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Ontogenetic changes in the diet of L. forbesi: insights from fatty acid and stable isotope analysis.

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The diet of *L. forbesi* in Scottish waters was subject to ontogenetic changes as shown by fatty acid and stable isotope analysis. Crustaceans were more frequently found in stomachs of immature squid smaller than 150 mm mantle length. With increasing size (> 150 mm) and maturity fish became more prominent in the diet. Seasonal differences in the diet were also found but seemed to be linked to seasonal changes in squid size. Prey type and species composition also varied slightly between different regions. Differences between size and region were reflected in fatty acid profiles of mainly the digestive gland tissue. High levels of the saturated fatty acid 16:0 and the polyunsaturated fatty acids 20:5n-3 and 22:6n-3, which are typical for fatty acid signatures of many crustacean species, were found in tissues of smaller immature squid. Higher levels of the monounsaturated fatty acids 16:1n-7, 20:1* and polyunsaturated fatty acids of the linoleic family (C18), which are typical for fish prey species, were found in squid of larger sizes and higher maturity stages. Regional differences found for fatty acid profiles of the digestive gland suggested a significant difference in origin of fatty acids thus indicating different diets. In comparing the fatty acid profiles of squid to those of putative prey species of *L. forbesi*, it was apparent that gadid species *Trisopterus minutus, Micromesistius poutassou* and *Gadus morhua*, were an important component of the diet of *L. forbesi*. With increasing size of the predator however the composition of fish species in the diet shifted more towards *Gadiculus argenteus, Trachurus trachurus* and *Sebastes marinus*. Results of quantitative fatty acid analysis on the estimate of the contribution of each prey species to the diet also reflected this shift in the importance of different prey species with increasing predator size.

Due to slower turnover rates in muscle, changes in carbon and nitrogen stable isotope ratios with diet were more pronounced in this tissue. Smaller squid showed the lowest $\delta^{15}N$ ratios thus feeding on the lowest trophic level of all squid examined. $\delta\ ^{13}C$ ratios were the most depleted for small

squid indicating that small squid feed on prey closer to the carbon source in the food chain. Stable isotope analysis also showed that squid with fish remains in their stomachs showed higher ratios of nitrogen and less depleted carbon ratios than squid feeding on crustaceans. Comparisons of isotope levels of squid and putative prey species identified blue whiting and silvery pout as putative prey species of bigger sized squid. Small squid seemed to feed on the same trophic level and all other prey species examined were always similar or higher in isotope ratios than squid of any size.

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Introduction

Although numerous feeding studies exist, the importance of many cephalopod species as predators is still not well known, in part reflecting problems in data collection and partly reflecting bias introduced through methodology.

Much of our existing knowledge on the feeding of squid is based on information gained through stomach content analysis and to a lesser extent observation in the field and the laboratory (Rodhouse & Nigmatullin, 1996). Through the application of conventional stomach content analysis it was possible to determine general feeding strategies for many squid species. It was found that neritic as well as pelagic squid species feed on a mixture of fish, crustacean and cephalopod species in varying proportions throughout their life cycle (e.g. Breiby & Jobling, 1985, Castro & Guerra, 1990, Rasero et *al.*, 1996). Many species showed ontogenetic shifts in their diets with juveniles feeding predominantly on crustacean prey with an increase in fish and cephalopod prey occurring with increasing squid size (e.g. Breiby & Jobling, 1985, Pierce et *al.*, 1994a). Furthermore daily feeding migrations have been reported for neritic squid species with squid feeding on the bottom during the day and in the water column and near the surface during the night (e.g. Tanaka, 1993, Sánchez et *al.*, 1996, Lapthikovsky, 2002). Prey species found in stomach contents could often be identified and tentative pictures of trophic relationships could be drawn. However there are several problems associated with these kinds of studies.

Due to morphological restrictions cephalopods are not able to swallow big lumps of prey, but have to macerate prey during ingestion. When eating relatively large fish prey this may lead to the rejection of the head (Porteiro et *al.*, 1990) and consequently the lack of identifiable hard parts such as otoliths and jaw bones. The lack of hard parts may consequently lead to underestimating certain prey in the diet (Nixon, 1987). Also rapid digestion rates (Bidder, 1966) will influence what is left in stomach contents and bias will result from the moment in time the animal has been caught. Indeed in many studies high proportions of stomachs are empty and therefore won't contribute to any dietary information. In cases where stomach remains are found identification requires the researcher to have a good taxonomic knowledge of organisms found in the system and sufficient reference material, which might not always be accessible.

In cases where animals are caught by trawl further bias might be introduced by net-feeding and stomach contents analysed might not reflect the animals natural diet (Breiby & Jobling, 1985). There is also the danger of secondarily ingested prey being considered as consumed directly and therefore overestimated in prey species composition. Most importantly however results obtained from stomach content analysis will only ever reflect single feeding events thus giving an indication of the prey spectrum and feeding behaviour but do not provide information about assimilation of food and long-term dietary trends.

These limitations have led to the development of more sophisticated methods, which do not only support results from conventional stomach content analysis but also have the further potential of being applied for quantitative analysis. These methods involve the use of fatty acid profiles and stable isotope signatures for the reconstruction of diets in the field.

The use of fatty acids as biomarkers is based on the assumption that many fatty acids in the marine environment, particularly polyunsaturated fatty acids can only be biosynthesized by certain phytoplankton and macroalgae species and become essential dietary components to higher trophic levels (Sargent et *al.*, 1976). It is also generally accepted that animals have only a limited capacity to desaturate or chain-elongate fatty acids and therefore many fatty acids are stored relatively unchanged in the predators tissue and can be useful as dietary tracers in food web analysis (Dunstan et *al.*, 1996).

The use of fatty acids as biomarkers does have the further potential to provide information as to which habitat contributes the majority of prey for an organism. Large concentrations of polyunsaturate (PUFA) 20:5n-3 for example are typically found in diatom-based food webs whereas high proportions of PUFA 22:6n-3 mostly derive from food chains based on a predominance of dinoflagellates in the phytoplankton. On the other hand high proportions of 20:4n-6 are indicative of benthic food webs as they are primarily produced by benthic algae and C18 PUFA and monounsaturates of the C20 and C22 family are typically enriched in copepod based food webs (Sargent & Whittle, 1981). The influence of freshwater and marine habitats on the other hand can be distinguished through elevated levels of C16 and C18 PUFA levels freshwater organisms in

contrast to higher C20 and C22 MUFA found in marine organisms (Ackman, 1967). Feeding studies in fish, molluscs and marine mammals, that used fatty acids as dietary tracers, have clearly shown that fatty acids of the prey will be reflected in the predators tissue and can be a viable tool for the reconstruction of diets in the field (e.g. Olsen et *al.*, 1991, Kirsch et *al.*, 1998, Navarro et *al.*, 2000).

The use of stable isotopes as dietary tracers is based on the principle that isotopic concentrations of consumer diets can be related to those of consumer tissues in a predictable fashion (Hobson & Clark, 1992). It has been extensively applied in the investigation of trophic relationships in various marine ecosystems (e.g. Rau et *al.*, 1983, Hobson & Welch, 1992, Petersen, 1999) and has been used to determine feeding migrations in cephalopods and birds (Takai, 1998, Cherel et *al.*, 2000). The small fractionation of carbon in a predator relative to its prey (1 ‰) suggests that ^{13}C in the predator will reflect the isotopic composition in the prey and that ^{13}C values can successfully be used to identify carbon pathways and sources of primary productivity (DeNiro & Epstein, 1978) whereas the stepwise enrichment of nitrogen between trophic levels (3 - 4 ‰) will indicate the trophic position of species in the food web investigated (Minagawa & Wada, 1984). The analysis of more than one stable isotope will also allow greater segregation of species than the use of a single isotope (Hobson, 1993).

The conservative transfer of carbon isotopic compositions to the animal from the diet can also be useful in tracing food webs in systems where there are food sources with large differences in ^{13}C values, such as terrestrial versus marine, benthic versus pelagic, inshore versus offshore and latitudinal differences (e.g. Schoeninger & DeNiro, 1984, DeNiro &Epstein 1978, Rau et *al.*, 1982, Fry, 1988). In effect laboratory studies have shown that organisms fed on an isotopically distinct diet will, in time, approach dietary values as the animal grows and tissues turn over (DeNiro & Epstein, 1978, Fry & Arnold, 1982, Hobson & Clark, 1992, Tominaga et *al.*, 2003) thus suggesting that the use of stable isotopes as dietary tracers is a valid approach to investigate feeding in cephalopod diets.

The application of both fatty acid and stable isotope analysis offer three main advantages over the use of stomach content analysis alone: (a) they can be applied to any animal, even if the stomach is

empty (b) they provide information on "average" diet, integrated over a period of time and (c) through the use of tissues with different metabolic rates provide information on diet integrated over different lengths of time (Hobson, 1993).

The life cycle of *L. forbesi*, has been relatively well-studied in European waters. Previous studies on the feeding of *L. forbesi* have covered various parts of its range (e.g. Ngoile, 1987; Porteiro *et al.*, 1990; Rocha *et al.*, 1994; Collins & Pierce, 1996; Pierce & Santos, 1996) and were exclusively based on stomach contents analysis.

In most locations studied, fish was found to be the main prey item with crustacean, cephalopod and polychaete species present in the diet to varying degrees. In all studies, *L. forbesi* was found to consume a wide variety of fish and crustacean species. The most prominent fish species present in the diet belonged to families Gadidae, Clupeidae, Ammodytidae and Gobiidae (e.g. Rocha *et al.*, 1994, Collins & Pierce, 1996, Pierce & Santos, 1996). In Scottish waters *Loligo forbesi* appeared to primarily feed on gadid and ammodytid species (Pierce *et al.*, 1994b). Of crustaceans found in the diet of *L. forbesi* from Scottish waters, decapod and euphausiid families were the ones identified most frequently. Cephalopod prey consisted mainly of loliginid, sepiolid and octopodid species (Pierce et al, 1994b, Collins & Pierce, 1996).

Apart from regional differences, diets showed seasonal variation (e.g. Pierce *et al.*, 1994a, Collins *et al.*, 1994) and were found to be dependent on squid size (Rocha *et al.*, 1994; Collins & Pierce, 1996). Ontogenetic shifts occurred from a crustacean dominated diet in juvenile squid to a predominance of fish in the diet of adult squid. No significant differences were found between the diets of male and female *L. forbesi* (Pierce *et al.*, 1994a; Rocha *et al.*, 1994) or animals of different maturity stages (Rocha *et al.*, 1994).

Lipid and fatty acid analysis has previously been applied to loliginid species, primarily investigating squid energy requirements and growth and their nutritional value for human consumption (e.g. de Koning, 1993, Hayashi, 1996; Kunisaki, 2000; Navarro & Villanueva, 2000). Only one study examining the lipid and fatty acid composition of Southern Ocean squid (i.e. *Sepioteuthis australis*) used fatty acid data to investigate food web interactions (Phillips *et al.*, 2002). *Loligo bleekeri* is the

only loliginid species to date for which stable isotope ratios were examined. This study however, was concerned with investigating biological and geographical differences between several cephalopod species rather than trophic interactions between cephalopods and their prey (Takai *et al.*, 1998).

The aim of this study was to use a combined approach of conventional and alternative techniques to explore possible ontogenetic and seasonal shifts in the diet of a relatively well studied cephalopod species (*Loligo forbesi*) and assess their usefulness in studies in the field.

Material & Methods

Sampling

A total of 107 specimens of *L. forbesi* were sampled from research cruises and commercial fishing vessels over a 10 months period in order to cover all four seasons. Samples of *Loligo forbesi* obtained in May 1999 (n = 39) were collected during a demersal trawling survey on board the FRV *Scotia*, operated by the FRS Marine Laboratory Aberdeen, in Scottish shelf waters (Fig. 1). Further samples of *L. forbesi* collected in May (n = 10), August (n = 20) and November 1999 (n = 20) and March 2000 (n = 20), were landed as by-catch from commercial fishing vessels landing at Kinlochbervie market (Northwest Highlands, Scotland). For samples collected in August, November and March, gear type and exact geographical location, other than approximate region of catch are unknown, although most landings in Kinlochbervie are from trawlers fishing the “Minch” (i.e. marine straight between the Scottish West coast and the Outer Hebrides, Fig.1). Samples of putative prey species (Table 1) were collected on several consecutive cruises (2000 – 2001) of the research vessel FRV *Scotia* in Scottish and Irish waters. All squid and prey samples collected during these surveys were bottom trawled with trawls lasting between 30 minutes and 1 hour.

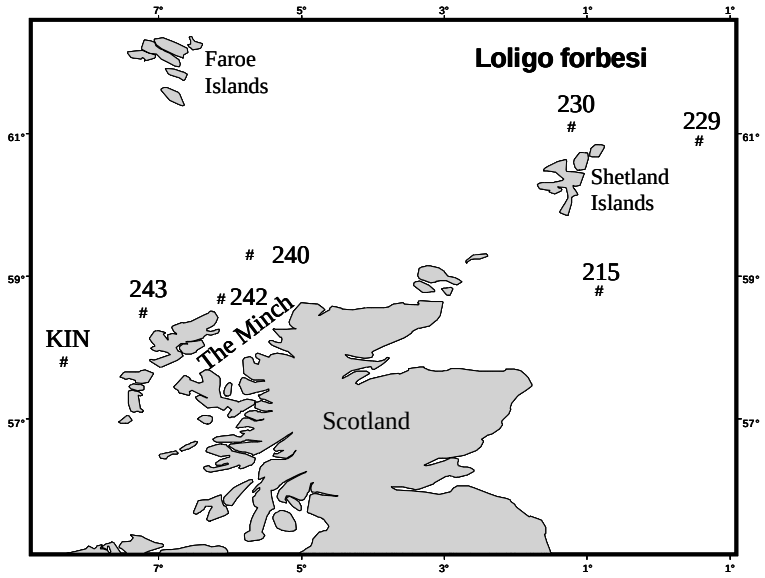


Fig.1: Location of catch of *Loligo forbesi* in May 1999. Numbers indicate hauls. KIN = market sample Kinlochbervie.

Apart from commercial samples (i.e. market samples) all squid and fish were dissected immediately after capture. In squid pieces of muscle tissue were taken from the ventral side of the mantle whereas digestive gland and stomachs were sampled whole. Muscle tissue samples of fish were taken from the anterior dorsal side of the body. Crustacean prey was collected whole. Tissue samples were packed individually in polythene bags and frozen at – 20 °C. Commercial samples of *L. forbesi* were stored on ice for up to 3 days at sea prior to dissection in the laboratory. Upon dissection, squid were measured, sexed and a maturity stage was assigned. Dorsal mantle length (DML) was measured from the anterior tip of the middorsal point to the posterior body tip, measured to the nearest centimetre (Boyle & Ngoile, 1993). Size classes chosen for morphometric and dietary comparisons were selected according to frequency distribution of mantle lengths and resulting adequate sample size, resulting in eight size categories of 30 mm DML. To determine sexual maturity in *L. forbesi* a modified scale was used (Pierce & Guerra, 1994) based on Lipinski's (1979) universal maturity scale for cephalopods.

Stomach content analysis

All 107 squid specimens sampled were investigated for stomach contents. Stomach samples were defrosted at room temperature and stomach fullness assessed. The mass of stomach contents was estimated on a subjective scale adapted from Pierce et *al.* (1994) and assigned 5 different categories from empty = 0 to full = 4.

In order to prevent contamination with fish scales, cephalopod suckers or skin from dissection procedures, stomachs were rinsed on the outside prior to opening. Using scissors, stomachs were then cut open from their proximal to their distal end and contents washed in tap water over a 0.355-mm sieve. In cases where undigested flesh made the cleaning of remains difficult, stomach contents were soaked in a detergent solution (Bio-tex; Blumøller Ltd., High Wycombe) prior to washing (Pierce & Boyle, 1991; Pierce et *al.*, 1994d). Material remaining in the sieve was recovered and transferred into petri dishes. Prey remains were then examined under a binocular microscope fitted with a calibrated eyepiece graticule. All hard parts found in stomachs were stored in 70% ethanol until further analysis. Prey remains were initially sorted into major prey taxa i.e. fish, crustacea, cephalopoda and subsequently identified to the lowest taxon possible.

Identification of fish prey was based on retrieval of hard parts such as otoliths and fish bones. These were identified using reference material held at the Department of Zoology, University of Aberdeen, and published guides (Harkønen, 1986; Watt et *al.*, 1997). Other remains, including eye lenses, scales, flesh and skin, were used to confirm the presence of fish in the diet. Crustacean remains consisted of exoskeleton fragments such as mandibles, chelae, telsae and rostrae, which were identified using a published guide (McLaughlin, 1980). Cephalopod prey could be identified from beaks, eye lenses, skin fragments and radulae. Beaks were the only remains that allowed further analysis. Beak identification was achieved with reference material, published literature (Clarke, 1986) and the expertise of Dr. Maria B. Santos, University of Aberdeen. Other invertebrate remains, such as parasitic nematodes and bivalves, were noted as present but not considered to be prey and thus excluded from numerical analysis. Diet was quantified through frequency of occurrence (FO), which expresses the composition of the diet as a percentage of all stomachs containing food remains. Summarised data of food categories in some cases exceeds 100 % due to more than one food species present in the stomachs.

Table 1: Specimens of *Loligo forbesi* and prey species analysed. FA = fatty acid analysis, SI = stable isotope analysis, SC = stomach content analysis. * results courtesy to Jennifer Learmonth, University of Aberdeen.** results derive from a later study on the diet of deep sea fish carried out by the authors and funded by NERC.

Species	*Common name*	*n*	*Location (haul)*	*Season*	*Analyses*
Loligo forbesi	Veined squid	1	215	May	FA, SI, SC
		8	229	May	SI, SC
		10	230	May	FA, SI, SC
		14	240	May	FA, SI, SC
		3	242	May	FA, SC
		1	243	May	FA, SC
		10	KIN	May	FA, SC
		20	Scotland	August	SI, SC
		20	Scotland	November	SI, SC
		20	Scotland	March	SI, SC
Prey species					
Clupea harengus	Herring	10	Scotland	July	FA
Ammodytes sp.	Sandeel	10	Scotland	June	FA*
Argentina silus	Greater argentine	10	Scotland	September	FA
Gadiculus sp.	Silvery pout	6	Scotland	September	FA, SI
Gadus morhua	Cod	9	Scotland	January	FA, SI
Melanogrammus aeglefinus	Haddock	5	Scotland	January	SI
Micromesistius poutassou	Blue whiting	10	Scotland	September	FA, SI
Trisopterus minutus	Poor cod	8	Scotland	July	FA
Sebastes marinus	Redfish	8	Scotland	August	FA
Lepidorhombus whiffiagonis	Megrim	7	Scotland	January	FA, SI
Limanda limanda	Dab	4	Scotland	January	FA, SI
Trachurus trachurus	Horse mackerel	10	Irish Sea	August	FA
Meganyctiphanes sp.		10	Irish Sea	October	FA**

Lipid and fatty acid analysis

Due to storage difficulties only the samples obtained in May 1999 (n = 39) were suitable to be used for the lipid and fatty acid analysis of mantle and digestive gland tissue. Lipid was extracted from the mantle and digestive gland of 39 specimens of *L. forbesi* and from the muscle of 12 prey species (Table 1). Squid and fish tissue were homogenised prior to lipid extraction and a subsample (5 – 10g) taken for further processing.

Lipid was extracted using a chloroform-methanol-water solvent mixture (2 : 2 : 1.4 v/v/v, Bligh & Dyer, 1959, as modified by Hanson & Olley (1963) and applied according to the standard operating procedure (SOP) for lipid analysis, FRS Marine Laboratory, Aberdeen). Lipids were transesterified overnight (incubated at 50 ºC) using methanol containing sulphuric acid (1% v/v). The resulting methyl esters were extracted into iso-hexane and stored over anhydrous sodium sulphate at –20 ºC until further analysis.

Fatty acid methyl esters (FAME) were analysed by gas chromatography with flame ionisation detection (GC-FID) on a Hewlett Packard 5890 Series II gas chromatograph, fitted with a fused silica capillary column (0.25 mm i.d. x 30 m length) coated with a polar DB-23 phase (J & W Scientific Inc., California, USA) using nitrogen as the carrier gas. The detector temperature was set at 300° C. To achieve optimum separation of components the following temperature programme was used. The column temperature was ramped from 60° C initial oven temperature at 25° C min^{-1} to 150° C, then ramped at 1° C min^{-1} up to 200° C and held for 10 min, then ramped at 10° C min^{-1} to 220° C and finally held at 220° C for 5 min. Total running time was 59.60 min. On – column injection (1 μl) was by means of a Hewlett Packard 7673 automatic injector.Data were recorded by Perkin Elmer 600 Series Link Box connected to a Turbochrome Data Station and analysed using Turbochrome Navigator Software Version 6.1.

Twenty-five fatty acids were identified through reference to a standard of known composition (E023), containing the fatty acid population chosen for this assessment. Amounts present are expressed as percentages of the summed area for the 25 fatty acids (NA, normalised area percentage). Since chromatographic separation between isomers of monounsaturated fatty acids 18:1 and 20:1 was not always complete, the groups 18:1* (18:1n-9 and 18:1n-7) and 20:1* (20:1n-11 and 20:1n-9) were defined for all further analysis. A group of 17 fatty acids, which comprised the most abundant and dietary relevant fatty acids (i.e. abundant in potential prey species), was chosen for statistical analysis. In all further analyses these were classed as major and minor fatty acids, based on their level of contribution to the fatty acid profile, with minor fatty acids being < 5 % (single fatty acids).

Quantitation of data was checked by running the standard "E023" at the start of each analysis and after every 6th sample. E023 is a standard developed in an EU intercalibration study in order to assess laboratory competency in the determination of the fatty acid composition of fish oil (1988, Torry Research Station, Aberdeen). Comparability of data was tested with "Shewhart chart" quality control. In this analysis it utilised normalised area percentage of selected fatty acids (palmitic, eicosapentaenoic and docosahexaenoic acids) derived from the repeated analysis of E023.

Stable isotope analysis

Of the 20 samples initially analysed for each season and tissue (i.e. N = 80 per tissue), 61 and 70 samples for digestive gland and muscle tissue respectively yielded viable results. The discrepancy in measured values between duplicates of the remaining samples exceeded set limits (2 ‰) and these data therefore had to be discarded. Accepted samples, as distributed across seasons, are shown in Table 1.

Tissue samples of squid and putative prey species were placed into Eppendorf vials and freeze-dried (Edwards Super Modulyo freeze dryer, UK). Dried samples were powdered with a mortar and pestle and approximately 1-2 mg was loaded into a 8 x 4 mm tin capsule (Europa Scientific Ltd, Crewe, UK). All further processing of samples was carried out by Mr. Peter Tompson, under the auspices of Dr. John Speakman, Zoology Department, University of Aberdeen. Duplicate samples were combusted in a Carlo Erba Na 1500 NC Elemental Analyzer at 1800° C flash combustion temperature. Resulting gases (CO_2 and $N_{2)}$ were analysed for Carbon and Nitrogen stable isotope ratios respectively using a dual inlet mass spectrometer (VG Micromass OPTIMA).
Stable-isotope concentrations were expressed in δ notation as parts per thousand (‰) differences from a standard reference material:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$$

, where X is ^{13}C or ^{15}N, R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (for the sample or standard) and δ is the measure of the ratio of heavy to light isotope in the sample.

Values measured were raw mass spectrometry δ estimates relative to laboratory working standards and had to be adjusted to international standards. Internal working standards used were pea, maize and spelt flour. δ estimates were adjusted to international standards IAEA CH-6, NBS-19 and IAEA CO-1 (calibrated against carbon standard material Peedee belimnite (PDB)) and international standard IAEA 305 N (calibrated against atmospheric N_2 (AIR)), respectively. Analytical error for carbon was between 0.4 ‰ and 0.7 ‰ and 0.2 ‰ to 0.7

‰ for nitrogen, depending on the standard used. All carbon samples analysed produced negative values, due to samples being isotopically lighter (depleted in ^{13}C) compared to standards used. Tissue samples were reanalysed if the difference between duplicates was more than 2 ‰.

Statistical analysis

Chi square tests (χ^2) were used to investigate differences in stomach contents between groups with Yates' Correction for Continuity being applied in tests with only two categories present. Differences in the distribution of maturity stages were tested by using Mann-Whitney nonparametric tests. Correlations between maturity and size were tested by applying the Spearman-Rank correlation coefficient. A group of 17 fatty acids, which comprised the most abundant and dietary relevant fatty acids (i.e. abundant in potential prey species), was chosen for statistical analysis. Analyses were performed using 14:0, 15:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, 20:1n-11, 20:1n-9, 20:4n-6, 20:5n-3, 22:1n-11, 22:5n3 and 22:6n-3.

One-way ANOVA on arcsine-transformed fatty acid data was applied to test for variations in selected fatty acids in relation to stomach contents. To examine differences in fatty acid signatures with stomach content and between squid species, principal component analysis (PCA) on arcsine-tranformed fatty acid data was used. Statistical results are presented for selected fatty acids only. Two-way multivariate analysis of variance (MANOVA) on arcsine-transformed stable isotope data was applied to test for intra- and interspecific differences. All statistical analyses was carried out using MINITAB 12.23 for Windows (Minitab Inc., State College, Pennsylvania).

Quantification model for fatty acid analysis (QFASA)

In order to quantitatively determine the importance of selected prey species in the diet of a given predator a model for quantitative fatty acid signature analysis (QFASA) was developed by Dr. Graham Pierce (University of Aberdeen).

The model uses fatty acid signatures of predator and prey to quantitatively estimate the contribution of certain prey species to predator tissue composition. A subset of identified fatty acids was used that comprised of fatty acids that were of either dietary relevance to the predator or indicative of differences between prey species analysed (n = 18). Data on prey species were summarised and expressed by their mean fatty acid signatures.

The model calculates the difference between the observed proportion of each fatty acid in the predator from an estimated proportion of same fatty acid in the diet, the latter being based on some hypothetical combination of different prey species, and uses these distances to identify the most likely diet composition. The procedure is repeated for multiple hypothetical diets each comprising different combinations of proportions of the various prey species. Iverson *et al.* (2004) describes such a model, referring to its application as "quantitative fatty acid signature analysis" (QFASA). The present model was coded in BASIC by G.J. Pierce.

Following the notation of Iverson et *al.*, (2004), the goodness of fit (GOF) measure is based on the sum of the squared differences:: $\sum_j = (y_{ij} - \hat{y}_{ij})^2$

, where y_{ij} defines the proportion of the jth fatty acid of the ith predator and $\hat{y}_{ij}$ defines the mean proportion of the jth fatty acid of the ith prey species.

The number of comparisons of randomly selected proportions of different prey species was set at 10^4. Results derived from this analysis are expressed as best fit scenarios, i.e. those diets with the smallest GOF value.

Results

Population Indices

More than one size mode and maturity stage was present in most seasons. In accordance with breeding seasons found for *L. forbesi* in Scottish waters (e.g. Pierce & Boyle, 2003), body size as well as maturity increased in autumn and winter for both sexes. The small immature specimens caught during spring are indicative of recruitment in the months leading up to summer (e.g. Collins et al. 1999). For each of the seasons the number of females caught exceeded that of males (Table 2). The males were generally larger than the females with dorsal mantle lengths (DML) ranging from 100 to 520 mm for males and from 110 to 290 mm for female squid. However, only within the summer sample were male squid significantly larger than females ($p < 0.01$).

Table 2: Specimens of *L. forbesi* grouped according to sex, maturity stage and average size for each quarter of the year. DML = dorsal mantle length, SD = standard deviation. [a] Index defined by Lipinski (1979).

	Males			*Females*		
Season	n	DML ± SD (mm)	Maturity [a]	n	DML ± SD (mm)	Maturity [a]
Spring (05/99)	20	146 ± 9	range 1 – 2 median 1	27	134 ± 20	range 1 – 2 median 2
Summer (08/99)	9	232 ± 22	2	11	182 ± 34	2
Autumn (11/99)	7	287 ± 50	range 1 – 5 median 5	13	229 ± 124	range 1 – 5 median 3
Winter (03/00)	7	246 ± 20	range 2 – 5 median 5	13	201 ± 93	5

Both male and female squid varied significantly in size between seasons (female, $p < 0.01$ and male, $p < 0.01$). Specimens of both sexes were smallest in spring and reached peak in size in autumn. Maturity in both sexes was most advanced in autumn and winter. A moderate correlation existed between maturity and size in both male and female *L. forbesi* (male $r = 0.632$, $p < 0.01$, female $r = 0.668$, $p < 0.01$).

Diet composition

The diet was significantly different between *L. forbesi* smaller than 150 mm and *L. forbesi* larger than 150 mm ($p < 0.01$), with crustacean remains occurring more frequently in stomach contents of the two smallest size classes (DML 91 – 120 and 121 – 150 mm, table 3a). The occurrence of fish in the diet increased with increasing mantle length. The same trend applied to maturity stages, with lower maturity stages showing crustacean prey in their stomach contents more frequently than higher maturity stages, although differences were not significant (table 3b).

Related to the ontogenetic shift in diet of this species, the prey type composition was significantly different between seasons ($p < 0.01$). There was a clear seasonal trend with crustacean and cephalopod remains present only in spring and summer and fish replacing all other prey in autumn and winter (table 3 c). A combined analysis on the influence of season and size on the diet (i.e. stomach contents) showed no significant effect for either of the independent variables, suggesting

that these effects could not be separated from each other (ANOVA, general linear model, $p > 0.05$ for all factors).

Table 3 a,b,c: Diet of *L. forbesi* from Scottish waters. Shown are the number of empty and non-empty stomachs examined in each (a) size class, (b) maturity stage and (c) season, also the percentages of non-empty stomachs containing the following prey types: fish (F), crustaceans (Cr), cephalopods (C) and mixtures thereof (F+Cr). DML = dorsal mantle length.

(3a) DML (mm)	n		Percentage of non-empty stomachs containing			
	empty	non-empty	F	Cr	F+Cr	C
91 - 120	6	3	66.7	33.3	33.3	33.3
121 – 150	13	22	100.0	72.7	68.2	0.0
151 – 180	12	6	83.3	0.0	0.0	16.7
181 – 210	8	13	100.0	15.4	15.4	0.0
211 – 240	4	4	100.0	0.0	0.0	0.0
241 – 270	2	2	100.0	0.0	0.0	0.0
271 – 300	2	3	100.0	0.0	0.0	0.0
> 300	2	5	100.0	0.0	0.0	0.0
Total	49	58	94.8	32.8	31.0	3.4

(3b)Maturity stage	n		Percentage of non-empty stomachs containing			
	empty	non-empty	F	Cr	F+Cr	C
1	15	14	92.9	57.1	0.0	50.0
2	18	27	92.6	40.7	7.4	40.7
3	1	4	100.0	0.0	0.0	0.0
4	1	1	100.0	0.0	0.0	0.0
5	14	12	100.0	0.0	0.0	0.0
Total	49	58	94.8	32.8	3.4	31.0

(3c) Season	n		Percentage of non-empty stomachs containing			
	empty	non-empty	F	Cr	F+Cr	C
Spring (05/99)	17	30	93.3	60.0	56.7	3.3
Summer (08/99)	12	8	87.5	12.5	12.5	12.5
Autumn (11/99)	7	13	100.0	0.0	0.0	0.0
Winter (03/00)	13	7	100.0	0.0	0.0	0.0
Total	49	58	94.8	32.8	31.0	3.4

Of the 107 stomachs analysed, 46 % (n = 49) were found to be empty. Of the stomachs containing prey remains (n = 58), the majority (83 %; n = 48) contained only small traces of food (stomach fullness 1, Chapter 2). Only ten specimens were recorded with stomachs half or three quarters full.

Empty stomachs occurred less frequently in spring and autumn, although differences to the remaining seasons turned out not to be significant. No significant relationship was found between stomach emptiness and sex, maturity or size class.
Of animals with food in their stomach, 69 % (n = 40) contained a single prey type. Except for three stomachs in which only crustacean or cephalopod remains were found, the term “single prey” is indicative of teleost remains. The remaining “non-empty” stomachs (n = 18) contained multiple prey items, which were primarily found in squid of smaller sizes and lower maturity stages (Table 3 a,b). Because more than one prey category was present, in these cases the sum of the percent occurrences exceeds 100 %. The majority of prey remains were not identifiable to species or genus but only to family. Prey identified for samples of *L. forbesi* are listed in Table 4.

The most frequently identified fish were gadoid species such as *Trisopterus* sp. and silvery pout (*Gadiculus argenteus*), and gobiid species such as transparent goby (*Aphia minuta*). Many samples contained numerous very small prey remains. Identification of these remains was impeded by their less distinctive and more fragile structures. Therefore fish prey found in 52 % (n = 30) of non-empty stomachs remained unidentified.

Based on mandible and telson morphology, three families of crustaceans could be identified. Most crustacean prey belonged to decapod or euphausiid species and one copepod of the order Calanoida was found. Many remains though were too fragmented to allow identification other than “crustacean”.

Cephalopod prey were identified through the presence of suckers and skin in stomach remains but, since no beaks were found, no further identification was possible. Insect remains were found in one stomach but, due to the small number, these were excluded from the statistical analysis. Significant seasonal differences were observed in the occurrence of prey species ($p < 0.05$). Gadid species were more important in the diet during winter and spring and were less frequently found in stomachs collected in summer and autumn. Gobids and clupeids were found only in stomachs collected in spring and autumn respectively. Crustacean prey seemed to be restricted to spring and summer months, with decapods only found in spring. Prey species variation seemed to be highest in spring.

Owing to the difficulty in identifying prey remains, the importance of certain prey species might have been under- or overestimated.

Table 4: Prey of *L. forbesi* from Scottish waters Shown are frequencies of occurrence (%) of prey in non-empty stomachs per season.

Prey	**Spring**	**Summer**	**Autumn**	**Winter**
	n = 30	n =8	n = 13	n = 7
	Frequency			
Family Clupeidae				
Clupea harengus			15.4	
Family Gadidae				
Trisopterus spp.	10.0		7.7	
Gadiculus argenteus			15.4	
Unidentified Gadidae	40.0	12.5		42.9
Family Gobiidae				
Aphia minuta	10.0			
Unidentified Gobiidae	33.3			
Flatfish	10.0			
Unidentified fish	40.0	75.0	61.5	57.1
Total Fish	93.3	87.5	100.0	100.0
Class Copepoda				
Order Calanoida	3.3			
Order Euphausiacea	3.3	12.5		
Order Decapoda				
Pandalidae	3.3			
Unidentified Decapoda	3.3			
Unidentified Crustacea	46.7			
Total Crustacea	60.0	12.5		
Total Cephalopoda	3.3	12.5		
Nematoda	3.3		15.4	28.6
Insecta	3.3			

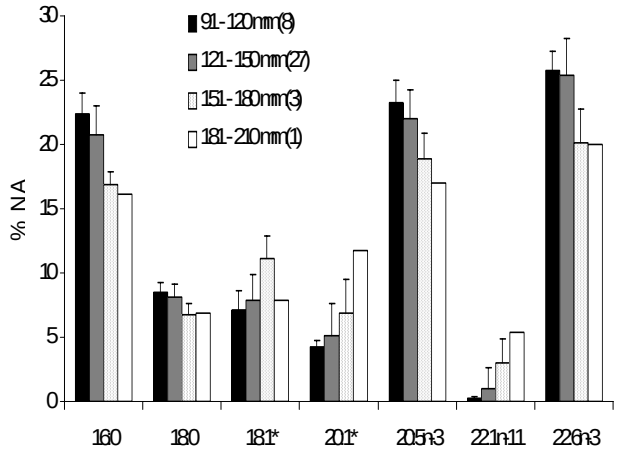
Squid fatty acid profiles

There was significant variation in the majority of fatty acids in digestive gland samples (18:0, 18:1*, 18:3n-3 and 20:1* $p < 0.05$, all other FA $p < 0.01$) between size classes (Fig. 3a,b). Squid of

mantle length ≤ 150 tended to show higher amounts of saturated fatty acids 16:0, 18:0 and PUFAs 20:5n-3 and 22:6n-3 in digestive gland tissue. In contrast bigger animals (> 150 mm) showed significantly higher amounts of saturated 14:0, all monounsaturates, PUFAs of the linoleic family (C18) and PUFA 22:5n-3.

Comparing fatty acid signatures of squid by maturity stage, statistical analysis showed that digestive gland tissue of less mature squid (maturity stage 1) showed lower proportions of saturated fatty acids 14:0, 16:0, monounsaturates 16:1n-7, 20:1*, 22:1n-11 and PUFAs of the linoleic family as well as PUFA 22:5n-3 (Fig 4 a,b).

3a.)



3b.)

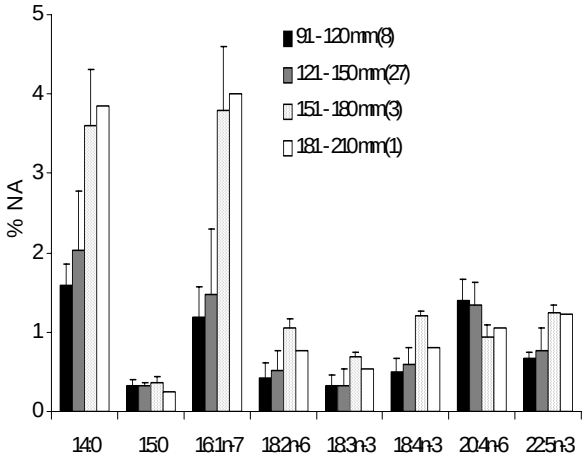


Figure 3 a, b.: Normalised area percentage (% NA) of a) major and b) minor fatty acids (< 5 %) in digestive gland tissue of *L. forbesi*. Shown are means per size class (dorsal mantle length, mm) + standard deviation. Legend: numbers in parenthesis indicate number of squid per group.

4a.) 4b.)

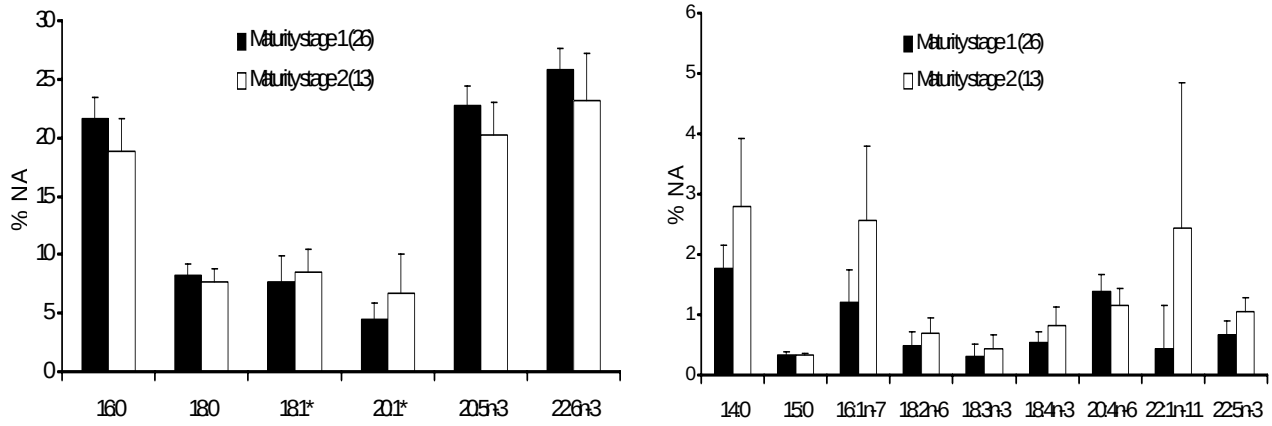


Figure 4 a, b.: Normalised area percentages (NA%) of a) major and b) minor abundant fatty acids (< 5 %) in digestive gland tissue of *L. forbesi*. Shown are means per maturity stage (1 and 2) + standard deviation. Legend: numbers in parentheses indicate numbers of squid per group.

As found for smaller sized animals, squid of lower maturity stages were characterised by high proportions of PUFAs 20:5n-3 and 22:6n-3 in their digestive gland tissue. In muscle samples, only saturated fatty acid 18:0 showed a significant variation in proportion, with bigger ($p < 0.05$) and more mature animals storing higher concentrations in their tissue. In muscle tissue squid of maturity stage 1 showed significantly lower amounts of saturated fatty acid 18:0 ($p < 0.01$).

Fatty acid profiles of putative prey species

All fatty acids were significantly different between prey species ($p < 0.01$ for all FAs). The most prominent differences were found for fatty acids 14:0, 18:1*, 20:1*, 18:4n-3, 20:5n-3 and 22:6 n-3 (Table 7).

Clupea harengus and *Ammodytes* sp. showed comparatively high levels of saturated fatty acid 14:0 monounsaturates 20:1* and 22:1n-11 and PUFAs of the linolenic family. Proportions of saturated fatty acid 16:0 and PUFAs 20:5n-3 and 22:6n-3 were lower than in any of the other species

examined. *Argentina silus* and *T. trachurus* were close in fatty acid signature and mainly separated by greatly differing proportions of monounsaturates 18:1*, 20:1* and 22:1n-11 and PUFA 22:6n-3.

Gadid and flatfish fish showed similar fatty acid signatures and were separated from other fish species mainly by their low levels of saturated 14:0 and monounsaturates 20:1* and 22:1n-11. The main difference between flatfish and gadid species was based on significantly higher levels in PUFAs 20:4n-6, 20.5n-3 and 22:5n-3 found in flatfish and higher proportions of PUFA 22:6n-3 found in gadids. Variation in fatty acid profiles within Gadiformes and flatfish were mainly due to differences in minor fatty acids (with % NA < 5). *Gadiculus* sp. was significantly different to all other Gadiformes and showed fatty acid profiles rather similar to those of *S. marinus*. Both species shared large proportions of 20:1* and 22:1n-11 and relatively low proportions of highly unsaturated fatty acids. Fatty acid signatures of the crustacean *Meganyctiphanes* sp. were characterised by comparatively high proportions of PUFAs of the linoleic family (C18), PUFAs 20:5n-3 and 22:6n-3, very low levels of saturates, and low levels of monounsaturates (except 18:1*).

Table 7: Fatty acid profiles of putative prey species. Shown are the most abundant and dietary relevant fatty acids (expressed in normal area percentage) of each species ± standard deviation.

	C. harengus n = 10	*Ammodytes sp.* n = 10	*A. silus* n = 10	*Gadiculus sp.* n = 6	*G. morhua* n = 9	*M. poutassou* n = 10	*T. minutus* n = 8
Fatty acid							
14:0	8.2 ± 0.9	6.3 ± 0.6	4.9 ± 0.8	3.5 ± 0.8	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
15:0	0.5 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
16:0	13.5 ± 1.1	13.8 ± 1.2	17.9 ± 0.8	14.4 ± 1.5	18.4 ± 0.6	18.6 ± 1.2	19.6 ± 0.4
16:1n-7	4.3 ± 0.6	5.2 ± 1.0	2.4 ± 1.1	2.8 ± 0.5	1.3 ± 0.2	1.1 ± 0.1	1.2 ± 0.1
18:0	1.1 ± 0.2	1.6 ± 0.1	4.8 ± 0.4	3.1 ± 0.3	4.2 ± 0.3	4.4 ± 0.3	4.8 ± 0.1
18:1*	9.0 ± 1.6	6.7 ± 0.8	24.9 ± 4.1	9.7 ± 0.8	10.7 ± 0.5	13.0 ± 1.1	10.2 ± 0.9
18:2n-6	1.6 ± 0.2	2.1 ± 0.4	0.9 ± 0.1	1.2 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
18:3n-3	1.3 ± 0.2	1.6 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
18:4n-3	3.8 ± 0.7	5.3 ± 0.5	0.9 ± 0.2	2.6 ± 0.5	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.1
20:1*	13.0 ± 2.1	11.4 ± 1.2	8.0 ± 1.0	9.9 ± 2.4	0.7 ± 0.4	1.3 ± 0.2	1.4 ± 0.3
20:4n-6	0.6 ± 0.1	0.6 ± 0.0	1.2 ± 0.1	1.1 ± 0.2	5.0 ± 1.5	2.3 ± 0.5	2.8 ± 0.5
20:5n-3	7.5 ± 1.1	9.9 ± 0.8	6.1 ± 0.6	8.7 ± 1.1	16.7 ± 1.6	10.8 ± 2.4	14.4 ± 1.4
22:1n-11	20.7 ± 2.4	19.0 ± 1.6	7.1 ± 2.3	14.6 ± 3.4	0.1 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
22:5n-3	0.8 ± 0.1	0.7 ± 0.0	2.1 ± 0.4	0.9 ± 0.1	2.3 ± 1.1	1.3 ± 0.2	1.9 ± 0.2
22:6n-3	10.8 ± 1.8	11.0 ± 0.7	14.4 ± 2.8	23.5 ± 4.1	36.7 ± 2.5	41.9 ± 3.4	38.6 ± 2.9

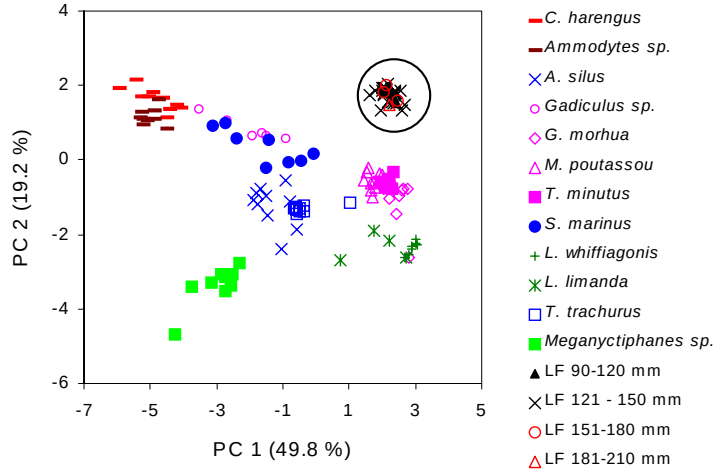
Table 7 continued	*S. marinus* n = 8	*L. whiffiagonis* n = 7	*L. limanda* N = 4	*T. trachurus* n = 10	*Meganyctiphanes* sp. n = 10
Fatty acid					
14:0	3.9 ± 1.1	1.0 ± 0.2	1.5 ± 0.8	4.5 ± 0.9	1.5 ± 0.2
15:0	0.3 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	1.3 ± 0.2
16:0	15.9 ± 1.8	22.4 ± 1.0	18.7 ± 2.8	19.3 ± 0.6	8.3 ± 0.8
16:1n-7	3.4 ± 0.7	1.5 ± 0.1	3.5 ± 0.9	1.9 ± 0.3	5.1 ± 0.5
18:0	3.7 ± 0.5	5.6 ± 0.3	6.0 ± 0.8	6.3 ± 0.4	2.1 ± 0.2
18:1*	9.6 ± 2.4	8.0 ± 0.7	10.8 ± 1.7	10.0 ± 1.6	27.9 ± 2.6
18:2n-6	1.6 ± 0.1	0.8 ± 0.1	1.2 ± 0.4	1.8 ± 0.2	1.8 ± 0.1
18:3n-3	0.8 ± 0.2	0.2 ± 0.0	0.4 ± 0.3	1.1 ± 0.2	1.6 ± 1.1
18:4n-3	1.8 ± 0.7	0.2 ± 0.0	0.5 ± 0.3	3.2 ± 0.7	3.2 ± 0.3
20:1*	9.2 ± 2.3	1.2 ± 0.2	2.2 ± 0.2	0.5 ± 0.2	3.1 ± 0.7
20:4n-6	1.6 ± 0.5	7.9 ± 1.7	5.9 ± 1.5	0.7 ± 0.1	1.1 ± 0.1
20:5n-3	11.3 ± 2.1	17.9 ± 1.6	19.3 ± 4.1	9.1 ± 0.3	11.3 ± 0.5
22:1n-11	9.7 ± 3.8	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	1.1 ± 0.3
22:5n-3	1.2 ± 0.2	4.5 ± 0.6	2.7 ± 0.7	1.2 ± 0.1	0.7 ± 0.1
22:6n-3	22.7 ± 5.6	26.7 ± 2.6	23.3 ± 3.3	36.2 ± 2.7	26.3 ± 2.2

Comparison of squid and prey

Principal component analysis clearly showed groupings in prey species as well as some similarities between squid and prey (Fig. 6a). Fatty acid profiles of squid muscle tissue were clearly distinguishable from all prey species. The first principle component, which accounted for 49.8 % of variance in the data, separated squid tissue from all species but gadids (*G. morhua*, *T. minutus* and *M. poutassou*) and flatfish (*L. limanda* and *L. whiffiagonis*). The biggest distance between squid and prey fatty acid signatures was found for *C. harengus*, *Ammodytes* sp. and the euphausiid crustacean *Meganyctiphanes* sp.

Separation along the first principle component axis was mainly due to saturates 16:0 and 18:0 and the major PUFAs 20:5n-3 and 22:6n-3. Fatty acid signatures of gadid and flatfish muscle were the closest, in levels of the saturates 16:0 and 18:0 and the major PUFAs 20:5n-3 and 22:6n-3 to muscle tissue of *L. forbesi*. The second principle component (PC2), which accounted for 19.2 % of variation in the data, separated squid muscle tissue from all prey species but *C. harengus* and *Ammodytes* sp. Separation here was mainly due to fatty acids 14:0, 20:1* and 22:1*, similar levels of which both *C. harengus* and *Ammodytes* sp. shared with muscle tissue of *L. forbesi*.
High variation between digestive gland samples caused increased separation as demonstrated through PC1 (50.8 %) (Fig. 6b).

6a.)



6b.)

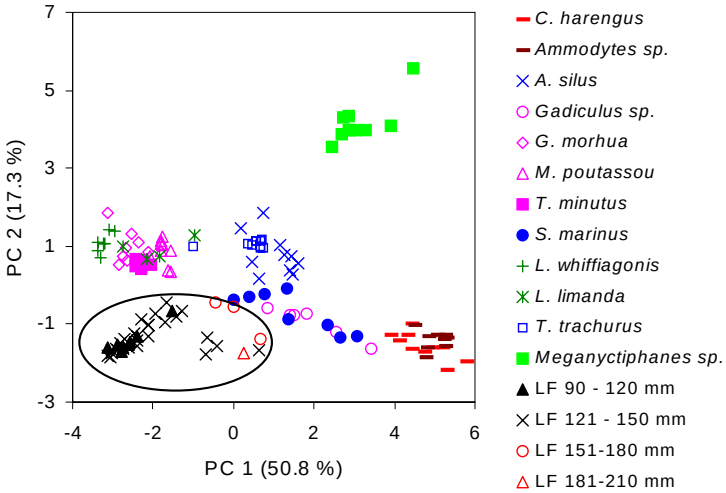


Figure 6a,b: PCA of fatty acid signatures of *L. forbesi* (by size class) and putative prey species. Encircled data indicate results found for *L. forbesi* muscle (6a) and digestive gland tissue (6b).

Fatty acid signatures of digestive gland tissue showed more similarities to fatty acid signatures of prey species than found for muscle tissue. The majority of digestive gland samples were close to gadid and flatfish species in their fatty acid profiles. However, with increasing size of the squid the digestive gland samples showed similarities in fatty acid signatures with *Gadiculus* sp., *T. trachurus*, *S. marinus* and *A. silus* (PC 1). These similarities between squid and fish related to high

levels of saturate 16:0, monounsaturate 22:1n-11, and PUFAs of the linoleic family (C18). Further separation along PC 2 (17.3 %) was mainly due to fatty acids 15:0 and 18:1*, levels of which were (as for muscle) similar between bigger sized squid and the pelagic fish species.The strongest separation from squid tissue was still found for *C harengus*, *Ammodytes* sp. and *Megayctiphanes* sp.

Quantitative fatty acid analysis

Data for a number of selected fatty acids (n = 17) of muscle and digestive gland tissue samples were run against data on prey fatty acids and best fit predicted prey composition determined for each squid specimen. Results presented are based on an average of the first twenty best fit scenarios (out of 10^4 combinations tested).

The composition of the predicted diet was different for squid of different sizes and maturity stages in digestive gland tissue. With increasing size of the predator, the proportions of the flatfish and gadid species decreased and species such as argentine, redfish, herring and sandeel seemed to contribute more to the diet (Table 8a,b). More variation was found for data from muscle samples, with less pronounced changes in estimated diet with increasing size of the squid. Only horse mackerel and redfish showed a clear increase in predicted proportion with predator size. Sample sets for the bigger sized animals were however comparatively small (151 – 180 mm, n = 3, 181 – 210 mm, n = 1) and changes could therefore be under – or overestimated.

Table 8 a,b: Prey composition in a.) digestive gland and b.) muscle tissue of *L. forbesi* as determined by quantitative fatty acid analysis. Shown are average percentage results for individual samples based on the first 20 best-fit scenarios grouped by size and maturity stage, including group mean and standard deviation.

Digestive gland	n	*C. harengus*	*Ammodytes* sp.	*A. silus*	*Gadiculus* sp.	*G. morhua*
Size classes (mm)						
91 - 120	8	0.0 ± 0.0	0.2 ± 0.6	0.0 ± 0.0	0.1 ± 0.4	7.6 ± 2.8
121 – 150	27	0.8 ± 2.8	2.2 ± 5.2	0.6 ± 1.6	0.6 ± 1.2	7.1 ± 4.5
151 – 180	3	3.7 ± 2.4	11.0 ± 8.8	7.1 ± 8.1	1.7 ± 2.0	3.0 ± 4.5
181 - 210	1	7.3 ± 0.0	15.2 ± 0.0	0.0 ± 0.0	3.8 ± 0.0	0.7 ± 0.0
Maturity stages						
1	26	0.0 ± 0.0	0.6 ± 1.9	0.6 ± 1.6	0.5 ± 1.1	7.5 ± 3.4
2	13	3.2 ± 4.1	7.2 ± 8.2	1.7 ± 4.5	1.0 ± 1.6	5.1 ± 5.6

Table 8a continued

Digestive gland	n	*M. poutassou*	*T. minutus*	*S. marinus*	*L. whiffiagonis*	*L. limanda*	*T. trachurus*
Size classes (mm)							
91 - 120	8	0.0 ± 0.0	7.4 ± 2.4	4.8 ± 3.8	32.4 ± 7.5	45.9 ± 4.8	1.6 ± 1.8
121 – 150	27	0.0 ± 0.0	8.5 ± 6.6	8.7 ± 9.0	25.6 ±10.1	44.7 ± 5.8	1.3 ± 1.6
151 – 180	3	0.4 ± 0.7	1.8 ± 3.1	15.7 ± 6.6	11.3 ± 8.9	44.4 ± 4.8	0.0 ± 0.0
181 - 210	1	0.0 ± 0.0	0.0 ± 0.0	29.7 ± 0.0	7.5 ± 0.0	35.8 ± 0.0	0.0 ± 0.0
Maturity stages							
1	26	0.0 ± 0.0	8.3 ± 4.8	6.3 ± 6.6	29.2 ± 8.4	45.6 ± 5.3	1.4 ± 1.8
2	13	0.1 ± 0.3	6.1 ± 8.0	14.3 ±10.7	17.8 ± 11.5	42.8 ± 5.9	0.8 ± 1.0

Table 8b

Muscle	*n*	*Ammodytes sp.*	*Gadiculus sp.*	*G. morhua*	*M. poutassou*	*T. minutus*
Size classes (mm)						
91 - 120	8	0.0 ± 0.0	4.0 ± 1.9	10.9 ± 4.7	4.0 ± 1.9	39.1 ± 11.2
121 – 150	27	0.0 ± 0.0	4.9 ± 2.4	12.9 ± 2.2	4.9 ± 2.4	41.8 ± 5.1
151 – 180	3	0.2 ± 0.4	3.7 ± 4.0	5.3 ± 3.0	3.7 ± 4.0	30.0 ± 18.8
181 - 210	1	0.0 ± 0.0	4.6 ± 0.0	11.4 ± 0.0	4.6 ± 0.0	45.1 ± 0.0
Maturity stages						
1	26	0.0 ± 0.0	4.8 ± 2.1	13.0 ± 2.5	4.8 ± 2.1	42.1 4.6
2	13	0.1 ± 0.2	4.4 ± 2.8	9.7 ± 4.2	4.4 ± 2.8	37.1 12.7

Table 8b continued

Muscle	*n*	*S. marinus*	*L. whiffiagonis*	*L. limanda*	*T. trachurus*
Size classes (mm)					
91 - 120	8	0.5 ± 1.3	30.9 ± 8.7	3.7 ± 9.8	10.8 ± 5.2
121 – 150	27	0.8 ± 3.2	28.0 ± 4.9	0.3 ± 0.6	11.2 ± 3.9
151 – 180	3	4.0 ± 7.0	37.0 ± 10.6	8.2 ± 13.4	11.4 ± 6.0
181 - 210	1	0.0 ± 0.0	26.9 ± 0.0	0.0 ± 0.0	11.9 ± 0.0
Maturity stages					
1	26	0.2 ± 1.1	28.6 ± 4.8	0.3 ± 0.7	11.0 ± 3.8
2	13	2.4 ± 5.2	30.7 ± 9.2	4.1 ± 9.7	11.6 ± 4.9

Stable isotope analysis

δ ^{13}C abundance

δ ^{13}C ratios in muscle tissue (- 16.2 ± 1.4 ‰) were on average 4 ‰ more depleted than in digestive gland tissue (-20.5 ± 1.5 ‰). Season was the only factor influencing δ ^{13}C levels in digestive gland tissue (Table 9). δ ^{13}C values of muscle tissue on the other hand varied with size ($p < 0.01$), maturity ($p < 0.05$) and season ($p < 0.01$) (Table 9).

Squid of smaller sizes and the majority of squid of maturity stages 1 and 2, had muscle tissue that was more depleted in δ ^{13}C than did squid of larger sizes and maturity stages. On a seasonal basis δ ^{13}C ratios derived from muscle tissue fell into two groups: squid caught in winter and summer had similar values, implying feeding on similar carbon sources, as did squid caught in spring and autumn. Squid caught in spring were up to 3 ‰ more depleted in δ ^{13}C than squid caught in summer. δ ^{13}C levels in digestive gland tissue of samples from squid caught in the summer were more enriched in δ ^{13}C compared to all other seasons.

δ ^{15}N abundance

Overall muscle and digestive gland tissue showed similar mean δ ^{15}N ratios (muscle 8.6 ± 1.7 ‰, digestive gland 8.3 ± 1.5 ‰). Within tissues both muscle and digestive gland showed significant variation between size classes, maturity stages and seasons (all categories $p < 0.01$)(Table 9).

When comparing δ ^{15}N values for different size classes, squid smaller than 151 mm DML showed significantly lower values in both muscle and digestive gland than squid of bigger mantle lengths. In squid of lower maturity stages (1 and 2), tissues were more depleted in δ ^{15}N than in higher (3 to 5) maturity stages. Muscle and digestive gland δ ^{15}N values were influenced by season, with squid caught in spring showing on average less enriched δ ^{15}N values than squid caught in any other season. The biggest seasonal differences in δ ^{15}N abundance were found between animals caught in winter and spring. δ ^{15}N values for each tissue were similar for squid caught in summer and autumn.

Table 9: Isotopic composition of muscle and digestive gland tissue of L. forbesi. Given are mean abundances ± standard deviation for $\delta^{13}C$ and $\delta^{15}N$ per size class, maturity stage and season.

		$\delta^{13}C$		$\delta^{15}N$	
	n	*Digestive gland*	*Muscle*	*Digestive gland*	*Muscle*
Size classes					
≤ 150 mm	20	-20.5 ± 1.1	-17.2 ± 1.1	6.2 ± 0.9	7.1 ± 1.1
> 150 mm	50	-20.5 ± 1.5	-15.8 ± 1.4	9.0 ± 0.8	9.2 ± 1.4
Maturity stages					
1	15	-20.6 ± 0.9	-17.0 ± 1.0	6.2 ± 1.0	7.0 ± 0.9
2	11	-20.5 ± 1.3	-16.6 ± 1.9	7.7 ± 1.2	7.9 ± 1.9
3	5	-19.5 ± 1.0	-15.3 ± 1.2	8.8 ± 0.4	8.6 ± 0.5
4	2	-20.0 ± 0.0	-14.1 ± 0.8	8.7 ± 0.0	9.2 ± 0.1
5	37	-20.6 ± 1.6	-16.0 ± 1.3	9.1 ± 0.9	9.5 ± 1.4
Season					
Winter	16	-20.5 ± 1.3	-15.8 ± 1.0	9.7 ± 0.8	9.9 ± 0.9
Spring	19	-20.7 ± 0.8	-17.5 ± 1.0	6.1 ± 0.7	6.9 ± 0.9
Summer	20	-19.6 ± 0.7	-14.9 ± 1.2	8.7 ± 0.5	8.8 ± 1.1
Autumn	15	-21.2 ± 2.0	-16.9 ± 0.8	8.7 ± 0.7	9.2 ± 1.9
Stomach contents					
E		-20.2 ± 1.2	-16.1 ± 1.3	8.4 ± 1.5	8.6 ± 1.6
C		-18.9 ± 0.0	-14.4 ± 0.0	8.6 ± 0.0	8.9 ± 0.0
Cr		-20.1 ± 0.0	-16.9 ± 0.0	6.2 ± 0.0	7.6 ± 0.0
F		-21.0 ± 1.6	-16.1 ± 1.4	8.8 ± 1.1	9.3 ± 1.7
FCr		-20.6 ± 0.9	-17.4 ± 1.5	6.4 ± 1.2	7.2 ± 1.1

Comparison to prey species

Stomach contents

Animals feeding on a mixed diet of fish and crustaceans (FCr) together with the specimen for which only crustacean remains (Cr) were found in the stomach, showed more depleted $\delta^{13}C$ ratios in their muscle tissue than the rest of the feeding groups.

Squid feeding on a mixed diet of fish and crustaceans (FCr) were found to have lower $\delta^{15}N$ ratios than animals for which no prey (E) or only fish remains (F) were found in the stomach (Table 9). The two specimens that had only crustacean or cephalopod remains in the stomachs showed $\delta^{15}N$ ratios similar to those found for squid feeding on a mixed or fish diet respectively. Due to lower within-group variation, differences between groups were more pronounced in results from the digestive gland.

Putative prey

Of prey species analysed for stable isotope profiles only *M. poutassou*, *Gadiculus sp.*, *G. morhua*, *M. aeglefinus*, *L. limanda* and *L. whiffiagonis* were of relevance as putative prey to species *L. forbesi* (Fig. 7a,b).

$\delta^{13}C$ ratios of squid muscle tissue were either similar to prey species or less enriched by 1-2 ‰. Significant differences in $\delta^{13}C$ were only found between *L. forbesi* and *G. morhua*. Apart from one sample taken in autumn, digestive gland tissue was always enriched, by at least 1 ‰ and up to 7 ‰, $\delta^{13}C$ relative to any of the prey species (Fig. 7a,b).

$\delta^{15}N$ abundance in squid tissues (both muscle and digestive gland) was significantly different from values in prey species ($p < 0.01$) (Fig. 7a,b). On average squid tissue was enriched in $\delta^{15}N$ relative to pelagic species *Gadiculus* sp. and *M. poutassou*. *Gadus morhua, M. aeglefinus, L. limanda* and *L. whiffiagonis* were on average enriched relative to squid tissues. However, due to high variation in values for muscle tissue it was not possible to distinguish between seasons in relation to possible food preferences. Squid smaller than 150mm DML (collected in spring), showed similar or lower 15N values when compared to pelagic fish species *M. poutassou* and *Gadiculus* sp.

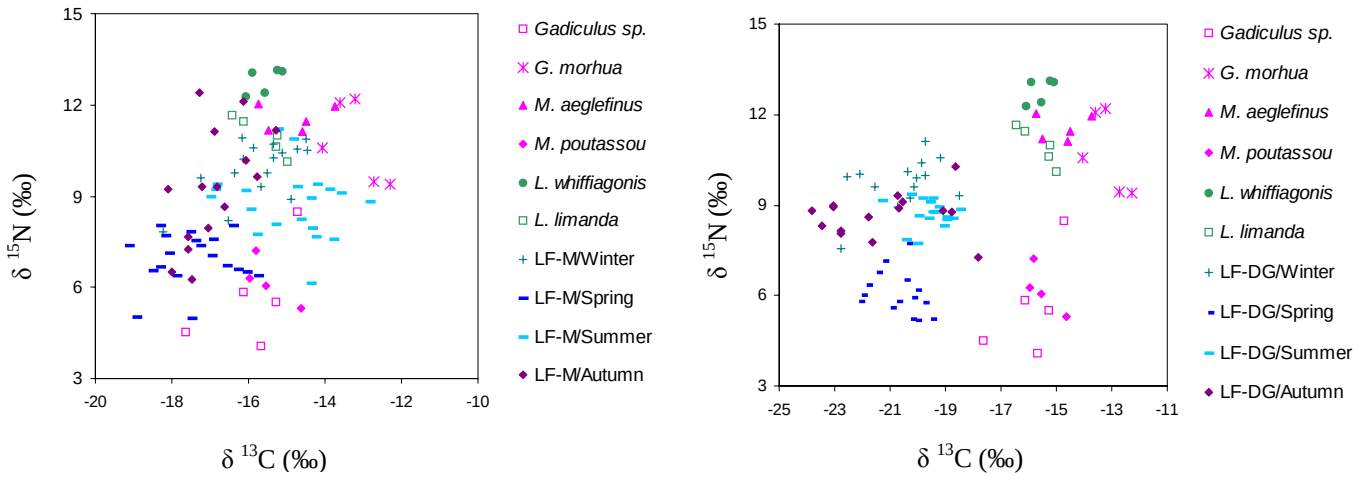


Figure 7a,b: Nitrogen (δ ^{15}N) and carbon (δ ^{13}C) stable isotopes of *L. forbesi* muscle (a) and digestive gland (b) and putative prey species. LF/M = *L. forbesi* muscle, LF/DG = *L. forbesi* digestive gland tissue.

Discussion

Stomach contents, fatty acid profiles and stable isotope signatures varied considerably between squid sizes, seasons and areas studied, demonstrating that *L. forbesi* does exploit a wide spectrum of prey across its distribution and life cycle.

Stomach content analysis

Stomach content analysis clearly indicated that the diet of *L. forbesi* changes from a diet containing high proportions of crustacean prey in small juvenile specimens to a more fish-dominated diet in squid of bigger sizes. These ontogenetic shifts in diet are known for many squid species (e.g. Rodhouse & Nigmatullin, 1996) and have also been found in previous feeding studies of *L. forbesi* (e.g. Ngoile, 1987, Rocha *et al.*, Collins & Pierce, 1996). *Loligo forbesi* is considered to be a primarily piscivorous species (e.g. Collins et *al.*, 1994, Pierce et *al.*, 1994b), which agrees with current findings that in squid of all seasons and sizes present, fish was always the predominant prey type (67 – 100 % FO). Although squid tend to feed on larger prey with increasing mantle length, bigger squid still consume small prey throughout their life cycle (e.g. Collins & Pierce, 1996).

The prey species composition in the present study was broadly similar to diets previously found for *L. forbesi* in Scottish and Irish waters (Pierce et *al.*, 1994, Collins et *al.*, 1994, Collins & Pierce 1996). Prey composition changed from a more variable diet in spring dominated by gadid and gobiid species to a less varied diet with decreasing proportions of gadids over the summer and autumn and the presence of clupeids in autumn in the diet. Due to its short life cycle of just over one year, squid size and maturity are strongly linked to season. Seasonal variation found in the diet composition is therefore most likely a function of ontogenetic shifts in the diet.

Ngoile (1987) and Pierce et *al.* (1994b) found that, next to gadid species, ammodytid species (sandeel) played an important part in the diet of *L. forbesi* in Scottish waters. No sandeels were identified from stomach remains in specimen of *L. forbesi* in this study. Due to the scarcity of otoliths and the high level of fragmentation of stomach remains a big proportion of fish remained unidentified and thus the presence of sandeel in the diet could have been missed. The small sample size analysed and prey availability for each season might also have had an influence on the distribution of results.

Of crustacean species identified in this study, euphausiids comprised the biggest proportion in the diet. If identified, crustacean prey in previous studies usually belonged to euphausiid and decapod families (Collins et *al.*, 1994, Pierce et *al.*, 1994b, Pierce & Santos, 1996). Cannibalism and predation on other cephalopod species has previously been reported in *L. forbesi* (Ngoile, 1987, Rocha et *al.*, 1993, Collins et *al.*, 1994, Pierce et *al.*, 1994b, Pierce & Santos, 1996). Although studies exist in Scottish waters where cephalopod prey was taken on a more frequent basis than found in this study, those data are localised and are likely due to the availability of prey in the area (Pierce et *al.*, 1994b).

Fatty acids

Fatty acid analysis of digestive gland tissue clearly indicated dietary differences between squid below and above 150 mm mantle length. Increased levels of saturated and highly polyunsaturated fatty acids were found for squid of smaller size classes whereas higher levels of C18 PUFA and MUFA were characteristic for fatty acid signatures of bigger squid.

There are significant differences as to the origin of these fatty acids. Large concentrations of PUFA 20:5n-3 are typically found in diatom-based food webs and are usually elevated relative to 22:6n-3 in herbivorous and benthic higher trophic levels. In contrast, high proportions of 22:6n-3 mostly derive from food chains based on dinoflagellates and are found in higher proportions in marine omnivorous and carnivorous animals (Sargent & Whittle, 1981). Both elevated levels of C18 PUFA and monounsaturates, are typical for pelagic food webs. C18 PUFA usually originate from phytoplankton and can reach high relative proportions in wax esters of copepods and their predators (Sargent, 1976). Monounsaturates (MUFA) C20:1 and C22:1 on the other hand are biosynthesised by calanoid copepod species and abundant in the alcohols of their wax esters (Lee et *al.*, 1971). Higher trophic levels rich in these MUFAs (e.g. *C. harengus, Ammodytes* sp., *Meganyctiphanes norvegica*), are thought to assimilate fatty alcohols from the wax esters of their prey and directly convert them into fatty acids (Ratnayake & Ackman, 1979, Falk-Petersen et *al.*, 1982). Although generally found in small proportions in marine organisms, saturated fatty acid 18:0 can be elevated in benthic feeders since marine detritus contains substantial quantities of saturated fatty acids between 14 and 18 carbon chain length (Perry et *al.*, 1979).

Accepting the general assumption that fatty acids stored in digestive gland tissue are mainly of dietary origin rather than being biosynthesised (Blanchier & Boucaud-Camou, 1984, Clarke et *al.*, 1994, Semmens, 1998, 2002), the distribution of fatty acid proportions across size classes therefore indicates that smaller squid fed predominantly in benthic habitats whereas squid of larger sizes fed increasingly on prey of pelagic origin.

As in previous feeding studies of *L. forbesi* the stomach remains found in this study showed an ontogenetic shift in the diet with changes from a crustacean dominated diet to a fish dominated diet

with increasing size of the squid. The overall scarcity of copepod remains agrees on the one hand with low proportions of MUFA in tissues of smaller squid and suggests that high levels of MUFA in bigger squid originate from copepod predators such as herring or sandeel rather than the crustacean itself. Crustacean species found in the stomachs, such as euphausiids and decapods can, depending on their habitat, have fatty acid signatures resembling demersal fish species high in PUFA and saturated fatty acids (Morris & Sargent, 1973). Thus the predominance of high proportions of PUFA and SAT over MUFA in smaller squid does not exclude the presence of crustaceans in the diet.

Fatty acid profiles showed gradual differences between size classes, which is in concordance with findings of bigger squid still feeding on small proportions of crustaceans and smaller squid feeding on small fish species. Differences in fatty acid profiles do not directly indicate the change from a crustacean to a fish dominated diet but rather a difference between benthic and pelagic feeding. The swap from a more benthic to a more pelagic diet with increasing size might be due to squid of bigger sizes being faster and therefore able to catch faster prey (Macy, 1982).

The fatty acid composition of muscle tissue remained largely unchanged with increasing mantle length and indicates a similar feeding history between size classes investigated in this study.

Multivariate analysis grouped the majority of digestive gland samples close to demersal gadid and flatfish species. This agrees with results from stomach content analysis, where all identifiable remains belonged to demersal gadid and flatfish species, and suggests that demersal species were the main prey in recent feeding. However *L. forbesi* is known to take a wide variety of prey species and pelagic fish quite frequently are also part of the diet (Pierce et *al.*, 1994b).

Although not identified from stomach contents in spring samples PCA grouped pelagic and benthopelagic species *M. poutassou*, *Gadiculus* sp., *T. trachurus*, *S. marinus* and *A. silus* with part of the squid samples analysed. All of these prey species have previously been identified from stomach remains of *L. forbesi* in Scottish waters (Pierce et *al.*, 1994b) even if in low frequencies. Results from fatty acid analysis suggest that these species might on a short-term local basis have

played an important part in the diet of *L. forbesi* collected in this study and emphasises the usefulness of fatty acids to support conventional stomach content analysis.

Ammodytes sp. and *C. harengus* were not identified from stomach content and together with *Meganyctiphanes* sp. were grouped furthest away from squid fatty acid profiles in PCA. Although the analysis of individual fatty acids in squid tissues suggests a significant contribution of *Ammodytes* sp. or *C. harengus* these species were not identified from stomach content analysis and multivariate analysis (PCA) did not group them together with squid tissue samples. Fish species that store their lipids in the muscle tissue such as *C. harengus*, *Ammodytes* sp., *T. trachurus* and *Sprattus sprattus* (sprat) are subject to seasonal changes in their meat lipid deposits. A study on *S. sprattus* in British waters found that the proportions of saturates and MUFA increased and the proportion of PUFAs decreased as the season progressed (Hardy & Mackie, 1969). Therefore fish samples collected in the summer will likely have lower PUFA and higher saturates and MUFA levels in their tissues than the fish prey the squid samples in spring will have encountered. Digestive gland samples were separated from *Ammodytes* sp. and *C. harengus* only through the first principal component and the main separation factor was indeed the difference in highly polyunsaturated fatty acids. Euphausiid species in the North-east Atlantic are also subject to seasonal changes in their lipid storage (Morris, 1971). The separation between *Meganyctiphanes* sp. and squid tissues is likely due to species originating from different geographical areas and seasons and the unlikelihood of squid analysed in this study having encountered this species.

Fatty acid signatures of muscle tissue were well separated from all putative prey species but as for digestive gland did show the least separation to demersal gadid and flatfish species (2^{nd} PC). Proposing that diet will to a small but significant extent affect the fatty acid composition of muscle tissue this indicates that these squid fed predominantly on demersal species over an extended period of time which also supports the assumption of a predominantly benthic lifestyle of this squid species.

Quantitative fatty acid analysis

The development of a statistical model using fatty acid data of predator and prey allowed us to quantitatively estimate the proportions of putative prey species in the diet of *L. forbesi*.

As expected the predicted prey composition for muscle tissue was less varied than for the digestive gland due to smaller differences in fatty acid profiles within the muscle. As for the analysis of individual fatty acids the predicted diet did change from high proportions of benthic species in the diet of smaller squid to a more pelagic oriented diet in squid of bigger sizes.

Iverson et *al.* (2004) noted that, to be able to quantitatively estimate a predators' diet, it is necessary to accurately differentiate between different prey fatty acid signatures, evaluate their variation due to environmental factors and understand how and if ingested fatty acids are deposited and metabolised in the predators' tissue.

The authors found that, the bigger the prey sample size and the more fatty acids were included into the analysis, the more representative their estimates were of the true diet, and that fits were better and more consistent where diet items were easily distinguished. By applying a calibration coefficient, which compensated for biomodification of fatty acids in the tissues results were further improved. This was also found in a feeding study on *L. brevis* (unpublished data), where through the application of a calibration coefficient the estimated diet reflected true prey considerably closer than when no calibration coefficient was used.

It is currently not known to which extent fatty acids are deposited in the tissues of *L. forbesi* and no calibration coefficient could be established for this species. Diet was therefore estimated solely based on the use of prey fatty acid signatures without considering biochemical changes due to metabolic activity in the tissues.

A general limitation to fatty acid analysis, is the similarity of prey fatty acid signatures, which also arose in this study. Results from stomach content analysis in previous studies showed that flatfish in contrast to gadids played only a minor part in the diet of *L. forbesi*. Quantitative analysis however

attributed flatfish a rather prominent contribution to the diet in muscle and digestive gland (30% and 75% respectively). This might to a small part be due to flatfish species generally being underestimated in stomach content analysis but is to the biggest part due to highly similar fatty acid signatures for demersal gadid and flatfish species. As shown in multivariate analysis these similarities also apply to fatty acid signatures of blue whiting and to a small extent to horse mackerel. A control analysis in which individual prey were designated as predators, and QFAA run, did also show that demersal gadid and flatfish species were the only species that were correctly identified through the model. The within-species variation in the remaining prey caused misidentification and subsequently led to prey species being under-represented in the predicted diet. Furthermore the variation in fatty acid signatures of squid tissue samples (mainly muscle) might not be big enough for the model to be able to find minor effects of other more different prey species.

The model was however able to show trends in feeding with increasing size of the predator and did offer an estimate as to which ecosystem provided most of the squids' diet. Even if no calibration coefficients can be established for this species, bias could be minimised in future applications by sampling more significantly different prey species, by sampling bigger numbers within these prey species to reduce within species variation and to eliminate environmental influences prey species should also be chosen from similar seasons and areas as the predator.

Stable isotopes

Both carbon and nitrogen analysis have demonstrated that *L. forbesi* shows ontogenetic and seasonal changes in feeding.

Significant differences were found between $\delta\ ^{13}C$ of muscle and digestive gland tissue with muscle showing carbon values always enriched by 4 ‰ relative to the digestive gland. Feeding studies on marine mammals and various invertebrate species have shown that the deposition of stable isotopes in the predators' tissue is dependent on the turnover (i.e. metabolic activity) of the respective tissue and the selective incorporation of biochemical fractions such as proteins or lipids into these tissues (Hobson & Clark, 1992b, Pinnegar & Polunin, 1999). Tissues with a slower turnover (e.g. muscle) tend to integrate a range of isotope values with varying diets, whereas tissues with faster turnover (e.g. liver) tend to reflect the isotope content of the most recent diet (Tieszen et *al.*, 1983).

Differences in turnover rates for carbon between different tissues were shown in a feeding study of squid *Lolliguncula brevis* (unpublished data), in which digestive gland showed quicker and higher turnover rates than muscle. The differences in ratios found between tissues in this study are therefore likely a function of different turnover rates. We would therefore expect that the stable isotope data gathered in this study will provide us with dietary information integrated over a longer period of time and data gained from the analysis of digestive gland tissue will provide us with information on diet most recently ingested.

However compared to putative prey species analysed in this study carbon ratios of the digestive gland were always significantly depleted relative to the prey. Previous studies have shown that lipids are isotopically lighter in ^{13}C compared to other biochemical fractions in the body and that lipid-rich tissues can therefore be depleted rather than enriched in ^{13}C relative to the diet (McConnaughey & Mc Roy, 1979, Tieszen et *al.*, 1983). Lipids were not removed prior to stable isotope analysis and since lipid is ^{13}C poor the high lipid content typical for digestive gland tissue will have depleted carbon ratios. Carbon ratios were therefore of limited use in this study for the interpretation of trophic relations regarding the influence of individual prey species on the diet but the data could be applied to detect general trends such as the impact of seasonal and ontogenetic changes in feeding on the diet. However the extraction of lipids as a precaution has to be considered carefully since the removal of lipids can result in greater variance of the data and thus poorer resolution of dietary relationships (Pinnegar & Polunin, 1999).

Stomach content analysis for cephalopods in general, and for *L. forbesi* in this and previous studies, has shown that the prey species consumed by cephalopods vary seasonally with changing foraging locations, seasonal prey availability and changes in feeding due to cephalopod size (e.g. Nixon, 1987, Pierce et *al.*, 1994b, Collins & Pierce, 1996 Rodhouse & Nigmatullin, 1996). The isotopic distribution across seasons showed summer samples to be significantly enriched to all other seasons with the most depleted ratios found within autumn and winter samples. However both winter and autumn showed also a wide range of values within seasons. Primary production in the North East Atlantic is highest during spring and summer. A study on the temporal variations of carbon ratios in particulate organic matter (POM) in the Southern North Sea found a similar seasonal trend in carbon ratios to this study and suggested that due to the fact that algae preferably use isotopically

light DIC (dissolved inorganic carbon) the organic matter synthesized by these algae can also become isotopically enriched and this enrichment will then be carried along the food chain (Megens et *al.*, 2001). The relative high enrichment in summer and spring samples could therefore been caused by phytoplankton blooms offering enriched carbon to higher trophic levels in these seasons. Since *L. forbesi* is a short-lived species of approximately one year its growth is closely linked to season. Therefore the high variation within autumn and winter samples is likely due to bigger sized squid feeding on a bigger variety of prey and thus carbon sources. This would also explain the relative enrichment of some bigger squid from winter samples compared to smaller squid collected in spring.

Apart from feeding on a wider variety of prey with increasing size *L. forbesi* is also known to move offshore during summer and autumn and return to coastal areas in winter and spring for spawning purposes (Pierce et *al.*, 1994b). More depleted carbon ratios are usually associated with offshore and pelagic habitats whereas coastal and benthic ecosystems show comparatively less depleted carbon ratios (Hobson, 1993, Hobson et *al.*, 1994). The majority of squid in the winter did show less depleted carbon ratios which suggests coastal, more benthic feeding. The more depleted ratios in autumn on the other hand suggest more pelagic feeding, which is supported by the fact that stomach contents analysed from this season were the only ones containing remains of pelagic fish species.

Muscle tissue showed similar trends in the distribution of carbon ratios across sizes and seasons, the differences to digestive gland tissue being apparent in a bigger variation in ^{13}C within seasons and the shift of autumn samples to more enriched values in relation to the other seasons. This suggests that the long-term effect on the diet is dominated by ontogenetic differences in feeding with bigger squid taking bigger prey further removed from the carbon source. The distribution of carbon ratios across seasons and sizes demonstrates the omnivorous feeding behaviour of *L. forbesi*.

Comparing carbon ratios of squid to stomach contents, carbon ratios followed the trend of being more depleted in specimens that had crustacean remains in their stomachs than squid containing fish or no remains in their stomachs. Across the seasons samples taken in autumn showed less depleted values only to pelagic small gadid species *M. poutassou* and *Gadiculus sp*. Summer and winter

samples were the only squid showing carbon ratios close to demersal gadid and flatfish species thus confirming previously found trends in feeding across the seasons.

Again as for carbon different fractionation factors are usually found between tissues as nitrogen deposition depends on protein levels in the diet and metabolic activity of the tissue (Owen, 1987). Feeding studies on marine mammals and birds did show that different tissues had different fractionation factors depending on the diet with fractionation being higher for tissues of higher metabolic activity (DeNiro & Epstein, 1981, Tieszen et *al.*, 1983). A feeding study on rainbow trout found liver to be slightly less enriched relative to the diet than muscle and the authors considered the difference in structural amino acids between tissues to be responsible for these differences (Pinnegar & Polunin, 1999). Since the muscle tissue of squid species is high in structural proteins compared to predominantly dietary proteins in the digestive gland (Kunisaki, 2000), the relative higher enrichment of 0.6 ± 1.4 ‰ compared to digestive gland tissue is also likely due to differences in protein storage between tissues.

The reconstruction of trophic pathways through stable isotope analysis has in many field studies been based on the generalisation that a nitrogen fractionation of 3.4 ‰ occurs with every step in the food chain. Many feeding studies however found that these enrichments can vary considerably within one species and that especially in food webs with different nitrogen sources and high occurrence of omnivory the separation into individual species contributing to a diet will be difficult (Adams & Sterner, 2000, Post, 2002).

Post (2002) also noted that the mean trophic fractionation of 3.4 ‰ is a valid approximation of trophic fractionation only when applied to entire food webs, involving multiple trophic pathways and many species. Since prey data was limited to only a few species in this study and no source data was available, no fractionation factor relating to trophic levels could be established for *L. forbesi* and information gathered on the feeding over different trophic levels must be limited to investigating the contribution of benthic and pelagic and offshore and coastal species to the diet.

Differences in nitrogen ratios were mainly found with increasing body size in and were less pronounced between seasons. Increases in ^{15}N with increasing weight or size and therefore trophic

position have previously been found for fish and have been attributed to changes in prey type and size of prey with increasing body size of the predator (Jennings, 2002). The feeding study on *L. brevis* (unpublished data) did also show that feeding on a species of lower calorific value and lower trophic level in squid will result in lower trophic level of the predator tissue. Therefore changes with increasing size found for *L. forbesi* are likely due to feeding on different and more varied trophic levels with increasing body size.

Clear seasonal separation in nitrogen ratios of the muscle was only found between spring and winter with ^{15}N being considerably enriched in winter samples. Unsurprisingly considering the development of squid across the seasons, winter samples consisted mainly of big pre-spawning squid whereas spring samples comprised mostly juvenile small squid. Higher nitrogen ratios in winter samples therefore are indicative of feeding on a higher trophic level in bigger squid.

The considerable variation found for nitrogen ratios over all seasons in muscle tissue suggests that long-term feeding is varied in this species and supports previously discussed findings of omnivorous feeding in this species. Also previous results on bigger squid feeding on a wider variety of prey are supported by the fact that isotopic variation is more pronounced for squid bigger than 150 mm than for smaller squid.

The same trends are found for recent diet (i.e. digestive gland) with smaller squid feeding on prey of lower trophic levels and squid taken from the spring sample showing the lowest nitrogen ratios. Variation is also much lower within size classes and seasons which suggests that most recent feeding, although still varied, is more selective and that muscle tissue retains a more mixed isotopic memory of previous diets integrated over a longer period of time.

Big carnivorous fish (*G. morhua* and *M. aeglefinus*) and benthic feeders (flatfish) analysed in this study showed higher nitrogen ratios than more pelagic offshore species such as *M. poutassou* and *Gadiculus* sp. The similarity of nitrogen ratios for big gadids and flatfish suggest that although big squid might not feed on these species directly they feed on the same trophic level as piscivorous fish. Small squid were similar in nitrogen ratios to small pelagic gadids although hardly ever enriched relative to these fish indicating feeding on prey of lower trophic levels in general and

possibly the same type of prey these small gadids feed. Although absolute values between studies are difficult to compare due to differences in methodology general trends can still be evaluated. Jennings et *al.* (2002), in a study on links between size and trophic level through isotope analysis, also found *L. forbesi* linked to benthic feeding.

As squid grew, dietary changes were observed in stomach contents involving increased importance of fish in the diet. These changes were reflected in increases in ^{15}N in squid showing predominantly fish remains in their stomachs. ^{15}N of squid with no food in their stomachs followed the same trend, which suggests that for these specimens fish was also the main prey.

Conclusions

Stomach content data, fatty acid analysis and stable isotope analysis indicated that *L. forbesi* is mainly associated with the benthic food web and that prey type and prey variability changed with increasing body size. The application of all three methods made it also possible to follow general movement patterns of the species from offshore into more coastal waters and most importantly made it possible to suggest the diet of animals where no food was found in the stomachs.

Both fatty acid and stable isotope analysis did however show that without considering factors influencing the deposition of fatty acids and stable isotopes in the tissues of the predator it will be necessary to supplement data with information gained from conventional dietary analysis.

The results gained from the application of these methods to a species where the life cycle is relatively well known has validated their usefulness for studies on the trophic ecology of little known species. The strengths of these findings however will always depend on the complexity of feeding relationships considered, i.e. numbers of species involved in the food web sampled, and total numbers of specimens sampled to minimise the effect of within species variation.

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