Chapter 2
Seasonal analysis of fattyacids in
*Sardinella longiceps* and
*Sardinella fimbriata*

2.1 Introduction

Fattyacids in sardines are known to show seasonal fluctuations in their composition and yield. Earlier studies in temperate regions on species like *Sardinops sagax* (Gamez-Mesa *et al.* 1999), *Sardina pilchardus* (Bandarra *et al.* 1997) and *Sardinops melanostictus* (Shirai *et al.* 2002) have clearly demonstrated a seasonal fluctuation influenced by temperature of sea water, food availability and sexual state of the animal. There has been a seasonal study in the Indian seas for *S. longiceps* (Gopakumar 1965), however this study did not delve at the granularity of individual fattyacids but considered PUFA as one component. There has been no seasonal fattyacid profiling on *S. fimbriata*, except the information available for a
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They catalogued the fatty acid composition of 31 marine fish species including these two species.

In the present investigation, fattyacid composition of the two species of sardines is compared four seasons. Though phylogenetically close, the feeding pattern of these two sardines are known to be slightly different; S. longiceps favouring phytoplankton diet (Nair 1953) while S. fimbriata being more partial to zooplanktons (Chacko 1956). Hence, the fattyacid profiles of these two fishes are expected to show deviations. Specific focus is on the variation of EPA and DHA composition of these fish oils as this is of high relevance in the subsequent bioactivity studies. Seasonal variations of these two important PUFA were analysed keeping in mind the commercial implication of such a variation for pharmaceutical and nutritional industry. Potential reasons for such a variation across seasons and across species in relation to the chief factors influencing the fattyacid composition is also elaborated.

2.2 Materials and Methods

2.2.1 Fish samples

Freshly caught samples of S longiceps and S fimbriata, were collected from the Kaalamukku landing centre (9°58’55”N, 76°14’33”E) at Kochi. Samples were washed in sterile water and brought to the laboratory in an ice box. Fishes were identified for their maturity stages (Antony Raja 1971) and their lengths measured and their stomach content analyzed (Wallace 1981). Sampling was done on the 15th day of four months
representative of four seasons in the Indian tropics (McKnight and Hess 2000) – September (post-monsoon), December (winter), March (summer) and June (monsoon).

2.2.2 Analysis of fattyacids:

Fattyacids were analyzed according to the method of AOAC (1975). In this method, fatty acids were made volatile by converting them into methyl esters. The esters were identified and quantified by GC by comparing with a set of standard esters. Lipid content of the tissues was estimated by the method of Folch et al. (1957). Methyl esters of fattyacids (FAME) from animal and vegetable origin having 8-24 atoms are separated and detected by gas chromatography. Methyl esters of the fattyacid thus obtained were separated by gas liquid chromatography equipped with a capillary column and a flame ionization detector. Fattyacids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fattyacids. Measurement of peak areas and data processing were carried out by Thremo Chrom card software. Individual fattyacids were expressed as mg/g and then converted to percentage of total fattyacids.

2.2.3 Extraction of Total Lipids (Folch et al. 1957)

One g of tissue was subjected to lipid extraction using chloroform-methanol mixture (2:1). The lipid extracts were transferred to a separating funnel and added with, 20% of water and left overnight. It was drained through filter paper containing anhydrous sodium sulphate and was collected in round bottom flask and evaporated to dryness in an evaporator. The lipid in the round bottom flask was made up to 10ml with
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chloroform, taken into a pre-weighed vial and allowed to dry in warm temperature to constant weight. Total lipid content was calculated from the difference in weight and the result expressed as mg/g of total lipid of fresh tissue.

Satellite Imagery of the collection site (Courtesy: Google maps)
2.2.4 Fattyacid Methyl Ester Method (AOAC 1975)

Lipid weighing about 300-500 mg was taken in a round bottom flask, and was added with 6 ml of methanolic NaOH and boiling chip. The condenser was attached and refluxed under nitrogen (10 min) until fat globules disappeared. To this, 6 ml of Borone trifluoride (BF₃) solution was added through condenser and continued boiling for 2 minutes. Heat was removed and 15 ml saturated NaCl solution is added. Stoppered flask was shaken vigorously for 15 sec while solution was still tepid. Aqueous phase was transferred to 250 ml separating funnel and extracted with two 30 ml portions of petroleum ether. The combined extracts were washed with 20 ml portion of water, dried over anhydrous sodium sulphate, filtered and solvent evaporated. The content was made up to 1 ml with PE and separated by gas liquid chromatography.

Table 2: Gas Liquid Chromatography Characteristics

<table>
<thead>
<tr>
<th>Type and Dimensions of Column</th>
<th>10% OV275 on Chromosorb HP (30m long and 0.54mm diameter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Flame Ionization Detector</td>
</tr>
<tr>
<td>Injector temperature</td>
<td>260°C</td>
</tr>
<tr>
<td>Detector temperature:</td>
<td>275°C</td>
</tr>
<tr>
<td>Column temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Flow rate of Carrier Gas</td>
<td>0.8 ml/min</td>
</tr>
</tbody>
</table>
2.4.5 Presentation of Measures

Individual fattyacids were expressed in mg/g tissue. This is a measure of absolute yield and is relevant for the viability of a nutritional industry. Additionally, the same values were expressed in percentage of total FA/PUFA and compared. This is a relative yield and is an indication of the quality of the product. It is emphasized that both measures are complimentary to each other and serve different purposes. Specific focus has been given to important fattyacids in fish oil and aggregates like Saturated Fattyacids (SFA), Mono-unsaturated Fattyacids (MUFA) and Poly-unsaturated Fattyacids (PUFA).

2.3 Results

Table 3: Sampling Details of *S. longiceps* and *S. fimbriata*

<table>
<thead>
<tr>
<th>Season</th>
<th>Notes on Catches and Sampling</th>
<th><em>S. longiceps</em> (length in mm)</th>
<th><em>S. fimbriata</em> (length in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>September</td>
<td>Mostly Running (6) or Partially spent ones (7a) in the catch. Rarely Immatures (1). Selected samples were in stage 6.</td>
<td>178.8±5.11</td>
<td>142.6±3.91</td>
</tr>
<tr>
<td>December</td>
<td>Mostly immatures (1) and rarely Spent (7b) or Spent Resting (2b). Selected samples were in stage 1.</td>
<td>131.4±3.13</td>
<td>105.2±5.71</td>
</tr>
<tr>
<td>March</td>
<td>Mostly immatures (1) and developing virgin (2a) stages. Selected samples had both 1 and 2a stages</td>
<td>138.6±5.85</td>
<td>111.2±10.63</td>
</tr>
<tr>
<td>June</td>
<td>Mostly maturing (4) and sometimes mature (5). Selected samples were in stage 4</td>
<td>165.6±7.3</td>
<td>129.2±5.54</td>
</tr>
</tbody>
</table>
Table 3 provides all details on the sampling of both species, the maturity period of the fishes used for experiments and their lengths expressed as a mean and SD.

Total FA content in mg/g meat for both species across seasons is illustrated in Figure 3. *S. longiceps* has a higher concentration of FA across all four seasons. The statement is also valid for all the three variants of FA. FA concentration is highest during December (165.71 mg/g and 90.38 mg/g) and lowest during June and September for both species.

In both species, PUFA dominate the profile followed by SFA and MUFA (Figure 4 & 5). It is also clear that the concentration of PUFA peaks during the winter (December-March) and falls during the spawning (June-September). However, MUFA has a complementary increase during this period.

![Fatty Acid Profile](image)

*Fig. 3: Seasonal variation in fatty acid profile of *S. longiceps* and *S. fimbriata*
The seasonal trend of seven dominant Fattyacids in these sardines are shown (Table 4).

### Table 4: Variation of Important Fattyacids in *S. longiceps* and *S. fimbriata* across seasons

<table>
<thead>
<tr>
<th>FA</th>
<th>FA Name</th>
<th>In percentage of total fatty acids</th>
<th><em>Sardina longiceps</em></th>
<th><em>Sardinella fimbriata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sept</td>
<td>Dec</td>
<td>Mar</td>
</tr>
<tr>
<td>C14</td>
<td>Tetradecanoic Acid</td>
<td>10.93</td>
<td>12.33</td>
<td>7.68</td>
</tr>
<tr>
<td>C16:1</td>
<td>Palmitoleic Acid</td>
<td>7.40</td>
<td>2.41</td>
<td>8.30</td>
</tr>
<tr>
<td>C18</td>
<td>Stearic Acid</td>
<td>7.85</td>
<td>5.31</td>
<td>6.19</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>Oleic Acid</td>
<td>18.30</td>
<td>14.18</td>
<td>8.99</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>Docosahexaenoic Acid</td>
<td>6.86</td>
<td>14.88</td>
<td>18.42</td>
</tr>
<tr>
<td>Σ SFA</td>
<td></td>
<td>38.33</td>
<td>35.43</td>
<td>36.35</td>
</tr>
<tr>
<td>Σ MUFA</td>
<td></td>
<td>25.91</td>
<td>16.75</td>
<td>17.79</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td></td>
<td>33.77</td>
<td>46.35</td>
<td>44.08</td>
</tr>
<tr>
<td>% EPA in PUFA</td>
<td></td>
<td>66.31</td>
<td>49.59</td>
<td>45.29</td>
</tr>
<tr>
<td>% DHA in PUFA</td>
<td></td>
<td>20.32</td>
<td>32.10</td>
<td>41.78</td>
</tr>
</tbody>
</table>

Palmitic Acid (C:16) is the dominant SFA in both species followed by Tetradecanoic Acid (C14) and Stearic Acid (C18) respectively. Among MUFA, Oleic Acid (C18:1n-9) seems to be the most dominant followed by Palmitoleic Acid (C16:1) while among PUFA, EPA was the dominant FA in *S. longiceps* while DHA showed dominance in the FA of *S. fimbriata* and there was a noticeable trend across seasons in both these PUFA.

Though December happens to be the month with maximum yield for both the PUFA in the Sardines, the relative concentrations of EPA and DHA in terms of PUFA seems to vary much across seasons (Fig 6 & 7). Months of June and September seems to have a higher concentration of EPA and
lower concentrations of DHA in *S. longiceps*. Similarly during the same months, the concentration of DHA is higher in *S. fimbriata*.

![Seasonal variation of different types of fatty acids present in Sardinella longiceps](image1)

**Fig. 4:** Seasonal variation of different types of fatty acids present in *Sardinella longiceps*

![Seasonal variation of different types of fatty acids present in Sardinella fimbriata](image2)

**Fig. 5:** Seasonal variation of different types of fatty acids present in *Sardinella fimbriata*
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**Sardinella longiceps - EPA-DHA**

![Graph showing variations in EPA and DHA for Sardinella longiceps](image)

**Fig. 6: Variations in EPA and DHA in *Sardinella longiceps***

**Sardinella fimbriata - EPA-DHA**

![Graph showing variations in EPA and DHA for Sardinella fimbriata](image)

**Fig. 7: Variations in EPA and DHA in *Sardinella fimbriata***
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Fig. 8: Variation in EPA concentration in *S. longiceps* and *S. fimbriata*

Fig. 9: Variation in DHA concentrations in *S. longiceps* and *S. fimbriata*
Hence, in both species, the DHA-EPA concentrations are complementary – when DHA values are less, EPA values are more and vice versa. Among the species, EPA values and DHA values are themselves complementary – when EPA values of *S. longiceps* are high, it is low for *S. fimbriata* and vice versa; the same holds good for DHA too (Figure 8 & 9).

Bray-Crutis analysis of similarity indices (Zar 1984) for both species clearly indicates two clusters in terms of fatty acid variations (Figure 10 & 11). Fatty acid composition of June and September months seems to be more similar and divergent from the months of March and December.
Fig. 11: Bray-Curtis similarity index for FA from *Sardinella fimbriata*

### 2.4 Discussion

Total FA content in mg/g meat for both species are highest during December-March and lowest in June-September. June to September happens to be the spawning period for both species (Hornell and Nayudu 1924, Chidambara and Venkataraman, 1946). This could be attributed to the result of fat mobilization associated with gametogenesis (Hady and Keay 1972). During winter, the sea water temperature falls to 25-26 °C as compared to 30-31°C and hence FA concentration in fishes like sardines increase to sustain the lowered temperature. This has also been observed in several prior studies on Sardines elsewhere (Gamez-Mesa *et al.* 1999, Bandarra *et al.* 1997, Shirai *et al.* 2001) including one on *S. longiceps* in the tropic coasts (Gopakumar 1965). A secondary reason could be the dominance of juveniles and immatures in the catch during winter – these individuals are heavy feeders and accumulate large quantities of fat during
winter. The high concentrations of FA and particularly PUFA in the winter are an interesting nutrition aspect as the PUFA intake per sardine goes up significantly. The same is also important from an industrial production perspective as their yield would be significantly better during winter.

In both species, PUFA dominate the profile and peaks during winter and falls during spawning while MUFA has a complementary increase during the spawning. This inverse relation has also been noted in prior studies in a variety of fishes in the west coast (Reena et al. 1997), however its biochemical significance is unknown.

Palmitic Acid (C:16), the most dominant SFA in both species, has no noticeable trend across seasons and this seems to be true for other species of sardines too (Bandarra et al. 1997). This compound is also hypothesized as not influenced by the diet (Ackman 1964, Ackman 1966). Among MUFA, Oleic Acid (C18:1n-9) seems to be the most dominant, and this is in accordance with findings by Ackman (1982) who pointed out that the main MUFA detected in marine lipids usually contained 18 carbon atoms. The difference in the EPA and DHA content between S. longiceps and S. fimbriata of these two species of sardines is also present in the values tabulated in prior studies (Reena et.al. 1997) though its relevance was not highlighted then.

The variation in the EPA and DHA amount in both species can be explained by the maturity stage prevalent in the catch during the season and its dietary patterns. The dietary patterns of the catches were confirmed in current study by analysis of gut contents of these fishes prior to the experiments. Months of June and September are characterized by the
presence of adults in the catch during the spawning season of *S. longiceps*. Adult *S. longiceps* are exclusive phytoplankton feeders (Nair 1953) and hence have an EPA-rich diet. However, immatures found abundantly during the winter are carnivorous with varying amounts of zooplankton entering into its diet. The gill-rakers of the immatures are either imperfectly developed or under developed and their carnivorous tendency is actually an indirect selection for the large sized items by their inefficient filtering mechanism while the predominantly phytoplanktonic diet of the adult is due to their efficient sieving of the minute organisms (Bensam 1964). Hence, it is the presence of zooplankton in the diet of immatures that increases the DHA content during winter. The situation is directly the opposite for *S. fimbriata*. Adults found during June-September are exclusive zooplankton feeders (Chacko 1956) and hence PUFA concentrations show high DHA content during this period. However, the immatures found during December-March intake varying amount of phytoplankton also (Basheeruddin and Nayar 1961) and hence EPA reaches higher levels for this species in winter. Hence, in both species, the DHA-EPA concentrations are complementary – when DHA values are less, EPA values are more and vice versa.

The variation in DHA-EPA composition in these two *Sardinella* species is a classical case of trophic upgradation in the seas. Interestingly, this has a large influence on the nutritional aspects of dietary fish intake in humans - food products based on *S. fimbriata* would enhance DHA intake in its consumers. Zooplanktons, the main food source of *S. fimbriata*, feed on microplanktons – and the microplanktons in turn feed on phytoplankton which also happens to be the chief food of *S. longiceps*. However, these
microplanktons, also known as heterotrophic protists, are known to trophically improve poor algal quality for subsequent use by higher trophic organisms (Klein Breteler et al. 1999). As an intermediate prey, they improve the quality and quantities of the types of fatty acids in the food web there by forming fatty acids like DHA which have higher levels of unsaturation (Kleppel et al. 1998; Klein Breteler et al. 1999). Thus, higher concentrations of DHA in zooplankton are a consequence of this preferential assimilation in planktonic food webs by the microplanktons. Hence, it has to be realized that the fisheries of the zooplankton feeding *S. fimbriata* are of great importance in food and nutrition.

The result that EPA and DHA values are complementary among the species is particularly interesting from the perspective of a pharmaceutical industry as the result has direct implication on the quality of the fatty acids extracts. For EPA rich drugs, the industry has to base its operations on *S. longiceps* while *S. fimbriata* can be a source for DHA rich drugs. Across seasons, the quality of the PUFA extract also varies and hence additional processes need to be put in place to maintain a steady concentration of EPA or DHA, as the case may be.

Bray-Crutis analysis clearly indicated two clusters in terms of fatty acid profile - one cluster during the spawning period (June-September) dominated by mature individuals and another during the winter period (December-March) dominated by immature individuals. Despite variations in the yield, the fact that the fatty acid profile across season shows a high degree of similarity (> 50%) is encouraging as it provides a minimum
assurance on the levels of various individual fattyacids present in the extract throughout the year.

2.5 Conclusion

In summary, the two dominant sardines, *S. longiceps* and *S. fimbriata* in the Cochin coast represent an excellent source of essential fattyacids like EPA and DHA. Their PUFA profiles are complementary and suite the pharmaceutical and food processing industries to harness this resource and generate specialized drugs or PUFA-enriched food products. Though there is a strong seasonal variation in their FA profile, mainly between the spawning season and winter season, this knowledge about the variations in its key constituents across seasons will greatly help in the design of such an industry. Reasons for the seasonal fluctuations in FA profile are attributed to their respective food habits, lifecycle stages of these fishes and the temperature of the sea water.