (3060 µm for cultured and 1310 µm for wild plankton; van der Lingen 1994) and filter–feeds on large prey if these are present in high concentrations (van der Lingen 1994), and also other clupeids like herring (*Clupea harengus*) and mackerel (*Scomber japonicus*) whose feeding mode choice depends exclusively on prey density (O'Connell & Zwifel 1972, Batty et al. 1990).

Iberian sardines can retain particles as small as 4 µm (*i.e.* nanoplankton), as shown in experiments using cultured phytoplankton (Fig. 2.1), albeit with a relatively low filtering efficiency of 20% (Fig. 2.4). The filtering efficiency of Iberian sardines for small prey (23% for 4 to 12 µm prey) was similar to that shown by the obligate filter-feeder *Brevoortia tyrannus* (16.5% for 12 µm prey, Durbin & Durbin 1975), and higher than that shown by *Engraulis encrasicolus* which only retain particles larger than 90 µm (James & Findlay 1989) and by *Sardinops sagax* which had a filtering efficiency of 2% for particles of 13-17 µm. However, the reference value of maximum clearance rate calculated for *S. sagax* by van der Lingen (1994) was much higher than for *Sardina* *pilchardus*, since both mouth gape and swimming speed while filter-feeding of the former approximately double the values observed for *S. pilchardus* in this study. Previous stomach content analyses have indicated that the smallest prey retained by sardines was around 50 µm (Varela et al. 1988, Garrido 2003). However, small (2-20 µm) phytoplankton groups such as cocolithophores that have no resistant exteriors (in contrast to the silica test of diatoms and the carbonate skeleton of some dinoflagellates), may be ingested by sardines but difficult to identify because of rapid digestion by acids in the stomach. Given that nanoplankton species can frequently dominate primary production in upwelling systems such as the NE Iberian coast (e.g. Tilstone et al. 2003, Varela et al. 2003, Lorenzo et al. 2005) and that there are toxic species among nanoplankton that might affect fish behaviour and/or survival (Rodger et al. 1994, Wiegand & Pfulgmacher 2005), its importance in the sardines diet should be further examined.

The clearance rate for *Tetraselmis suecica* was not statistically different between the mixed assemblage experiment (0.15 l fish^{-1} min^{-1}) when fish were particulate-feeding, and the single prey experiment (0.08 l fish^{-1} min^{-1}) when fish were filter-feeding. This suggests that the shorter period that sardines spent with an open mouth whilst engaged in particulate-feeding was compensated for by their higher swimming speed. In other words, the clearance rates of small prey are similar whether they are obtained by incidental capture during particulate-feeding on larger prey items or obtained by filtering.

Filtration efficiency became close to maximum (>80 %) with prey larger than 200 µm (Fig. 2.4), which allows Iberian sardines to efficiently utilize all microplankton prey that dominate plankton abundance and sometimes biomass in coastal areas (Calbet et al. 2001, Turner 2004). This result is consistent with the composition of sardine stomach contents collected off the Portuguese coast which are volumetrically dominated by crustacean naupliar stages and small copepods, and numerically dominated by microplankton, especially chain-forming diatoms that can account for 99% of the of prey ingested during spring upwelling events (Garrido 2003). The dominance of microplankton in sardine stomachs suggests that filter-feeding is frequently used by sardines. This species appears to be well-adapted for filter-feeding compared to other clupeids because the small mesh of the gill-rakers means that Iberian sardines are able to retain small plankton, and this species also possesses one of the largest epibranchial organs (which is responsible for the concentration of food items from the gill rakers) among clupeids (Bode et al. 2003). However, the relatively short average feeding bout duration (0.5 s) shown by sardines when filter-feeding contrasts with previous suggestions that filter-bout duration is related to the degree of development of the epibranchial gland (van der Lingen 1994). According to that theory, species that lack an epibranchial gland would show bouts of short duration whereas obligate filter-feeders that have well-developed glands show long, almost continuous, filtering bouts.

Clearance rates obtained for sardines in these experiments were low during both filter- and particulate-feeding compared to results obtained in similar experiments conducted on other clupeids. The major reasons for this are the smaller mouth gape and lower swimming velocity of Iberian sardines (maximum observed swimming speed of 38 cm s^{-1}) compared to other clupeids; Atlantic menhaden of 260 mm swam during filter-feeding at speeds of up to 65 cm s^{-1} (Durbin & Durbin 1975), as did southern Benguela sardine of 230 mm (van der Lingen 1994), whereas Cape anchovy of 120 mm reached speeds of 55 cm s^{-1} (James & Findlay 1989). A degree of caution must be used when comparing clearance rates between species, since differences in techniques and analyses employed may have a strong impact on results obtained. For example, clearance rates for *S. sagax* were calculated using estimates of feeding intensity (the mean number of fish observed with open mouths at regular intervals during an experiment) whereas the total number of fish in the experimental tank was used in this study to estimate F, since in these experiments all sardines appeared to be engaged in feeding at the same time using the same feeding mode. Even if clearance rates in this study were calculated using the percentage of time sardines spent with their mouths open during each experiment (which is assumed to be similar to the calculation of feeding intensity), the clearance rates would still be substantially lower than those of *S. sagax* and other clupeids.

Clearance rate increased with prey length and was especially high when fish eggs were used as prey, particularly if provided at low densities. Prey density had no effect on the feeding mode used by sardines nor on clearance rates during filter-feeding. On the other hand, prey density affected both clearance rates and swimming speeds when particulate-feeding on a particular prey type, since both parameters increased with decreasing densities of food organisms (as shown in the single prey-type experiments using fish eggs).

Sardines generally removed larger prey at higher rates than small prey when feeding on a mixture of different sized organisms, which is indicative of selective feeding. When given in mixed assemblages fish eggs were preferred to all other prey, even larger fish larvae. Feeding selectivity was also observed in experiments using wild prey organisms, and again feeding selectivity was not exclusively related to prey size, since copepods were preferred to the larger doliolids and other gelatinous plankton. Selective feeding by planktivorous fish can have a marked impact on plankton communities (Brooks & Dodson 1965, James & Findlay 1989), both through the removal of particular zooplankton species (Koslow 1981) but also via predation on their own eggs and larvae (*i.e.* cannibalism) or of other species.

The clearance rate of sardines engaged in filter-feeding mostly depends on prey size, and is associated with different filtering efficiencies for different sized prey. While particulate-feeding, however, clearance rate depends on factors other than prey size or prey biomass. It is likely that a complex relationship, that is affected by prey detectability (itself a function of shape, colour, chemical properties as taste and smell, and motion), previous experience with that particular prey, and differential escape abilities of prey, exists between predator and prey during particulate-feeding (Lazzaro 1987). Fish eggs are motionless, as opposed to the other large prey that sardines particulate-fed on, and this is likely to have increased their vulnerability to capture compared to mobile prey that are able to escape predators. On the other hand, prey mobility may increase detectability; sardines avidly pursued the highly mobile *Sparus aurata* larvae but not *Solea senegalensis* larvae that barely moved and were positioned near to the water's surface. Sources of variation impacting on the feeding behaviour of *Sardina pilchardus*, such as selectivity unrelated to prey size, the effect of prey density, and the ambient prey composition, illustrate some of the difficulties in assigning clearance rate values for a given assemblage of food. However, this kind of information is essential for the construction of trophic models for coastal areas.

This study has provided important knowledge of the feeding behaviour of *Sardina pilchardus*, which will allow recognition of favourable feeding conditions for this species. Iberian sardine has a highly efficient filtering mechanism, and is able to retain the smallest food particles yet described for studies of this type. This ability of sardines (including both *Sardina* and *Sardinops*) to utilize very small particles, together with their well developed migration capabilities and serial spawning habits, have been suggested as characteristics that enable sardines to feed and spawn under conditions of relatively low productivity, and hence seek out environmental loopholes in predation fields that may not be available to anchovy, which are not able to entrap small prey and have reduced migratory capabilities compared to sardine (Bakun & Broad 2003). On the other hand, sardines also seem to be well adapted to feed on large plankton particles, and behave in a similar manner to species that are primarily particulate-feeders such as *E. mordax* and *E. encrasicolus*, namely particulate-feeding at high food densities of large prey and with mixtures of several prey sizes. This fact shows the extremely opportunistic feeding behaviour of Iberian sardines, which are highly adapted to different feeding conditions. We propose that, contrary to what was formerly thought, particulate-feeding may be frequently used by sardines, at least during the day, preying directly on bigger organisms if encountered. Although several experiments revealed the high visual acuity of several clupeids, that are able to particulate feed during bright moonlight nights, particulate feeding is proposed to be a visual predation, therefore it is not possible to occur during dark nights. Therefore, during the period of the night, when a visual predation is not possible or when large prey are scarce, sardines are able to filter feed on small prey with high filtering efficiencies. In fact, empty sardine stomachs have not yet been detected in our studies (Garrido 2003, Cunha et al. 2005) that involved a large number of individuals collected at night. However, feeding mode shifts also depend on energetic output, and experiments to examine the relative energetic costs of each feeding mode, as have been conducted for Cape anchovy (James & Probyn 1989) and Benguela sardine (van der Lingen 1995), should be done on Iberian sardine in order to permit the construction of energetic models for this species.

2.6 – References

Bakun A & Broad K (2003) Environmental “loopholes” and fish population dynamics: comparative pattern recognition with focus on El Niño effects in the Pacific. Fish Oceanogr 12: 458-473

Batty RS, Blaxter JHS, Richard JM (1990) Light intensity and the feeding behaviour of herring *Clupea harengus*. Mar Biol 107 (3): 383-388

Bode A, Carrera P, Lens S (2003) The pelagic foodweb in the upwelling ecosystem of Galicia (NW Spain) during spring: natural abundance of stable carbon and nitrogen isotopes. ICES J Mar Sci 60:11-22

Bode A, Alvarez-Ossorio MT, Carrera P, Lorenzo J (2004) Reconstruction of the trophic pathways between plankton and the North Iberia sardine (*Sardina pilchardus*) using stable isotopes. Sci Mar 68: 165-178

Borges MF, Santos AMP, Crato N, Mendes H, Mota B (2003) Sardine regime shifts off Portugal: a time series analysis of catches and wind conditions. Sci Mar 67: 235-244 Suppl 1

Brooks JL & Dodson SI (1965) The effect of a marine planktivore on lake plankton illustrates theory of size, competition, and predation. Science 150 (3692): 28-35

Calbet A, Garrido S, Saiz E, Alcaraz M, Duarte C.M (2001) Annual zooplankton succession in coastal NW Mediterranean waters: the importance of the smaller size fractions. J Plankton Res 23: 319-331

Costa PR & Garrido S (2004) Domoic acid accumulation in the sardine (*Sardina pilchardus*) and its relationship to *Pseudo-nitzschia* diatom ingestion. Mar Ecol Progr Ser 284: 261-268

Cunha ME, Garrido S, Pissarra J (2005) The use of stomach fullness and colour indices to assess *Sardina pilchardus* feeding. J Mar Biol Ass UK, 85: 425-431

Durbin AG & Durbin EG (1975) Grazing rates of Atlantic menhaden *Brevoortia tyrannus* as a function of particle size and concentration. Mar Biol 33 (3): 265-277

Friedland KD, Haas LW, Merriner, JV (1984) Filtering rates of the juvenile Atlantic menhaden *Brevoortia tyrannus* (Pisces: Clupeidae), with consideration of the effects of detritus and swimming speed. Mar Biol 84 (2): 109-117

Garrido S (2003) Alimentação de *Sardina pilchardus* (Walbaum, 1792) ao largo da costa continental portuguesa e implicações da condição nutricional das fêmeas na qualidade dos oócitos. MS thesis, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Porto

Gibson RN & Ezzi IA (1990) Relative importance of prey size and concentration in determining the feeding behaviour of the herring *Clupea harengus*. Mar Biol 107 (2): 357-362

Gibson RN & Ezzi IA (1992) The relative profitability of particulate-feeding and filter-feeding in the herring, *Clupea harengus* L. J Fish Biol 40 (4): 577-590

Grant WS & Bowen BW (1998) Shallow population histories in deep evolutionary linkages of marine fishes: Insights from sardines and anchovies and lessons for conservation. J Hered 89: 415-426

Guisande C, Vergara AR, Riveiro I, Cabanas JM (2004) Climate change and abundance of the Atlantic-Iberian sardine (*Sardina pilchardus*). Fish Oceanogr 13 (2): 91-101

Harvey WH (1937) Note on selective feeding by *Calanus*. J Mar Biol Ass UK 22: 97-100

ICES (2006) Report of the working group on the assessment of mackerel, horse mackerel, sardine and anchovy. International Council for the Exploration of the Sea (CM Papers and Reports) CM 2006/ ACFM 08: 631p

James AG & Findlay KP (1989) Effect of particle size and concentration on feeding-behaviour, selectivity and rates of food ingestion by the Cape anchovy *Engraulis capensis*. Mar Ecol Progr Ser 50 (3): 275-294

James AG & Probyn T (1989) The relationship between respiration rate, swimming speed and feeding behaviour in Cape anchovy *Engraulis capensis* Gilchrist. J Exp Mar Biol Ecol 131: 81-100

Janssen J (1976) Feeding modes and prey size selection in alewife (*Alosa pseudoharengus*). J Fish Res Bd of Canada 33 (9): 1972-1975

Koslow JA (1981) Feeding selectivity of schools of northern anchovy, *Engraulis mordax*, in the southern California bight. Fish Bull 79 (1): 131-142

Lazzaro X (1987) A review of planktivorous fishes - their evolution, feeding behaviours, selectivities, and impacts. Hydrobiol 146 (2): 97-167

Leong RJH & O'Connell CP (1969) A Laboratory study of particulate and filter feeding of northern anchovy (*Engraulis mordax*). J Fish Res Bd Canada 26 (3): 557-582

Lorenzo LM, Arbones B, Tilstone GH, Figueiras FG (2005) Across-shelf variability of phytoplankton composition, photosynthetic parameters and primary production in the NW Iberian upwelling system. J Mar Syst 54 (1-4): 157-173

Mathisen OA, Thorne RE, Trumble RJ, Blackburn M (1978) Food consumption of pelagic fish in an upwelling area. In: Boje R. and Tomczak M. (eds.) Upwelling Ecossystems. Springer-Verlag, New York: 111-123

O'Connell CP & Zweifel JR (1972) Laboratory study of particulate and filter feeding of pacific mackerel, *Scomber japonicus*. Fish Bull (Wash DC) 70 (3): 973-981

Parrish RH, Nelson CS, Bakun A (1981) Transport mechanisms and reproductive success of fishes in the California current. Biol Oceanogr 1(2): 175-203

Peleteiro B, Marçalo A, Olmedo M, Pousão-Ferreira P, Garrido S, Porteiro C, Stratoudakis Y (2004) Sardine tagging off the Iberian Peninsula: Laboratory experiments and operations at sea. ICES CM 2004/Q20

R Development Core Team (2005) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org

Rodger HD, Turnbull T, Edwards C, Codd GA (1994) Cyanobacterial (blue-green algal) bloom associated pathology in brown trout, *Salmo trutta* L, in Loch Leven, Scotland. J Fish Diseases 17 (2): 177-181

Schwartzlose RA, Alheit J, Bakun A, Baumgartner TR, Cloete R, Crawford RJM, Fletcher WJ, Green-Ruiz Y, Hagen E, Kawasaki T, Lluch-Belda D, Lluch-Cota SE, MacCall AD, Matsuura Y, Nevárez-Martínez MO, Parrish RH, Roy C, Serra R, Shust KV, Ward MN, Zuzunaga JZ (1999) Worldwide large-scale fluctuations of sardine and anchovy populations. S Afr J Mar Sci 21: 289-347

Stratoudakis Y, Bernal M, Borchers DL, Borges MF, Cardador F (2003) Changes in the distribution of sardine eggs and larvae off Portugal, 1985-2000. Fish Oceanogr 12 (1): 49-60

Tilstone GH, Figueiras FG, Lorenzo LM, Arbones B (2003) Phytoplankton composition, photosynthesis and primary production during different hydrographic conditions at the Northwest Iberian upwelling system. Mar Ecol Progr Ser 252: 9-104

Turner JT (2004) The importance of small planktonic copepods and their roles in pelagic marine food webs. Zool Studies 43 (2): 255-266 van der Lingen CD (1994) Effect of particle size and concentration on the feeding behaviour of adult pilchard *Sardinops sagax*. Mar Ecol Prog Ser 109:1-13

van der Lingen CD (1995) Respiration rate of adult pilchard *Sardinops sagax* in relation to temperature, voluntary swimming speed and feeding behaviour. Mar Ecol Prog Ser 129: 41-54.

Varela M, Larrañaga A, Costas E, Rodríguez B (1988) Contenido estomacal de la sardina (*Sardina pilchardus*, Walbaum) durante la campaña Saracus 871 en las plataformas Cantábrica y de Galicia en Febrero de 1987. Bol Inst Esp Oceanogr 5:17-28

Varela M, Alvarez-Ossorio MT, Valdés L (1990) Método para el estudio cuantitativo del contenido estomacal de la sardina. Resultados preliminares. Bol Inst Esp Oceanogr 6:117-126

Varela MM, Barquero S, Bode A, Fernández E, González N, Teira E (2003) Microplanktonic regeneration of ammonium and dissolved organic nitrogen in the upwelling area of the NW of Spain: relationships with dissolved organic carbon production and phytoplankton size-structure. J Plankton Res 25:719-736

Venables WN & Ripley BD (2002) Modern Applied Statistics with S. Fourth Edition. Springer, New York. ISBN 0-387-95457-0

Wiegand C & Pflugmacher S (2005) Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. Toxicol and Appl Pharmacol 203 (3): 201-218

CHAPTER 3

SPATIAL AND TEMPORAL VARIATION IN DIETARY COMPOSITION AND FEEDING INTENSITY OF SARDINE (*Sardina pilchardus*) OFF PORTUGAL AND RELATIONSHIPS WITH SATELLITE-DERIVED CHLOROPHYLL DATA*

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*Garrido S, Ben-Hamadou R, Oliveira PB, Cunha ME, Chícharo MA, van der Lingen CD. Spatial and temporal variation in dietary composition and feeding intensity of sardine (*Sardina pilchardus*) off Portugal and relationships with satellite-derived chlorophyll data. *Accepted in Mar Ecol Progr Ser.*

3.1 – Abstract

Spatial and temporal variability in the diet and feeding intensity of sardine *Sardina pilchardus* off Portugal was examined through analysis of the stomach contents of 3600 fish collected fortnightly from the fishing ports of Peniche (West Portugal) and Portimão (South Portugal) during 2003 and 2004. Dietary composition of the modal sardine length class (18cm) was assessed by determining the frequency of occurrence and carbon content of identified prey, and these two parameters were combined to estimate a modified index of relative importance (*mIRI*) of dominant prey items. Feeding intensity was estimated using stomach content weight data. The most important prey for sardines were zooplankton, comprising crustacean eggs, copepods (adults, copepodites and nauplii, especially of the genus *Euterpina*, *Oncaea*, *Centropages* and *Temora*), decapods (mostly Brachyuran zoea), cirripeds and fish eggs, dinoflagellates (especially *Ceratium* and *Protoperidinium*), and diatoms (particularly the toxin producer genus *Pseudo-nitzschia*), which together accounted for more than 90% of estimated dietary carbon. Dietary composition throughout the year was similar for both areas except that the contribution of phytoplankton was higher for fish from the W Portuguese coast where upwelling events are recurrent during spring and summer months compared to south Iberia where upwelling is weaker. The numerical predominance of prey <200µm (particularly dinoflagellates and chain-forming diatoms) and the high importance of prey <750µm in sardine diet suggests that filter-feeding is the dominant feeding mode used in the wild. Feeding intensity was similar for both sexes and for fish of different length classes and was higher in Peniche (W coast) than in Portimão (S coast), which is probably related to the higher productivity of the W coast. Although there was high inter-annual variability in feeding intensity this

parameter was highest for both areas during spring and particularly for the west coast during the winter. Temporal variability in satellite-derived chlorophyll *a* is similar to the temporal variability in the dietary contribution by phytoplankton, suggesting that satellite data could be used to proxy sardine feeding intensity.

3.2 – Introduction

Sardine is the most abundant pelagic fish in Iberian waters and represents the main target of purse-seine fisheries of Portugal and Spain (ICES 2006). Variations in the abundance, condition and recruitment of the Atlanto-Iberian sardine stock observed over the last decades were found to be related to changes in environmental conditions (Borges et al. 2003, Guisande et al. 2004, Silva et al. 2006), and within the area of distribution of this stock there are distinct oceanographic regimes that might explain geographic differences in sardine morphometric characteristics, age structure, population dynamics and maturation patterns (ICES 2000, Carrera & Porteiro 2003, Silva 2003, Silva et al. 2006). Environmental discontinuities such as temporal and geographical variations in food availability have been proposed to explain differences in sardine maturation patterns between fish in the Mediterranean and southern Iberian Atlantic waters compared to those from western Iberia (Silva et al. 2006). It is likely that sardines living in areas where recurrent upwelling events provide rich food environments will have different feeding strategies compared to sardines living in less productive environments. Sardines have a highly plastic/ flexible feeding behaviour and are able to use filter- or particulate-feeding over a broad prey size-spectrum with high efficiency (Garrido et al. 2007), indicating that they are adapted to feeding in a wide variety of environments. This suggests that their key prey species probably vary geographically, and that differences in feeding intensity and food quality may help in explaining regional differences in sardine ecology, namely growth and reproduction.

The west and south Iberian coasts have different oceanographic conditions which influence plankton abundance and composition throughout the year. Seasonal variation in plankton communities off the Portuguese coast seem to be due to coastal upwelling, river run-off, seasonal cycles in thermal stratification (summer and late autumn) and vertical convection (winter and early spring) (Cunha 2001), and the seasonal influence of oligotrophic oceanic waters over the shelf (Moita 2001). The west coast of Portugal is under the influence of a northerly wind regime that leads to coastal upwelling events from July to September, although sporadic upwelling events of smaller intensity than in the summer may also occur during December-January (Fiúza et al. 1982). Off the south coast the effects of upwelling are reduced, although upwelling does occasionally occur there under the influence of local westerly winds, or when upwelled waters from the west coast intrude over the southern shelf break following an easterly extension of the equatorward upwelling current (Fiúza 1983, Relvas & Barton 2002). As a consequence, maxima in phytoplankton biomass off the Portuguese coast are generally found during all seasons off the W coast (Moita 2001). Moreover, in that region, the shelf is larger, flatter and has higher thermo-haline stratification than in the south, which is the major reason for the N-S differences in plankton composition (Cunha 2001), although inshore-offshore variations in phytoplankton composition appear to be higher than latitudinal variations (Moita 2001).

Sardine condition peaks during late summer (Bandarra et al. 1997) and it is empirically assumed that feeding intensity also peaks during this season. However, examination of the seasonal cycle in sardine feeding and identification of seasonal changes in the relative importance of sardine prey has not yet been performed, and such a study would enable identification of the season responsible for high fish condition in summer that influences the following spawning season.

Finally, the observed correspondence between the ambient food environment and sardine stomach contents (Varela et al. 1990, Bode et al. 2003) suggests that this species is essentially a filter-feeder whose diet reflects local plankton composition. If this assumption is true then routine measures of plankton productivity, such as remotely-sensed chlorophyll a, could be used to study spatial and temporal variability in sardine feeding intensity, which would be an important tool since stomach content analysis represents a laborious and time-consuming task that is difficult to perform at a large-scale (Cunha et al. 2005).

The objective of this study was to examine the diet of sardine from two areas off the Portuguese coast characterized by different hydrological regimes, with the intention of (i) identifying the most important prey items; (ii) studying seasonal variation in sardine diet; (iii) examining sardine feeding intensity in relation to age and sex; and (iv) assessing whether remotely-sensed chlorophyll a data provides a good estimation of sardine feeding intensity, particularly of phytoplankton prey.

3.3 – Material and methods

Samples comprising ≈70 sardine were obtained every fortnight from purse-seiners fishing off west (Peniche) and south (Portimão) Portugal from January 2003 to March 2004 (Fig. 3.1). The location of the trawls was provided by the fisherman, and data on total length, gutted weight (fish weight minus viscera), sex, gonad maturity stage and indices of fat content were recorded for each sardine.

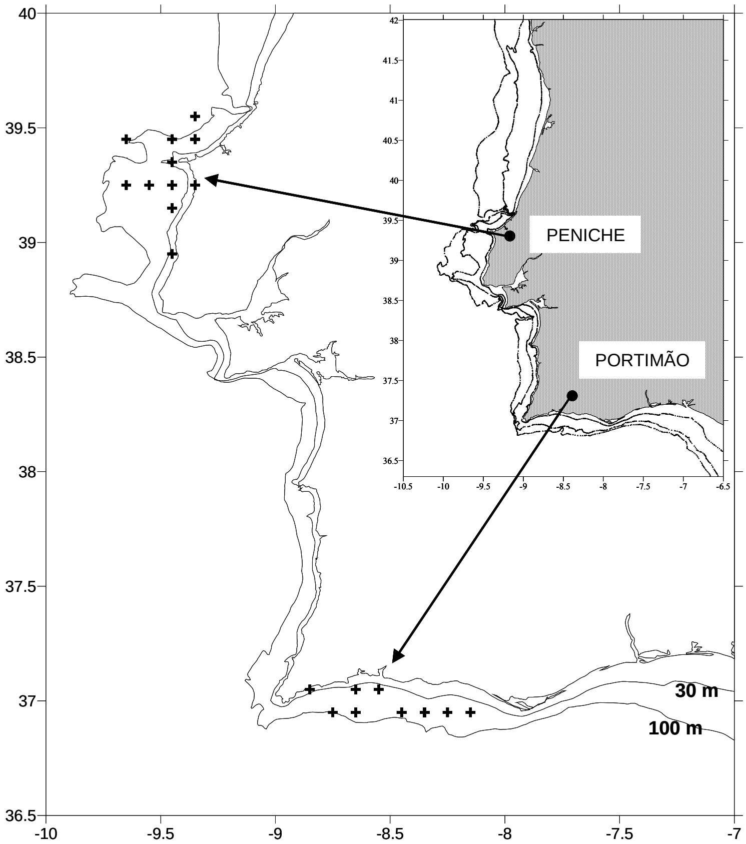

Figure 3.1 – Map of the Portuguese continental coast, showing the sampling locations of Sardina pilchardus in the fishing areas of Peniche and Portimão (note that several samples were taken from the same location).

The stomach of each fish was removed and weighed, cut open and the contents removed and weighed, and the empty stomach weighed again. All weight measurements were made to the nearest 0.1 mg. Stomach contents from each fish were then individually deep-frozen for subsequent prey analysis. Microscopic analysis of food items was conducted for subsamples of 10 stomachs per sample and was restricted to sardines of the modal length class in the fishery (18 cm) in order to avoid size-based differences in diet. Contents from the 10 subsamples were combined and an aliquot comprising 50% of this was used to identify and count prey items. The aliquot was first weighed, then diluted in a known volume of water (proportional to the weight of the aliquot) and filtered through a 200 µm sieve in order to facilitate the identification of the food items, and the two size fractions were analysed separately. All prey was identified to the lowest possible taxa; those <200 µm were identified using an inverted microscope at a magnification of 400x whereas those >200 µm were examined using a stereomicroscope at a magnification of 80x. Digital pictures of prey were taken using the software Zeiss KS100 3.0 on the inversion microscope and the software Leica IM 500 on the stereomicroscope, and the major and minor axis of each prey item were recorded and converted into volumetric estimates using equations that approximated the appropriate shape (generally spherical or ellipsoidal). In order to determine the relative importance of prey, their carbon content was estimated using equations given in van der Lingen (2002) for phytoplankton, zooplankton and fish eggs, and Lindley (1998) for decapods.

The maximum dimension of identified prey was used to group prey items into size-classes; chain forming diatoms were assigned to a size class based on the maximum dimension of single cells, since most phytoplankton cells were found as individuals in stomach contents and the length of the chain was unknown. The frequency of occurrence, percentage contribution by number, and percentage contribution to dietary carbon of each prey size-class were then calculated.

A modified index of relative importance of prey (*mIRI*; Hayse 1990) was used to examine spatial and temporal variability in sardine dietary composition:

$$mIRI_i = \%O_i.\%C_i \qquad (1)$$

where $\%O$ is the frequency of occurrence (determined as the number of stomachs samples in which a given prey was found versus the total number of stomachs samples),

and $\%C$ is the percentage contribution to total carbon content of a given prey in the stomach samples.

For this analysis, copepod eggs, copepod egg sacs, and eggs of other crustaceans were grouped together, and the different species of *Temora* and *Centropages* were grouped to genus. Sardine and other spherical fish eggs were grouped together whilst *Engraulis* eggs were recorded separately.

Seasonality in dietary composition at the two sample sites (Peniche and Portimão) was assessed using hierarchical clustering analysis of the fortnightly relative contribution of prey groups to dietary carbon, with Bray-Curtis similarities and Euclidean distances being calculated and the flexible linkage agglomeration considered following Legendre & Legendre (1998). Dominant prey groups were selected for each area and an empirical threshold criterion was applied to cumulative *mIRI* values over seasons. First, prey groups were ordered by their *mIRI* value (decreasing order) and the tangent measured. Most important prey groups were selected until the tangent of the accumulated *mIRI* go bellow 45º.

Feeding intensity (*FI*) was calculated using the following expression:

$$FI = \frac{stomach\ \ content\ \ weight}{(total\ \ weight - gonad\ \ weight)} \times 100\% \qquad (2)$$

Feeding intensity was standardized in order to account for differences in the size of the stomach in relation to fish size. To investigate the effect of sex on feeding intensity, separate contingency matrices for males and females that contained the average stomach content weight for each length class and each fortnight sample were created, and the unfolded matrices were compared using the Mantel test (Legendre & Legendre 1998). To identify differences of stomach content weight of fish of different length classes, a contingency matrix was built with each entry being the average stomach content weight for each length class and fortnight sample. Length classes corresponding to 12, 13, 14 and 23 cm were ignored due to insufficient data, and the Spearman rank correlation statistic was used to compare pairs of either length classes or sexes, and tested by 10

000 permutations. Temporal differences in sardine feeding intensity at each sampling location were investigated using an ANOVA conducted on normalised (log-transformed) stomach weight data from fortnightly samples, and an *a posteriori* Tukey HSD test ($p < 0.05$) for unequal sample sizes was used to identify significant differences in mean values.

In order to assess whether remotely-sensed chlorophyll could be used to proxy sardine feeding intensity and food quality, we extracted chlorophyll data from 9km resolution SeaWiFS Chlorophyll a concentration standard mapped images (L3SMI product, Thomas & Franz 2005) for the fishing areas from which sardine were sampled. Five-day Chlorophyll a averages computed from daily images were compared with (1) total carbon content of autotrophic phytoplankton cells identified in the stomachs, and (2) average values of feeding intensity for each fortnightly sample using linear regression model. When chlorophyll data was not available for the five day period in a particular fishing location the value of the nearest neighbourhood (closest 9 km block) was used. The Spearman correlation coefficient was used to assess significance of derived linear regressions and was tested by 10000 permutations of the chlorophyll averages.

All data and statistical analyses were performed using MATLAB software (version 6.5; MathWorks Inc. 2002).

3.4 – Results

3.4.1 – Diet composition

3.4.1.1 – Relative importance of prey

The mean length, frequency of occurrence (%O), contribution to dietary carbon (%C) and mean index of relative importance (*mIRI*) for all prey identified from sardine stomachs at Peniche and Portimão are given in Table 3.1. Phytoplankton and zooplankton prey occurred in every stomach sampled, and fish eggs occurred in 69% of Portimão samples and 67% of Peniche samples. Zooplankton was the most important prey at both locations (*mIRI* values of 6300 and 5600 for Peniche and Portimão, respectively), with phytoplankton the second most important prey group at Peniche (*mIRI* of 1941) and fish eggs the second most important prey group at Portimão (*mIRI* of 2065).

Unidentified crustacean eggs plus copepod eggs and copepod egg sacs not only had the highest frequency of occurrence, but were also the most abundant in stomach contents and had the highest relative importance to sardine diet of all zooplankton prey groups in both areas (Table 3.1). Copepods (adults and copepodites) occurred in every stomach sample, and calanoid copepods (represented by a higher diversity of genera than the other copepod orders but comprising mainly *Centropages* spp.) were the most important group, followed by harpacticoid copepods (primarily *Euterpina spp.*) and poecilostomatoid copepods (*Oncaea* spp.). Cirripeds followed copepods in importance to sardine diet, while other zooplankton groups had very low significance.

Fish eggs were present in sardine stomach contents in low numbers but their high relative carbon content resulted a high *mIRI* for this prey (Table 3.1). Eggs of *Engraulis encrasicolus* were only present in samples from Portimão, where they made a low average contribution to sardine diet. Eggs clearly identified as sardine eggs occurred in both areas but had a higher relative importance in samples from Portimão.

Dinoflagellates and diatoms occurred in every stomach sampled but dinoflagellates made a larger contribution to sardine diet than did diatoms for both areas (Table 3.1), and the importance of diatoms and dinoflagellates to the diet was higher in fish from Peniche than from Portimão. The most frequently encountered (%O) dinoflagellates were *Ceratium* spp., *Dinophysis caudata* and *Protoperidinium* spp. Whereas *Protoperidinium* and *Ceratium* were the most important dinoflagellates in terms of dietary carbon in both regions, dinoflagellate cysts were only important to the diet of fish from Portimão. The diatom *Paralia sulcata* had the highest frequency of occurrence for fish from both areas, followed by *Thalassiosira*, *Pseudo-nitzschia* and *Coscinodiscus* in Peniche and *Diploneis* and *Thalassiosira* in Portimão. *Pseudo-nitzschia* made the largest contribution to dietary carbon and had the highest *mIRI* value for both areas, although its importance was much higher in Peniche than in Portimão.

Table 3.1 – Average length, frequency of occurrence (%O), relative contribution to prey carbon content (%C), and modified index of relative importance (*mIRI*) estimated for prey species identified from January 2003 to March 2004 in stomach contents of sardines collected off Peniche and Portimão.

	Length	Peniche (W)			Portimão (S)		
Prey item	(µm)	%O	%C	*mIRI*	%O	%C	*mIRI*
Phytoplankton							
Unident.	29.3	22.2	0.0	0.2	7.1	0.0	0.0
Dinophyceae							
Unident.	37.3	100.0	1.0	99.2	96.4	1.2	119.8
Ceratium spp.	119.7	55.6	5. 5	302.7	67.9	1.8	121.6
Dinophysis acuta	70.5	33.3	0.4	14.0	32.1	0.2	5.4
Dinophysis caudata	87.4	63.0	0.5	33.3	60.7	0.3	19.2
Dinophysis acuminata	54.0	37.0	0.0	1.6	7.1	0.0	0.1
Dinophysis rotundata	40.9	7.4	0.0	0.0	10.7	0.0	0.1
Diplopsalis sp.	61.7	0.0	0.0	0.0	3.6	0.0	0.0
Goniodoma polyedricum	60.5	7.4	0.0	0.1	28.6	0.1	2.8
Ornithocercus spp.	62.2	0.0	0.0	0.0	7.1	0.0	0.2
Pyrocystis lunula	129.5	0.0	0.0	0.0	7.1	0.0	0.0
Prorocentrum micans	43.7	25.9	0.0	0.3	28.6	0.0	0.8
Protoperidinium spp.	91.2	92.6	6.0	557.1	82.1	7.5	613.3
Protoperidinium diabolum	70.9	11.1	0.0	0.0	0.0	0.0	0.0
Protoperidinium divergens	128.3	3.7	0.0	0.1	3.6	0.0	0.0
Protoperidinium oceanicum	119.2	7.4	0.1	1.0	7.1	0.1	0.5
Noctiluca sp.	77.4	0.0	0.0	0.0	7.1	0.0	0.0
Scripsiella sp.	25.3	29.6	0.1	3.3	17.9	0.0	0.2
Cyst	30.1	81.5	0.2	14.0	93.1	1.6	145.6
Total Dinophyceae		100	13.8	1376.1	100	12.8	1280.7
Bacillariophyceae							
Centric diatom	32.2	55.6	0.0	1.2	60.7	0.0	1.5
Pinullate diatom	80.7	44.4	0.0	0.3	28.6	0.0	0.1
Biddulphia alternans	32.5	37.0	0.0	0.2	7.1	0.0	0.0
Cerataulina pelagica	25.0	0.0	0.0	0.0	3.6	0.0	0.0
Coscinodiscus spp.	55.0	51.9	0.0	1.7	53.6	0.2	10.5
Diploneis spp.	39.9	22.2	0.0	0.0	67.9	0.0	0.2
Lauderia annulata	35.1	0.0	0.0	0.0	3.6	0.0	0.0
Melosira spp.	13.3	7.4	0.0	0.0	0.0	0.0	0.0
Navicula spp.	69.3	22.2	0.0	0.1	57.1	0.0	1.0

Paralia sulcata	17.4	88.9	0.0	2.2	78.6	0.0	1.9
Planktionella sol	55.2	3.7	0.0	0.0	0.0	0.0	0.0
							(cont.)
Podosira sp.	42.0	0.0	0.0	0.0	3.6	0.0	0.0
Pleurosigma/Girosigma	160.9	7.4	0.0	0.0	53.6	0.0	1.8
Nitzschia spp.	45.0	0.0	0.0	0.0	3.6	0.0	0.0
Pseudo-nitzschia spp.	69.3	63.0	5.5	345.2	17.9	0.7	13.1
Stephanopyxis turris	21.0	0.0	0.0	0.0	3.6	0.0	0.0
Surirella spp.	47.5	0.0	0.0	0.0	42.9	0.0	0.6
Thalassiosira spp.	38.2	66.7	0.0	1.0	60.7	0.2	9.7
Thalassionema spp.	39.9	3.7	0.0	0.0	10.7	0.0	0.0
Rhizosolenia spp.	86.0	0.0	0.0	0.0	10.7	0.0	0.0
Trachyneis spp.	106.0	7.4	0.1	0.3	0.0	0.0	0.0
Total Bacillariophyceae		100	5.6	564.0	100	1.2	122.4
Prymnesiophyceae							
Phaeocystis sp.	62.2	0.0	0.0	0.0	3.6	0.0	0.0
Dictyochophyceae							
Dictyocha specullum	23.8	3.7	0.0	0.0	0.0	0.0	0.0
Total Phytoplankton		100	19.4	1941.1	100	14.0	1403.3
Zooplankton							
Tintinoinea	57.2	74.1	0.1	8.5	64.3	0.5	30.6
Radiolaria	138.8	0.0	0.0	0.0	7.1	0.0	0.0
Foraminifera	143.9	11.1	0.0	0.0	10.7	0.0	0.0
Globligerina boloides	137.2	7.4	0.0	0.0	14.3	0.0	0.0
Lamelibranchia veligera	347.8	14.8	0.0	0.5	3.6	0.0	0.0
Gastropoda	248.1	14.8	0.1	0.8	0.0	0.0	0.0
Crustaceans							
Unident.	255.0	11.1	0.0	0.4	10.7	0.0	0.1
Cladocera	300.0	3.7	0.0	0.1	0.0	0.0	0.00
Podon spp.	750.0	3.7	0.0	0.0	0.0	0.0	0.0
Crustacean eggs	116.8	96.3	19.3	1855.0	89.3	14.4	1287.5
Crustacean nauplii	250.0	7.4	0.1	0.7	0.0	0.0	0.0
Copepod eggs	74.2	18.5	0.4	7.7	17.9	2.4	43.6
Copepod egg sacs	202.3	48.1	2.8	134.9	57.1	4.8	272.2
Copepod nauplii	165.5	70.4	0.3	23.2	57.1	0.3	17.9
Unident. copepods	639.6	40.7	4.0	164.3	50.0	4.3	213.4
Euterpina spp.	482.1	92.6	6.4	589.1	85.7	3. 6	305.6
Microsetella spp.	607.2	44.4	1.8	78.1	42.9	0.8	34.9
Aeogistus spp.	600.0	3.7	0.1	0.4	0.0	0.0	0.0
Total Harpacticoida		96.30	8.2	791.17	96.55	4.4	422.9
Oncaea spp.	345.5	88.9	5.2	463.8	92.9	6.7	617.4
Corycaeus spp.	675.5	44.4	0.2	7.0	14.3	0.1	0.7
Total Poecilostomatoida		96.30	5.4	517.58	96.55	6.7	646.6
Cyclopoida: *Oithona* spp.	383.5	33.3	0.0	0.5	21.4	0.0	0.3
Calanus spp.	952.2	44.4	1.4	62.6	39.3	1.5	58.6
Calanus helgolandicus	1131.4	14.8	0.3	4.6	17.9	0.4	7.5
Paracalanus spp.	770.0	3.7	0.0	0.0	7.1	0.2	1.4
Clausocalanus spp.	1130.0	7.4	0.1	0.3	0.0	0.0	0.0
Eucalanus spp.	2390.0	0.0	0.0	0.0	3.6	0.6	2.2
Euchaeta spp.	1055.3	14.8	0.1	1.4	14.3	1.2	16.8
Temora spp.	876.7	29.6	0.5	15.7	21.4	1.1	23.3
Temora stylifera	1026.4	14.8	0.2	2.6	25.0	1.8	43.8
Temora longicornis	849.1	29.6	0.5	14.9	10.7	0.5	5.5
Centropages spp.	1162.6	63.0	7.5	474.8	53.6	3.4	180.8
Centropages chierchiae	1132.0	3.7	0.0	0.1	17.9	1.0	18.1
Candacia spp.	1670.0	3.7	0.1	0.4	0.0	0.0	0.0
Acartia spp.	954.4	29.6	0.2	5.9	32.1	1.7	55.4
Pleurommama spp.	1500.0	3.7	0.0	0.1	0.0	0.0	0.0
Isias clavipes	900.0	3.7	0.0	0.0	0.0	0.0	0.0
Labidocera spp.	1030.0	3.7	0.0	0.1	0.0	0.0	0.0

Total Calanoida		96.30	11.0	1060.0	93.10	13.4	1245.7
Total copepods		100	24.6	2461.5	100	24.5	2447.2
							(cont.)
Cirripedia	534.8	66.7	8.0	530.1	50.0	4.4	221.7
Anfipoda	1640.0	3.7	0.0	0.0	0.0	0.0	0.0
Euphausiacea	1150.0	3.7	0.0	0.0	0.0	0.0	0.0
Decapoda	1793.3	11.1	0.1	1.4	3.6	0.3	1.2
Brachyura zoe	1567.5	25.9	3.1	79.5	7.1	0.1	0.6
Apendicularia	1900.0	3.7	0.1	0.2	0.0	0.0	0.0
Total Zooplankton	-	100	63.0	6300.3	100	56.0	5600.6
Fish eggs							
Unident. Fish eggs	895.4	63.0	13.5	846.6	57.1	19.0	1086.3
Engraulis encrasicolus eggs	1370.0	0.0	0.0	0.0	3.6	0.0	0.1
Sardina pilchardus eggs	1339.8	7.4	4.0	29.5	21.4	10.9	233.8
Total Fish eggs	895.4	66. 7	17.4	1162.1	69.0	30.0	2065.8
Trematode parasites	1165.9	66.7	-	-	92.9	-	-
Terrestrial pollen	82.1	63.0	-	-	60.7	-	-

3.4.1.2 – Prey size-classes in stomachs

The relative contribution to sardine dietary carbon by prey size (Fig. 3.2) showed that two modes (50-150 µm and 1000-1500 µm) clearly dominated stomach contents of fish from both Peniche and Portimão. Small prey 50-150µm made the highest contribution to dietary carbon, contributing 40% on average to dietary carbon for fish from Peniche and 30% for those from Portimão. These small size-classes represented 85% on average of the number of prey in stomachs of fish from Peniche, and 79% of prey in fish from Portimão, and correspond mainly to phytoplankton of the genera *Ceratium* (±120 µm in length), *Protoperidinium* (±90 µm in length) and *Pseudo-nitzschia* (±69 µm in length), in addition to crustacean eggs (±117 µm in length). The larger mode (900-2000 µm) comprises primarily the copepod *Centropages* and fish eggs, and contributed 24% of dietary carbon in sardine from Peniche and 34% for fish from Portimão.

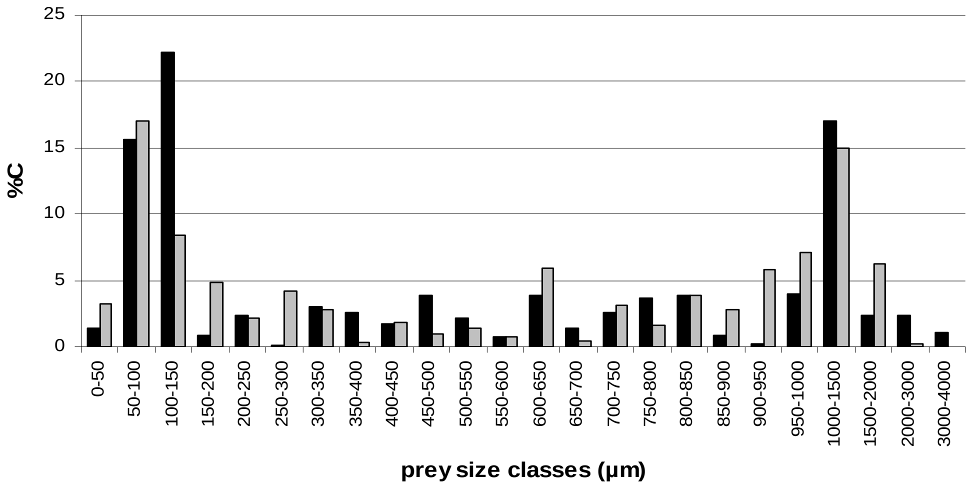

Figure 3.2 – Contribution to dietary carbon (%C) by size class of identified prey in the stomachs of ≈18 cm sardines collected fortnightly from Peniche (black bars) and Portimão (grey bars) over the period January 2003 to March 2004.

3.4.1.3 – Seasonal variation in prey composition and relative dietary importance

Sardine prey composition shows seasonal variation at both Peniche and Portimão, particularly regarding the relative importance of phytoplankton to dietary carbon which was more important over summer (June-September), mainly at Peniche (Fig. 3.3). Prey groups that made a major contribution to dietary carbon throughout the year included copepods (adults, copepodites and nauplii) which contributed 24% ± 5.3 of dietary carbon, crustacean (including copepod) eggs (23%± 5.2), fish eggs (23% ± 8.0), dinoflagellates (14% ± 4.7), cirripeds (6% ± 3.2), diatoms (4%± 2.0) and decapods (2% ± 1.8). Together, these groups accounted for >90% of the estimated carbon content of identified prey in sardines from both areas.

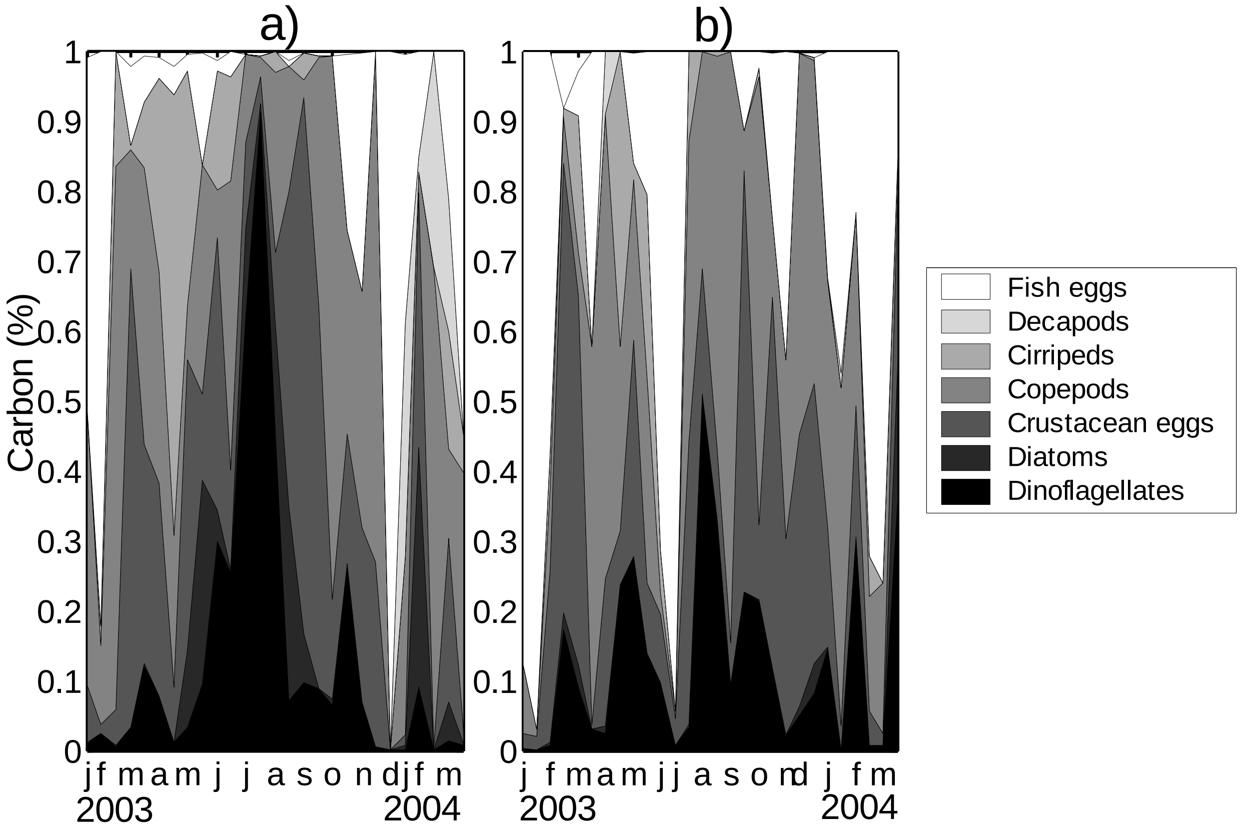

Figure 3.3 – Cumulative percentage contribution to dietary carbon (%C) by prey category in the stomachs of ≈18 cm sardines collected fortnightly from Peniche (a) and Portimão (b).

The cluster analysis conducted on the percentage contribution to dietary carbon by the various prey types identified 9 categories for Peniche (Fig. 3.4 a) and 8 categories for Portimão (Fig. 3.4 b), these categories representing groups of fortnightly samples that were similar in terms of prey percentage contribution to dietary carbon.

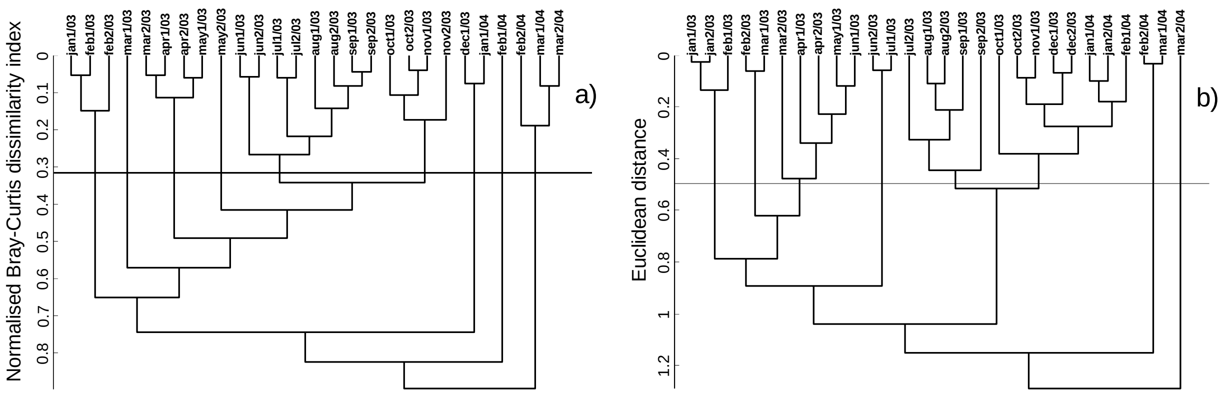

Figure 3.4 – Cluster analysis of prey composition in terms of contribution to dietary carbon for stomach samples collected fortnightly (“1” represents the first and “2” the second fortnight of each month) from Peniche (a) and Portimão (b) over the period January 2003 to March 2004.

The modified index of relative importance was estimated for each prey type for each category, which allowed identification of the most important prey per category for each area. At Peniche, 11 prey types were identified as the most important during at least one of the categories (Table 3.2), whilst 9 prey types were identified as the most important at Portimão (Table 3.3). *Protoperidinium*, *Pseudo-nitzschia*, crustacean eggs, *Euterpina*, *Oncaea*, *Centropages*, cirripedes and fish eggs were identified as the most important prey for both areas, and *Ceratium*, unidentified copepods and decapod brachyura zoea were important for sardines collected off Peniche whilst *Temora* was important for sardines collected off Portimão.

Table 3.2 – Percent modified index of relative importance (*MIRI*) of the most important prey groups in the diet of *S. pilchardus* collected from Peniche between early January 2003 (Jan1) and late March 2004 (Mar2)

***mIRI* Peniche (W)**	**Jan-Feb2**	**Mar1**	**Mar2-May1**	**May2**	**Jun1-Sep2**	**Oct1-Nov2**	**Dec1-Jan1**	**Feb1**	**Feb2-Mar2**
Ceratium spp.	0.0	0.0	19.3	82.7	1481.5	170.6	0.0	18.4	0.0
Protoperidinium spp.	3.2	96.4	371.3	432.8	1394.8	628.3	0.0	427.1	39.1
Pseudo-nitzschia spp.	0.0	0.2	207.5	2905.6	845.2	0.0	14.5	3427.2	121.1
Crustacean eggs	481.6	6541.4	2783.6	1230.4	3199.7	2101.5	83.0	3625.3	848.2
Copepods n.i.	825.4	1674.8	4.2	405.8	57.6	219.8	0.0	0.0	168.5
Euterpina spp.	192.5	9.5	1072.4	19.1	1196.0	309.8	3.2	17.6	385.1
Oncaea spp.	1924.1	0.7	51.2	2.3	171.8	1579.5	0.0	15.5	89.3
Centropages spp.	0.0	17.1	98.8	2837.9	3.8	2181.1	1097.1	103.6	1314.7
Cirripedes	424.6	59.6	3329.6	0.0	431.5	0.0	0.2	0.0	744.4
Brachyura zoea	9.2	0.0	0.0	0.0	0.0	0.0	1673.6	215.9	1037.8
Fish eggs	2944.4	1137.5	409.1	1585.0	27.6	740.3	6910.6	1516.5	1690.8

Fish eggs were one of the most important prey types during winter at both locations, whereas prey composition was more variable during spring. In 2003, samples collected at Peniche during spring were grouped into three categories; crustacean eggs, unidentified copepods and fish eggs dominated sardine diet in early March, cirripedes and crustacean eggs dominated from late March to early May, and the diatom *Pseudo-nitzschia* and copepods of the genus *Centropages* were the most important prey during late May. During the spring of 2004 the prey composition of fish from Peniche differed from that seen in spring 2003, and was dominated by fish eggs, decapod *Brachyura* zoea and *Centropages*.

Table 3.3 – Percent modified index of relative importance of the most important prey groups in the diet of *S. pilchardus* collected from Portimão between early January 2003 (Jan1) and late March 2004 (Mar2)

***mIRI* Portimão (S)**	**Jan1-Feb1**	**Feb2-Mar1**	**Mar2-Jun1**	**Jun2-Jul1**	**Jul2-Set2**	**Oct1-Feb1**	**Feb2-Mar1**	**Mar2**
Protoperidinium spp.	0.5	1020.8	754.5	196.9	1423.4	420.1	11.6	3047.2
Pseudo-nitzschia spp.	0.0	42.6	0.0	0.1	0.0	0.0	0.0	1886.8
Crustacean eggs	960.2	5852.2	1405.0	675.4	2683.1	2622.4	326.4	2540.1
Euterpina spp.	84.3	33.8	601.0	133.8	418.6	242.8	314.4	35.2
Oncaea spp.	52.4	10.6	698.5	16.2	1629.6	776.9	114.4	14.4
Temora spp.	0.0	0.0	78.0	0.0	179.0	604.6	606.5	179.3
Centropages spp.	169.2	78.3	707.2	7.6	23.3	504.0	483.5	0.0
Cirripedes	58.9	1031.6	846.3	314.9	109.5	9.1	144.3	0.0
Fish eggs	8059.2	158.8	943.1	8240.1	45.9	1608.6	7410.1	1498.5

Dietary samples collected from Portimão during the spring of 2003 were grouped into two categories, the first taken in February and early March and dominated by crustacean eggs, cirripedes and *Protoperidinium*, whereas fish eggs, *Centropages*, *Oncaea* and *Euterpina* were also important from late March to early June. Samples collected during the spring of 2004 were also grouped into two groups according to prey composition; the diet in early spring (late February and early March) being dominated by fish eggs, *Centropages* and *Temora*, while in late March fish eggs and crustacean eggs had a similar relative importance to the dinoflagellate *Protoperidinium* and the diatom *Pseudo-nitzschia*.

Prey composition during the autumn months in Peniche was dominated by crustacean eggs and the copepods *Centropages* and *Oncaea* and was quite different from the prey composition observed in winter months. In contrast, the prey composition of fish from Portimão sampled during autumn and winter was similar and was dominated by fish and crustacean eggs, followed by several copepod genera (*Oncaea*, *Temora*, *Centropages* and *Euterpina*). During summer (June until September), crustacean eggs, three phytoplankton genera (the diatom *Pseudo-nitzschia* and the dinoflagellates *Protoperidinium* and *Ceratium*) and the copepod *Euterpina* were the most important prey. The prey composition of sardines from Portimão varied during summer, with fish eggs dominating the diet until early July, and crustacean eggs, *Oncaea* and *Protoperidinium* being the most important prey from late July until September.

Digenetic trematode parasites were frequently found in sardine stomach contents from both regions (frequency of occurrence of 93% and 67% for Portimão and Peniche, respectively), as was terrestrial pollen (frequency of occurrence of approximately 60% for both areas; see Table 3.1).

The numerical abundance of identified prey was highly variable and ranged from 147 to 7466070 prey items per stomach. There was a significant relationship between average stomach content weight and prey carbon content when samples from both areas were combined ($p < 10^{-5}$, $r^2 = 0.73$, $n=56$), with zooplankton prey being primarily responsible for this relationship, followed by ichthyoplankton prey, whereas the carbon content of ingested phytoplankton was not significant in explaining variation in the stomach content weight.

3.4.2 – Feeding intensity

Sardine feeding intensity (*FI*) was examined for a total of 3599 fish, and its relation to sex and fish length was assessed. The Spearman rank correlation showed that there was no difference in *FI* between sexes ($r = 0.89$ for Peniche and $r = 0.94$ for Portimão) or for fish of different length classes (from 14 to 22 cm; $r = 0.80$ for Peniche and $r = 0.90$ for Portimão). Results of the analysis of variance indicated that *FI* varied significantly ($p<0.001$) for both areas (Fig. 3.5). Feeding intensity in fish from Peniche was higher than in those from Portimão for all seasons, and the seasonal variation in *FI* was higher at Peniche than Portimão.

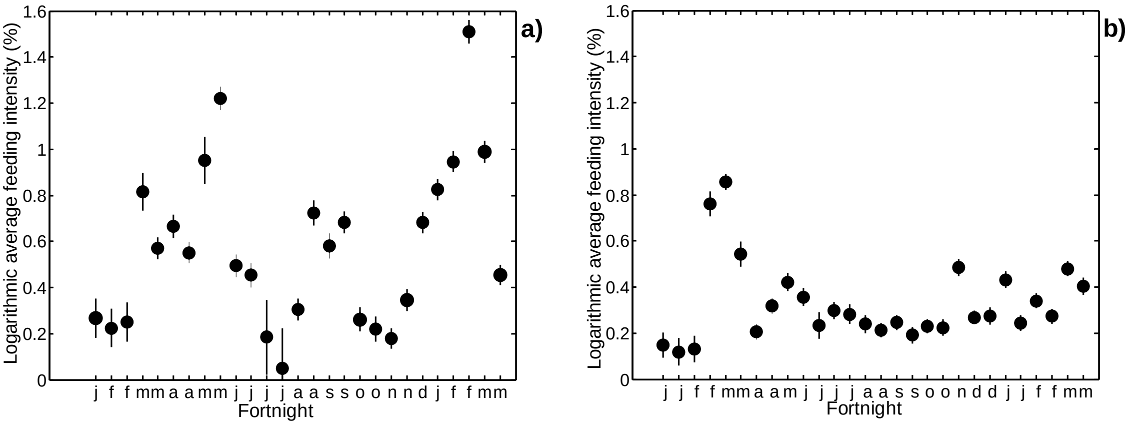

Figure 3.5 – Tukey test results of the fortnightly variation of the feeding intensity ($\log(x+1)$) in Peniche (a) and Portimão (b) from January 2003 to March 2004.

Maximum feeding intensity values were obtained during spring and winter, particularly during late February 2004 and late May 2003 in fish from Peniche when copepods and decapods for February and *Pseudo-nitzschia*, *Centropages*, crustacean eggs and fish eggs for May dominated sardine stomach contents (Table 3.2), and from end-February to March 2003 for Portimão when dietary carbon was dominated by crustacean eggs, *Protoperidinium* and cirripedes (Table 3.3). Minimum feeding intensity values were obtained during winter of 2003 for both areas, and feeding intensity during winter of 2004 was clearly higher than during winter the preceding year in both areas.

3.4.3 – Feeding intensity, ingested phytoplankton and satellite-derived chlorophyll data

Estimates of chlorophyll *a* concentration obtained from satellite remote sensing for the same spatio-temporal locations as trawls from which sardine samples were collected were compared with the calculated carbon content of autotrophic phytoplankton in sardine stomachs (Fig 3.6a), and with the average feeding intensity (Fig 3.6b) of fortnightly samples collected at Peniche and Portimão. Significant positive relations were found between chlorophyll *a* concentration and the carbon content of autotrophic phytoplankton (r=0.55 p=0.0006), as well as between chlorophyll *a* concentration and average feeding intensity (r=0.49 p=0.0021), indicating that chlorophyll data can be used to proxy the amount of plankton ingested by sardines (Fig 3.6). As expected, the correlations between the variables decreased, but was still significant when data for the same location but in a different 5 day period (either before or after the trawl) was used (r=0.44 p=0.0007 for carbon content and r=0.32 p=0.0086 for feeding intensity in relation to chlorophyll data) and when data from the nearest location for the same 5-day period was used (r=0.35 p=0.0007 for carbon content and r=0.33 p=0.014 for feeding intensity in relation to chlorophyll data).

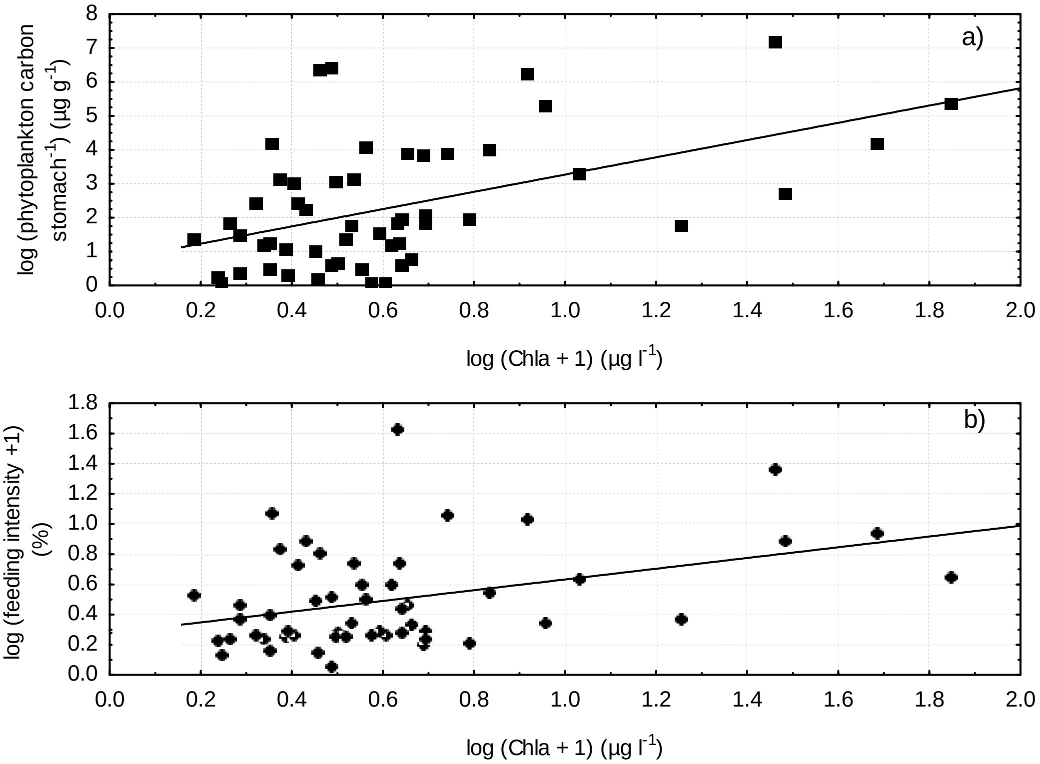

Figure 3.6 – Regression between remotely-sensed chlorophyll a concentration and (a) the carbon content of phytoplankton prey in sardine stomachs, and (b) the feeding intensity of sardines collected from Peniche and Portimão (combined) over the period January 2003 to March 2004.

3.5 – Discussion

Results from this study show that Iberian sardines have a diverse diet, with phytoplankton, zooplankton and fish eggs all making significant contributions to dietary carbon of fish off both the west and south coasts of Iberia and the relative importance of these prey groups depending on area and season. These results are in accordance with previous stomach content (numerical and volumetric estimates) and stable isotope studies of Iberian sardine diet (Varela et al. 1988, 1990, Bode et al. 2003, Garrido 2003), although the dietary contribution of fish eggs was significantly higher in this than in earlier studies. In general, zooplankton are an important dietary component throughout the year, whereas fish eggs are mostly important during winter and phytoplankton are particularly important during spring and summer for sardines living in the upwelling region off the Iberian west coast. Differences in dietary composition between spring 2003 and spring 2004 for fish from both areas and differences of feeding intensity between winter 2003 and winter 2004 shows that sardine diet has significant inter-annual variability.

Dinoflagellates, diatoms, crustacean eggs, copepods (adults, copepodites and nauplii), decapods, cirripedes and fish eggs accounted for more than 90% of the dietary carbon of the identified prey in every stomach sample from both areas. The modified index of relative importance of prey (*mIRI*) identified the most important prey groups for sardine diet as crustacean eggs, the copepods *Euterpina*, *Oncaea*, *Centropages* and *Temora*, fish eggs, the dinoflagellates *Ceratium* and *Protoperidinium*, the toxin producer diatoms of the genus *Pseudo-nitzschia*, cirripeds and the decapods Brachyura zoea.

Zooplankton was a significant source of food throughout the year with similar importance for sardine diet for both areas, and crustacean eggs, copepods and cirripedes contributed the majority of zooplankton dietary carbon. The high contribution of crustacean eggs to dietary carbon seems to indicate that this type is the most important prey for sardines, although their contribution might be overestimated since copepod eggs have been reported to survive passage through clupeoid digestive tracts (Flinkman et al. 1994).

Fish eggs were one of the most important prey types for sardines in both areas, especially during winter which is the sardine spawning season. Fish eggs were also reported in sardine stomachs by previous studies (Silva 1954, Varela et al. 1988, Garrido 2003). It was not possible to identify the majority of the fish eggs found, but most ranged from 900 to 1000 µm and probably correspond to unfertilized or Stage 1 sardine eggs since the eggs of co-habiting species such as *Trachurus trachurus*, *Scomber scombrus*, *S. japonicus*, *Macrorhamphosus* spp and *Capros aper* are generally smaller.

Egg cannibalism has been proposed as a density-dependent, self-regulatory mechanism in other clupeoid species (Szeinfeld 1991). However, the increased importance of eggs in winter, when zooplankton availability is lower, particularly in the south, suggests that sardines may be able to compensate for reduced food availability by feeding on their own eggs and/or the eggs of other fish species. During the spawning season (October until March) sardines had an average of 42 eggs per stomach, equivalent to 688 eggs per kg of fish. The average number of eggs eaten per kg of sardine per day can be estimated using the equation proposed by Hunter & Kimbrell (1980):

$$C = EE.g.t \qquad (3)$$

where C = mean number of eggs eaten per kg of fish during time t; EE = mean number of eggs observed per kg of fish mass, g is the gastric evacuation rate for eggs, and t is the duration of feeding. If sardines show a similar gastric evacuation rate for eggs to that reported for northern anchovy *Engraulis mordax* fed their own eggs ($-0.701.h^{-1}$; Hunter & Kimbrell 1980) they would be able to complete egg digestion in approximately 2 hours. Assuming that sardines use particulate-feeding to prey on fish eggs (Garrido et al. 2007) and do so only during daylight hours, they would have the ability to ingest at least four times the average amount of eggs per day estimated from stomach contents during the spawning season (there are about 9 hours of daylight during winter off the Portuguese coast), hence the average number of eggs eaten per kg of sardines per day is estimated as 4344 $eggs.kg^{-1}$ fish.

Using estimates of sardine reproductive parameters obtained for fish collected off the Portuguese coast in recent years, namely a batch fecundity of 14255 $eggs.spawn^{-1}$, a spawning fraction of 10% and a proportion of females in the adult population of 0.61 (ICES 2003), enables the calculation of egg mortality arising from cannibalism exerted by adult sardines. The resultant value of 30% is within the range of estimates calculated for various anchovy species; Hunter & Kimbrell (1980) estimated that 32% of natural mortality of northern anchovy eggs was due to cannibalism; Alheit (1987) estimated a value of 22% for Peruvian anchovy (*Engraulis ringens*), and Valdés et al. (1987) estimated a value of 70% for Cape anchovy *Engraulis capensis* (this very high value may have arisen from limited sampling in an area of unusually high egg density; a later study estimated an average value of 6%, Szeinfeld 1991). However, the first two of these studies conducted on anchovy considered that feeding occurred over the entire 24 hour period, and the third study that feeding occurred over a 15 hour period, not only during daylight as we did for sardine. Assuming 24 hour feeding by sardine would lead to an estimated 81% of sardine egg mortality being due to cannibalism.

If, however, the gastric evacuation rate of Iberian sardines is slower than $-0.701.h^{-1}$ documented for northern anchovy (evacuation rates ranging from -0.09 to $-0.27.h^{-1}$ have been reported for *Sardinops sagax* off South Africa; van der Lingen 1998), then the estimated number of eggs eaten per kg per day (4344) will be an overestimate, as will the estimate of cannibalism-induced egg mortality. The dependence of these estimations upon feeding duration and gastric evacuation rate of Iberian sardine strongly suggest that further work be conducted on this species in order to refine such estimates.

The assessment of the impact of cannibalism by adult sardines on their eggs is highly important because this might be a significant source of mortality. Previous laboratory experiments (Garrido et al. 2007) have shown that sardines select fish eggs in preference to other prey, including larger fish larvae (that were not seen in stomach contents examined during this study), when fed cultured, mixed prey assemblages. Sardine also showed selection for copepods and decapods over other zooplankton prey when fed wild, mixed prey assemblages (Garrido et al. 2007), and the selection of preferred food items by sardines can have a marked impact on plankton communities (Brooks & Dodson 1965).

Phytoplankton prey were especially important for sardine off the west coast of Portugal during summer months, when the occurrence of diatom (mostly of the genus *Pseudo-nitzschia*) blooms that are associated with upwelling events is concomitant with stratified waters that stimulate the growth of dinoflagellates (Moita 2001). This resulted in phytoplankton contributing 74-93% of the dietary carbon of sardine collected from Peniche during July 2003, and phytoplankton had a higher contribution for sardine diet throughout the year in this area than compared to fish from the south Iberian coast.

The high concentrations of the toxin-producing diatom *Pseudo-nitzschia* observed in sardine stomachs collected off Peniche from May to late September have been related to the accumulation of domoic acid in sardine viscera, making them potential vectors of this toxin to higher trophic levels (Costa & Garrido 2004). Stomachs of sardines collected off Portimão do not show such high concentrations of *Pseudo-nitzschia* since the Portuguese south coast does not experience such strong and frequent upwelling as is observed off the west coast. However, phytoplankton such as the dinoflagellates *Dinophysis acuta* and *D. acuminata* that are known toxin producers are accumulated in high concentrations in sardine stomachs from both regions (see Table 3.1), implying that sardines might also function as vectors of Diarrhetic Shellfish Poison (DSP) for higher trophic levels, which should be further examined.

Some phytoplankton groups such as dinoflagellate cysts were frequently encountered and abundant in sardine stomachs, especially for fish from south Iberia. Although it is generally assumed that the digestion by fish of resting stages of phytoplankton is not possible, dinoflagellate cysts could be a good food source because of their lipid reserves, and further work to examine whether predators are able to assimilate cysts is required (Persson 2000).

Contrary to previous perceptions we propose that summer months do not represent the main feeding season for Iberian sardines, but instead the season when fish increase their fat reserves because they are not involved in reproduction. In our study summer did not correspond to a period of increased plankton availability for either the west or south coasts of Portugal as shown by both remotely-sensed chlorophyll data, nor to a peak in sardine feeding intensity. This was also observed during 2002 when sardines collected during spring months had higher feeding intensity than those collected during the summer (Garrido 2003). Variation in feeding intensity is more strongly related to plankton production than to fish reproductive condition, which is confirmed by the lack of difference in feeding intensity between sexes and length classes, and also explains why the feeding intensity of sardines from the west coast (an upwelling area) is higher than in those from the south coast.

Results from this study suggest that sardines of the west coast of Portugal seem to have a higher food availability throughout the year, which is related to increased production as a consequence of upwelling events and higher river runoff there, than do sardines of the south coast. However, studies on the regional differences in reproduction and growth of sardines indicate that sardines grow faster and reach maturity earlier off the south coast (Silva 2003, Silva et al. 2006), which could indicate a higher food availability but is in contrast to the results of this study. This disparity could mean that growth is not controlled by food availability but by other variables such as temperature. The numerical dominance of small prey in the stomachs and the high importance of prey <750 µm to sardine dietary carbon suggests that filter-feeding is the main feeding mode employed by sardines in the wild. This implies that the composition and abundance of stomach contents would reflect ambient plankton availability, an assumption that enabled us to compare sardine diet and remotely-sensed chlorophyll a concentration. Temporal variability in remotely-sensed chlorophyll a appears to follow the same pattern of temporal variability in the phytoplankton portion of sardine diet, indicating that satellite data could be used to proxy the contribution made by phytoplankton to sardine diet. Additionally, although phytoplankton are not the only important dietary component of sardines, phytoplankton are also a rough estimate of the secondary production on which sardines also feed, and the match between the temporal variability in sardine stomach mass and the remotely-sensed chlorophyll data indicates that satellite data are also a good proxy of sardine feeding intensity.

3.6 – References

Alheit J (1987) Egg cannibalism versus egg predation: their significance in anchovies. S Afr J Mar Sci 5: 467-470

Bandarra NM, Batista I, Nunes ML, Empis JM, Christie WW (1997) Seasonal changes in lipid composition of sardine (*Sardina pilchardus*). J Food Sci 62 (1): 40-42

Bode A, Carrera P, Lens S (2003) The pelagic foodweb in the upwelling ecosystem of Galicia (NW Spain) during spring: natural abundance of stable carbon and nitrogen isotopes. ICES J Mar Sci 60:11-22

Borges MF, Santos AMP, Crato N, Mendes H, Mota B (2003) Sardine regime shifts off Portugal: a time series analysis of catches and wind conditions. Sci Mar 67: 235-244 Suppl.1

Brooks JL & Dodson SI (1965) The effect of a marine planktivore on lake plankton illustrates theory of size, competition, and predation. Science 150 (3692): 28-35

Carrera P & Porteiro C (2003) Stock dynamic of the Iberian sardine (*Sardina pilchardus*, W.) and its implication on the fishery off Galicia (NW Spain). Sci Mar 67: 245-258 Suppl. 1

Costa PR & Garrido S (2004) Domoic acid accumulation in the sardine (*Sardina pilchardus*) and its relationship to *Pseudo-nitzschia* diatom ingestion. Mar Ecol Prog Ser 284: 261-268

Cunha ME (2001) Physical control of biological processes in a coastal upwelling system: comparison of the effects of coastal topography, river run-off and physical oceanography in the northern and southern parts of Portuguese coastal waters. PhD Dissertation, University of Lisbon, Lisbon, 292 pp

Cunha ME, Garrido S, Pissarra J (2005) The use of stomach fullness and colour indices to assess *Sardina pilchardus* feeding. J Mar Biol Ass UK 85: 425-431

Fiúza AFD, DeMacedo ME, Guerreiro MR (1982) Climatological space and time-variation of the portuguese coastal upwelling. Oceanol Acta 5 (1): 31-40

Fiúza A (1983) Upwelling Patterns Off Portugal. In Nato Conference Series IV Marine Science (10a) "Coastal Upwelling: Its Sediment Record", E. Suess & J. Thiede (eds): 85-98.

Flinkman J, Vourinen I, Christiansen M (1994) Calanoid copepod eggs survive passage through fish digestive tracts. ICES J Mar Sci (51): 127-129

Garrido S (2003) Alimentação de *Sardina pilchardus* (Walbaum, 1792) ao largo da costa continental portuguesa e implicações da condição nutricional das fêmeas na qualidade dos oócitos. MS thesis, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Porto

Garrido S, Marçalo A, Zwolinski J, van der Lingen CD (2007) Laboratory investigations on the effect of prey size and concentration on the feeding behaviour of *Sardina pilchardus*. Mar Ecol Prog Ser 330: 189-199

Guisande C, Vergara AR, Riveiro I, Cabanas JM (2004) Climate change and abundance of the Atlantic-Iberian sardine (*Sardina pilchardus*). Fish Oceanogr 13 (2): 91-101

Hayse JW (1990) Feeding habits, age, growth, and reproduction of Atlantic spadefish *Chaetodipterus faber* (Pisces, Ephippidae) in Souh Carolina. Fish Bull 88 (1): 67-83

Hunter JR & Kimbrell CA (1980) Egg cannibalism in the northern anchovy *Engraulis mordax*. Fish Bull 78 (3): 811-816

ICES (2000) Report of the Study Group on the estimation of Spawning Stock Biomass of Sardine and Anchovy. ICES CM 2000/ACFM:5, 546pp

ICES (2003) Report of the Study Group on the estimation of Spawning Stock Biomass of Sardine and Anchovy, Malaga, Spain, 23–27 June 2003. ICES CM 2003/ G17, 107pp

ICES (2006) Report of the working group on the assessment of mackerel, horse mackerel, sardine and anchovy. International Council for the Exploration of the Sea (CM Papers and Reports) CM 2006/ ACFM 08, 631pp

Legendre P & Legendre L (1998) Numerical Ecology. 2nd English edition. Elsevier Science, Amsterdam. 853 pp

Lindley JA (1998) Dry weights, carbon and nitrogen content of decapod larvae from the plankton. J Mar Biol Ass UK 78: 341-344

Moita MT (2001) Estrutura, variabilidade e dinâmica do fitoplâncton na costa de Portugal continental. PhD Thesis, University of Lisbon, Lisbon

Persson A (2000) Possible predation of cysts - a gap in the knowledge of dinoflagellate ecology? J Plankton Res 22 (4): 803-809

Relvas P & Barton ED (2002) Mesoscale patterns in the Cape São Vicente (Iberian Peninsula) upwelling region. J of Geophys Res 107 (C10) 3164 doi:10.1029/2000JC000456

Silva A (2003) Morphometric variation among sardine (*Sardina pilchardus*) populations from the northeastern Atlantic and the western Mediterranean. ICES J Mar Sci 60 (6): 1352-1360

Silva A, Santos MB, Caneco B, Pestana G, Porteiro C, Carrera P, Stratoudakis Y (2006) Temporal and geographic variability of sardine maturity at length in the northeastern Atlantic and the western Mediterranean. ICES J Mar Sci 63 (4): 663-676

Silva E (1954) Some notes on the food of the pilchard *Sardina pilchardus* (Walb.) of the Portuguese coasts. Rev Fac Cienc Lisboa, 2ª Série (4) 2:281-294.

Szeinfeld EV (1991) Cannibalism and intraguild predation in clupeoids. Mar Ecol Prog Ser (79): 17-26

Thomas D & Franz B (2005) Overview of SeaWiFS Data Processing and Distribution, URL: http://oceancolor.gsfc.nasa.gov/DOCS/SW_proc.html

Valdés ES, Shelton, PA, Armstrong MJ, Field JG (1987) Cannibalism in South African anchovy: egg mortality and egg consumption rates. S Afr J Mar Sci 5: 613-622

van der Lingen CD (1998) Gastric evacuation, feeding periodicity and daily ration of sardine *Sardinops sagax* in the southern Benguela upwelling ecosystem. In Benguela Dynamics: Impacts of Variability on Shelf-Sea Environments and their living resources. Pillar SC, Moloney CL, Payne AIL and Shillington FA (eds) S Afr J Mar Sci (19): 305-316

van der Lingen CD (2002) Diet of sardine *Sardinops sagax* in the Southern Benguela Upwelling Ecosystem. S Afr J Mar Sci 24: 301-316

Varela M, Larrañaga A, Costas E, Rodríguez B (1988) Contenido estomacal de la sardina (*Sardina pilchardus*, Walbaum) durante la campaña Saracus 871 en las plataformas Cantábrica y de Galicia en Febrero de 1987. Bol Inst Esp Oceanogr 5:17-28

Varela M, Alvarez-Ossorio MT, Valdés L (1990) Método para el estudio cuantitativo del contenido estomacal de la sardina. Resultados preliminares. Bol Inst Esp Oceanogr 6:117-126

CHAPTER 4

DOMOIC ACID ACCUMULATION IN THE SARDINE (*Sardina pilchardus*) AND ITS RELATIONSHIP TO *Pseudo-nitzschia* DIATOM INGESTION[*]

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[*] Costa P & Garrido S (2004) Domoic acid accumulation in the sardine (*Sardina pilchardus*) and its relationship to *Pseudo-nitzschia* diatom ingestion. Mar Ecol Progr Ser 284: 261-268

4.1 – Abstract

Planktivorous fish are key potential vectors for the phycotoxin domoic acid (DA), produced naturally by diatoms from the genus *Pseudo-nitzschia*. The Atlantic sardine (*Sardina pilchardus*) diet is largely dominated in number by microplanktonic species such as chain forming diatoms, which makes it particularly susceptible to accumulate and transfer this toxin to the higher trophic levels. DA concentration in sardine tissues and *Pseudo-nitzschia* ingestion were monitored fortnightly during 2002 and 2003 off the W coast of Portugal, where seasonal upwelling events are responsible for the occurrence of algal blooms. Sardine stomach content analysis showed that *Pseudo-nitzschia* prey reached concentrations as high as 7.8 x 10^6 cells g^{-1}; in some cases this diatom genus represented more than 99% of the phytoplanktonic prey identified in the stomachs. Four different diatom species were distinguished using scanning electron microscopy (SEM): *P. australis, P. pungens, P. pseudodelicatissima* and *P. delicatissima*. DA accumulation in sardines was linearly dependent on *P. australis* consumption. Toxin content per individual cell was estimated by relating DA and *P. australis* concentrations in the stomach contents. DA production by *P. australis* in the summer months was significantly higher than during the spring. In both years, DA in sardine guts was initially detected in May and peaked several times until late summer. Toxin distribution in the different tissues was also determined, with the highest DA levels detected in the intestine. The maximum toxin concentration observed in sardine guts was 128.5 µg DA g^{-1}. No DA was found in the sardine muscle; consequently implications to human health appear minimal.

4.2 – Introduction

Phytoplankton is the basis of marine food webs, supporting directly filter-feeding organisms. Some species of dinoflagellates and diatoms produce toxins that can cause poisoning in vertebrates. The accumulation and transfer of these toxins through the food chain can cause intoxication of consumers at higher trophic levels. Massive deaths of sea birds and marine mammals due to their predation on planktivorous fish have been reported in California, USA (Work et al. 1993, Sierra Beltrán et al. 1997, Lefebvre et al. 1999, Scholin et al. 2000). It was shown that the northern anchovy (*Engraulis mordax*), a planktivorous filter-feeder, was responsible for such events, acting as a domoic acid vector (Fritz et al. 1992, Lefebvre et al. 2001, 2002).

Domoic acid (DA), known as the amnesic shellfish toxin, is produced by some species of the diatom genus *Pseudo-nitzschia* (Subba Rao et al. 1988, Bates et al. 1989, Buck et al. 1992, Garrison et al. 1992). The illness referred to as Amnesic Shellfish Poisoning (ASP) was first recognised in 1987 in Canada, where at least 3 elderly people died and more than 100 became ill, suffering from varying degrees of gastrointestinal and neurological illnesses, due to consumption of contaminated blue mussels (Quilliam & Wright 1989, Todd 1993). The toxin is a water-soluble amino acid that binds irreversibly to glutamate receptor sites, causing destructive neuronal depolarisation (Debonnel et al. 1989) and permanent short-term memory loss in mammals (Perl et al. 1990, Todd 1993).

Although there is great concern on the effects of phycotoxins to higher organisms including marine mammals, sea birds and also humans, the trophic links between these organisms should be elucidated. In Europe, there has been extensive research focusing on DA accumulation in bivalves, however studies on toxin transfer through the pelagic food chain are scarce. Recent studies in California indicate that planktivorous fish are potentially more effective vectors than bivalves (Scholin et al. 2000, Lefebvre et al. 2002).

During a period of shellfish contamination in 2000 off Portugal, sardine (*Sardina pilchardus*) was found to accumulate high levels of DA (Vale & Sampayo 2001). In Portuguese waters, this omnivorous species is the most abundant fish with high commercial value. Sardines are primarily filter-feeders, whose diet composition is closely related to plankton availability (Varela et al. 1988, 1990). In terms of biovolume sardine diet is mostly supported by mesozooplankton organisms, which are assimilated more efficiently than phytoplankton (van der Lingen 1998, Bode et al. 2004). However, it is numerically dominated by organisms smaller than 200 µm, consuming large quantities of phytoplankton (Garrido 2003). Diatoms are especially abundant in the Portuguese coast during spring months (Moita 2001), when they can account for more than 90% of the total number of prey in sardine stomachs (Garrido 2003), suggesting that they can act as an important vector of the phycotoxin domoic acid.

The objective of this work is to study the seasonality and transfer of DA from the toxin producers to a direct consumer. To accomplish this objective, DA levels in sardine tissues were monitored during 2002 and 2003 in the fishing area of Peniche (Portuguese W coast), a region where seasonal upwelling events give rise to algal blooms, including *Pseudo-nitzschia* spp. Sardine diet was analysed, identifying and quantifying microplankton prey in stomach contents, with particular attention to toxic phytoplankton species, in order to establish a link between *Pseudo-nitzschia* ingestion and DA occurrence.

4.3 – Materials and methods

4.3.1 – Sample collection

Sardine samples were obtained fortnightly during 2002 and 2003 from purse seiners fishing off Peniche (W Portuguese coast), in a depth range of 12-70 m and 6 to 25 Km from the coast. Sampling was intensified in late spring 2003, when the first *Pseudo-nitzschia* cells in sardine stomach contents were observed in high concentrations (7 samples in May and 4 in June).

4.3.2 – Sardine stomach contents analysis

Stomach content analysis was performed in sardines collected during 2003. Analysis showed low coefficient of variation of the mean stomach contents weights (*CV* <25%) between individuals from the same sample date and length class, which led us to assume minimal differences in their diet. In each sample, 10 stomach contents from sardines of 18 cm total length (representative of the most abundant length class in the fishery) were pooled and homogenized prior to the analysis. A portion of the homogenate was used for DA determination (1 g), the remaining was sieved (200 µm) and a subsample was taken, excluding large prey (>200 µm), for identification and quantification of the microplankton under a light microscope (400x).

4.3.3 – Scanning electron microscopic (SEM) analysis

When *Pseudo-nitzschia* cells were present in stomachs, a subsample of the stomach content was used for their identification and enumeration, using SEM. Diatoms frustules were cleaned using the von Stosch’s method (Hasle & Syvertsen 1996). Samples were coated with gold and examination was carried out using a JEOL JSM-5200, usually at 20 kV accelerating voltage. The percentage of each *Pseudo-nitzschia* species was determined in a total count of at least 100 cells. To distinguish these species from each other, diverse characters were observed, such as the shape of the valve (which is asymmetrical in the case of the *P. australis* as opposed to the symmetric shape of the strongly silicified *P. pungens*), the width of the cell, the number and size of poroids and also the presence of a central nodulus (Hasle & Syvertsen 1996, Hasle et al. 1996, Skov et al. 1999). *P. pseudodelicatissima* and *P. delicatissima* were identified according to the previous authors, although they are under taxonomic revision (Lundholm et al. 2004).

4.3.4 – Toxin extraction and HPLC analysis

Sardines were dissected 5-10 hours after the capture while kept in fresh (4-7ºC), to minimize DA leakage and contamination across tissues; whole guts (the digestive tract and all internal organs including the gonads) of 5 individuals were removed and homogenized with a Polytron PT 3100. A 5g aliquot was kept at –20ºC for subsequent DA determination. Three naturally contaminated samples were selected to study the DA distribution in the different sardine tissues. Three sets of 5 individuals in each sample were used to determine DA concentrations in the following tissues: (1) intestine, (2) stomach, (3) liver, (4) gonads, (5) brain and (6) muscle.

Extracts for analysis were prepared according to Quilliam et al. (1995) with some modifications (Vale & Sampayo 2001). The extraction was performed with aqueous 50% methanol (ratio 1:4) at 20.000 rpm with a homogeniser probe for 1 min, followed by 10 minutes centrifugation at 4000 rpm. The supernatant was filtered (0.22 µm) and the equivalent of 1.0 mg extract (5 µl) was injected onto the column without any further clean-up.

HPLC analysis was performed on a Hewlett-Packard (HP) Model 1100 equipped with an in-line degasser, quaternary pump, autosampler, oven and diode-array detector (DAD); data collection and treatment of results were performed by the HP *Chemstation* software. The column used was a Nucleosil 100-5C-18 (125x3 mm, 5 µm), with a guard-column *Lichrospher* 100 RP-18 (4x4 mm, 5 µm). Detection wavelength was set at 242 nm with a 10 nm bandwidth, and reference wavelength at 450 nm with a 100 nm bandwidth. A confirmatory wavelength at 262 nm was used.

Calibration was performed with a full set of standards of DA (0.5, 2, 4, and 10 µg ml^{-1}). A single point calibration, with a working solution of 4 µg ml^{-1} DA in 10% acetonitrile was performed after six consecutive samples. DACS-1D certified DA standard was purchased from the National Research Council of Canada (NRC). Under these conditions the detection limit was 0.04 µg ml^{-1} that corresponded to 0.2 µg g^{-1} in tissue.

4.3.5 – Statistics

A least squares linear regression analysis was applied to data, using the statistical software R version 1.9.1. (www.r-project.org). At first, DA concentration in each stomach was considered to be a function of the interaction between *P. australis* cell counts and season and *P. pseudodelicatissima* concentration (which occurred only during the summer), through a multiple regression analysis. Two seasons were considered, spring (March until May) and summer (June until September), since no DA production was found in the rest of the year. Through backward stepwise regression analysis, the variables that were not significant to the model were removed (Draper & Smith 1998).

4.4 – Results

4.4.1 – Domoic acid in sardine

Domoic acid was found in sardine guts during both 2002 and 2003 (Fig. 4.1). In 2002, DA was detected from early May until late August, reaching its peak concentration (43 µg g^{-1}) in the first week of July. In 2003, a small amount of DA (1.3 µg g^{-1}) was detected during March in the sardine guts. In the same year, several DA peaks were detected from May to middle September, reaching a maximum concentration of 128.5 µg DA g^{-1} in late June.

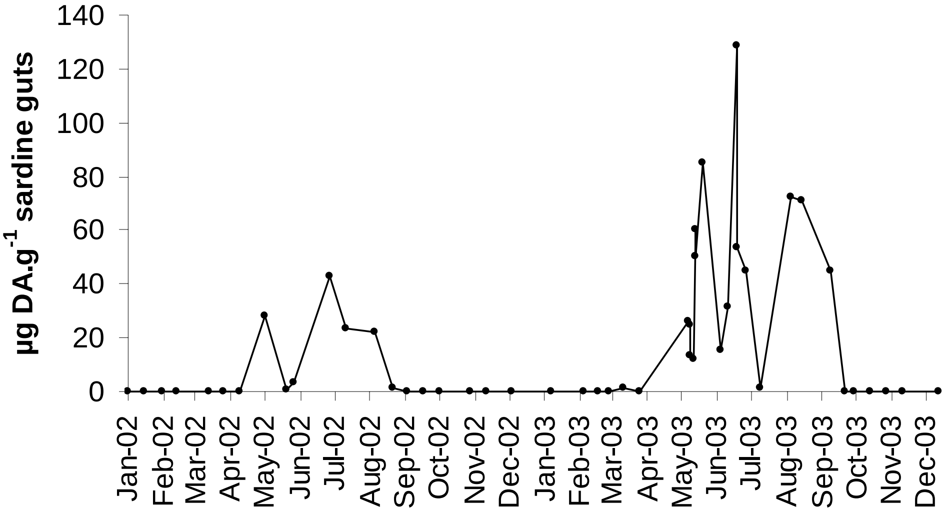

Figure 4.1 – Domoic acid concentration (µg g^{-1}) detected fortnightly in sardine guts collected during 2002 and 2003

Distribution of DA in different sardine tissues (Fig. 4.2) showed higher concentrations of the toxin in the intestine, followed by the stomach, liver and gonads. The intestine presented levels about 3 times higher than the stomach, and about 9 and 15 times higher than the liver and gonads, respectively. DA was not detected in the brain nor in the sardine muscle.

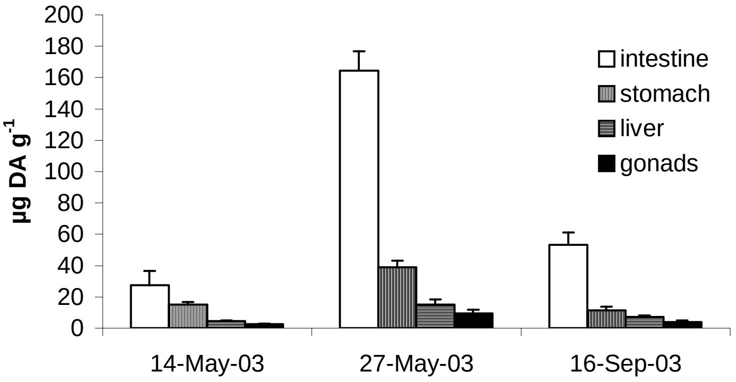

Figure 4.2 – Domoic acid concentration (µg g^{-1}, mean ± SD) in the different sardine tissues of 3 selected samples (5 individuals each)

4.4.2 – Sardine stomach content analysis

The seasonal pattern of occurrence for *Pseudo-nitzschia* and DA was similar (Fig. 4.3). During 2003, *Pseudo-nitzschia* were absent until mid March, when low concentrations were for the first time observed. During this period, sardine microplanktonic prey identified in the stomach contents consisted primarily of dinoflagellate species (such as *Scrippsiella spp.*) and the most abundant diatom was the chain forming benthic *Paralia sulcata*.

During the first half of May there was a sudden rise in the abundance of *Pseudo-nitzschia*, with densities 3 orders of magnitude above that registered for all other phytoplankton. High concentrations of these diatoms in sardine stomachs were observed until mid September. The maximum concentration was observed in late May, when 7.8 x 10^6 cells g^{-1} of sardine stomach content were recorded. In 75% of the stomach contents where *Pseudo-nitzschia* spp. occurred, this diatom was the most abundant phytoplankton prey, making up to 99% of the total number of phytoplankton cells found in the stomachs during May and August (Fig. 4.4).

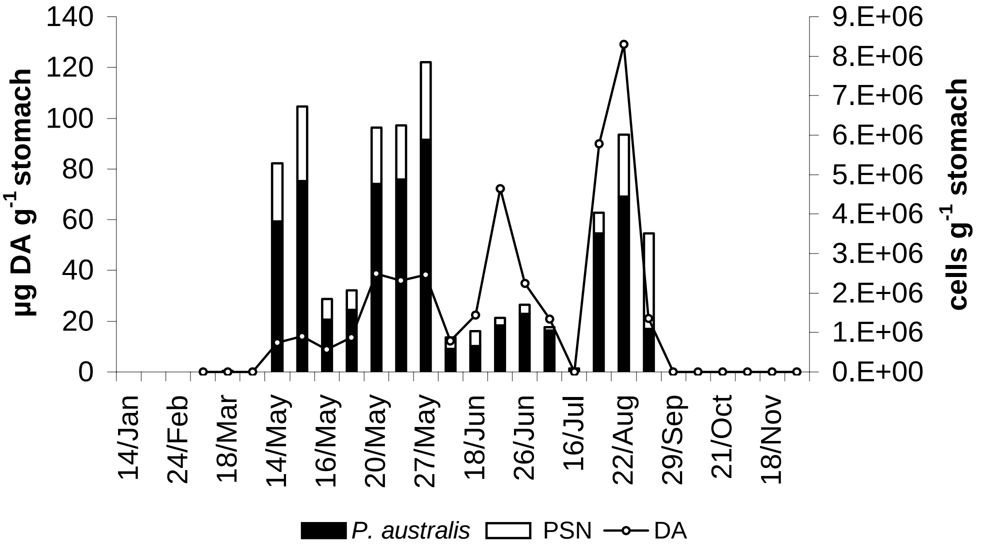

Figure 4.3 – Domoic acid (µg g^{-1}), *Pseudo-nitzschia australis* (cells g^{-1}) and the remaining species of the *Pseudo-nitzschia* genus (PSN) concentrations, observed in stomach contents of sardines collected during 2003

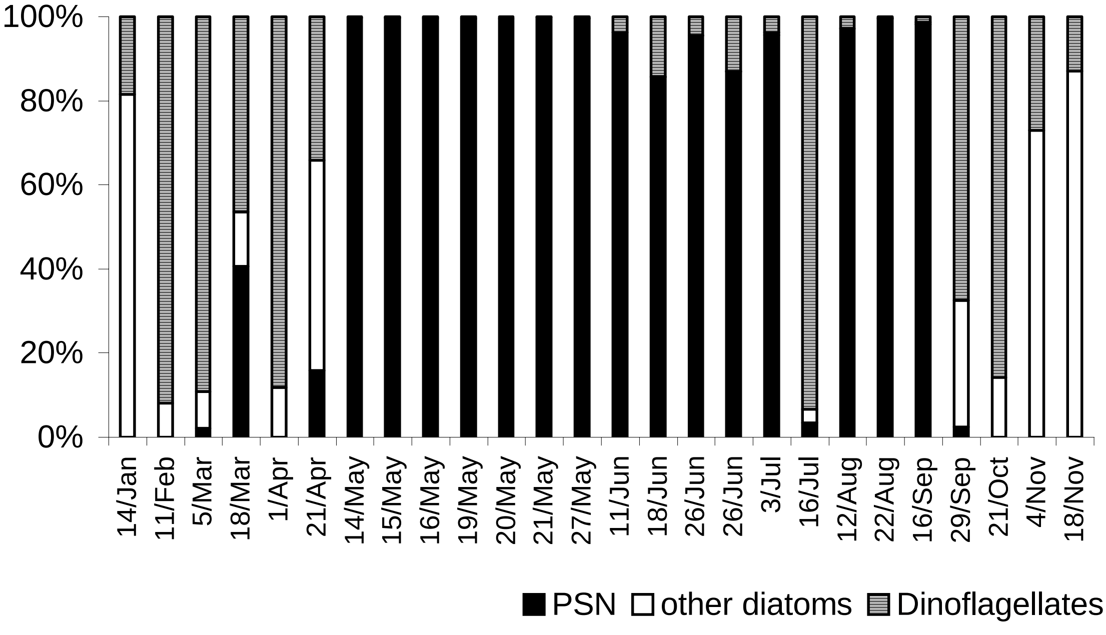

Figure 4.4 – Relative abundance (%) of *Pseudo-nitzschia* species (PSN), other diatoms and dinoflagellates found in sardine stomach contents during 2003

Four species of the diatom genus *Pseudo-nitzschia* were identified using SEM (Table 4.1). Whenever *Pseudo-nitzschia* spp. occurred, *P. australis* and *P. pungens* were present, and in 80% of the cases *P. australis* accounted for more than 60% of the

Pseudo-nitzschia species. *P. pseudodelicatissima* and *P. delicatissima* occurred during the summer, from June until September.

Table 4.1 – Relative abundance (%) of different Pseudo-nitzschia species in stomach contents during 2003.

Date	*P. australis*	*P. pungens*	*P. delicatissima*	*P. pseudodelicatissima*
14 May	72	28	0	0
15 May	72	28	0	0
16 May	72	28	0	0
19 May	76	24	0	0
20 May	77	23	0	0
21 May	78	22	0	0
27 May	75	25	0	0
11 Jun	66	10	0	24
18 Jun	63	9	28	0
26 Jun	83	14	0	3
16 Jul	8	22	49	21
12 Aug	86	6	0	8
22 Aug	74	8	0	18
16 Sep	31	8	3	58

Each time DA was detected in sardines, *Pseudo-nitzschia* cells were found in their stomachs. Moreover, *P. australis* cells were consistently identified during the toxic periods (Fig. 4.3). *P. pseudodelicatissima*, which is also considered toxic in some regions (review in Bates 2000) was present in the stomach contents during the summer. These results highlighted a link between DA and the number of *P. australis* cells in the stomach content. Through a linear regression model, DA content was related to the interaction between cell counts and season. As the intercept was not significant, regression lines were forced to cross zero. No statistically significant relationship was found between *P. pseudodelicatissima* cell counts and DA concentration in the stomachs. Significant differences were found between seasons ($p<0.001$), generating two different slopes. The final regression model related DA concentration with the interaction between *P. australis* cell counts and season ($r^2=0.93$) (Fig. 4.5). The estimation of DA content per *P. australis* cell showed higher values during the summer, ranging from 19 to 34 pg DA cell^{-1} (however, a concentration of 61pg DA cell^{-1} was detected for a single sample) while spring values ranged from 3 to 9 pg DA cell^{-1}. The average DA content per cell estimated from the model was 30 and 6 pg during the summer and spring, respectively.

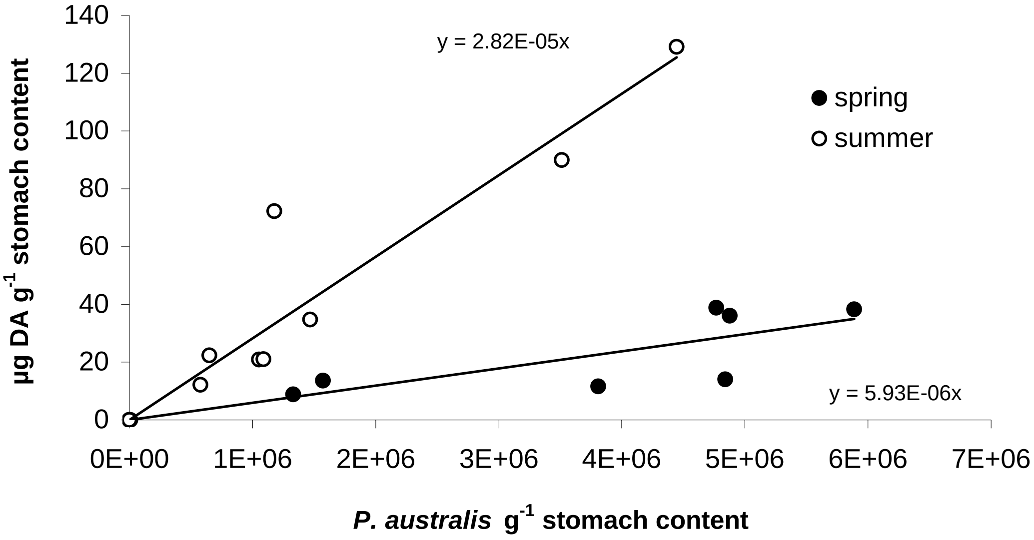

Figure 4.5 – Linear regression between DA concentration and the number of *Pseudo-nitzschia australis* cells in the stomach content samples during the spring (closed circles, n=9) and the summer (open circles; n=10). The equations are of the form y=ax, since the intercept was not significant. As DA production was significantly different between seasons, two different slopes are presented. R^2 applyes for the total regression model.

4.5 – Discussion

The importance of pelagic fish on DA transfer to higher trophic levels has been proposed in several works (e.g. Lefebvre et al. 1999, Lefebvre et al. 2002, Scholin et al. 2000). Sardines were pointed out as a particularly relevant group since they are able to filter and digest phytoplankton species efficiently and can accumulate large quantities of DA in their guts. Off the Portuguese coast, *Sardina pilchardus* is the most abundant planktivorous fish being the target of a directed purse seine fishery of economic relevance. Over the years, several aspects of the biology and dynamics of this species have been discussed, however studies characterizing their feeding habits and diet composition are scarce. Observations on South African sardine (*Sardinops sagax*) drew attention to a strategy for maximizing the net energy through prolonged bouts of low-cost filter-feeding, despite being capable of particulate-feeding, resulting in the ingestion of a large number of phytoplanktonic cells (van der Lingen 1994, 1995). In fact, zooplankton is the major contributor in terms of biovolume but phytoplankton is numerically dominant in the stomach contents of adult sardines collected off Portugal (Garrido 2003).

Sardine phytoplanktonic prey were characterised in this work by the numerical dominance of diatoms from the genus *Pseudo-nitzschia* mainly during spring and summer months (99% of the total phytoplankton prey in some cases). Previous studies confirmed the occurrence in the Portuguese coast of higher densities of *Pseudo-nitzschia* spp during spring and summer months, associated with the occurrence of upwelling events (Abrantes & Moita 1999, Moita 2001). *Pseudo-nitzschia* cells in sardine stomachs were especially abundant during May 2003, with concentrations up to 7.8 x 10^6 cells g^{-1}, a higher value than the average levels found by McGinness et al. (1995) in stomach contents of Californian northern anchovies.

In this study, four species of *Pseudo-nitzschia* were identified. The relative abundance of *Pseudo-nitzschia* species showed that the toxic *P. australis* was dominant. In addition, a link was established between the concentration of DA and the number of *P. australis* cells in each stomach content. The observations presented here show that DA production by *P. australis* increased during the summer, reaching values one order of magnitude higher than the ones obtained during the spring. DA production is affected by several environmental conditions such as irradiance and temperature (Lewis et al.

1993, Bates 1998), pH (Lundholm et al. 2004) and mostly by nutrient availability (review in Bates 1998), namely silicate and phosphate as well as trace metals such as iron. Theories developed from laboratory studies, mainly on *P. multiseries*, have shown that production of DA is associated with physiological stress caused by silicate and phosphate limitation (Bates et al. 1991, Pan et al. 1996a, Pan et al. 1996b, Bates 1998, Pan et al. 1998). Although field measurements of nutrient availability were not carried out in this work, it has been reported in other surveys that during the summer, nutrients such as silicate and phosphate decrease sharply in surface waters, whereas in spring months there is a strong fluvial nutrient input (Bôto 1945, Moita 2001). Recent studies on the relationship between trace metal availability and DA production by toxigenic *P. multiseries* and *P. australis* suggested that production is directly induced by Fe-deficient or Cu stress conditions (Rue & Bruland 2001, Maldonado et al. 2002). Therefore, changes in trace metal conditions in coastal waters are likely to change intracellular DA concentration and consequently influence the toxic effect of these diatom species.

In some regions of the world, *P. pseudodelicatissima* was recognized to produce DA at low concentrations (7 fg DA cell^{-1}, Martin et al. 1990; 0.12 pg DA cell^{-1}, Rhodes et al. 1998; 36 fg DA cell^{-1}, Pan et al. 2001; up to 4.6 pg DA cell^{-1}, Trainer et al. 2002, review in Bates 2000). In this study, *P. pseudodelicatissima* occurred in sardine stomachs during the summer but was not related to DA production, which is similar to the results of Scholin et al. (2000). Furthermore, DA was not detected in sardine stomachs collected in middle July, when *P. pseudodelicatissima* cells reached a concentration of 1.9 x 10^{4} cells g^{-1}.

This work represents the first effort to estimate DA production by the diatom *P. australis* from cells found in stomach contents. *Pseudo-nitzschia* abundance and consequently DA content per individual cell might be slightly overestimated due to digestion. Moreover, *Pseudo-nitzschia* consumed by sardine zooplanktonic prey were not counted and might increase DA values detected by HPLC. Nevertheless, DA levels registered in this estimation (3-34 pg DA cell^{-1}) were comparable to concentrations measured in field populations of *P. australis* from the California coast, where most estimates ranged between 7-32 pg DA cell^{-1} and the highest cellular levels reached 75-78 pg DA cell^{-1} (Scholin et al. 2000, Trainer et al. 2000). Cell cultures of this species showed a similar DA production (12-37 pg cell^{-1}, Garrison et al 1992, 2 pg cell^{-1}, Rhodes et al. 1996, 26 pg cell^{-1}, Cusack et al. 2002). As suggested by Trainer et al. (2002), laboratory measurements of specific cellular toxicity do not necessarily reflect actual levels in the field, which emphasises the importance of studies using routine analysis under natural conditions.

Maximum DA concentration (128.5 µg g^{-1} sardine guts) registered in late June 2003 was within the range of the DA detected during toxic events in California that caused severe intoxication in marine mammals (30-110 µg g^{-1}, Scholin et al. 2000, 169-728 µg g^{-1}, Lefebvre et al. 2002). Furthermore, higher DA concentrations (492.4 µg DA g^{-1}) were recorded during 2000 in the guts of sardines collected off Portugal (Vale & Sampayo 2001). *Sardina pilchardus* is at the basis of the marine food web, so it can act as an important domoic acid vector to several predators such as dolphins, which are large consumers of sardines in Portuguese waters (Silva 1999). Human intoxications due to sardine ingestion are unlikely to occur since DA was not detected in the muscle, the edible part. As described for other vertebrates, just small amounts of the toxin cross the gastrointestinal tract or blood-brain barrier (Iverson et al. 1989, Preston & Hynie 1991). Only during acute toxic episodes, with particularly high toxin concentrations in the viscera, trace levels of DA were detected in sardine and anchovy muscle (> 500 µg

g^{-1}, Lefebvre et al. 2001, 2002). Consumption of sardine juveniles without evisceration might represent a risk to human health, although juveniles appear to be less effective in retaining phytoplankton than adults (Bode et al. 2003).

DA was mainly accumulated in the intestine, followed by the stomach. Some possible reasons might be the higher content:tissue ratio of the intestine (with a thinner wall than the stomach) and due to differential rates of prey digestion and assimilation and elimination, as reported by (van der Lingen 1998), which is likely to increase phyto:zooplankton proportion within the intestine. DA was absent in the brain tissue and detected in low levels in the gonads. Earlier studies demonstrated that high concentrations of DA might affect neurologically anchovies (Lefebvre et al. 2001) as it does to mammals, but there is no information whether it affects sardine reproduction though in this region, sardines mainly spawn during winter and spring months.

In the Portuguese coast upwelling events are responsible for the occurrence of algal blooms, including *Pseudo-nitzschia* species, during spring and summer months. Consequently, high concentrations of this diatom are found in sardine stomach contents for this period. Several environmental factors such as exhaustion of nutrients are probably responsible for the higher production of DA by *P. australis* during the summer observed in this study. Our results show that sardines accumulate high concentrations of *Pseudo-nitzschia* and DA in their guts, acting as important toxin vectors in the marine food web, mainly during summer conditions.

4.6 – References

Abrantes F & Moita MT (1999) Water column and recent sediment data on diatoms and cocolithophorids, off Portugal, confirm sediment record upwelling events. Oceanol Acta 22: 319-336

Bates SS (1998) Ecophysiology and metabolism of ASP toxin production. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) The physiological ecology of harmful algal blooms. Springer-Verlag, Heidelberg, p 405-426

Bates SS (2000) Domoic-acid-producing diatoms: another genus added! J Phycol 36: 978-985

Bates SS, Bird CJ, de Freitas ASW, Foxall R, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Odense P, Pocklington R, Quilliam MA, Sim PG, Smith JC, Subba Rao DV, Todd ECD, Walter JA, Wright JLC (1989) Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. Can J Fish Aquat Sci 46: 1203-1215

Bates SS, de Freitas ASW, Milley JE, Pocklinghton R, Quilliam MA, Simth JC, Worms J (1991) Controls on domoic acid production by the diatom *Nitzschia pungens f. multiseries* in culture: nutrients and irradiance. Can J Fish Aquat Sci 48: 1136-1144

Bode A, Carrera P, Lens S (2003) The pelagic foodweb in the upwelling ecosystem of Galicia (NW Spain) during spring: natural abundance of stable carbon and nitrogen isotopes. ICES J Mar Sci 60: 11-22

Bode A, Alvarez-Ossorio M.T, Carrera P, Lorenzo J (2004) Reconstruction of the trophic pathways between plankton and the North Iberia sardine (*Sardina pilchardus*) using stable isotopes. Sci Mar 68: 165-178

Bôto RG (1945) Contribuição para os estudos de oceanografia ao longo da costa de Portugal- Fosfatos e nitratos. Trav St Biol Marit Lisb 49: 1-102

Buck KR, Uttal-Cooke L, Pilskaln CH, Roelke DL, Villac MC, Fryxell GA, Cifuentes L, Chavez FP (1992) Autecology of the diatom *Pseudonitzschia australis* Frenguelli, a domoic acid producer, from Monterey Bay, California. Mar Ecol Prog Ser 84: 293-302

Cusack CK, Bates SS, Quilliam MA, Patching JW, Raine R (2002) Confirmation of domoic acid production by *Pseudo-nitzschia australis* (Bacillariophyceae) isolated from Irish waters. J Phycol 38: 1106-1112

Debonnel G, Beauchesne L, Demontigny C (1989) Domoic acid, the alleged mussel toxin, might produce its neurotoxic effect through kainite receptor activation- an electrophysical study in the rat dorsal hippocampus. Can J Physiol Pharmacol 67: 29-33

Draper NR & Smith H (1998) Applied regression analysis. 3rd edn. John Wiley & Sons, Inc., NY

Fritz L, Quilliam MA, Wright JLC, Beale A, Work TM (1992) An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudonitzschia australis*. J Phycol 28: 439- 442

Garrido S (2003) Alimentação de *Sardina pilchardus* (Walbaum, 1792) ao largo da costa continental portuguesa e implicações da condição nutricional das fêmeas na qualidade dos oócitos. MS thesis, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Porto

Garrison DL, Conrad SM, Eilers PP, Waldron EM (1992) Confirmation of domoic acid production by *Pseudonitzschia australis* (Bacillariophyceae) cultures. J Phycol 28: 604-607

Hasle GR & Syvertsen EE (1996) Marine diatoms. In: Tomas CR (ed) Identifying marine diatoms and dinoflagelates. New York, Academic Press, Inc. p 5-385

Hasle GR, Lange CB, Syvertsen EE (1996) A review of *Pseudo-nitzschia*, with special reference to the Skagerrak, North Atlantic and adjacent waters. Helgol Meeresunters 50: 131-175

Iverson F, Truelove J, Nera E, Tryphonas L, Campbell J, Lok E (1989). Domoic acid poisoning and mussel-associated intoxication: preliminary investigations into the response of mice and rats to toxic mussel extract. Fd Chem Toxic 27: 377-384

Lefebvre KA, Powell CL, Busman M, Doucette GJ, Moeller PDR, Silver JB, Miller PE, Hughes MP, Singaram S, Silver MW, Tjeerdema RS (1999) Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. Nat Toxins 7: 85-92

Lefebvre KA, Dovel SL, Silver MW (2001) Tissue distribution and neurotoxic effects of domoic acid in a prominent vector species, the northern anchovy *Engraulis mordax*. Mar Biol 138: 693-700

Lefebvre KA, Silver MW, Coale SL, Tjeerdema RS (2002) Domoic acid in planktivorous fish in relation to toxic *Pseudo-nitzschia* cell densities. Mar Biol 140:625-631

Lewis NI, Bates SS, McLachlan JL, Smith JC (1993) Temperature effects on growth, Domoic acid production, and morphology of the diatom *Nitzschia pungens f. multiseries*. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Amsterdam, Elsevier p 601-606

Lundholm N, Hansen PJ, Kotaki Y (2004) Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*. Mar Ecol Prog Ser 273: 1-15

Maldonado MT, Hughes MP, Rue EL, Wells M (2002) The effect of Fe and Cu on growth and domoic acid production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. Limnol Oceanogr 47: 515-526

Martin JL, Haya K, Burridge LE, Wildish DJ (1990) *Nitzschia pseudodelicatissima*- a source of domoic acid in the Bay of Fundy, eastern Canada. Mar Ecol Prog Ser 67: 177-182

McGinness KL, Fryxell GA, McEachran JD (1995) *Pseudonitzschia* species found in digestive tracts of northern anchovies (*Engraulis mordax*). Can J Zool 73: 642-647

Moita MT (2001) Estrutura, variabilidade e dinâmica do fitoplâncton na costa de Portugal continental. PhD Thesis, University of Lisbon, Lisbon

Pan Y, Subba Rao DV, Mann KH, Brown RG, Pocklinghton R (1996a) Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseries*. I. Batch cultures studies. Mar Eco Prog Ser 131: 225-233

Pan Y, Subba Rao DV, Mann KH (1996b) Changes in domoic acid production and cellular chemical composition of the toxigenic diatom *Pseudo-nitzschia multiseries* under phosphate limitation. J Phycol 32: 371-381

Pan Y, Bates SS, Cembella A (1998) Environmental stress and domoic acid production by *Pseudo-nitzschia*: a physiological perspective. Nat Toxins 6: 127-135

Pan Y, Parsons ML, Busman M, Moeller PDR, Dortch Q, Powell CL, Doucette GJ (2001) *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* - a confirmed producer of domoic acid from the northern Gulf of Mexico. Mar Ecol Prog Ser 220: 83-92

Perl TM, Bedard L, Kosatsky T, Hocklin JC, Todd EDC (1990) An outbreak of toxic encephalopathy by eating mussels contaminated with domoic acid. N Engl J Med 322: 1775-1780

Preston E & Hynie I (1991) Tranfer constants for blood-brain barrier permeation of the neuroexcitatory shellfish toxin, domoic acid. Can J Neurol Sci 18:39-44

Quilliam MA & Wright JLC (1989) The amnesic shellfish poisoning mystery. Anal Chem 61: 1053A-1060A

Quilliam MA, Xie M, Hardstaff WR (1995) Rapid extraction and cleanup for liquid chromatographic determination of domoic acid in unsalted seafood. J AOAC Internat 78:543-554

Rhodes L, White D, Syhre M, Atkinson M (1996) *Pseudo-nitzschia* species isolated from New Zealand coastal waters: Domoic acid production in vivo and links with shellfish toxicity. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and Toxic Algal Blooms. Paris, Intergov Oceanogr Comm, UNESCO, p 155-158

Rhodes L, Scholin C, Garthwaite I (1998) *Pseudo-nitzschia* in New Zealand and the role of DNA probes and immunoassays in the refining marine biotoxin monitoring programmes. Nat Toxins 6:105-111

Rue E & Bruland K (2001) Domoic acid binds iron and copper: a possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*. Mar Chem 76: 127-134

Scholin CA, Gulland F, Doucette GJ, BensonS, Busman M, Chavez FP, Cordaro J, DeLong R, De Vogelaere A, Harvey J, Haulena M, Lefebvre K, Lipscomb T, Loscutoff S, Lowenstine LJ, Martin RI, Miller PE, McLellan WA, Moeller PDR, Powell CL, Rowles T, Silvagnl P, Silver M, Spraker T, Trainer V, Van Dolah FM (2000) Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403: 80-84

Sierra Beltrán A, Palafox-Uribe M, Grajales-Montiel J, Cruz-Villacorta A, Ochoa JL (1997) Sea bird mortality at Cabo San Lucas, Mexico: evidence that toxic diatom blooms are spreading. Toxicon 35:447-453

Silva MA (1999) Diet of dolphins, *Delphinus delphis*, off the Portuguese continental coast. J Mar Biol Ass UK 79: 531-540

Skov J, Ludholm N, Moestrup Ø, Larsen J (1999) Potencially toxic phytoplankton. 4. The diatom genus *Pseudo-nitzschia* (Diatomophyceae/ Bacillariophyceae). ICES Identification leaflets for plankton, no 185, ICES, Denmark

Subba Rao DV, Quilliam MA, Pocklington R (1988) Domoic acid - a neurotoxic amino acid produced by the marine diatom *Nitzschia pungens* in culture. Can J Fish Aquat Sci 45: 2076-2079

Todd ECD (1993) Domoic acid and amnesic shellfish poisoning- a review. J Food Protect 56: 69-83

Trainer VL, Adams NG, Bill BD, Sther CM, Wekell JC, Moeller P, Busman M, Woodruff D (2000) Domoic acid production near California coastal upwelling zones, June 1998. Limnol Oceanogr 45: 1818-1833

Trainer VL, Hickey BM, Horner RA (2002) Biological and physical dynamics of domoic acid production off the Washington coast. Limnol Oceanogr 47: 1438-1446

Vale P & Sampayo MAM (2001) Domoic acid in Portuguese shellfish and fish. Toxicon 39: 893-904 van der Lingen CD (1994) Effect of particle size and concentration on the feeding behaviour of adult sardine *Sardinops sagax*. Mar Ecol Prog Ser 109: 1-13

van der Lingen CD (1995) Respiration rate of adult sardine *Sardinops sagax* in relation to temperature, voluntary swimming speed and feeding behaviour. Mar Ecol Prog Ser 129: 41-54

van der Lingen CD (1998) Nitrogen excretion and absorption efficiencies of sardine *Sardinops sagax* fed phytoplankton and zooplankton diets. Mar Ecol Progr Ser 175: 67-76

Varela M, Larrañaga A, Costas E, Rodríguez B (1988) Contenido estomacal de la sardina (*Sardina pilchardus*, Walbaum) durante la campaña Saracus 871 en las plataformas Cantábrica y de Galicia en Febrero de 1987. Bol Inst Esp Oceanogr 5: 17-28

Varela M, Alvarez-Ossorio MT, Valdés L (1990) Método para el estudio cuantitativo del contenido estomacal de la sardina. Resultados preliminares. Bol Inst Esp Oceanogr 6: 117-126

Work TM, Beale AM, Fritz L, Quilliam MA, Silver M, Buck K, Wright JLC (1993) Domoic acid intoxication of brown pelicans and cormorants in Santa Cruz, California, In: Smayda TJ, Shimizu Y, (eds) Toxic phytoplankton blooms in the sea. Amsterdam, Elsevier, p 643-650

CHAPTER 5

SPATIO-TEMPORAL VARIABILITY IN FATTY ACID TROPHIC BIOMARKERS IN STOMACH CONTENTS AND MUSCLE OF IBERIAN SARDINE (*Sardina pilchardus*) AND ITS RELATIONSHIP WITH SPAWNING*

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* Garrido S, Rosa R, Ben-Hamadou R, Cunha ME, Chícharo MA, van der Lingen CD. Spatio-temporal variability in fatty acid trophic biomarkers in stomach contents and muscle of Iberian sardine (*Sardina pilchardus*) and its relationship with spawning. *Submitted to Mar Ecol Progr Ser.*

5.1 – Abstract

Temporal variation in the fatty acid (FA) composition of stomach contents of Iberian sardines was compared to the relative contribution to dietary carbon made by different prey types for fish from two areas off Portugal that are characterized by different food environments. The effect of the FA content of the diet on sardine muscle fatty acid composition was also studied. Significant spatial differences in the FA composition of sardine diet occurred, although total FA concentrations were similar for the two areas. Concentrations of (n-3) polyunsaturated fatty acids, namely eicosapentaenoic acid (20:5n-3) and linoleic acid (18:3n-3), were significantly higher in the diet of sardines from the west coast, whilst the diet of sardines from the south coast was richer in monounsaturated fatty acids, namely the carnivory biomarker oleic acid (18:1n-9). These results are in agreement with the higher contribution made by diatoms and dinoflagellates to the diet of sardines off the west coast, which is related to the higher productivity there arising from seasonal upwelling events, and with fish eggs being more important in the diet of sardines from the south. Whereas fatty acid biomarkers for diatoms and carnivory were in accordance with results from stomach content analysis, FA biomarkers for dinoflagellates did not correspond to their abundance in sardine stomachs, and were probably masked by the highly diversified diet of the omnivorous sardines. Spatial variation in sardine dietary FAs was also detected in their muscle composition, specifically for eicosapentaenoic acid, and the eicosapentaenoic acid/ docosahexaenoic acid and (n-3)/(n-6) FA ratios, which were higher in sardines from the west coast. No difference in FA composition was detected between sexes, and the seasonal variability in sardine total FA concentration was primarily related to the seasonality of spawning. Sardines accumulate high concentrations of fatty acids during

the resting stage of reproduction when the feeding intensity is similar or lower to that observed during the spawning season. Additionally, sardines show a high selective retention of MUFAs and PUFAs (chiefly docosahexaenoic acid and eicosapentaenoic acid) throughout the year except at the beginning of the spawning season, when these FAs are largely invested in the formation of the gonads. Finally, spring upwelling events might be responsible for a further extension of the spawning season of sardines off the west coast.

5.2 – Introduction

Fatty acid (FA) catabolism is the major source of energy in many species of fish and plays a major role in growth, swimming and reproduction (Tocher 2003). Several studies have established that the FA composition of fish can be strongly affected by their diet (e.g. Linko et al. 1985, Iverson et al. 2002, Shirai et al. 2002), such that the lipid and FA composition of the predators resembles that of their prey (Dwyer et al. 2003). This linkage provides an opportunity to use FA signatures as biomarkers in the identification of trophic relationships in the marine environment, and the comparison of FA signatures with stomach content analyses might help to identify key climatically-driven processes that impact on ecosystem dynamics through bottom-up pathways (Dalsgaard et al. 2003).

In addition to being affected by dietary composition, the FA signature of marine fish will also vary with environmental and biological factors such as water temperature, salinity, incident light, nutrient availability, and the physiological stage of the plankton (Reuss & Poulsen 2002), as well as with changes in primary and secondary production (Budge et al. 2002), all of which reflect the spatial and temporal variability of the fish's physical environment. Planktivorous fish such as sardine *Sardina pilchardus*, which are situated close to the bottom of the pelagic food web and feed directly on available phytoplankton and micro- and mesozooplankton prey and show highly opportunistic feeding behaviour (Garrido et al. 2007), are likely to be more rapidly affected by spatial and temporal variability of the producers and first order consumers than would fish situated at a higher trophic level.

Stomach content analyses conducted on Iberian sardines collected off the western and southern coasts of Portugal have revealed that diet varies both seasonally and spatially (Garrido et al. *accepted*). The most important prey are zooplankton, namely crustacean eggs, fish eggs, and developmental stages of copepods, decapods and cirripeds, as well as dinoflagellate and diatom phytoplankton, which together account for more than 90% of estimated dietary carbon of sardines from both areas. The relative contribution to dietary carbon made by phytoplankton varies seasonally and is highest during the spring-summer upwelling season, and the main spatial difference in diet is the significantly higher contribution made by phytoplankton to fish from the west coast, where upwelling is stronger, than is the case off the south coast (Fiúza et al. 1982). Such spatial and temporal variations in food availability and composition may be one of the causes of discontinuities in biological characteristics, including morphometric characteristics, age structure, population dynamics, maturation patterns and spawning seasonality, that have been observed for Atlanto-Iberian sardine (ICES 2000, Carrera & Porteiro 2003, Silva 2003, Silva et al. 2006).

Seasonal variation in sardine fat content is linked to their temporal location in the spawning season and hence their gonad maturation state, but is probably also related to seasonality of their diet as well as to variations in water temperature (Bandarra et al. 1997). Sardines generally spawn from October to May-June in Portuguese waters

(Coombs et al. 2006), and this extended spawning season must have high energetic costs that are probably supported both by fat reserves accumulated during the resting stage of reproduction (Spring to Autumn), and by feeding activity during the spawning season, as in the case of northern anchovy (Hunter & Leong 1981).

Food availability during the resting stage of reproduction will exert a strong influence on the amount of fat accumulated by sardines prior to the spawning season and hence will impact on reproduction. The length-at-maturity of sardines from western Iberia and the Mediterranean has decreased over the past two decades (Silva et al. 2006), and this decrease coincided with an increase in the fat reserves and condition factor of fish during the months prior to the spawning season. Those authors also found a relationship between sardine weight-at-length, and macroscopically-determined percentage fat-at-length, with the length of first maturity, whilst in Japanese sardines (*Sardinops sagax*) accumulated fat reserves seem to be strongly related to the quantity and quality of eggs produced during the following spawning season (Morimoto 1991, 1996). Hence food availability and lipid nutrition may therefore substantially affect recruitment, both by impacting on adult spawning success, particularly the duration of spawning, but also by influencing the survival of the younger stages (Bell & Sargent 1996, Marshall et al. 1999).

The objectives of this work were to 1) study spatial and temporal variation in the fatty acid composition of sardine diet and evaluate the relationship between FA biomarkers and stomach content composition, and 2) examine the effect of prey FA composition on seasonal variation in the FA composition of sardine muscle, and relate this to the sardine spawning cycle.

5.3 – Material and methods

Samples for dietary analysis comprised ≈70 sardine obtained every fortnight over the period January 2003 to March 2004 from purse-seiners fishing off western (Peniche) and southern (Portimão) Portugal (Fig. 5.1). Data on total length (TL, cm), gutted weight (fish weight minus viscera, g), sex, and the macroscopic determination of gonad maturity stage (Pinto & Andreu 1957) and indices of fat content (Krvaric & Muzinic 1950) were recorded for each sardine.

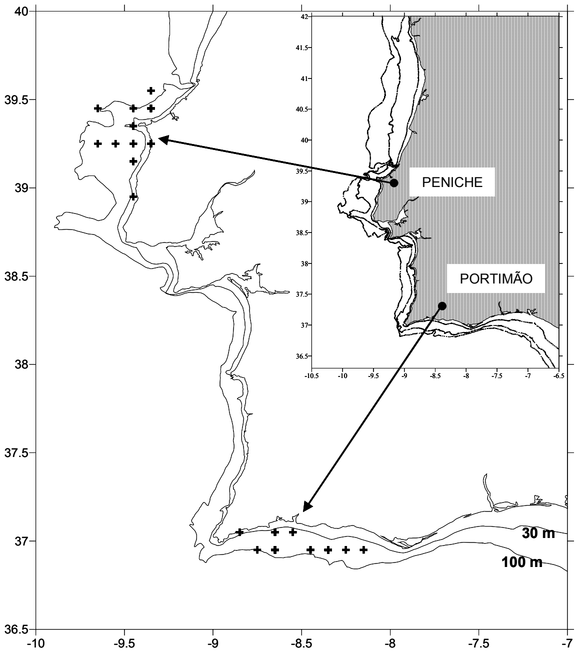

Figure 5.1 – Map of the Portuguese continental coast, showing the sampling locations of *Sardina pilchardus* in the fishing areas of Peniche and Portimão (note that several samples were taken from the same location).

Stomachs of sardine of the modal (18 cm) length class were removed and individually weighed, and prey analysis and estimation of the contribution by different prey types to dietary carbon, were conducted as described in detail by Garrido et al. (*accepted*).

Since the lipid digestion starts after the passage of food through the pyloric sphincter and does not occur in the fish stomach (Wedemeyer 1996), we were able to quantify fatty acids from the prey in the stomach contents. Stomach samples for fatty acid (FA) determination were collected monthly from April 2003 to March 2004 from both Portimão and Peniche, whilst muscle samples for FA determination were collected fortnightly during November 2002 from Portimão, and then from January 2003 until March 2004 from both Peniche and Portimão. Sardine muscle tissue was collected from the same fish as those on which stomach content analysis was performed, and muscle FA composition was determined separately for individuals classified as males or females, and as spawning (comprising fish in early phases of maturation-stage II, fish in advanced stage of maturation-stage III, in spawning condition-stage IV or recovering from recent spawning-stage VI), non-spawning (stage V) and immature (stage I). Fish were filleted and the skin removed from the fillet, which were then minced and the flesh thoroughly mixed before lipid extraction.

Determination of the FA composition of stomach contents was performed on sardines of 17-19 cm TL from each sample, since the 18 cm sardines had been used for stomach content analysis and there were insufficient fish of this size in samples for both types of analysis. It was assumed that the prey composition of sardines of 17 and 19 cm was similar to that recorded for 18 cm sardines, since feeding intensity was shown to be similar between length classes and sexes collected in the same sample (Garrido et al. *accepted*). The gonadosomatic index (gonad weight/fish gutted weight), and feeding intensity (stomach content weight/fish gutted weight), were estimated for 18 cm sardines from each sample.

The determination of FA profile was based on the experimental procedure of Lepage & Roy (1986) as modified by Cohen et al. (1988), with FA methyl esters being analysed in a CP 3800 Varian gas chromatograph equipped with an auto-sampler and fitted with a flame ionisation detector (FID). The separation was carried out with helium as the carrier gas in a DB-Wax Polyethylene Glycol column (30 m x 0.25 mm id) programmed to start at 180 ºC for 5 min, then heating at a rate of 4 ºC min^{-1} for 10 min and maintained at 220 ºC for 25 min, with detection conducted at 250 ºC using a split injector (100:1). Fatty acid methyl esters were identified by comparison of their retention times with those of Sigma chromatographic standards, peak areas were determined using the Varian software, and the FA 23:0 was used as an internal standard. Comparisons between the FA composition of stomachs and muscles between the two areas, between males and females, and between spawning, non-spawning, and immature sardines from each fortnightly-collected sample were performed using a t-test analysis for paired samples, with $p<0.05$ being used as the level of significant difference.

Seasonality in the FA composition of muscles from the two sample sites (Peniche and Portimão) was assessed using hierarchical clustering analysis with simple Euclidean distances being calculated, and considering flexible linkage agglomeration with a neighbourhood constraint (Legendre & Legendre 1998). Statistical analysis and graphics were performed using the open source software R 1.9 (R Development Core Team 2005), and MATLAB software (version 6.5; MathWorks Inc. 2002).

5.4 – Results

5.4.1 – Seasonal and spatial variability in prey carbon and fatty acid concentrations

Total prey fatty acid concentration in sardine stomachs was not significantly correlated with total prey dietary carbon as estimated from stomach content analysis. Prey dietary carbon was highest in sardines collected from the west coast in December 2003 and February 2004, when fish eggs and zooplankton, respectively, dominated stomach contents, whilst prey FA concentration was highest in June 2003 when prey carbon was dominated by zooplankton, followed by dinoflagellates and diatoms (Fig. 5.2a, c). The lowest prey FA concentrations for sardine from the western coast were recorded in October and coincided with the lowest prey carbon concentration. Off Portimão (south coast), prey FA concentrations were highest in late July and early August, when zooplankton and diatoms followed by dinoflagellates dominated prey carbon. The highest concentrations of prey carbon occurred during September and was related to the high concentration of zooplankton found in sardine stomachs from the south.

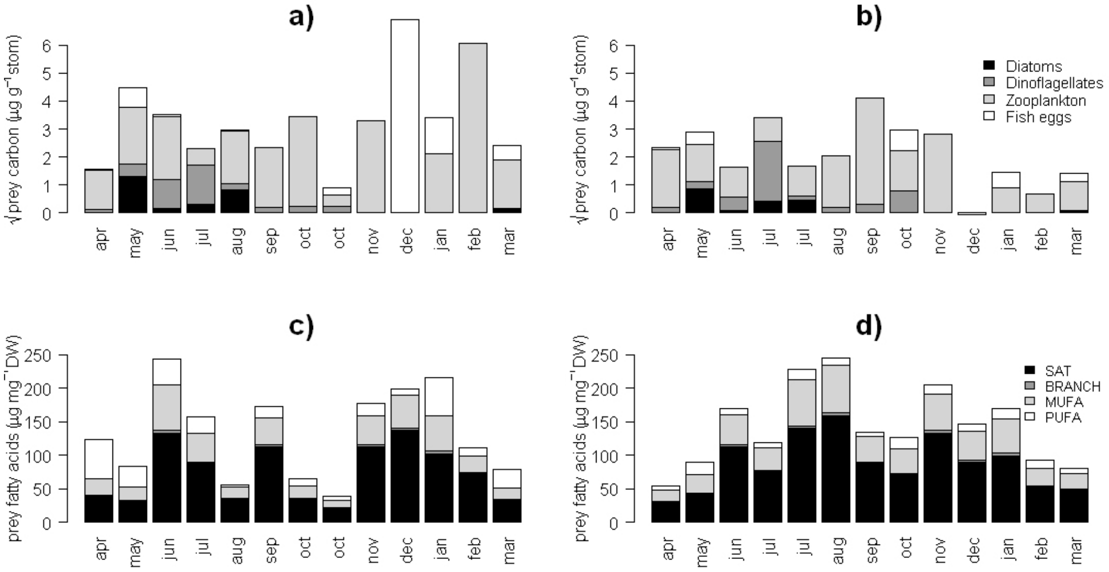

Figure 5.2 – Concentration of major prey types (upper graphs, values on the axis refer to the square root transformation of the summed carbon content of the prey with proportions between prey types maintained) and fatty acids classes (lower graphs) in stomach contents of sardines collected fortnightly from Peniche (a and c) and Portimão (b and d) from April 2003 to March 2004. For detailed information on sardine prey composition during this year for both areas see Garrido et al. (submitted).

Prey FA composition showed higher temporal variability in samples collected from the west coast compared to those collected from the south, which accords with the higher variability in prey species composition observed for the west (Garrido et al. *accepted*).

Prey concentrations of total and saturated FAs were similar for both areas (Fig. 5.3a, b), whereas prey of sardines collected from the west coast presented a significantly higher proportion of PUFAs (Fig. 5.3e), particularly in months when the relative contribution of phytoplankton prey was higher (Fig. 5.2). In contrast, the prey of sardines collected from the south coast showed significantly higher concentrations of MUFAs (Fig. 5.3d), but no differences in the concentration of total unsaturated FAs was detected between prey of sardines from both areas (Fig. 5.3c).

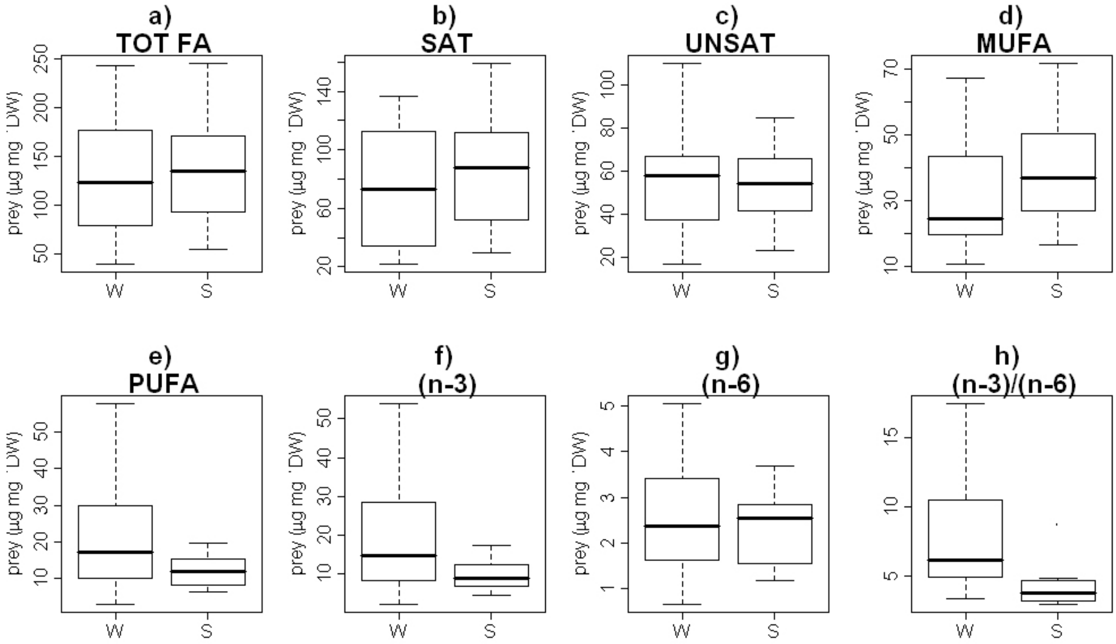

Figure 5.3 – Boxplots showing concentrations of a) Total, b) 18:1(n-9), c) Unsaturated, d) 18:3(n-3), e) EPA and f) (n-3), and g) (n-6) fatty acids, and h) the (n-3)/(n-6) fatty acid ratio, in stomach contents of sardines collected from Peniche and Portimão from April 2003 to March 2004. (Saturated: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0 and 22:0; Branched: Iso 14:0, Anteiso 14:0, Iso 16:0 and Anteiso 16:0; Monounsaturated: 16:1(n-7), 17:1(n-8), 18:1(n-9), 18:1(n-7) , 20:1(n-9), 20:1(n-7), 22:1(n-11) and 22:1(n-9); Polyunsaturated: 16:4(n-3), 18:2(n-6), 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:2(n-6), 20:4(n-6), 20:3(n-3), 20:4(n-3), 20:5(n-3), 22:4(n-6), 22:5(n-6), 22:5(n-3) and 22:6(n-3)).

5.4.2 – Stomach content analysis and fatty acid biomarkers

In order to compare prey FA signatures with results from stomach content analysis we used a combination of FAs and their respective ratios, because individual FAs cannot be used as taxonomic indicators (for more discussion regarding FA trophic markers see Dalsgaard et al. 2003 and Chapter 1, section 1.5). The concentration of (n-3) FAs and the (n-3)/(n-6) ratio were significantly higher in the diet of sardines from the western coast (Fig. 5.3f, h), one of the (n-3) FAs being linoleic acid 18:3(n-3) (Fig. 5.4d). The higher prevalence of phytoplankton prey (both dinoflagellates and diatoms) in sardines from the west coast (see Fig. 5.2) is in accordance with variations in diatom FA biomarkers, including the Σ16:1/Σ16 ratio (Fig. 5.4a), eicosapentaenoic acid EPA (Fig. 5.4e), and the ratio between EPA and docosahexaenoic acid (DHA) (Fig. 5.4f), all of which reached significantly higher concentrations in the diet of sardines from the west coast compared to those collected from the south (Fig. 5.4).

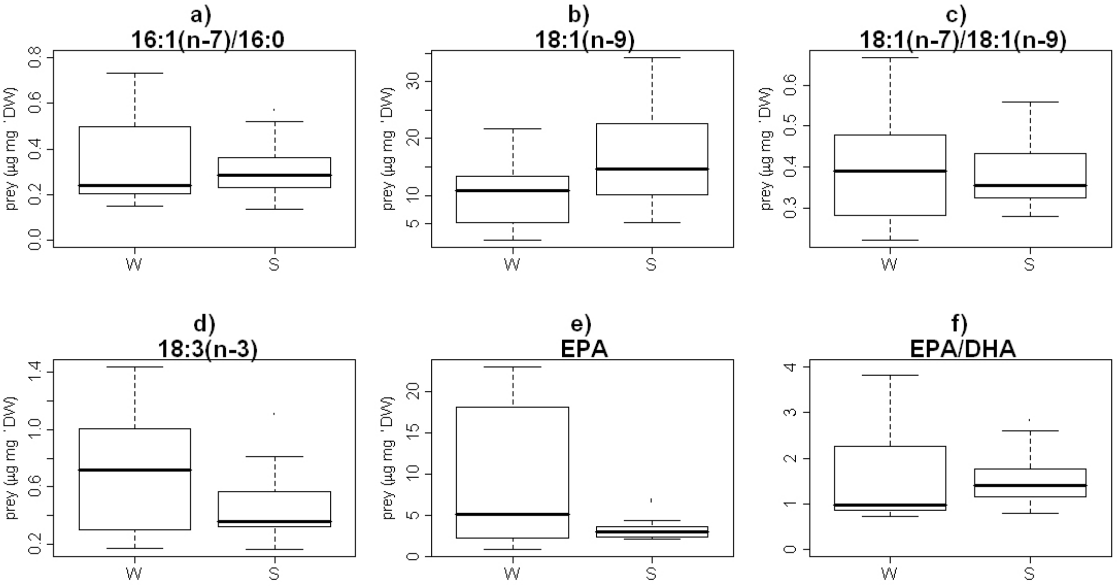

Figure 5.4 – Boxplots showing concentrations of biomarker fatty acids including a) the 16:1(n-7)/16:0 ratio, b) 18:1(n-9), c) the 18:1(n-7)/18:1(n-9) ratio, d) 18:3(n-3), e) EPA and f) the EPA/DHA ratio, in stomach contents of sardines collected from Peniche and Portimão from April 2003 to March 2004.

Prey EPA concentration was higher than the DHA concentration from April to July 2003 and again from January to March 2004 for both areas (Fig. 5.5), although the difference in concentrations for the west coast samples was significantly higher than for samples from the south. From August until December prey concentrations of DHA and EPA were very similar in samples from both areas. The highest concentrations of EPA in sardine prey were recorded during spring (Apr-Jun) in samples from the west coast; these concentrations were markedly higher than DHA concentrations, as shown by DHA/EPA ratios of 0.15 and 0.26 for May and June, respectively.

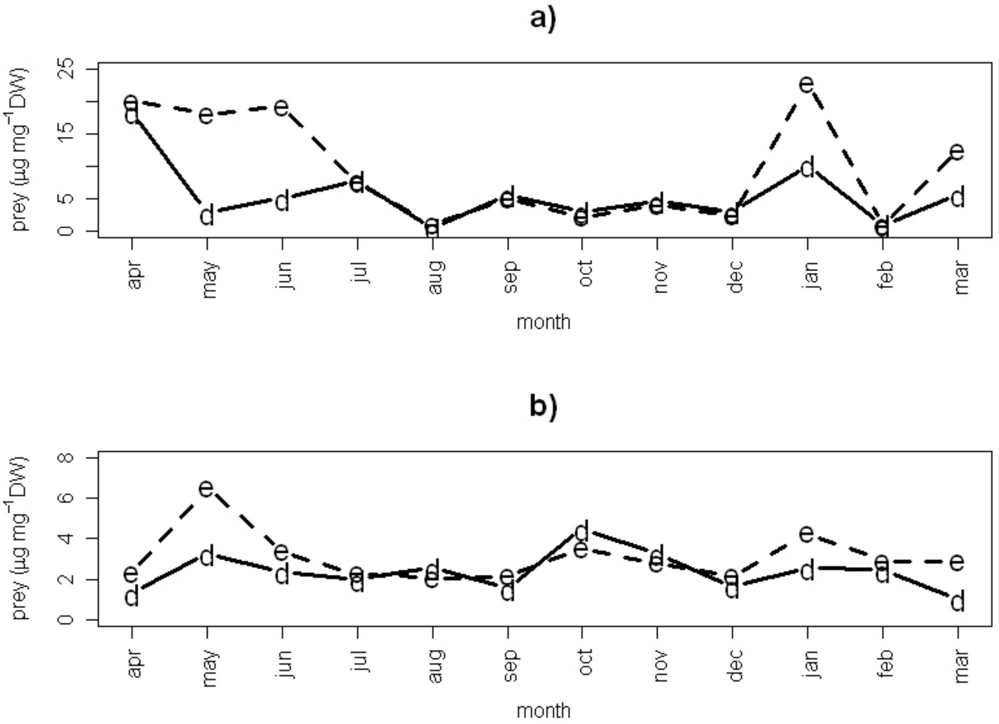

Figure 5.5 – Seasonal variation in the concentration of EPA (e symbols) and DHA (d symbols) in sardine stomach contents from (a) Peniche and (b) Portimão from April 2003 to March 2004.

For both areas, prey concentrations of the 16:0 FA reached their lowest value during late May (16.17 and 27.88 µg mg^{-1} DW for the west and south coasts, respectively), when phytoplankton carbon in the stomachs was highest (21.84 and 7.25 µg g^{-1} DW for west and south coasts, respectively). The biomarker for carnivory, 18:1(n-9), was higher in the diet of sardines from Portimão, while the biomarker for herbivory, the 18:1(n-7)/18:1(n-9) ratio, was higher for sardines from the west coast (Fig. 5.4b, c). Another herbivory biomarker, the PUFA/SFA ratio, was also higher in sardines from west coast compared to those from the south (0.26 ± 0.42 and 0.13 ± 0.10, respectively), particularly from April to June. Results from biomarkers are in agreement with those from stomach content analyses, which showed that phytoplankton is more important in the diet of sardines from west coast and fish eggs are particularly important for sardines from the south, whilst zooplankton prey are important in both areas (Fig. 5.2).

Significant positive correlations were obtained between the relative contribution of certain prey types to dietary carbon and several FA biomarkers. This was the case for the majority of diatom biomarkers, particularly between this algal group and the Σ16:1/Σ16 ratio (Pearson's r=0.74) but also with EPA concentration (Pearson's r=0.38), the EPA/DHA (Pearson's r=0.40), Σ16/Σ18 (Pearson's r=0.44), and Σ(n-3)/Σ(n-6) (Pearson's r=0.60) ratios. Whilst the relative contribution to dietary carbon from diatoms was positively correlated with FA C20 (Pearson's r=0.32), it was inversely correlated with FA C18 (Pearson's r=0.32). No significant correlation was found between the relative contribution of diatoms to dietary carbon and C16 PUFAs. FA trophic markers for dinoflagellates, namely DHA, Σ18, C22 PUFA, 18:0 and 18:1(n-9) FAs, were not correlated with their contribution to dietary carbon content, possibly because the concentration of dinoflagellates in sardine stomachs was small compared to other prey types rich in DHA, such as crustacean eggs which are one of the most important and frequently-encountered zooplankton prey items in sardine stomachs. Concentrations of the FAs 20:1(n-9) and 22:1(n-11), which are both biomarkers for herbivorous calanoid copepods, were not correlated with dietary carbon derived from such copepods. Fish eggs were significantly correlated with the SFA palmitic acid 16:0 (Pearson's r=0.37) and also with the DHA/EPA ratio (Pearson's r=0.37).

5.4.3 – Seasonal variability in fatty acid concentrations in sardine muscle and its relationship with diet and spawning

Muscle tissue of male and female sardines engaged in spawning and collected from the same sample showed no significant differences in total FA concentration and composition (Fig. 5.6). Similarly, sardines whose gonads were macroscopically classified as being in the resting stage of spawning showed no differences in muscle concentrations of total saturated or monounsaturated FAs compared to sardines engaged in spawning, but presented significantly (p=0.01, n=10) higher proportions of polyunsaturated FAs compared to reproductively active fish (Fig. 5.7a). Male and female sardines classified as immature showed significant differences (p=0.015, n=6) in total muscle FA concentration compared to mature sardines collected in the same sample (Fig. 5.7b), with immature sardines generally presenting higher FA concentrations which probably reflect the absence of investment in reproduction.

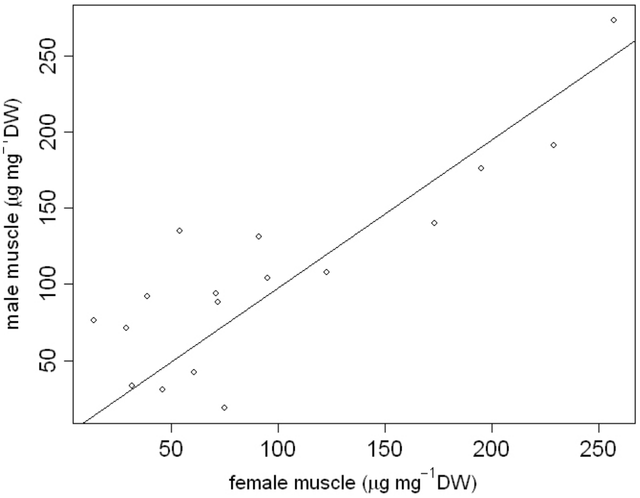

Figure 5.6 – Relationship between total fatty acids in the muscle of female and male sardines engaged in spawning and collected from the same sample (y= 0.98x, r^2= 0.90, p=1.3*10^{-9}).

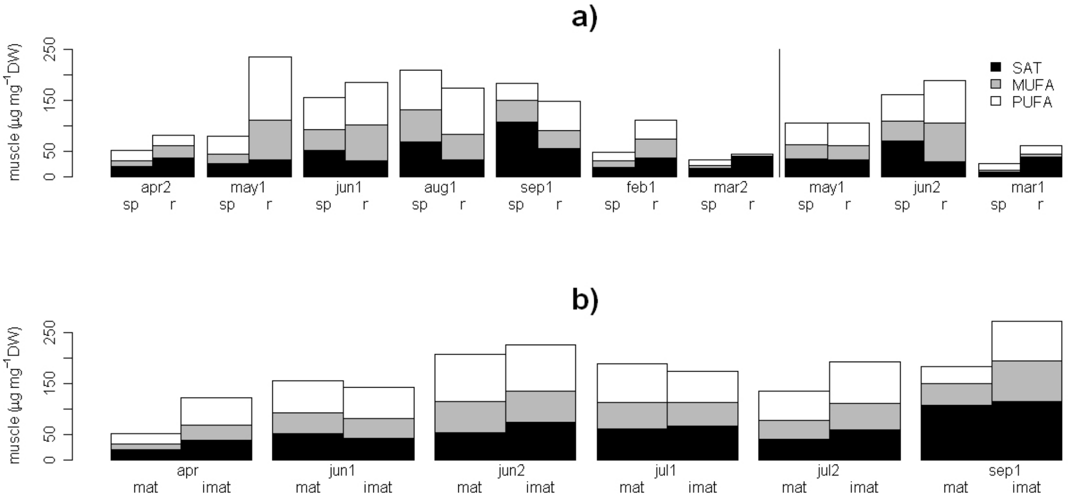

Figure 5.7 – Comparison of the concentration of Saturated, MUFA and PUFA fatty acids in the muscle of a) sardines engaged in spawning (“sp”) or at the resting stage of reproduction (“r”) and b) mature (“mat”) and immature (“imat”) sardines. The vertical line in a) shows the separation between samples collected from Peniche (left of the line) and Portimão (right), while in b) all samples when mature and immature sardines coincided were collected from Peniche. The numbers “1” or “2” after the month name refer to the first or second fortnight.

The main saturated FAs in sardine muscle were palmitic acid (16:0) followed by 14:0 and 18:0, the main MUFAs were 18:1(n-9) followed by 16:1(n-7) and 18:1(n-7), and the most important PUFA was DHA followed by EPA and 18:4(n-3) (Table 5.1), all of which corresponded to the major FAs of these classes detected in the diet. Significant differences in sardine muscle FA concentration and composition between the two areas were found for EPA and the EPA/DHA and (n-3)/(n-6) ratios, which were all higher for sardines from west coast compared to those from the south (Figs 5.8 and 5.9).

Table 5.1 – Mean ± S.D. Fatty acid composition (µg mg^{-1} dry weight) in the muscle of sardines collected in the west (W) and south (S) coasts of Portugal during one year. Months were grouped according to a cluster analysis with Eucledian distances.

Fatty acids	**Muscle**						
(µg mg DW)		**West**			**South**		
Months	**jan-aug1**	**aug2-oct2**	**nov1-mar2**	**nov1-jun2**	**sep1-sep2**	**nov2-feb2**	**mar2**
14:0	6.48 ± 4.91	13.53 ± 4.65	6.19 ± 5.75	8.04 ± 3.50	16.22 ± 0.40	3.80 ± 2.66	0.57
16:0	23.42 ± 11.84	52.36 ± 12.93	24.36 ± 20.14	30.58 ± 13.62	60.89 ± 1.50	20.59 ± 12.27	5.40
18:00	4.94 ± 2.32	10.65 ± 2.35	4.40 ± 3.34	6.69 ± 2.98	12.27 ± 0.82	4.04 ± 2.22	1.30
16:1(n-7)	6.77 ± 5.77	11.66 ± 3.91	2.38 ± 1.15	8.96 ± 4.99	2.52 ± 0.11	3.17 ± 2.13	0.59
18:1(n-9)	10.10 ± 7.32	20.19 ± 7.11	3.81 ± 1.82	12.92 ± 7.62	5.44 ± 0.81	5.92 ± 4.39	0.63
18:1(n-7)	4.48 ± 7.06	5.44 ± 2.03	1.58 ± 0.80	3.81 ± 1.67	1.84 ± 0.07	1.48 ± 0.68	0.31
20:1(n-9)	0.78 ± 1.63	2.95 ± 2.38	0.99 ± 0.36	0.37 ± 0.21	1.82 ± 0.01	1.67 ± 0.96	0.36
20:1(n-7)	2.99 ± 3.92	2.38 ± 3.18	0.18 ± 0.17	3.40 ± 2.28	0.77 ± 0.60	0.11 ± 0.06	0.02
22:1(n-11)	0.89 ± 1.89	1.60 ± 1.35	0.79 ± 0.30	0.53 ± 0.51	1.14 ± 0.47	1.23 ± 0.73	0.42
22:1(n-9)	1.57 ± 1.90	1.52 ± 1.40	0.19 ± 0.14	3.11 ± 2.80	0.68 ± 0.20	0.21 ± .10	0.04
16:4(n-3)	0.91 ± 1.01	0.99 ± 0.59	0.64 ± 1.24	1.09 ± 0.48	0.10 ± 0	0.40 ± 0.40	0.06
18:2(n-6)	1.11 ± 0.75	2.78 ± 2.80	0.45 ± 0.29	1.25 ± 0.78	0.24 ± 0.02	0.52 ± 0.17	0.17
20:4(n-6)	0.93 ± 0.57	1.05 ± 0.75	0.19 ± 0.14	1.25 ± 0.27	0.05 ± 0.03	0.50 ± 0.12	0.10
20:5(n-3)	12.47 ± 10.98	17.63 ± 14.04	2.27 ± 1.49	14.29 ± 5.18	0.60 ± 0.18	4.18 ± 1.95	0.87
22:6(n-3)	18.49 ± 7.01	25.76 ± 22.08	5.08 ± 3.03	20.73 ± 5.39	1.27 ± 0.21	8.08 ± 2.76	2.24
Σ Sat[1]	36.30 ± 19.26	80.68 ± 20.20	36.67 ± 30.56	47.24 ± 20.58	94.08 ± 2.57	29.92 ± 17.57	7.61
Σ Branch[2]	0.63 ± 0.57	1.87 ± 0.75	0.59 ± 0.46	0.74 ± 0.41	1.55 ± 0.09	0.49 ± 0.40	0.06
Σ MUFA[3]	27.96 ± 22.08	46.34 ± 16.71	10.11 ± 3.96	33.65 ± 16.81	14.62 ± 0.95	13.94 ± 8.80	2.40
Σ PUFA[4]	40.80 ± 24.60	60.25 ± 48.30	10.27 ± 5.04	46.42 ± 13.88	3.21 ± 0.18	16.29 ± 5.58	3.84
Σ HUFA	38.40 ± 22.98	54.75 ± 43.78	9.39 ± 4.83	43.64 ± 12.77	2.50 ± 0.31	15.09 ± 5.13	3.61
Σ Unsat	68.76 ± 45.91	106.59 ± 64.09	20.39 ± 7.40	80.07 ± 29.49	17.84 ± 0.77	30.23 ± 13.86	6.24
Σ (n-3)	37.50 ± 22.63	54.44 ± 43.60	9.19 ± 4.61	42.39 ± 12.74	2.38 ± 0.44	14.67 ± 5.20	3.48
Σ (n-6)	3.30 ± 1.88	5.80 ± 4.74	1.08 ± 0.48	4.03 ± 1.31	0.83 ± 0.25	1.61 ± 0.46	0.37
(n-3)/(n-6)	11.55 ± 1.88	9.00 ± 1.74	8.49 ± 2.69	10.75 ± 1.77	3.09 ± 1.47	9.31 ± 1.59	9.47
EPA/DHA	0.78 ± 0.21	0.69 ± 0.16	0.44 ± 0.08	0.68 ± 0.14	0.47 ± 0.06	0.58 ± 0.34	0.39
Σ Total FA	106.00 ± 63.93	189.15 ± 71.72	57.66 ± 29.47	128.06 ± 49.48	113.47 ± 3.43	60.65 ± 31.26	13.91

[1] Saturated: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0 and 22:0

[2] Branched: Iso 14:0, Anteiso 14:0, Iso 16:0 and Anteiso 16:0

[3] Monounsaturated: 16:1(n-7), 17:1(n-8), 18:1(n-9), 18:1(n-7) , 20:1(n-9), 20:1(n-7), 22:1(n-11) and 22:1(n-9)

[4] Polyunsaturated: 16:4(n-3), 18:2(n-6), 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:2(n-6), 20:4(n-6), 20:3(n-3), 20:4(n-3), 20:5(n-3), 22:4(n-6), 22:5(n-6), 22:5(n-3) and 22:6(n-3)

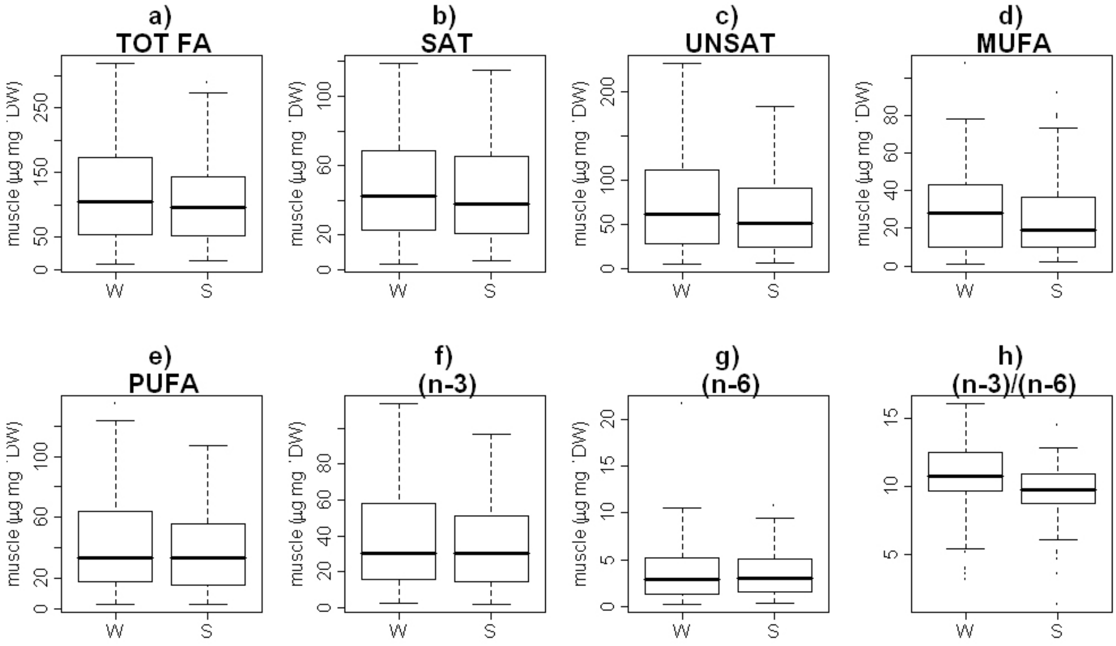

Figure 5.8 – Boxplots showing concentrations of a) Total, b) Saturated, c) Unsaturated, d) Monounsaturated, e) Polyunsaturated, f) (n-3), and g) (n-6) fatty acids, and h) the (n-3)/(n-6) fatty acid ratio, in the muscle of sardines collected from Peniche and Portimão from January 2003 to March 2004 (fatty acids contributing to each fraction are described in the caption to Figure 3).

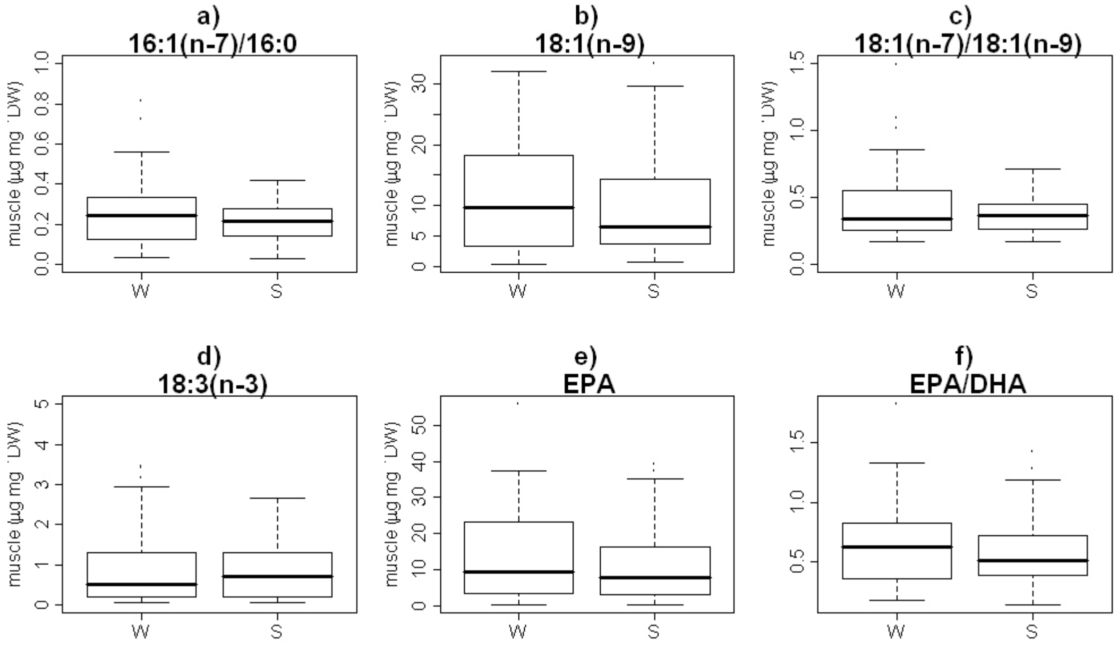

Figure 5.9 – Boxplots showing concentrations of biomarker fatty acids including a) the 16:1/(n-7)/16:0 ratio, b) 18:1(n-9), c) the 18:1(n-7)/18:1(n-9) ratio, d) 18:3(n-3), e) EPA and f) the EPA/DHA ratio, in the muscle of sardines collected from Peniche and Portimão from January 2003 to March 2004.

Seasonal variation in DHA and EPA in sardine muscle was synchronous for both areas, with maximum concentrations coinciding with the peak in fat content (Figs. 5.10 and 5.11). In contrast to their prey, sardine muscle concentrations of DHA were always higher than EPA concentrations with the exception of sardines collected during early and late June from the west coast and late March 2003 from the south. The concentration of 16:0 in the muscle was similar in fish from both areas and was higher from July until December for the west coast and from May until September for the south, and appeared to correspond to higher concentrations of this FA in the diet. The concentration of 16:1(n-7) in sardine muscle was highest in June for both areas.

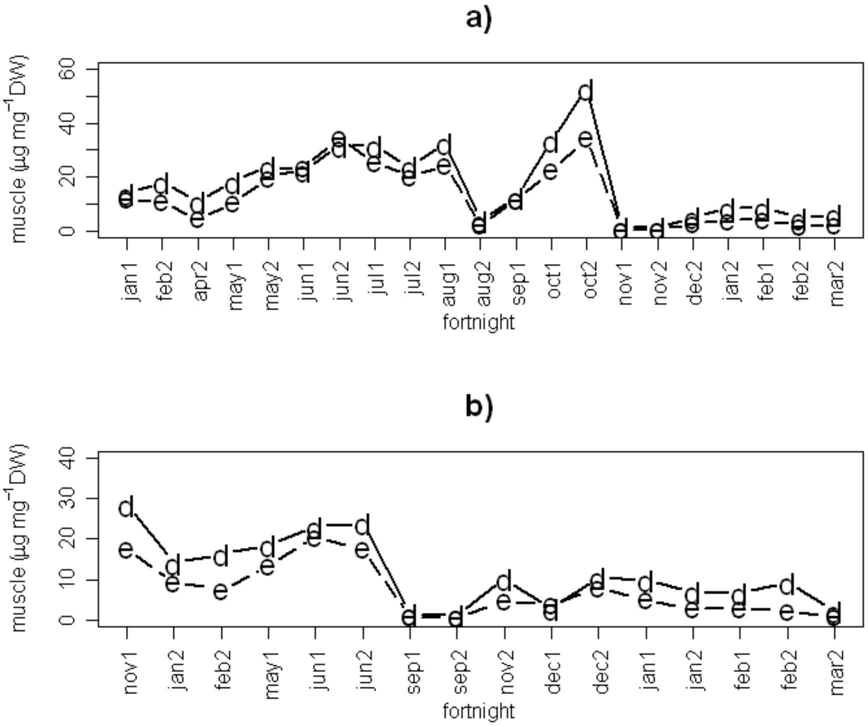

Figure 5.10 - Seasonal variation in the concentration of EPA (e symbols) and DHA (d symbols) in sardine muscle from (a) Peniche and (b) Portimão from April 2003 to March 2004.

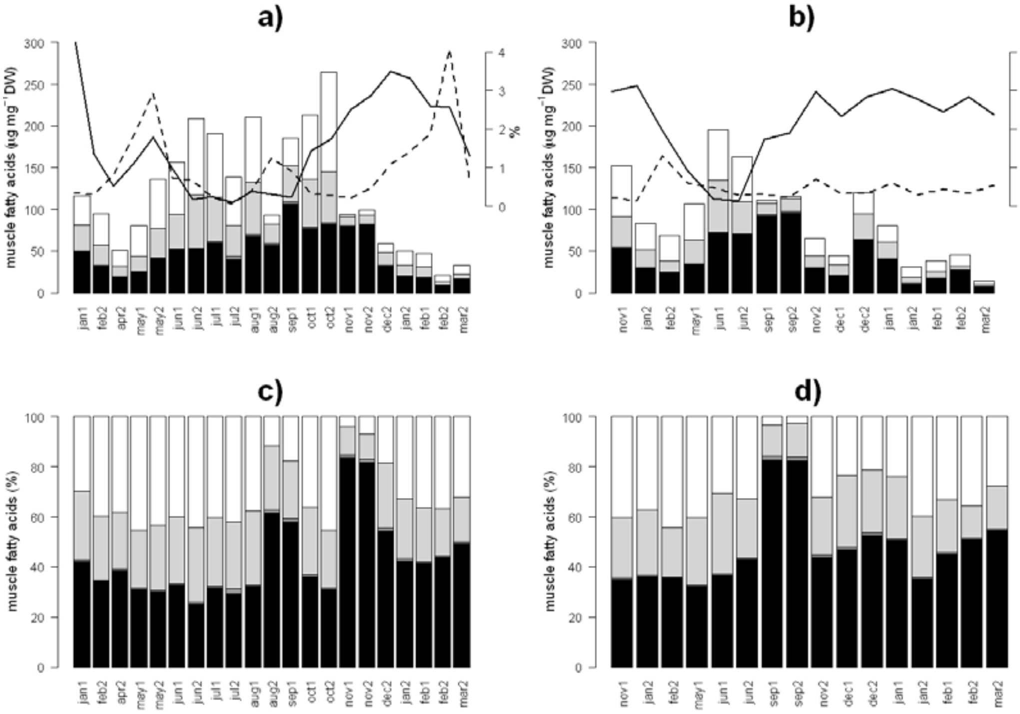

Figure 5.11 – Concentration (upper graphs) and percentage contribution (lower graphs) of fatty acid fractions in the muscle of sardines engaged in spawning and collected from Peniche (a) and c)) and Portimão (b) and d)) from November 2002 to March 2004. The gonadosomatic index (solid line; gonad weight/fish gutted weight*40, to fit in the same scale as the feeding intensity) and feeding intensity (dashed line; stomach content weight/fish gutted weight) are also shown in the upper graphs for both areas. The numbers “1” or “2” after the month name refers to the first or second fortnight.

Peak FA concentrations in the muscle of sardines collected from the west coast occurred during October (210.25 µg mg^{-1} DW), but decreased by over 50% in the following month (93.45 µg mg^{-1} DW) (Fig. 5.11a). The muscle FA concentration of sardines from Portimão was highest during June (195.01 µg g^{-1} DW), but was considerably lower in September (111.06 µg g^{-1} DW), indicating that sardines from the south coast start losing reserves earlier than do those from the west coast (Fig. 5.11b). There is a gap in the data for July and August for mature sardines from Portimão (only immature sardines of the selected size class were collected during those months), and their FA content may have continued to increase. However, the gonadosomatic index of sardines from the other size classes collected in the same sample increased by around 50% from late June (0.4743) to early July (0.9498) and reached 1.4574 during late August, indicating that the spawning season began earlier for sardines off the south than for those off the west coast. Unsaturated FAs decreased dramatically at the start of the spawning season in fish from both areas (Figs 5.11c, d), which changed the proportions of saturated, monounsaturated and polyunsaturated FA fractions that had been fairly constant during the rest of the year. Although the FA content for sardine muscle from both areas continued to decrease throughout the spawning season the relative proportions of saturated and unsaturated FAs recovered shortly after the initial decay in fish from both areas, since the decrease of MUFA and PUFA occurred immediately at the beginning of the spawning season whereas the saturated fraction decreased one month later for both areas. The lowest FA concentrations were recorded in late February 2004 (20.93 µg g^{-1} DW) for sardines from the west coast and late March 2004 (13.91 µg g^{-1} DW) for sardines from Portimão, near the end of the spawning season.

Sardines start accumulating fat reserves as soon as the spawning season ceases, as evidenced by the decrease in the gonadosomatic index (Fig. 5.11), which occurred during May for fish from Portimão and from March for those from the west coast. Sardine feeding intensity increased between April and May, probably corresponding to the development of spring blooms related to upwelling. This appears to influence spawning duration for fish from the west coast, since their gonadosomatic index that had decreased in the preceding months increased again in May, before finally reaching its lowest level in June.

5.5 – Discussion

In general, differences in the fatty acid (FA) composition of stomach contents of sardine from the two areas are in accordance with differences in their prey composition as determined from stomach content analyses, although some signals of prey composition were not detected by FA trophic biomarkers. The FAs described as biomarkers of diatoms (Dalsgaard et al. 2003) were positively correlated with the relative contribution to dietary carbon in sardine stomachs made by diatoms, particularly the 16:1(n-7)/16:0 and Σ(n-3)/ Σ(n-6) ratios but also eicosapentaenoic acid, the EPA/DHA ratio, and C20 FA. Diatom-derived dietary carbon was negatively correlated with concentrations of C18 FA, a biomarker for dinoflagellates, and concentrations of other dinoflagellate biomarkers such as docosahexaenoic acid and C22 PUFA did not match the relative contribution of this prey type to dietary carbon. Several explanations for this lack of correlation between dinoflagellates and their biomarkers are possible, including the underestimation of dinoflagellates in stomach content analysis arising from the rapid digestion of dinoflagellate cells that had no exterior coverage. Additionally, crustacean eggs and fish eggs, both of which can make a major contribution to sardine dietary carbon, contain large amounts of DHA and C18 FA (Sargent & Falk-Peterson 1988), hence high DHA concentrations in stomach contents may reflect a high dietary contribution by these food types and not dinoflagellates. The total carbon content estimated for prey identified in sardine stomach contents was not correlated with the total FA concentration of stomach contents, which might be due to the different composition of the different prey types in terms of their lipid content and also due to the seasonal variation in the fat content of each prey class, including both phyto- and zooplankton species (e.g. Reuss & Poulsen 2002, Lee et al. 2006).

Total FA concentrations of prey in sardine stomachs determined over one year were similar for fish from the west and south coasts of Portugal, as was the concentration of saturated FAs, but significant differences in the unsaturated fraction were observed between sampling areas. The concentration of PUFAs (specially n-3 series), and the n-

3/n-6 ratio, were significantly higher in stomachs of fish from the west coast, and this spatial difference appears to have been caused by differences in prey composition. The concentration of PUFAs was particularly high in stomach contents of sardine collected from the west coast during the spring when the highest concentration of phytoplankton (both diatoms and dinoflagellates) was observed, and probably coincides with the beginning of the phytoplankton bloom associated with the seasonal upwelling events that occur in that area. In fact, only phytoplankton is capable of biosynthesizing (n-3) and (n-6) PUFAs *de novo*. At the beginning of phytoplankton blooms the algae might have as much as 10-20% of their carbon content as fat (Reuss & Poulsen 2002), and the highest concentration of EPA, a FA produced by diatoms, was also registered during spring in sardine stomachs from both areas. The higher PUFA content of sardine prey off the west coast might also be related to the lower water temperatures (Dalsgaard et al. 2003) that occur for that area, arising from the higher latitude of that region as well as from the decreased temperatures at the surface due to the occurrence of seasonal upwelling events.

On the other hand, the relative contribution of MUFAs was significantly higher in the diet of sardines from the south coast, and this is probably linked to the fact that fish eggs, which are a major prey of sardines especially off the south coast, have high concentrations of MUFA in their oil globule (Wiegand 1996). However, the high relative contribution of MUFA in stomachs of sardines collected in Portimão is mainly due to the C18:1 FA, which would indicate a high degree of carnivory since this FA is characteristic of copepods (Lee et al. 2006). Moreover, the 18:1(n-7)/18:1(n-9) and PUFA/SAT ratios, which are also indicative of carnivory (Dalsgaard et al. 2003) were also higher for the south sardines than those from the west coast. Besides indicating carnivory, 18:1 FA is also associated with small autotrophic flagellates and

Prymnesophiceae (Reuss & Poulsen 2002), and as mentioned above, naked phytoplankton cells that are ingested by sardine might not be detected during stomach content analysis due to rapid digestion (Garrido et al. *accepted*). In fact, in a study carried off southern Portugal near Ria Formosa, naked dinoflagellates reach in some occasions 50% of phytoplankton biomass for that area (Barbosa 2006).

The mobilization of specific FAs for the fish depend on whether they are required solely for the provision of metabolic energy or destined for gonad development (Sargent et al. 1989). Internal metabolic processes of the fish such as the mobilization of particular FAs during gonadogenesis distort potential dietary signals (Dalsgaard et al. 2003), and this probably explains why most of the spatial differences in FA composition observed in sardine prey are not clear when examining the muscle FA composition. However, there were significant geographical differences of FA composition in sardine muscle that are probably related to the differences in their diet, such as a higher concentration of EPA, and the EPA/DHA and (n-3)/(n-6) ratios, for fish collected from the west coast compared to those from Portimao, and seasonal and spatial variations in EPA in sardine muscle varied according to variation of this FA in the plankton. However, as observed in other studies comparing the FA composition of clupeids and their diets (e.g. Linko et al. 1985, Shirai et al. 2002), the concentration of EPA in the plankton was higher than in sardine muscle and the EPA/DHA ratio was higher in the prey than in the muscle, which might indicate selective conservation of DHA, a crucial FA for the functioning of biomembranes (Sargent et al. 2002). Geographical differences in muscle fat composition, namely higher concentrations of EPA associated with spring upwelling events, have also been observed for other fish species (Gamez-Meza et al. 1999, Budge et al. 2002, Shirai et al. 2002, Uysal & Aksolar 2005, Nedenskov et al. 2007). PUFAs are important food quality indicators since the retention of this fraction in the muscle of fish would be an appropriate strategy for species or populations that migrate, or to provide energy for spawning activity (Wiegand 1996), which may constitute an advantage for sardines living in the western coast.

Saturated FAs in sardine muscle showed strong seasonal variability, and contributed 26.7 to 84.9% of total FA concentration in fish from the west coast and 25.7 to 85.3% in those from Portimão. These levels are considerably higher than those reported (25.8 to 28.8%) by a previous study in which the fat content of sardine from the west coast was determined monthly during 1994-1995 (Bandarra et al. 1997), and that study reported constant proportions of SAT, MUFA and PUFA fractions in sardine tissue. This is in contrast to results from this study where the proportions of the FA fractions of sardine muscle remained fairly constant during most of the year but changed dramatically in both areas at the start of the spawning season, when the proportion of SATs increased substantially whist the unsaturated FA proportion decreased (Fig. 5.11c, d). However, FA class relative proportions rapidly recovered to their "constant" levels after spawning had started in fish from both areas, and it may be that the unchanging FA class proportions reported by Bandarra et al. (1997) resulted from samples not being collected at the initiation of the spawning season. A decrease in muscle PUFA concentrations related to gonad maturation has also been observed for other fish species (e.g. Uysal & Aksolar 2005).

Data from stomach content analysis (Garrido et al. *accepted*) revealed that sardines continue to feed during their spawning season, and that feeding intensity is higher in winter than in summer when sardines accumulate FAs prior to initiation of the spawning season. However, FA concentrations in sardine muscle follow a seasonal pattern that is not clearly related to the seasonal pattern observed in feeding intensity, and seasonal patterns in muscle FA concentrations in fish from both areas are the opposite of the spawning season; the lipid content of sardine muscle increased when sardines were in the resting stage of reproduction and sharply decreased when they started to reproduce. Additionally, increases in the FA content of sardine muscle seem to be more dependent on their reproductive stage than on their feeding conditions, since fish increase their FA content during the summer which is one of the seasons during which they show a lower feeding intensity. However, at the end of the spawning season (March-April) it appears that a direct relationship between feeding and spawning exists for sardine from the west coast, since a sudden increase in their gonadosomatic index occurred after a period of high availability of food, which opposed the decreasing tendency for spawning observed in the previous month (Fig. 5.11a). In fact, lipids are an important carbon-rich product of the spring bloom that are relatively easily metabolizable (Parrish 1998).

The extension of the spawning season of sardine off the Portuguese south coast is similar to the fish off the central western coast, and generally the extension of spawning increases when moving south for the entire sardine distribution area. Since the southern coast of Portugal is not as rich an environment as the west coast in terms of plankton availability, food is obviously not the only factor explaining the extended spawning season of fish in the south, and other factors such as temperature (Coombs et al. 2006) or the avoidance of the stronger upwelling period in summer off the west coast when eggs might be transported offshore are likely contributing factors.. However, long-term time series of sardine energy density (ED) caught off the west coast (Rosa et al. *submitted*) demonstrate that the seasonal cycle in environmental forcing largely determines the temporal variability in ED (explaining 70.13% of the observed variation), and that after removing this seasonal cycle patterns of oceanographic variability corresponding to spring phytoplankton bloom conditions (before the late summer energy peak of sardine body energy) explained over 50% of the residual energy variance (rED), which had clear repercussions on the fishery productivity of the upcoming year.

The impact of plankton availability as a determinant of Iberian sardine spawning season duration off the Portuguese coast has recently been questioned (Coombs et al. 2006), since peak spawning occurs during the winter months which are not considered to be seasons of high productivity in that area. However Garrido et al. (*accepted*) showed that spring and winter (despite high inter-annual variability) were the seasons when sardines showed the highest feeding intensity, in fish from both the west and south coasts of Portugal, although that study only covered a year and a half, and the high plankton availability off the west coast during winter has also been mentioned by other authors in broader-scale studies using remotely-sensed chlorophyll data (Peliz & Fiúza 1999, Ribeiro et al. 2005). Nutrients obtained from feeding during the spawning season are probably immediately mobilized to the gonads without being accumulated in the muscle, as proposed for sardines from the Mediterranean (Ganias et al. 2003). However, the mobilization of muscle FAs for reproduction seems to be particularly high at the beginning of the spawning season, when the concentration of MUFA and PUFA sharply decreases in the muscle of sardines from both areas, which was also proposed for Mediterranean sardines (Ganias et al. 2003). The balance between stored reserves and current supplies of fats derived from feeding will affect the sensitivity to short-term plankton variability of spawning sardines (Somarakis et al. 2006).

The seasonality in fat composition was similar for male and female sardines, indicating similar feeding strategies and a similar investment in reproduction. The fact that sardines in the resting stage of reproduction always presented significantly higher concentrations of PUFA than during the spawning season, while concentrations of SAT and MUFA fractions remained similar, indicates that the PUFA fraction from the diet is probably being directly mobilised to the gonads during the spawning season, and also that sardine diet is very rich in those FAs, which allows individuals to rapidly recover from the extensive investment in reproduction. Fatty acids, particularly PUFAs, are the major source of metabolic energy for reproduction (Tocher 2003), which explains the large decrease in fatty acid concentrations prior to and during the spawning season.

Sardine spawning off the west coast might be advantaged by the composition of prey available during the resting stage of reproduction (summer and autumn months). Although feeding intensity was lowest during summer, summer was the season when the relative contribution of phytoplankton to the diet was highest, due to high concentrations of diatoms associated with summer upwelling events and dinoflagellates favoured by the thermal stratification that occurs during that season. These phytoplankton groups are the producers of two of the main FAs required for fish reproduction, namely docosahexaenoic acid and eicosapentaenoic acid, both of which are directly linked to the nervous system and visual acuity of the larvae as well as to the production of eicosanoids, which are essential hormones for fish (Sargent et al. 2002).

Sardine dietary composition varies seasonally and geographically and depends to some extent on the prevailing food conditions, and these differences are strong enough to be reflected in fish body composition. Sardines living in upwelling areas benefit not only from higher food availability but also from the higher nutritional value of phytoplankton, particularly the (n-3) series and eicosapentaenoic acid produced by diatoms, which are both linked to the well-being of the fish and to a higher food quality for human consumption.

5.6 – References

Bandarra NM, Batista I, Nunes ML, Empis JM, Christie WW (1997) Seasonal changes in lipid composition of sardine (*Sardina pilchardus*). J Food Sci 62 (1): 40-42

Barbosa A (2006) Estrutura e dinâmica da teia alimentar microbiana na Ria Formosa. PhD Thesis, University of the Algarve, Algarve

Bell MV & Sargent JR (1996) Lipid nutrition and fish recruitment. Mar Ecol Progr Ser 134 (1-3): 315-316

Budge SM, Iverson SJ, Bowen WD, Ackman RG (2002) Among- and within-species variability in fatty acid signatures of marine fish and invertebrates on the Scotian Shelf, Georges Bank, and southern Gulf of St. Lawrence. Can J Fish and Aquat Sci 59 (5): 886-898

Carrera P & Porteiro C (2003) Stock dynamic of the Iberian sardine (*Sardina pilchardus*, W.) and its implication on the fishery off Galicia (NW Spain). Sci Mar 67: 245-258 Suppl 1

Cohen Z, Von Shak A, Richmond A (1988) Effect of environmental conditions on fatty acid composition of the red algae *Porphyridium cruentum*: correlation to growth rate. J Phycol 24:328–332

Coombs SH, Smyth TJ, Conway DVP, Halliday NC, Bernal M, Stratoudakis Y, Alvarez P (2006) Spawning season and temperature relationships for sardine (*Sardina pilchardus*) in the eastern North Atlantic. J Mar Biol Ass UK 86 (5): 1245-1252

Dalsgaard J, St John M, Kattner G, Muller-Navarra D, Hagen W (2003) Fatty acid trophic markers in the pelagic marine environment. Adv Mar Biol 46: 225-340

Dwyer KS, Parrish CC, Brown JA (2003) Lipid composition of yellowtail flounder (*Limanda ferruginea*) in relation to dietary lipid intake. Mar Biol 143 (4): 659-667

Fiúza AFD, DeMacedo ME, Guerreiro MR (1982) Climatological space and time-variation of the portuguese coastal upwelling. Oceanol Acta 5 (1): 31-40

Gamez-Meza N, Higuera-Ciapara I, de la Barca AMC, Vazquez-Moreno L, Noriega-Rodriguez J, Angulo-Guerrero O (1999) Seasonal variation in the fatty acid composition and quality of sardine oil from *Sardinops sagax caeruleus* of the Gulf of California. Lipids 34 (6): 639-642

Ganias K, Somarakis S, Machias A, Theodorou AJ (2003) Evaluation of spawning frequency in a Mediterranean sardine population (*Sardina pilchardus sardina*). Mar Biol 142 (6): 1169-1179

Garrido S, Marçalo A, Zwolinski J, van der Lingen CD (2007) Laboratory investigations on the effect of prey size and concentration on the feeding behaviour of *Sardina pilchardus*. Mar Ecol Prog Ser 330:189-199

Garrido S, Ben-Hamadou R, Oliveira PB, Cunha ME, Chícharo MA, van der Lingen CD. Spatial and temporal variation of sardine (*Sardina pilchardus*) diet composition and feeding intensity off Portugal: relationship with remote sensing chlorophyll data. *Accepted in Mar Ecol Progr Ser*

Hunter JR & Leong R (1981) The spawning energetics of female northern anchovy, *Engraulis mordax*. Fish Bull 79 (2): 215-230

ICES (2000) Report of the Study Group on the estimation of Spawning Stock Biomass of Sardine and Anchovy. ICES CM 2000/ACFM:5, 546pp

Iverson SJ, Frost KJ, Lang SLC (2002) Fat content and fatty acid composition of forage fish and invertebrates in Prince William Sound, Alaska: factors contributing to among and within species variability. Mar Ecol Progr Ser 241: 161-181

Krvaric J & Muzinic R (1950) Investigation into the fat content in the sardine tissues (*Clupea pilchardus* Walb.) Acta Adriatica, 33(8): 1-26

Lee RF, Hagen W, Kattner G (2006) Lipid storage in marine zooplankton. Mar Ecol Progr Ser 307: 273-306

Legendre P & Legendre L (1998) Numerical Ecology. 2nd English edition. Elsevier Science, Amsterdam. 853 pp

Lepage G & Roy CC (1986) Direct transesterification of all classes of lipids in one-step reaction. J Lipid Res 27: 114–119

Linko RR, Kaitaranta JK, Vuorela R (1985) Comparison of the fatty acids in Baltic herring and available plankton feed. Comp Biochem and Physiol B 82 (4): 699-705

Marshall CT, Yaragina NA, Lambert Y, Kjesbu OS (1999) Total lipid energy as a proxy for total egg production by fish stocks. Nature 402 (6759): 288-290

Morimoto H (1991) Relationship between reproductive ability and nutritional body condition in Japanese sardine. ICES CM 1991/H:19

Morimoto H (1996) Effects of maternal nutritional conditions on number, size and lipid content of hydrated eggs in the Japanese sardine from Tosa Bay, southwestern Japan. In Survival Strategies in Early Life Stages of Marine Resources. Watanabe, Y., Yamashita, Y. and Y. Oozeki (Eds). Rotterdam; A.A. Balkema: 3-12

Nedenskov KJ, Jacobsen C, Neilsen HH (2007) Fatty acid composition of herring (*Clupea harengus* L.): influence of time and place of catch on (n-3) PUFA content. J. Sci.of Food and Agric. *Published online* 10.1002/jsfa.2776

Parrish CC (1998) Lipid biogeochemistry of plankton, settling matter and sediments in Trinity Bay, Newfoundland. I. Lipid classes. Organic Geochem 29 (5-7): 1531-1545

Peliz AJ & Fiúza AFG (1999) Temporal and spatial variability of CZCS-derived phytoplankton pigment concentrations off the western Iberian Peninsula. Intern J of Remote Sensing 20 (7): 1363-1403

Pinto JS & Andreu B (1957) Echelle pour la caractérisation des phases evolutives de l'ovaire de sardine (*Sardina pilchardus*, Walb.) en rapport avec l'histophisiologie de la gonade. Proc Techn Pap Gen Fish Counc Mediterr 4

R Development Core Team (2005) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org

Rosa R., Broitman, BR, Nunes ML. Fish energetics link ecosystem dynamics and fisheries. *Submitted to Proc Natl Acad Sci USA*

Reuss N & Poulsen LK (2002) Evaluation of fatty acids as biomarkers for a natural plankton community. A field study of a spring bloom and a post-bloom period off West Greenland. Mar Biol 141 (3): 423-434

Ribeiro AC, Peliz A, Santos AMP (2005) A study of the response of chlorophyll-a biomass to a winter upwelling event off Western Iberia using SeaWiFS and in situ data. J of Mar Syst 53 (1-4): 87-107

Sargent JR & Falk-Peterson S (1988) The Lipid Biochemistry of calanoid copepods. Hydrobiol 167: 101-114

Sargent JR, Henderson RJ, Tocher DR (1989) The Lipids. In *Fish Nutrition*, 2nd ed. (ed JR Halver): 152-218. San Diego: Academic Press.

Sargent JR, Tocher DR & Bell JG (2002) The Lipids. *In Fish Nutrition*, 3rd ed. (eds. JR Halver & RW Hardy) pp 182-259. London: Academic Press.

Shirai N, Terayama M, Takeda H (2002) Effect of season on the fatty acid composition and free amino acid content of the sardine *Sardinops melanostictus*. Comp. Biochem. And Physiol. Part B 131: 387-393

Silva A (2003) Morphometric variation among sardine (*Sardina pilchardus*) populations from the northeastern Atlantic and the western Mediterranean. ICES J Mar Sci 60 (6): 1352-1360

Silva A, Santos MB, Caneco B, Pestana G, Porteiro C, Carrera P, Stratoudakis Y (2006) Temporal and geographic variability of sardine maturity at length in the northeastern Atlantic and the western Mediterranean. ICES J Mar Sci 63 (4): 663-676

Somarakis S, Ganias K, Siapatis A, Koutsikopoulos C, Machias A, Papaconstantinou C (2006) Spawning habitat and daily egg production of sardine (*Sardina pilchardus*) in the eastern Mediterranean. Fish Oceanogr 15 (4): 281-292

Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci 11 (2): 107-184

Uysal K & Aksoylar MY (2005) Seasonal variations in fatty acid composition and the N-6/N-3 fatty acid ratio of pikeperch (*Sander lucioperca*) muscle lipids. Ecol Food and Nutrition 44 (1): 23-35

Wedemeyer GA (1996) Physiology of fish in intensive culture systems. New York: Chapman & Hall, 1996. 232p.

Wiegand MD (1996) Composition, accumulation and utilization of yolk lipids in teleost fish. Rev in Fish Biol and Fish 6 (3): 259-286

CHAPTER 6

EFFECT OF MATERNAL FAT RESERVES ON THE FATTY ACID COMPOSITION OF SARDINE (*Sardina pilchardus*) OOCYTES

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* Garrido S, Rosa R, Ben-Hamadou R, Cunha ME, Chícharo MA, van der Lingen CD. Effect of maternal fat reserves on the fatty acid composition of sardine (*Sardina pilchardus*) oocytes. *Accepted in Comp Biochem Physiol B*

6.1 - Abstract

We compared the fatty acid composition of the muscle and gonads of female Iberian sardines with hydrated oocytes collected at two different times (November and February) during the sardine spawning season off the southern and western Portuguese coasts. A significant decrease in condition factor and total fatty acid concentration in sardine muscle occurred between the two sampling dates, while the gonadosomatic index was similar between samples. Saturated and polyunsaturated fatty concentrations in sardine gonads did not differ between samples, whereas mono-unsaturated content did differ. There was a significant relationship between fatty acid concentrations in female muscle and oocytes, including eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (AA; 20:4n-6), both of which are considered essential for normal larval development. The concentration of docosahexaenoic acid (DHA; 22:6n-3) in oocytes was not dependent on muscle concentration, probably as a result of its selective transfer and accumulation in the eggs. Ratios between DHA, EPA, and AA, which have been linked to egg quality, were examined for both tissue types. The EPA:DHA ratio was highly conserved in both muscle and oocytes of sardines, while DHA:AA and EPA:AA ratios varied significantly between tissue samples, mainly as a result of the high variation in AA. These results indicate that the fatty acid composition and concentration of eggs produced by sardines varies throughout the spawning season, egg fatty acid concentrations decreasing as females lose condition, and fatty acid composition also shows spatial variability. Both types of variability may have a significant impact on egg quality, in particular on the amount of reserves available to larvae which will affect their resistance to starvation, as well as on the appropriate fatty acid composition required for normal growth and development.

6.2 – Introduction

Fatty acids are the major source of energy for all life history stages of fish from eggs through to adults (Tocher 2003), and although there is a high selective metabolic pressure to maintain the composition of fish eggs (Wiegand 1996), fatty acids can be potential determinants of egg quality (Sargent et al. 2002). For teleosts, much of the energy required for oocyte development is derived from lipid reserves accumulated by the female prior to or during the spawning season (Wiegand 1996); in northern anchovy *Engraulis mordax*, for example, approximately two-thirds of the energy required for repeated spawning is derived from lipid reserves (Hunter & Leong 1981). Hence nutrition is vital for the production of high quality eggs and larvae, with optimal fatty acid compositions that will provide developing embryos and larvae the best chance of success (Tocher 2003).

Iberian sardines *Sardina pilchardus* show seasonal cycles of somatic lipid content, with the deposition of fat occurring before the reproductive season (which extends from Oct-Nov to Mar-Apr off the Portuguese coast) and its depletion from the onset until the end of spawning (Bandarra et al. 1997, Garrido et al. *submitted*). Sardine fatty acid reserves decline dramatically from nearly 300 μg g^{-1} DW at the start of spawning to 14 μg g^{-1} DW by the end of the reproductive season (Garrido et al. *accepted*) which might have an affect on the quality of eggs produced. For fish such as sardine that show indeterminate spawning (*i.e.* producing many batches of eggs during a single reproductive season) and have short periods of vitellogenesis between spawning events, it appears likely that egg quality will also be affected by the lipid content of food ingested during the spawning season, as has been shown for gilthead sea bream *Sparus aurata* (Fernández-Palacios et al. 1995). Sardines feed throughout their spawning season (Garrido et al. *accepted*), and the 6-7 month extent of reproduction undoubtedly involves an enormous investment in egg production that probably is not only dependent on reserves accumulated prior to spawning but also from feeding that takes place during spawning.

Little is known about how poor feeding regimes or a low level of female fat reserves will influence the egg composition of fish in the wild, although many experiments with farmed marine fish species have shown that parental diet affects egg fatty acid composition and that this in turn impacts on the reproductive success and egg quality (e.g. Bell et al. 1997, Almansa et al. 1999, Bruce et al. 1999, Izquierdo et al. 2001, Mazorra et al. 2003, Tveiten et al. 2004, Salze et al. 2005). These studies have shown that fish require (n-3) highly unsaturated fatty acids (HUFAs) for their normal growth and reproduction, particularly the essential dietary fatty acids docosahexaenoic acid (DHA, 22:6(n-3), eicosapentaenoic acid (EPA, 20:5(n-3) and arachidonic acid (AA, 20:4(n-6), not only in sufficient amounts but also with the proper ratios between them (Sargent et al. 1999).

The impact of broodstock diet on egg quality and larval viability is probably more noticeable and important for farmed fish that are fed artificial diets of varying compositions than it is for fish feeding on their natural prey to which they are adapted. However, for Iberian sardine larvae in the wild it has been demonstrated that during the transition from endogenous to exogenous feeding both the quantity of yolk and its composition after hatching are related to larval survival (Riveiro et al. 2000). For Japanese sardine *Sardinops sagax*, a positive correlation between the lipid content of female muscle and that of the ovary was reported by Morimoto (1991), who later showed that female sardine with a higher muscle lipid content had larger ovaries and produced more eggs that had a higher yolk content than did females with a lower muscle lipid content (Morimoto 1996). Similarly, the total number of eggs produced by Barents Sea cod *Gadus morhua* was found to be related to the total lipid energy of spawning females, which varied with food availability (Marshall et al. 1999).

Iberian sardines show a high seasonal and geographical variability in their dietary fatty acid content and composition, which is reflected in their muscle composition (Garrido et al. *submitted*). Sardines accumulate fat reserves in the viscera, muscles and under the skin (Abdelmouleh & Hadj Ali Salem 1981) and the seasonal variation of the muscle and whole body of sardines is synchronous (Rosa et al. *submitted*). The fatty acid composition of roe of Pacific sardine (*Sardinops caerulea*) was closely related to adult fish diet (Lasker & Theilacker 1962), indicating that the fatty acid composition of the oocytes might also strongly depend on the quality of the diet. Iberian sardines living in the upwelling areas off the west coast of Portugal have significantly higher concentrations of (n-3) fatty acids in their muscle than do sardines living off the south coast of Portugal, suggested as arising from the higher contribution by phytoplankton to the diet of fish from the west compared to those in the south (Garrido et al. *submitted*). Hence the natural variability of sardine dietary composition probably affects their egg composition, particularly closer to the end of the spawning season when lipid reserves are depleted.

The objective of this work was to study the effect of female sardine fatty acid content on the quantity and composition of fatty acids in their oocytes, by comparing fatty acid profiles of females and their hydrated oocytes collected at different times during the spawning season and from two different locations (the west and south coasts) characterized by different feeding environment.

6.3 – Material and Methods

Sardine samples were collected during two research cruises conducted on board the RV *Noruega* (IPIMAR) off the Portuguese continental coast carried out over two different periods (November and February) during the 2002/03 spawning season. Biological data including total length (TL, cm), gutted weight (fish weight minus viscera, g), sex, and macroscopically-determined indices of gonad maturity stage (Pinto & Andreu, 1957) and fat content (Krvaric & Muzinic 1950) were recorded for each sardine. The gonadosomatic index (GSI, ovary mass per unit of ovary-free mass) and condition factor (CF, gutted mass divided by total length cube) were estimated for each fish. Female sardines with hydrated oocytes were then selected from samples, being collected off the south coast during November 2002 and off the south and west coasts during February 2003 (Fig. 6.1). A total of 51 female sardines with hydrated oocytes were collected from 12 different trawls. Muscle and gonad samples were collected from each hydrated female for fatty acid (FA) determination. These fish were filleted and the fillets skinned to provide muscle samples, and their gonads were also removed, and both tissues were immediately cryopreserved with liquid nitrogen for later analysis. In the laboratory, the muscle and gonad samples were thawed then minced before conducting lipid extraction and FA analysis.

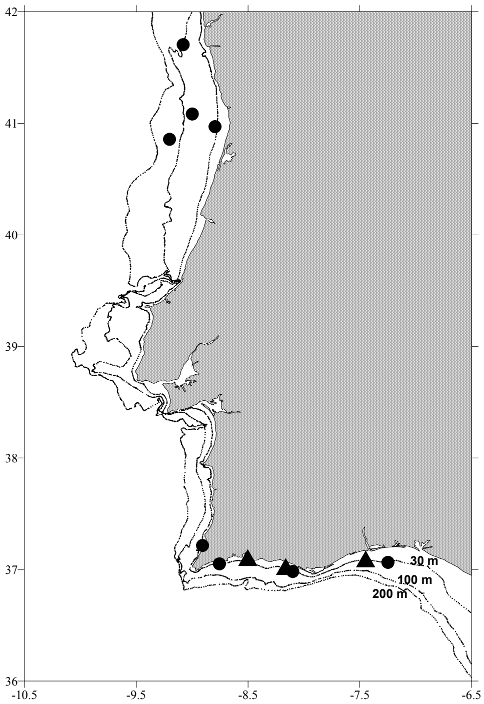

Figure 6.1 – Map of the Portuguese continental coast showing sampling sites where female sardines with hydrated oocytes were collected during November 2002 (triangles) and February 2003 (circles).

The determination of FA profiles was based on the experimental procedure of Lepage & Roy (1986) as modified by Cohen et al. (1988), with FA methyl esters being analysed in a CP 3800 Varian gas chromatograph equipped with an auto-sampler and fitted with a flame ionisation detector (FID). The separation was carried out with helium as the carrier gas in a DB-Wax Polyethylene Glycol column (30 m x 0.25 mm id) programmed to start at 180 ºC for 5 min, then heating at a rate of 4º C min^{-1} for 10 min and maintained at 220 ºC for 25 min, with detection conducted at 250 ºC using a split injector (100:1). Fatty acid methyl esters were identified by comparison of their retention times with those of Sigma chromatographic standards, peak areas were determined using the Varian software, and the FA 23:0 was used as an internal standard. We assumed that the FA composition determined from the gonads was representative of oocyte FA composition, since the contribution from ovarian tissue itself (as distinct from the actual oocytes) is assumed to be minimal once uptake of vitellogenin is well established (Wiegand 1996).

A comparison between the FA composition of fish muscle and oocytes between the three sampling period/area combinations (south coast November 2002, south coast February 2003 and west coast February 2003) was done using analysis of variance (ANOVA) and *a posteriori* Tukey analysis for unequal sample sizes, with $p<0.05$ being used as the level of significance difference.

The relationship between total FAs in muscle and oocytes was examined using a linear regression model. To minimize the influence of outliers, we fitted our data using a robust least squares regression (Bi-square weights) scheme which minimizes the weighted sum of squares by weighting each data point according to how far that point is from the fitted line (Cruz Ortiz et al. 2006). Points near the line get a full weight whereas those farther from the line get a reduced weight, and points that are farther from the line than would be expected by random chance get zero weight. This type of linear regression model was also used to compare the concentrations and relative proportions of the essential FAs (DHA, EPA and AA) in the muscle and oocytes. To obtain data of the water temperature during the 3 sampling periods we extracted Sea Surface Temperature (SST) data from the AVHRR Oceans Pathfinder through the online PO.DAAC Ocean ESIP Tool (PO.DAAC), NASA Jet Propulsion Laboratory, Pasadena, CA. http://podaac.jpl.nasa.gov/poet."

6.4 - Results

Female sardines with hydrates oocytes collected off the Portuguese coast (Fig. 6.1) ranged from 13.5 to 22.5 cm TL, but average length was not significantly different for fish from different samples (Fig. 6.2 a). Condition factor of fish off the south coast showed a significant decrease from November to February (Fig. 6.2 b) and was similar in February for fish from both the west and south coasts, while gonadosomatic index showed no significant difference between the three groups of females (Fig. 6.2 c). Total fatty acid concentration in female sardine muscle decreased from November to February and was similar in both areas in February, whereas total FA concentrations of the oocytes were intermediate between November and February muscle values and were very similar for the three samples (Fig. 6.2 d). Multiple regression analysis of muscle FA concentration as a function of length, area and month showed that month was the only significant ($p<0.001$) parameter in explaining differences in FA concentration.

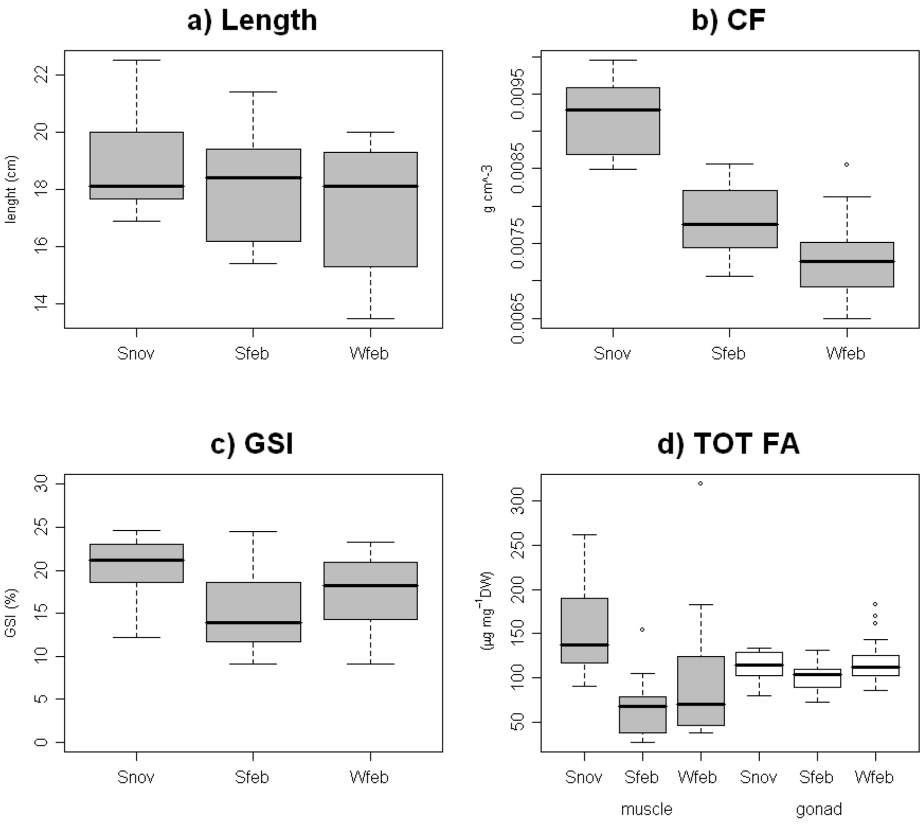

Figure 6.2 - Boxplots of the a) total length (cm), b) condition factor (total weight/length^3) and c) gonadosomatic index (gonad weight*100/total weight) and d) total fatty acid content (µg mg^{-1} DW) of female sardines with hydrated oocytes collected during November (“nov”) and February (“feb”) off the south (S) and west (W) coasts of Portugal. The median (horizontal line), inter-quartile range (shaded box), upper and lower hinges are shown for each sample.

The concentration of all fractions of fatty acids in sardine muscle decreased from November to February, although the decrease in monounsaturated fatty acids (MUFA) was larger than in saturated fatty acids (SAT) and polyunsaturated fatty acids (PUFA) (Fig. 6.3). Concentrations of SAT and PUFA in the oocytes showed no significant differences between the three samples, but oocyte MUFA concentrations in sardines collected from southern Portugal in February were significantly lower than in the other samples. Saturated fatty acids comprised around 35% of total FA concentration in both muscle and oocytes with little variation between samples (Fig. 6.3d), while the percentage of MUFA was more variable in muscle and oocytes and average values ranged from around 15-20% of total FAs. The percentage contribution of PUFA was similar and around 45% for both tissue types, although oocytes of sardines from the west coast of Portugal showed higher variability in %PUFA.

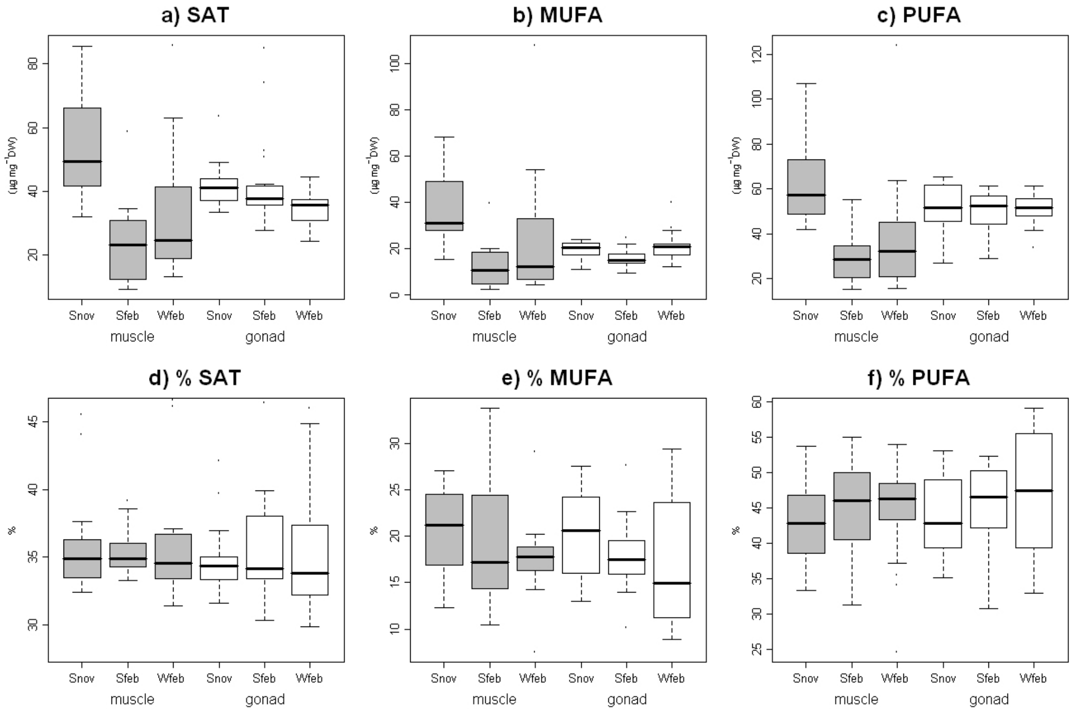

Figure 6.3 – Boxplots of fatty acid concentration (µg g^{-1} DW) of a) Unsaturated fatty acids, b) Monounsaturated fatty acids, c) Polyunsaturated fatty acids, d) % Unsaturated e) % Monounsaturated and f) % Polyunsaturated fatty acids in muscle and gonad of females collected during November (“nov”) and February (“feb”) off the south (S) and

west (W) coasts of Portugal. The median (horizontal line), inter-quartile range (shaded box), upper and lower hinges are shown for each sample.

Concentrations of the (n-3) and (n-6) FA series decreased sharply from November to February in sardine muscle, and while (n-3) series concentrations in sardine oocytes were not significantly different between samples the (n-6) series showed significantly higher concentrations in sardine oocytes collected in February off the south coast (Fig. 6.4). The ratio of (n-3) to (n-6) in sardine oocytes was significantly higher in fish from the west coast when compared to samples from the south coast.

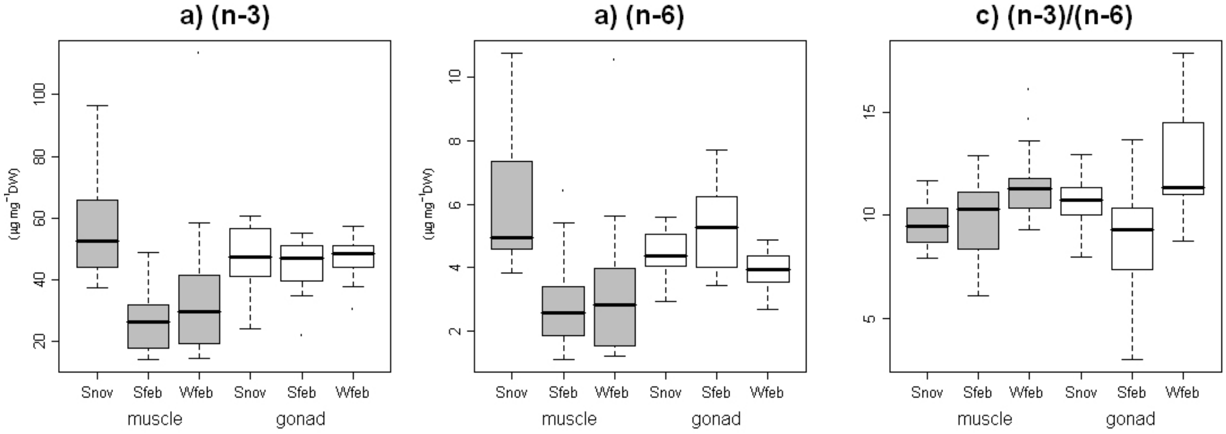

Figure 6.4 – Boxplots of fatty acid concentration (µg g^{-1} DW) of a) (n-3) fatty acids, b)(n-6) fatty acids and c) the (n-3):(n-6) fatty acid ratio in muscle and oocytes of females collected during November ("nov") and February ("feb") off the south (S) and west (W) coasts of Portugal. The median (horizontal line), inter-quartile range (shaded box), upper and lower hinges are shown for each sample.

The fatty acid composition of sardine oocytes closely resembled that of the muscle (Table 6.1), with both tissues containing high concentrations of saturated fatty acids, particularly 16:0, and very high concentrations of polyunsaturated fatty acids, particularly highly unsaturated fatty acids of the (n-3) series such as DHA and EPA. The most important monounsaturated FA was 18:1(n-9) followed by 16:1(n-7) (Table 6.1).

Table 6.1 – Mean ± S.D. fatty acid concentrations (µg mg^{-1} dry weight) in the muscle and oocytes of sardines collected off Peniche (W) and Portimão (S) at the beginning (November) and end (February) of the spawning season.

Fatty acids	**Muscle**			**Gonad**		
(µg mg DW)	S Nov	S Feb	W Feb	S Nov	S Feb	W Feb
14:0	8.96 ± 3.80	3.34 ± 2.56	5.84 ± 5.75	5.21 ± 1.41	3.40 ± 4.03	4.86 ± 2.69
16:0	34.52 ± 10.43	15.62 ± 7.63	20.75 ± 11.20	29.68 ± 6.06	25.37 ± 7.63	29.96 ± 12.18
18:00	7.91 ± 2.48	3.45 ± 2.02	4.23 ± 1.91	4.58 ± 0.86	4.33 ± 1.41	4.35 ± 1.00
16:1(n-7)	9.57 ± 4.54	2.92 ± 2.35	4.85 ± 5.93	6.57 ± 1.67	4.86 ± 4.17	6.51 ± 2.77
18:1(n-9)	15.85 ± 7.81	4.75 ± 4.29	8.45 ± 5.87	7.71 ± 1.66	5.78 ± 1.60	8.36 ± 2.80
18:1(n-7)	5.91 ± 6.95	1.97 ± 1.19	4.64 ± 9.48	3.37 ± 0.67	3.55 ± 7.29	3.81 ± 1.02
20:1(n-9)	0.14 ± 0.28	0.40 ± 0.90	0.68 ± 1.33	0.15 ± 0.33	0.06 ± 0.09	0.19 ± 0.51
20:1(n-7)	4.12 ± 1.95	1.17 ± 1.30	3.63 ± 5.19	0.89 ± 0.40	0.88 ± 1.77	0.93 ± 0.58
16:4(n-3)	1.06 ± 0.91	0.56 ± 0.47	0.70 ± 1.06	0.30 ± 0.19	0.36 ± 0.81	0.25 ± 0.18
22:1(n-11)	0.97 ± 1.00	1.09 ± 1.17	1.20 ± 2.29	0.18 ± 0.16	0.29 ± 0.46	0.27 ± 0.25
22:1(n-9)	0.74 ± 0.94	0.19 ± 0.24	0.56 ± 1.21	0.13 ± 0.08	0.12 ± 0.28	0.16 ± 0.16
18:2(n-6)	2.17 ± 0.69	0.78 ± 0.46	1.02 ± 0.73	1.20 ± 0.21	0.93 ± 0.59	0.90 ± 0.30
20:4(n-6)	1.80 ± 0.77	1.11 ± 0.60	0.94 ± 0.71	1.98 ± 0.45	3.03 ± 1.40	1.77 ± 0.37
20:5(n-3)	18.06 ± 9.48	7.16 ± 4.45	10.72 ± 12.36	13.47 ± 4.34	13.85 ± 6.81	14.33 ± 2.47
22:6(n-3)	28.59 ± 4.88	15.97 ± 4.44	18.27 ± 5.29	27.82 ± 5.83	24.03 ± 6.66	26.75 ± 6.62
∑ Sat[1]	54.18 ± 16.72	23.49 ± 12.87	32.41 ± 18.79	42.79 ± 12.32	34.72 ± 13.35	36.51 ± 13.69
∑ Branch[2]	0.83 ± 0.24	0.28 ± 0.19	0.37 ± 0.32	0.59 ± 0.22	0.45 ± 0.27	2.57 ± 12.05
∑ MUFA[3]	37.75 ± 15.85	12.68 ± 9.58	24.28 ± 24.99	23.42 ± 12.09	15.95 ± 13.55	20.47 ± 14.20
∑ PUFA[4]	62.24 ± 18.96	29.72 ± 12.11	37.42 ± 24.93	52.38 ± 13.33	50.17 ± 3.87	44.69 ± 16.39
∑ HUFA	57.79 ± 17.96	28.03 ± 11.05	35.34 ± 23.48	49.33 ± 12.55	47.90 ± 13.08	42.76 ± 14.71
∑ Unsat	99.99 ± 34.43	42.41 ± 20.91	61.71 ± 49.57	75.80 ± 24.50	66.13 ± 25.99	65.16 ± 27.97
∑ (n-3)	56.27 ± 17.04	26.82 ± 10.68	34.28 ± 22.73	47.70 ± 12.11	44.80 ± 13.02	41.25 ± 14.19
∑ (n-6)	5.96 ± 2.01	2.90 ± 1.52	3.13 ± 2.21	4.69 ± 1.36	5.37 ± 1.63	3.45 ± 1.34
(n-3)/(n-6)	9.57 ± 1.12	9.91 ± 1.97	11.39 ± 1.74	9.89 ± 1.26	8.77 ± 2.37	11.77 ± 2.48
DHA/EPA	1.84 ± 0.59	2.82 ± 1.47	2.91 ± 1.62	2.02 ± 0.52	1.82 ± 0.69	2.15 ± 1.09
∑ Total FA	155.00 ± 50.97	66.19 ± 33.63	94.50 ± 68.18	113.09 ± 15.96	101.31 ± 39.10	119.07 ± 26.95

[1] Saturated: 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0 and 22:0

[2] Branched: Iso 14:0, Anteiso 14:0, Iso 16:0 and Anteiso 16:0

[3] Monounsaturated: 16:1(n-7), 17:1(n-8), 18:1(n-9), 18:1(n-7) , 20:1(n-9), 20:1(n-7), 22:1(n-11) 22:1(n-9)

[4] Polyunsaturated: 16:4(n-3), 18:2(n-6), 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:2(n-6), 20:4(n-6), 20:3(n-3), 20:4(n-3), , 20:5(n-3), 22:4(n-6), 22:5(n-6), 22:5(n-3) and 22:6(n-3)

The concentration of AA in sardine muscle decreased from November to February in the south, but AA concentration in the oocytes was significantly higher in sardine from the south than from the west coast in February (Fig. 6.5a). EPA and DHA were conserved, with concentrations in the oocytes of both FAs similar to those observed in the muscle at the beginning (Nov) of the spawning season (Figure 6.5b, c). Concentrations of the monounsaturated FAs 18:1(n-9) decreased significantly both in the muscle and gonads from November to February in the south coast while 20:1(n-9) and 22:1(n-11) were present in lower concentrations in sardine oocytes than in muscle (Fig. 6.5d-e).

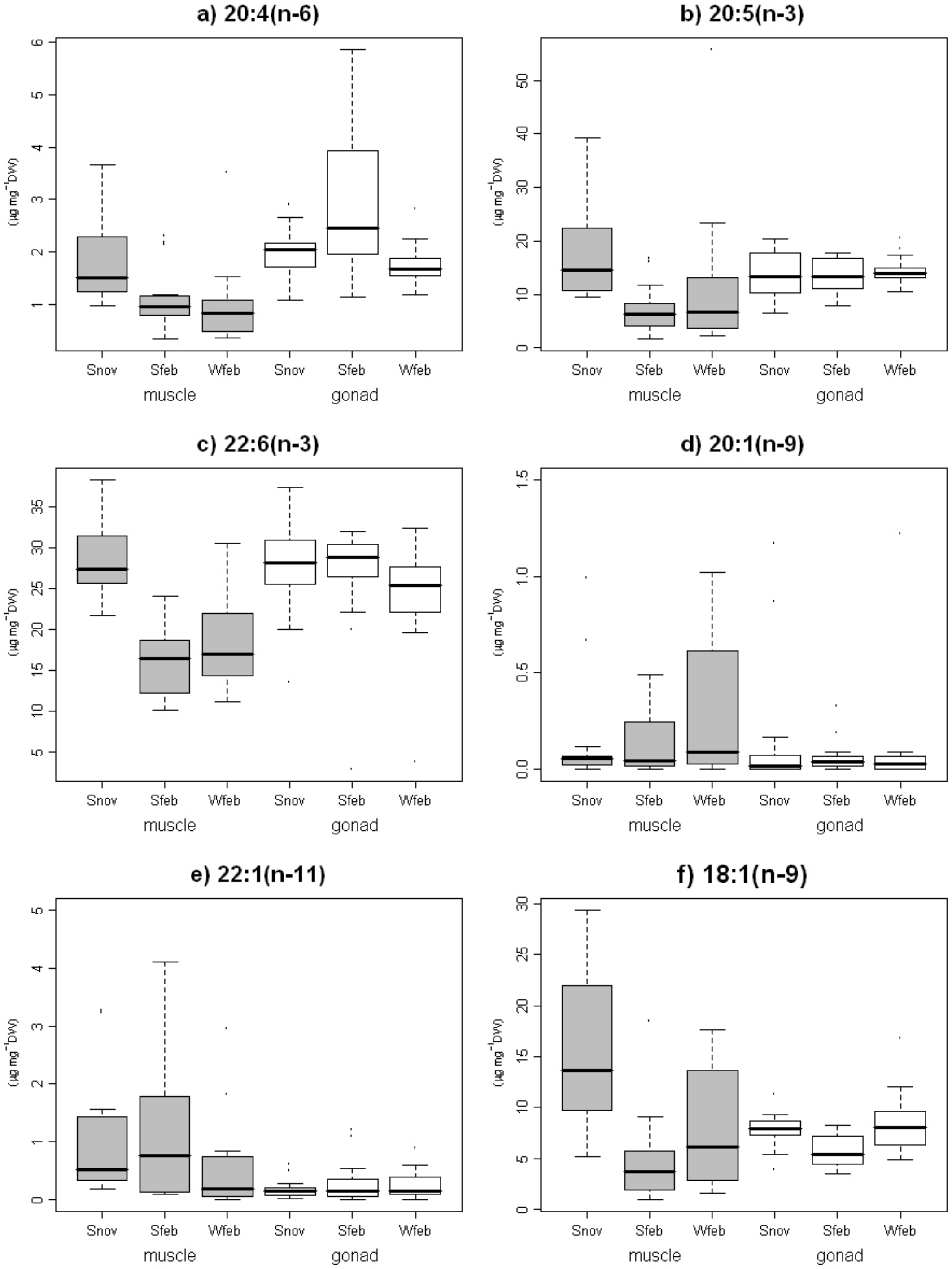

Figure 6.5 – Boxplots of the concentration (µg g^{-1} DW) of the fatty acids; a) 20:4(n-6), b) EPA 20:5(n-3), c) DHA 22:6(n-3), d) 20:1(n-9), e) 20:1(n-11), f) 18:1(n-9) in the muscle (gray boxes) and oocyts (white boxes) of females collected during November ("nov") and February ("feb") off the south (S) and west (W) coasts of Portugal. The median (horizontal line), inter-quartile range (shaded box), upper and lower hinges are shown for each sample.

Total fatty acid concentration, the concentration of most of the major FA fractions (unsaturated, MUFA and PUFA), and concentrations of some individual FAs in sardine oocytes were significantly positively correlated with their concentrations in female muscle (Figure 6.6 and Table 6.2). Whereas the concentrations of the saturated fatty acids 14:0, 16:0 and 18:0 in the oocytes were significantly correlated with muscle concentrations the total SAT fraction was not. Significant correlations between muscle and oocytes were observed for the most abundant MUFAs, 16:1(n-7) and 18:1(n-9), and PUFAs that showed the strongest correlations between oocyte and muscle tissue included arachidonic acid (20:4(n-6)), eicosapentaenoic acid (20.5(n-3) and the fatty acid 22:5(n-3). In addition to the fatty acids listed in Table 6.2, the fatty acids 12:0, 16:4(n-3), 18:3(n-6), 18:4(n-3), 20:1(n-9), 20:1(n-7), 20:2(n-6), 20:3(n-3), 20:4(n-3) and 22:5(n-6) in the oocytes were also positively correlated with their concentrations in the muscle.

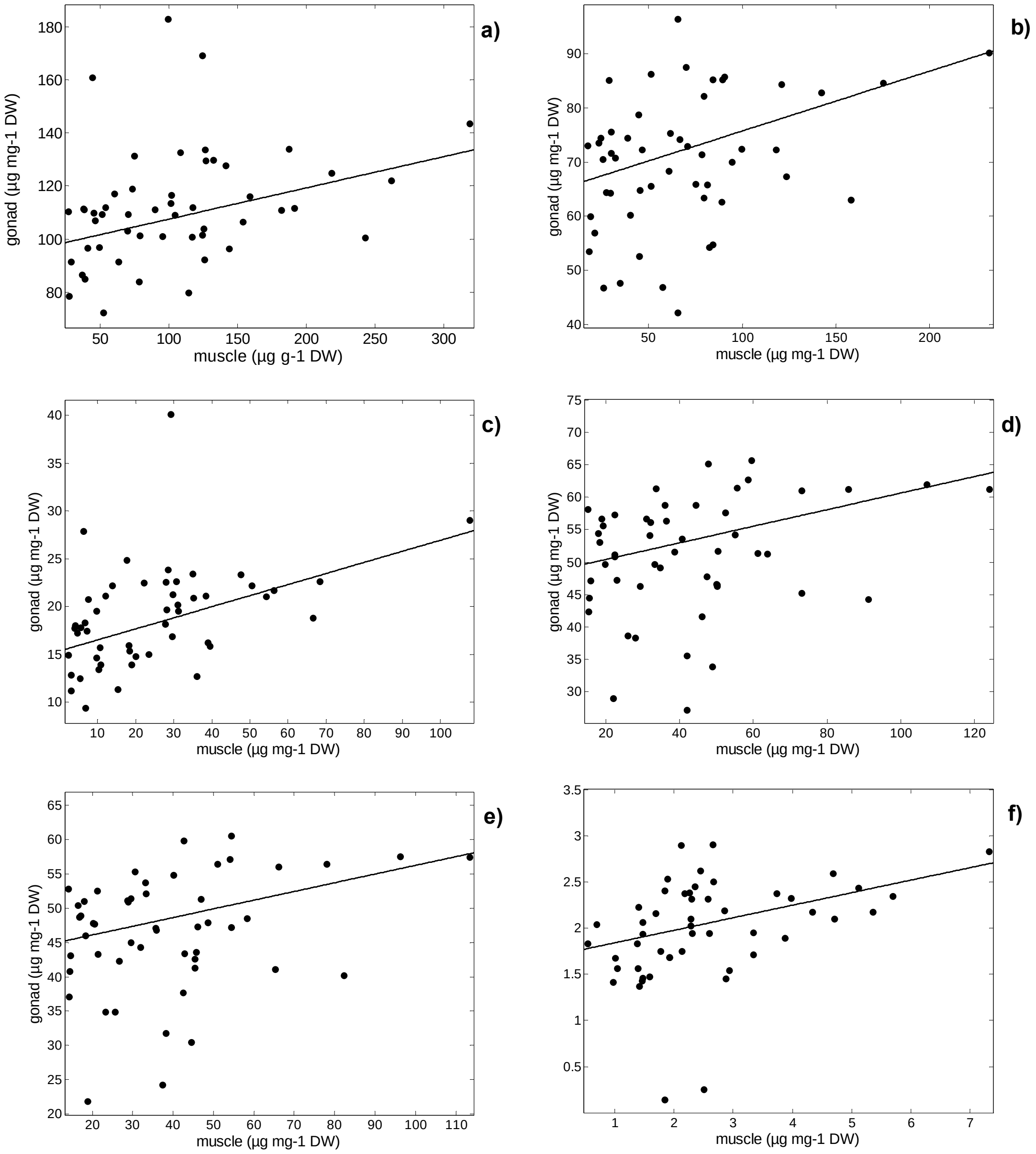

Figure 6.6 – Scatterplots showing relationships between the concentrations (µg mg^{-1} DW) of fatty acids in the muscle and oocytes of female sardines with hydrated oocytes: a) Total fatty acids, b) Unsaturated fatty acids, c) Monounsaturated fatty acids, d) Polyunsaturated fatty acids, and e) (n-3) fatty acids. Panel f) shows the relationship between DHA:EPA ratios in the muscle and oocytes of female sardines with hydrated oocytes. Linear regressions are shown on the plots, and the regression coefficients are presented in Table 2.

Table 6.2 – Results of the regression analysis between individual fatty acid content of females and oocytes. Significant regressions are marked with (*) for $p<0.05$ and (**) for $p<0.01$. (SSE= Sum of squares due to error; Adj.r^2= coefficient of determination and adjusted coef. of determination).

Fatty acid (μg g^{-1} DW)	**Coefficients**		**SSE**	**Adj. r^2**
	slope	**intercept**		
14:0	0.1633** (0.1027, 0.2239)	3.191** (2.715, 3.666)	47.67	0.3766**
16:0	0.1418** (0.05624, 0.2275)	23.93** (21.61, 26.24)	623.1	0.1806**
16:1(n-7)	0.1391** (0.06048, 0.2176)	5.314** (4.692, 5.936)	97.51	0.1992**
18:0	0.1659* (0.1001, 0.2317)	3.467** (3.087, 3.848)	15.22	0.3552*
18:1(n-9)	0.1365** (0.07179, 0.2012)	5.821** (5.03, 6.612)	134	0.2616**
18:1(n-7)	0.05304 (-0.1249, 0.231)	3.391 (2.82, 3.961)	39.38	-0.01352
18:2(n-6)	0.1021* (0.01796, 0.1862)	0.8597* (0.7253, 0.9942)	2.891	0.09556
18:3(n-3)	0.07485 (-0.05377, 0.2035)	0.6638 (0.5337, 0.7939)	3.353	0.00766
20:4(n-6)	0.2641* (0.008645, 0.5195)	1.737* (1.355, 2.12)	22.75	0.0648
20:5(n-3)	0.2145** (0.1064, 0.3225)	11.78** (10.39, 13.18)	359	0.2414*
22:1(n-11)	0.08332* (0.0209, 0.1457)	0.1657* (0.07119, 0.2601)	3.22	0.1124*
22:1(n-9)	0.08211** (0.03031, 0.1339)	0.09291** (0.06384, 0.122)	0.2788	0.1634**
22:5(n-3)	0.2513** (0.1199, 0.3827)	2.05** (1.714, 2.386)	26.44	0.2159**
22:6(n-3)	0.1518 (-0.00778, 0.3115)	24.36 (20.83, 27.89)	734.4	0.05368
∑ Unsat	0.1107* (0.05365, 0.1677)	64.68* (60.05, 69.3)	2647	0.2597**
∑ MUFA	0.1159* (0.06865, 0.1632)	15.34** (13.8, 16.88)	546.9	0.327**
∑ PUFA	0.1131* (0.03164, 0.1945)	48.2** (44.28, 52.12)	1815	0.1318
∑ (n-3)	0.1271* (0.04314, 0.211)	43.56* (39.89, 47.23)	1573	0.1559*
DHA/EPA	0.1357* (0.06, 0.2114)	1.704* (1.483, 1.925)	6.376	0.2001*
∑ FA	0.1179* (0.05436, 0.1814)	95.72* (87.85, 103.6)	9242	0.2161*

The ratio between the essential docosahexaenoic acid and eicosapentaenoic acid (DHA/EPA) was not significantly different in muscle and oocytes for all three samples, except the muscle and gonads from the west coast, presenting significant differences between them and with the muscle and gonads of February from the south coast. The ratio between eicosapentaenoic acid and arachidonic acid (EPA/AA) was significantly different for muscle and oocytes of the south coast in February in comparison with both muscle and gonads of the south coast in November and with muscle and gonads from February in the west coast. The ratio between docosahexaenoic acid and arachidonic acid (DHA/AA) in the ovary of females collected during February in the west coast was different to that in the muscle (Fig. 6.7). On the other hand, the concentrations of essential fatty acids were highly correlated in sardine muscle (Fig. 6.8 and Table 6.3), with DHA:EPA ($r^2 = 0.60$), EPA:AA ($r^2 = 0.84$) and AA:DHA ($r^2 = 0.57$) all showing significant ($p<0.01$) positive relationships In sardine oocytes the relationships between the concentration of these fatty acids were not as significant as in muscle, with DHA:EPA ($r^2 = 0.25$) and EPA:AA ($r^2 = 0.18$) being significantly related ($p=0.037$ and $p=0.048$, respectively), whilst DHA and AA concentrations showed no significant correlation.

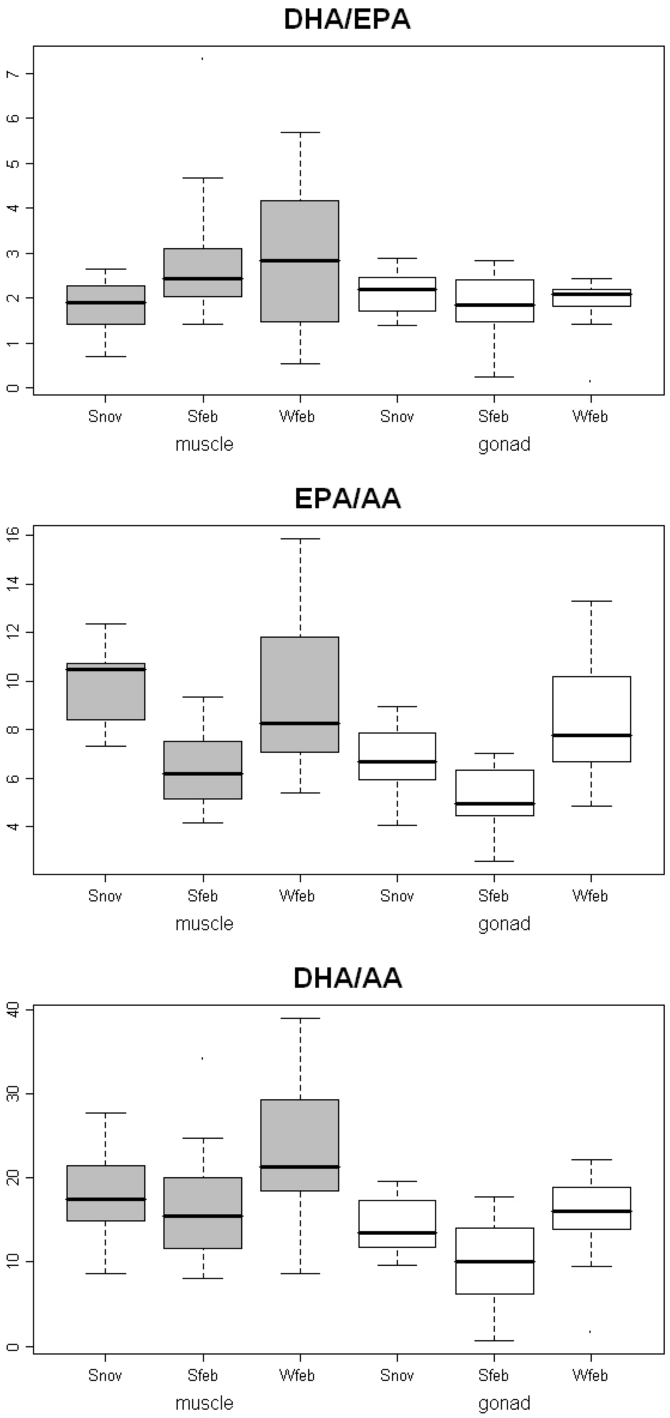

Figure 6.7 - Boxplots of the ratios of the concentrations (µg mg^{-1} DW) between the fatty acids; a) DHA:EPA, b) EPA:AA, c) DHA:AA in the muscle (gray boxes) and oocytes (white boxes) of females collected during November (“nov”) and February (“feb”) off the south (S) and west (W) coasts of Portugal. The median (horizontal line), inter-quartile range (shaded box), upper and lower hinges are shown for each sample.

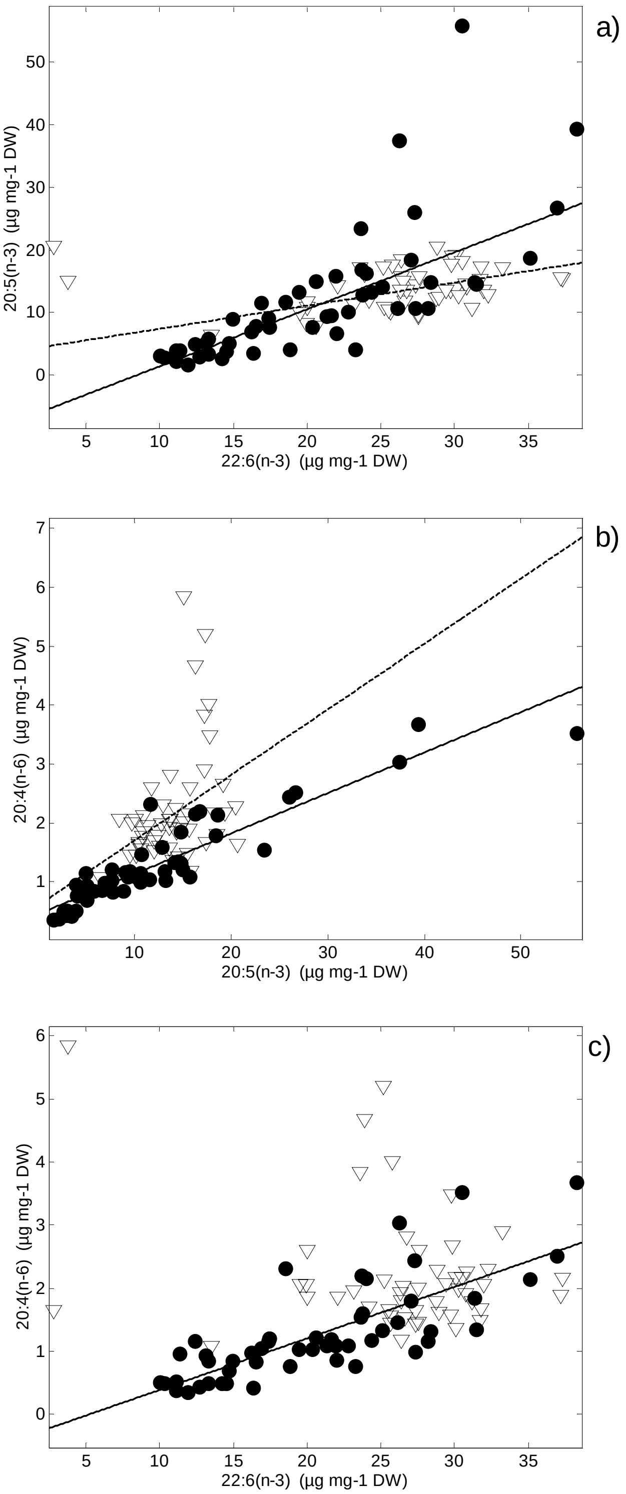

Figure 6.8 – Scatterplots showing relationships between the concentrations (µg mg^{-1} DW) of a) DHA and EPA, b) EPA and AA, and c) DHA and AA in the muscle (circles; solid line) and oocytes (triangles; dashed line) of female sardines collected off Portugal. Significant linear regressions are shown on the plots, and the regression coefficients are presented in Table 3.

In both tissues when the concentration of DHA increased the concentration of EPA also increased (Fig. 6.8 a), reflecting the general increase in total fatty acid concentration. However, the proportions of DHA and EPA in relation to the total fatty acid concentration were inversely related in both muscle and oocytes; i.e. when the proportion of one increased the proportion of the other decreased, hence maintaining a relatively constant proportion (approximately 45% for the oocytes and 40% for the muscle) of the total fatty acid concentration (Fig. 6.9a and Table 6.3). The proportions of EPA and AA to the total fatty acid concentration were correlated in the oocytes while were not for the muscle (Fig. 6.9b), as the case of the proportions of DHA and AA in the muscle and oocytes (Fig. 6.9c).

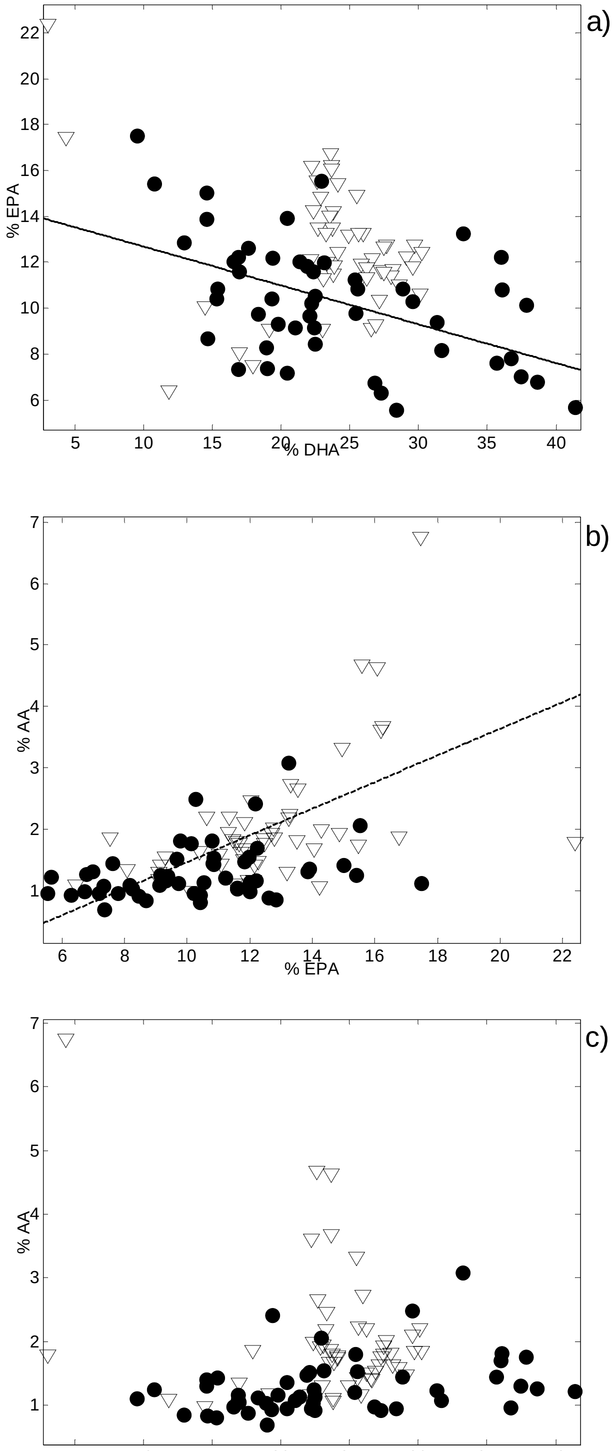

Figure 6.9 – Scatterplots showing relationships between the percentage contribution of essential fatty acids to total FA concentration for a) DHA and EPA, b) EPA and AA and c) DHA and AA in the muscle (crosses; solid line) and oocytes (circles; dashed line) of female sardines collected off Portugal. Significant linear regressions are shown on the plots, and the regression coefficients are presented in Table 3.

Table 6.3 – Results of the regression analysis ($y = ax + b$) between the concentrations (“c”) ($\mu g\ g^{-1}$ DW) and proportions (“p”) of DHA and EPA, EPA and AA, and DHA and AA in the muscle and oocytes of female sardines collected off Portugal. Significant regressions are marked with (*) for $p<0.05$ and (**) for $p<0.01$. (Adj.r^2= adjusted coefficient of determination).

Fatty acids		**type**	**tissue**	**Coefficients**		**Adj. r^2**
x	**y**			**slope**	**intercept**	
DHA	EPA	c	muscle	0.909	-7.701	0.603**
DHA	EPA	c	oocytes	0.368	3.697	0.246*
EPA	AA	c	muscle	0.070	0.451	0.837**
EPA	AA	c	oocytes	0.111	0.594	0.183*
DHA	AA	c	muscle	0.081	-0.427	0.570**
DHA	AA	c	oocytes	0.0003	2.137	-0.021
DHA	EPA	p	muscle	-0.169	14.37	0.236**
DHA	EPA	p	oocytes	0.153	8.493	0.044
EPA	AA	p	muscle	0.0419	0.797	0.089
EPA	AA	p	oocytes	0.217	-0.708	0.354**
DHA	AA	p	muscle	0.019	0.832	0.095
DHA	AA	p	oocytes	0.029	1.13	0.012

6.5 - Discussion

This study has shown that significant variation in the fatty acid concentration and composition of Iberian sardine (*Sardina pilchardus*) oocytes is caused by parental effects, namely the fat reserves of the female. A positive relationship between ovarian lipid content and female muscle lipid content was also shown for the Japanese sardines, *Sardinops melanostictus* (Morimoto 1991). Lasker & Theilacker (1962) showed that lipids in Pacific sardine (*Sardinops caerulea*) eggs collected from the plankton (*i.e.* spawned eggs) had a very similar composition to ovarian lipids, leading us to assume that the lipids determined in the oocytes are representative of those that will be available in the egg. These results show that the amount and composition of the fat reserves that sardines are able to accumulate prior to the spawning season will have a marked effect on the quality of the eggs produced during the spawning season.

The concentration of several saturated fatty acids in Iberian sardine oocytes was positively related to maternal concentrations, although no relationship between the total saturated fraction in muscles and oocytes was found. In contrast, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) concentrations in the oocytes were significantly related to their concentrations in female muscle. MUFAs are the primary source of energy for the developing embryo (Sargent et al. 2002), hence concentrations of these fatty acids in the mother at a given time will determine the MUFA concentrations of the eggs produced. Our results showed that MUFA concentrations were low in the muscle and oocytes of females collected from the south coast of Portugal during February, which could mean either that the amount of fat accumulated by sardines off the south coast was lower than for fish off the west coast, or that the spawning season of south coast sardines began earlier than for west coast fish, and that the reserves of south coast sardines were further depleted than their west coast counterparts.

The amount of fat reserves of both female and male sardines is closely linked to their temporal location in the spawning cycle; at the beginning of the spawning season sardines have probably accumulated enough fat content to ensure sufficient fatty acids of the composition required for normal egg development. As the spawning season advances reserves are depleted such that they may become insufficient for egg development, and the sardines probably become more dependent on fatty acids derived from feeding for successful gametogenesis. When this occurs ambient food concentrations will have a direct impact on gametogenesis, and sardines living in areas of higher food availability like the west coast of Portugal (Garrido et al. *accepted*) may be at an advantage compared to those living in the comparatively food-poor environment off the south coast. For gilthead seabream *Sparus aurata* it was shown that eggs spawned early in the spawning season were not affected by broodstock diet, whereas later in the season eggs were influenced by dietary fatty acids (Almansa et al.

1999). In European sea-bass (*Dicentrachus labrax*), improved egg quality and higher hatching rates were associated with broodstock diet during vitellogenesis, identifying this as the period where fatty acids are more effectively incorporated into the developing oocytes (Navas et al. 1997). Iberian sardines actively prey on their own eggs, and eggs were more important in the diet of fish from the south coast compared to the west coast (Garrido et al. *accepted*). Food availability appears to be lower on the south coast than the west coast, and the higher predation rate on eggs off the south coast might constitute a mechanism by which sardine reabsorb the energy of eggs during less favourable feeding periods.

Although concentrations of (n-3) FAs in sardine oocytes were similar for the three sampling periods and were related to the (n-3) FA concentration in female muscle, the (n-3)/(n-6) ratio in the oocytes was higher for sardines from the west coast compared to those from the south coast. There is a high requirement for (n-3) HUFAs in developing egg and larvae because of their preponderance in neural and visual tissues, which predominate in early stages of development (Bruce et al. 1999). Any deficiency in these particular fatty acids can cause abnormalities in the neural system and may affect the success of larvae as visual predators at the onset of first feeding (Bell & Sargent 1996). One of the essential (n-3) HUFAs, docosahexaenoic acid (DHA), was conserved in sardine oocytes which showed higher DHA concentrations than in the muscle, even when muscle DHA concentrations were significantly reduced in both areas in February. No relationship between DHA concentrations in the oocytes and the muscle was observed. Selective incorporation of essential fatty acids, particularly DHA, in fish eggs, and selection against 20:1(n-9) and 22:1(n-11), as was observed in this study (see Table 1), have been previously described for a large number of fish species (Sargent et al. 2002).

On the other hand, oocyte concentrations of the other two (n-3) HUFAs considered as being essential for fish, arachidonic acid (AA) and eicosapentaenoic acid (EPA), were dependent on their concentrations in female muscle, although both were selectively transferred to the oocytes which had higher AA and EPA concentrations than in the muscle. The dependence of these particular fatty acids on the female reserves might have serious implications for the quality of the egg.

Due to the competitive metabolism of HUFAs, muscle and egg requirements of these fatty acids must be considered both in absolute amounts but also in relative concentrations, particularly the ratios between these HUFAs. Appropriate ratios of these HUFAs in broodstock diet have been shown in several fish species to meet the requirements for neural functioning as well as optimizing the functioning and production of eicosanoids, which are responsible for a wide range of physiological roles including osmoregulation, cardiovascular functions, neural control and the functioning of reproductive systems (e.g. Bell et al. 1997, Bruce et al. 1999, Sargent et al. 1999, Mazorra et al. 2003, Salze et al. 2005). The DHA:EPA ratio was strongly preserved in both tissues examined in this study, and their combined relative contribution to the total fatty acid concentration showed a constant proportion of 40 and 45% for muscle and oocytes, respectively. The correlation between concentrations of EPA and AA was higher for the muscle than for the oocytes, while DHA and AA concentrations were only correlated in the muscle but not in the oocytes. Both EPA:AA and DHA:AA presented significant differences between samples, suggesting that there is a natural variability of the proportion of the essential fatty acids in sardine eggs that might have an effect on their quality. Understanding the dietary and tissue relationships between DHA, EPA and AA is essential for illuminating the complex interactions between nutrition, behaviour and environmental stress (Sargent et al. 1999).

For the larvae of several fish species, it has been demonstrated that the principal energy source during embryogenesis is a pool of free amino acids, whilst the oil droplet (in these eggs that possess one or more) is utilized after hatching (e.g. Rønnestad et al. 1994, Ohkubo et al. 2006). This probably reflects the greater energy demands of the mobile, free-swimming yolk-sac larvae compared to the embryonic phase (Sargent et al. 2002). In fact, Riveiro et al. (2000) found a relationship between Iberian sardine egg protein percentage and larval survival time. The amount of lipid reserves would determine the period that larvae would be able to survive before first-feeding while the proper concentration of essential fatty acids will guarantee normal larval development and growth.

Fatty acids indices in female gonad might be appropriate for assessing health status of first-feeding larvae (Navarro & Sargent 1992). High levels of essential fatty acids positively influence condition as well as development of the brain, nervous, pigmentary tissues development and schooling behaviour and escape ability from predators in larval stages, key factory determining survival (Navarro & Sargent 1992, Ishizaki et al. 2001, Nakayama et al. 2003). Therefore, the accumulation of essential fatty acids in the early developmental stages of fishes is potentially a key factor in fish recruitment fluctuations (Bell & Sargent 1996).

The biochemical composition of Iberian sardine eggs, in particular their protein content, was negatively related to water temperature for sardine eggs collected off the north-west Iberian coast (Riveiro et al. 2000), although a previous study conducted further south off the west coast found that whereas eggs with lower dimensions (considering egg size as proxy of the amount of reserves) were coincident with lower water temperatures no clear relationships between sardine egg dimensions and environmental factors (temperature, salinity and oxygen) were observed (Ré 1986). Eggs with higher lipid and protein reserves seem to be associated with lower temperatures, and lower temperatures would result in a decreased development rate and hence increase the time that larvae have to subsist on their yolk reserves (Ré 1986). In our study water temperature was higher during November than during February (averaging 18ºC for November off the south coast and 14ºC and 12-13ºC for the south and west coast during February, respectively), which means that eggs with higher fatty acid concentrations occurred in the month when temperatures were also higher. Contrary, a decrease of egg size during the spawning season observed for the anchoveta *Engraulis ringens* was coincident with an increase in the water temperature (Llanos-Rivera & Castro 2004).

The production of smaller eggs late in the spawning season by northern anchovy *Engraulis mordax* was considered to be an energy-sparing mechanism whereby fecundity is maintained at a constant level but at lower reproductive effort (Hunter & Leong 1981). For sardines, the end of the spawning season occurs at a time of high food availability for both the west and south coasts (Garrido et al. *accepted*), hence the production of eggs with lower reserves could be an adaptive mechanism since larvae would have more food available at that time than compared to the beginning of the spawning season, when sardine feeding intensity is lower (Garrido et al. *accepted*). The same temporal variation of food availability and egg size during the spawning season was described for the anchoveta *Engraulis ringens* (Llanos-Rivera & Castro 2004) and the production of larger eggs is related to larger larvae that resist better to starvation (Llanos-Rivera & Castro 2006).

Fish annual egg production and/or reproductive effort can be modulated by changes in batch fecundity (the number of hydrated eggs per spawn), the number of spawnings during the spawning season, female size at first maturity, egg cannibalism and egg biochemical content (Hunter & Leong 1981), and variation in these characteristics means that a direct relationship between spawner biomass and the number of eggs produced is seldom, if ever, observed (Marshall et al. 1999). Egg quality induced by maternal effects is only one of the factors that may affect spawning success, but the extent of its impact on recruitment is not known. Although there is a strong tendency to preserve the fatty acid composition of fish eggs, the fatty acid composition of Iberian sardine oocytes was strongly related to the amount and composition of female fat reserves, which shows significant changes both seasonally and spatially. This indicates that the relationship between female diet and the quality of offspring produced should be further investigated for this species, particularly the importance of egg quality on larval survival, but also how the impact of environmental changes on the variability of feeding conditions may ultimately effect recruitment.

6.6 - References

Abdelmouleh A & Hadj Ali Salem M (1981) Etude préliminaire sur les variations des lipides dans différentes parties du corps de la sardine: *Sardina pilchardus* (Walbaum, 1792) de la région de Bizerte (Tunisie). Bull. Inst. Natn. Scient. Tech. Océanogr. Pêche Salammbó 8: 53-58.

Almansa E, Perez MJ, Cejas JR, Badia P, Villamandos JE, Lorenzo A (1999) Influence of broodstock gilthead seabream (*Sparus aurata* L.) dietary fatty acids on egg quality and egg fatty acid composition throughout the spawning season. Aquaculture 170 (3-4): 323-336

Bandarra NM, Batista I, Nunes ML, Empis JM, Christie WW (1997) Seasonal changes in lipid composition of sardine (*Sardina pilchardus*). J Food Sci 62 (1): 40-42

Bell JG, Farndale BM, Bruce MP, Navas JM, Carillo M (1997) Effects of broodstock dietary lipid on fatty acid compositions of eggs from sea bass (*Dicentrarchus labrax*). Aquaculture 149 (1-2): 107-119

Bell MV & Sargent JR (1996) Lipid nutrition and fish recruitment. Mar Ecol Progr Ser 134 (1-3): 315-316

Bruce M, Oyen F, Bell G, Asturiano JF, Farndale B, Carrillo M, Zanuy S, Ramos J, Bromage N (1999) Development of broodstock diets for the European Sea Bass (*Dicentrarchus labrax*) with special emphasis on the importance of n-3 and n-6 highly unsaturated fatty acid to reproductive performance. Aquaculture 177 (1-4): 85-97

Cohen Z, Von Shak A, Richmond A (1988) Effect of environmental conditions on fatty acid composition of the red algae *Porphyridium cruentum*: correlation to growth rate. J Phycol 24: 328–332

Cruz Ortiz M, Sarabia LA, Herrero A (2006) Robust regression techniques: A useful alternative for the detection of outlier data in chemical analysis. Talanta 70 (3): 499-512

Fernández-Palacios H, Izquierdo MS, Robaina L, Valencia A, Salhi M, Vergara JM (1995) Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L). Aquaculture 132 (3-4): 325-337

Garrido S, Ben-Hamadou R, Oliveira PB, Cunha ME, Chícharo MA, van der Lingen CD. Spatial and temporal variation of sardine (*Sardina pilchardus*) diet composition and feeding intensity off Portugal: relationship with remote sensing chlorophyll data. *Accepted to Mar Ecol Progr Ser*

Hunter JR & Leong R (1981) The spawning energetics of female northern anchovy, *Engraulis mordax*. Fish Bull 79 (2): 215-230

Ishizaki Y, Masuda R, Uematsu K Shimizu K, Arimoto M, Takeuchi T (2001) The effect of dietary docosahexaenoic acid on schooling behaviour and brain development in larval yellowtail. J Fish Biol 58: 1691–1703.

Izquierdo MS, Fernández-Palacios H, Tacon AGJ (2001) Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197: 24-42

Krvaric J & Muzinic R (1950) Investigation into the fat content in the sardine tissues (*Clupea pilchardus* Walb.) Acta Adriatica 33 (8): 1-26

Llanos-Rivera A & Castro LR (2004) Latitudinal and seasonal egg-size variation of the anchoveta (*Engraulis ringens*) off the Chilean coast. Fish Bull 102: 207–212

Llanos-Rivera A & Castro LR (2006) Inter-population differences in temperature effects on *Engraulis ringens* yolk-sac larvae. Mar Ecol Progr Ser 312: 245-253

Lasker R & Theilacker GH (1962) Fatty acid composition of the lipids of some Pacific sardine tissues in relation to ovarian maturation and diet. J of Lipid Res 3 (1): 60-64

Lepage G & Roy CC (1986) Direct transesterification of all classes of lipids in one-step reaction. J Lipid Res 27: 114–119

Marshall CT, Yaragina NA, Lambert Y, Kjesbu OS (1999) Total lipid energy as a proxy for total egg production by fish stocks. Nature 402 (6759): 288-290

Mazorra C, Bruce M, Bell JG, Davie A, Alorend E, Jordan N, Rees J, Papanikos N, Porter M, Bromage N (2003) Dietary lipid enhancement of broodstock reproductive performance and egg and larval quality in Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 227 (1-4): 21-33

Morimoto H (1991) Relationship between reproductive ability and nutritional body condition in Japanese sardine. ICES CM 1991/H:19

Morimoto H (1996) Effects of maternal nutritional conditions on number, size and lipid content of hydrated eggs in the Japanese sardine from Tosa Bay, southwestern Japan. In Survival Strategies in Early Life Stages of Marine Resources. Watanabe, Y., Yamashita, Y. and Y. Oozeki (Eds). Rotterdam; A.A. Balkema: 3-12

Nakayama S, Masuda R, Takeuchi T, Tanaka M (2003) Effects of highly unsaturated fatty acids on escape ability from moon jellyfish *Aurelia aurita* in red sea bream *Pagrus major* larvae Fish Sci 69 (5): 903–909

Navarro JC & Sargent JR (1992) Behavioural differences in starving herring *Clupea harengus* L. larvae correlate with body levels of essential fatty acids. J Fish Biol 41: 509-513

Navas JM, Bruce M, Thrush M, Farndale BM, Bromage N, Zanuy S, Carrillo R, Bell JG, Ramos J (1997) The impact of seasonal alteration in the lipid composition of broodstock diets on egg quality in the European sea bass. J Fish Biol 51 (4): 760-773

Ohkubo N, Sawaguchi S, Hamatsu T, Matsubara T (2006) Utilization of free amino acids, yolk proteins and lipids in developing eggs and yolk-sac larvae of walleye pollock *Theragra chalcogramma*. Fish Sci 72 (3): 620-630

Pinto JS & Andreu B (1957) Echelle pour la caractérisation des phases evolutives de l'ovaire de sardine (*Sardina pilchardus*, Walb.) en rapport avec l'histophisiologie de la gonade. Proc. Techn. Pap. Gen. Fish. Counc. Mediterr. 4

Ré P (1986) Ecologia da postura e da fase planctónica de *Sardina pilchardus* (Walbaum, 1792) na região central da costa portuguesa. Bol Soc Port Ciênc Nat XXIII: 5-81

Riveiro I, Guisande C, Lloves M, Maneiro I, Cabanas JM (2000) Importance of parental effects on larval survival in *Sardina pilchardus*. Mar Ecol Progr Ser 205: 249-258

Rønnestad I, Koven WM, Tandler A, Harel M, Fyhn HJ (1994) Energy metabolism during development of eggs and larvae of gilthead sea bream (*Sparus aurata*). Mar Biol 120 (2): 187-196

Rosa R, Broitman, BR, Nunes ML. Fish energetics link ecosystem dynamics and fisheries. *Submitted to Proc Natl Acad Sci USA*

Salze G, Tocher DR, Roy WJ, Robertson DA (2005) Egg quality determinants in cod (*Gadus morhua* L.): egg performance and lipids in eggs from farmed and wild broodstock. Aquaculture Res 36 (15): 1488-1499

Sargent J, Bell G, McEvoy L, Tocher D, Estevez A (1999) Recent developments in the essential fatty acid nutrition of fish. Aquaculture 177 (1-4): 191-199

Sargent JR, Tocher DR & Bell JG (2002) The Lipids. In Fish Nutrition, 3rd ed. (eds. JR Halver & RW Hardy) pp 182-259. London: Academic Press

Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev. In Fish. Sci. 11 (2): 107-184

Tveiten H, Jobling M, Andreassen I (2004) Influence of egg lipids and fatty acids on egg viability, and their utilization during embryonic development of spotted wolf-fish, *Anarhichas minor* Olafsen. Aquaculture Res 35 (2): 152-161

Wiegand MD (1996) Composition, accumulation and utilization of yolk lipids in teleost fish. Rev in Fish Biol and Fisher 6 (3): 259-286

CHAPTER 7

CONCLUSIONS

This dissertation has provided detailed information on the feeding ecology of Iberian sardine *Sardina pilchardus* in the Northeast Atlantic upwelling system. Mechanisms of prey selection used by this species are described, and ingestion rates for different prey types and concentrations quantified. Temporal variations in diet composition of fish collected from two areas off Portugal characterized by different food environments are examined, and routine measurements of plankton productivity, particularly remotely-sensed chlorophyll data, were tested as estimators of sardine feeding intensity. In the area where algal blooms occur frequently as a result of upwelling events, the ingestion of diatoms of the genus *Pseudo-nitzschia*, which are natural producers of the toxin domoic acid, was compared to the accumulation of this toxin in sardine tissues. Temporal variation in the fatty acid composition of sardine stomach contents and sardine muscle from the two areas was assessed, the effect of different dietary composition on sardine muscle fatty acid composition was examined, and seasonal patterns in fat accumulation by sardine were related to seasonality of their spawning. Finally, maternal effects, namely the concentration and composition of lipid reserves accumulated by females, on oocyte fatty acid composition, were investigated.

Results obtained demonstrate the highly opportunistic and flexible feeding ability of sardines derived from their utilization of either of two different feeding modes. Filter-feeding is used to capture small prey items down to nanoplankton size, and particulate-feeding is used to actively select, pursue and capture individual, larger prey items. Which feeding mode is used depends exclusively on prey size and is independent of prey concentration: for prey <720 µm sardines use filter-feeding while for prey >780 µm they use particulate-feeding. The clearance rates and swimming speeds of fish engaged in particulate-feeding were higher than values estimated for filter-feeding fish. The filtering efficiency of sardines is the highest reported for a clupeid to date; Iberian sardines were able to retain particles as small as 4 µm at a filtering efficiency of 20%, and filtering efficiency reached >80% when feeding on prey >230 µm. However, the clearance rates of Iberian sardines were generally low compared to other clupeids, a consequence of their smaller mouth gape and slower swimming speed.

Stomach content analysis of sardines from the western and southern coasts of Portugal revealed that sardines are omnivorous, with the most important prey types for sardine in both areas being zooplankton (mainly crustacean eggs, copepods, decapods, and cirripeds), fish eggs, and phytoplankton (both diatoms and dinoflagellates). Together, these prey types represented >90% of the total dietary carbon in every stomach sampled. The contribution of different prey sizes to sardine dietary carbon showed two modes, one for prey of 50-150 µm and other for prey of 1000-1500 µm, and these two modes probably correspond to the two feeding modes used by sardines.

These results demonstrate that regional and seasonal variations in the food environment must be taken into account in order to understand the influence of environmental factors on the dynamics of small pelagic fish species such as sardine. Geographical differences in biological characteristics and stock dynamics observed for Atlanto-Iberian sardines (see the Introduction) can be explained by differences in the food web structure and environmental forcing in different areas. Whilst zooplankton were important prey year-round, fish eggs were mainly important during the winter and phytoplankton were mainly important during the spring and summer, particularly off the west coast. The major dietary differences between fish from the two areas were the higher importance of phytoplankton to sardines off the west coast while fish eggs were more important for sardines from the south. The highest feeding intensity occurred during spring and the winter of 2004 in both areas, and was higher during all seasons for the west coast compared to the south, this difference being related to upwelling events and riverine inputs off the west coast that enhance productivity there.

The most important diatoms for sardines from both areas were species of the genus *Pseudo-nitzschia*, which is particularly abundant and persistent off the west coast. These species naturally produce the phycotoxin domoic acid, which is the principal toxin responsible for the human intoxication known as Amnesic Shellfish Poisoning (ASP). Domoic acid was detected in sardine stomach contents from May until September in 2002 and 2003, and its accumulation in sardine was related to occurrence of *P. australis*. The production of domoic acid was higher during summer than during spring, which is probably related to the exhaustion of nutrients in the water during summer. Domoic acid accumulated mainly in fish guts and not in their flesh, meaning that effects to human health appear to be minimal. However, the high accumulation of *Pseudo-nitzschia* and domoic acid in the guts suggests that sardine can act as an important toxin vector to higher trophic levels, and stomach content analysis should be conducted during periods when *Pseudo-nitzschia* are high (i.e. toxic bloom events) in order to confirm whether the toxin elimination mechanism of sardine is efficient at higher toxin concentrations. Furthermore, other toxin-producing algae such as *Dynophisis acuta* and *D. acuminata* were frequently detected in stomach contents of sardine from both areas, implying that sardines might also function as vectors of Diarrhetic Shellfish Poisoning (DSP). Because they are efficient filterers sardines accumulate large concentrations of phytoplankton cells, including producers of phycotoxins, in their stomachs, and hence are not only susceptible to toxins produced by such harmful algae but can also function as vectors for higher trophic levels. Moreover, sardines might exert top-down control on harmful algal blooms or initiate new blooms by resuspending algal cysts, which were important and common prey for sardines off the south coast. Hence the role of sardines should be considered when studying the dynamics of toxic events in upwelling areas.

Laboratory experiments showed that sardines are able to feed selectively, preferring fish eggs when given mixtures of cultured plankton and showing higher clearance rates for eggs compared to other cultured prey types, and preferring copepods and decapod larvae when given mixtures of natural zooplankton. The preference for fish eggs by sardines in the wild was demonstrated by the high contribution that fish eggs made to sardine diet, particularly off the south coast. Cannibalism by a species on its own eggs can be a self-regulatory, density-dependent mechanism as has been proposed for other clupeids, or a way to reabsorb energy in periods of low plankton availability. Alternatively, preying on eggs of fish species that at later life history stages prey on sardine, might be way for small pelagics to control the density of their predators. These questions suggest that further investigations should be conducted to investigate the impact of egg cannibalism by sardine, and their predation on the eggs of other co-habiting species, to sardine survival and recruitment.

Temporal variability in the feeding intensity of sardines was correlated with remotely-sensed chlorophyll a concentrations in the areas where the fish were collected and remotely-sensed chlorophyll a concentrations explained a portion of the observed variance in sardine feeding intensity, suggesting that these data could be used to proxy sardine feeding intensity. Stomach content analysis is laborious, time–consuming, and very difficult to perform in large scale studies, and alternative methods to evaluate sardine feeding intensity and diet composition should be investigated in order to obtain a sufficiently large data set that permits further study of the influence of environmental factors on sardine feeding, and the influence of sardine feeding on their population dynamics. The relationship between remotely-sensed chlorophyll a concentration and sardine feeding intensity suggests that further investigations of this nature should be conducted, such as a regional approach to investigate relationships between chlorophyll a and sardine distribution and recruitment strength. Recently, a method to rapidly evaluate sardine feeding was developed, in which stomachs are classified by two indices according to stomach fullness and colour (the latter index being a proxy for a more herbivorous or a more carnivorous diet; Cunha et al., 2005), and this method is now being used in Portuguese and Spanish sardine biological sampling, and provides large-scale data on sardine feeding in Iberian waters. Additionally, detailed studies of the dynamics of phyto- and zooplankton communities that are preyed on by sardines would help understand the variations of sardine distribution and abundance.

Whereas alternative, low resolution methods of assessing sardine feeding have been developed and can be used to provide information at a large-scale, the qualitative description of sardine diet in terms of different compounds, such as fatty acids, has proved to be essential in order to fully evaluate food quality. Fatty acid biomarkers were useful in this regard and detected differences in dietary composition between sardines from the south and west coasts, in addition to being related to the degree of carnivory of the diet, and correlated with prey carbon content in the case of diatoms. However, biomarkers were not able to distinguish the relative contribution made by dinoflagellates to sardine diet, most probably because of masking of the dinoflagellate biomarker by other prey types such as crustacean or fish eggs that also contain high concentrations of docosahexaenoic acid.

The fatty acid concentrations of sardine diet was similar for fish from both areas but the fatty acid composition was significantly different, with prey from the west coast presenting higher concentrations of polyunsaturated fatty acids (PUFAs) and fatty acids of the (n-3) series, including eicosapentaenoic acid and linoleic acid. This is in agreement with the higher contribution made by both dinoflagellates and diatoms to the diet of sardines from the west coast as seen from stomach content analysis, since phytoplankton have relatively high levels of PUFAs. In contrast, the diet of sardines from the south coast was richer in monounsaturated fatty acids (MUFAs), namely the biomarker for carnivory 18:1(n-9), which is in agreement with the higher prevalence of fish eggs and zooplankton in the diet of sardines from the south.

Differences in the dietary composition of sardines from the west and south coasts were reflected in the fatty acid composition of their muscle, with sardines from the west having higher concentrations of eicosapentaenoic acid (EPA), and higher EPA/DHA and (n-3)/(n-6) ratios compared to those from the south. The importance of EPA and these fatty acid ratios to fish well-being and reproduction is well documented, showing that in order to evaluate food quality for the fish the study of specific nutrients contained within prey items, and not only stomach content analysis, is required.

Feeding intensity and fatty acid composition of sardine muscle was similar for females and males, and seasonality in sardine fatty acid composition in fish from both areas is primarily driven by the seasonal cycle in spawning. Sardines accumulate high concentrations of fatty acids during the resting stage of reproduction, when the feeding intensity is similar to or lower than values observed during spawning. The concentration of the fatty acids in sardine muscle was maintained at fairly constant levels year round except at the beginning of the spawning season, when primarily PUFAs and MUFAs were invested in gametogenesis, but the proportion of these fractions in the muscle recovered shortly after initiation of the reproductive season. The enhanced productivity of early spring upwelling events coincident with the last months of spawning might cause an extension of the spawning season.

A significant relationship between the fatty acid composition of female muscle and the fatty acid composition of their oocytes was observed, particularly for two fatty acids considered to be essential for normal growth and for survival of the eggs and larvae, namely EPA and arachidonic acid (AA). Concentrations of DHA in oocytes were independent of the concentration of this fatty acid in female muscle. The ratios between DHA and EPA was kept constant for both muscle and oocytes, but the ratio between these two and AA varied significantly for both tissues, which might have significant implications for egg quality and ultimately on the recruitment of this species.

Small pelagic clupeids around the world experience high interannual fluctuations in abundance, and their recruitment is known to depend strongly on environmental conditions. Feeding dynamics will play an important role in determining the relative success of sardines under particular environmental conditions, and spatial differences in diet composition can impact on the reproduction of sardines, since the reserves accumulated by females prior to spawning influences egg composition, which probably has direct implications for larvae survival and recruitment. However sardines are able to accumulate large fat reserves even in seasons of low feeding intensity, suggesting that the main effect of food availability is not felt in adult survival but in spawning success. The recovery of small pelagic fish stocks is generally based on the formation of one or a few powerful year classes, and since body condition is essentially the integration of a sardine's trophic environment over time, a trophically-favourable environment for sardine will result in good-condition fish that produce eggs of higher quality that probably have a higher probability of survival than eggs from poor-condition fish. Hence trophodynamically-mediated successful spawning, both in terms of egg numbers and egg quality, could assist in the formation of strong year classes. Since food availability and seasonal abundance changes from area to area, future studies relating regional food-web variability and structure to recruitment success should help clarify the relationship between environmental forcing and reproduction success for sardines.

References

Cunha ME, Garrido S, Pissarra J (2005) The use of stomach fullness and colour indices to assess *Sardina pilchardus* feeding. J Mar Biol Ass UK 85: 425-431