Using archived otoliths to determine stock structure and early life history of plaice (*Pleuronectes platessa* L.)

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Introduction

European plaice (*Pleuronectes platessa)* is a commercially important flatfish occurring on the sandy bottoms of the European shelf. It is widely distributed in shallow waters (≤100m) from western Mediterranean to Iceland. The species spawns in offshore waters. This usually occurs in the North Sea from December to March (Bergman *et al.* 1988), while in the Irish Sea, Nash *et al.* (2000) reported spawning from late January to early May. The eggs and larvae are pelagic for three to four months. After the larvae hatch, they are distributed to local nursery grounds by residual currents. These nursery grounds are shallow, sandy habitats. The number of plaice larvae reaching suitable beaches is determined by drift and diffusion of eggs from offshore grounds where spawning takes place (Steele & Edwards 1970). At the end of the larval period, metamorphosis takes place. The fish move from a relatively dilute three dimensional environment to a relatively concentrated two-dimensional environment (Nash *et al.* 2000). This metamorphosis is termed as settlement and from then on the fish adopts a benthic way of life. Adults exhibit seasonal migration patterns from spawning grounds to feeding grounds (De Veen 1978).

Begg *et al.* (2000) defined a fish stock as an "intraspecific group of randomly mating individuals with temporal of special integrity". Stock discrimination of plaice is extremely important in forming the basis for fisheries management. Each stock may have unique demographic properties and responses to exploitation or rebuilding strategies (Begg *et al.* 1999b). Each must be managed separately to optimize yield and to ensure sustainable recruitment (Grimes *et al.* 1987). Stock identification is the process that seeks to discern these definite units of individuals. At the 1999 Flatfish symposium van der Veer *et al.* (2001) stated that one of the bigger issues discussed was the lack of information on stock structure. The need for more insight into the methodologies for determining and describing stock structure was highlighted.

Currently stocks can be identified using genetics or phenotypic characters. The basic principle of genetics is to use inherited stable markers to identify genotypes that characterise fish populations (Coyle 1998). Many physiological, morphometric, meristic and calcareous characters have been used to identify fish stocks. They can provide information on stock membership, spatial distribution and phylogeny of stocks (Coyle 1998).

Otoliths are structures composed of calcium carbonate and are ideal for use in stock identification. Variation in growth rate produces corresponding variation in otolith microstructure and shape (Gauldie & Jones 2000). Otoliths grow throughout the lifetime of the fish and are less variable than the growth seen in scales and bones. Once deposited, otolith material is unlikely to be reabsorbed or altered (Campana & Neilson 1985) and, therefore, is unaffected by changes in fish condition which can confound body morphometrics.

The microstructure of the otolith has several components. The hatch check marks the hatching of the fish from the egg. Daily increments are then laid down during the larval period under normal growing conditions. Each ring represented one day's growth (Pannella 1971). When the fish reaches metamorphosis and changes to a demersal stage of life, it lays down 4-6 accessory primordia (AP). These are secondary growth centres and they allow a reader to back calculate the date of settlement (Modin *et al.* 1996, Allen 2004). Otolith increment number is age-dependent whereas increment width is growth dependent. The analysis of daily increments provides important information about early life events and population dynamics and is an important tool in the study of fish populations and stock structure

The thesis presented here uses the microstructure and shape of plaice otoliths to test a number of hypotheses about spatial and temporal variation in early life history parameters, the stock structure of plaice, and the scales of variability between otolith readers.

In chapter two, otolith microstructure is examined to determine if stock structure exists in terms of larval growth and duration between larval plaice from the North Sea, east coast of

Ireland and the west coast of Ireland. The study also determines if larval growth and duration of these stocks vary over time.

Chapter three uses the same adult archived otoliths as chapter two to determine if otolith shape varies between stocks and if so can this variation can help determine if stock structure exists between plaice from the same three ICES areas as chapter two.

Finally, otolith reader variation is examined in chapter four. The identification of increments within prepared otoliths is not an exact science and variation may occur between readers and laboratories. Beamish *et al.* (1983) noted that only 66% of 500 publications reporting fish age estimates even attempted to corroborate the accuracy of their ages. A mere 3.4% were successful in doing so. As quality control monitoring is an important factor in ageing projects, an intra-lab reader experiment was conducted to look at inter-reader ageing bias and precision. The implications of such variation for the interpretation of results from aging studies are discussed.

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Spatio-temporal variation in larval life history of plaice (*Pleuronectes platessa* L.) determined using otolith microstructure in ICES area IVb, VIIa and VIIb

2.1 Abstract

Plaice (*Pleuronectes platessa)* are an economically important flatfish in European waters. However, there remains much to be learned about the life history of this fish especially for the earliest stages. This chapter presents an analysis of temporal and spatial variation in two aspects of the early life history of plaice: length of the larval life and larval growth rate. Archived otoliths (earstones) from adult fish were used for the investigation. Samples were obtained from collections held by the Marine Institute in Ireland, Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in England and the Animal Science Group in Wageningen University in the Netherlands. Three randomly selected year classes of plaice were examined; 1990, 1997 and 2001. Three areas in Western Europe were chosen for the investigation of spatial variability. These regions were based on international fishing divisions; ICES area VIIa (Irish Sea), VIIb (west of Ireland) and IVb (North Sea). Otoliths were polished and image analysis conducted. Statistical analysis of the otolith microstructure revealed that there was no significant temporal difference in larval growth rate and duration over the ten year study period at any location. With regards to spatial differences, there was no significant difference between plaice from the Irish Sea and west of Ireland for both larval growth rate and duration. However, VIIa (Irish Sea) and VIIb (west of Ireland) differed significantly from the North Sea plaice. Larval duration is shorter and growth rate is faster in plaice from VIIa and VIIb. A possible link with sea temperature and food quantity is explored and the results are discussed in the context of climate change.

2.2 Introduction

A stock is a group of individuals for which population parameters can be meaningfully estimated for specific management applications, typically fishery stock assessments (Begg & Waldman 1999).

Plaice in Ireland are treated as different stocks for the purpose of allocating TAC's (total allowable catch) i.e. Celtic Sea, West of Ireland, Irish Sea and Southwest of Ireland. However, this separation is based on the geography of fishing activity rather than any scientific justification.

Lumping together of harvested populations with little gene flow can have a detrimental local effect. Bailey (1997) stated that complex population structure has major implications for management. Ovenden (1990) also observed that the homogenous exploitation of a resource composed of numerous unidentified populations can lead to the erosion of structure and possible extinction of a section of the resource.

There are two general methods by which stocks are defined: by genotype and by phenotype. Generally, subpopulations of marine fish populations show low genetic differentiation (Ward & Grewe 1994). This is probably because in the marine environment there are fewer barriers to dispersal than in terrestrial or freshwater environments, so there is greater genetic exchange between subpopulations. However, the level of gene flow that can make subpopulations genetically indistinct is low compared to the amount of exchange that would be required to allow replenishment of one subpopulation by another. In the absence of genetic variation, phenotypic markers remain important for identifying groups of fish that for management purposes should be treated as distinct stocks. The combined approach of multiple characters examining differing aspects of the spatial relationship of groups of individuals through time has led to the increasing use of a "holistic" approach, combining both genotypic and phenotypic concepts of stock identification (Hare 2005).

Plaice in the North Atlantic show high genetic homogeneity. However, the level of stock mixing needed to make them genetically homogenous may be too low to consider them as a single unit for management purposes. A study by Watts *et al.* (2004) looked at genetic differences between plaice within the Irish Sea and genetic variability in all samples was low. Hoarau *et al.* (2002) showed that European plaice consist of two genetically distinct entities: the continental shelf and Iceland.

Phenotypic traits such as morphology, growth, development rates and behaviour are all influenced by a combination of environmental and genetic factors. Persistent phenotypic differences between stocks, whether environmental or genetic in origin, can indicate limited mixing and may be sufficient cause for stock separation for management purposes (Swain & Foote 1999). Phenotypic stocks are most often defined using characters from juvenile and adult fish collected at different locations. Brophy *et al.* (2002) looked at herring otoliths from the Irish Sea and were able to separate stocks into autumn and winter spawned fish, based on otolith growth rates. Prolonged separation of postlarval fish in different environmental regimes can produce variation in early life history traits such as hatching and settlement times and larval and juvenile growth rates. Growth rates and larval duration are influenced by temperature and food availability (Folkvord *et al.* 1997). Hydrological characteristics such as fronts and eddies also have a large effect on the transport of plaice to nursery grounds and in maintaining stock separation (Wegner *et al.* 2003). Although phenotypic methods do not provide evidence for separate breeding populations, they can be more appropriate tools for defining stocks then genetic studies (Coyle 1998).

The studies referenced above and the current study all used otoliths to determine phenotypic differences between stocks. The reason is otoliths are good indicators of variation in early life history traits because they grow incrementally, throughout the life of the fish at a rate that is proportional to fish growth. Otolith daily increments provide a permanent record of past growth that is unaffected by the fish's metabolism (unlike scales and bones) (Campana &

Neilson 1985). When differences in the larval portion of the otolith are observed in juvenile and adult fish from different areas, it indicates that the groups were spatially or temporally distinct as larvae, and may indicate distinct spawning origin. Karakiri *et al.* (1991) compared larval growth, date of settlement and subsequent growth of 0+ plaice from two different locations in the Wadden Sea. They found differences in hatching times, larval period, settlement and growth rates between fish from different locations. This indicates that the fish had experienced different early life environments and therefore originated from different spawning grounds.

The current study uses archived collections of otoliths to investigate if adult plaice from three regions: ICES IVb (North Sea), VIIa (Irish Sea) and VIIb (west coast Ireland), display any variation in early life history traits (larval growth rate and duration) which would indicate that they are of different larval origin. Abiotic factors such as temperature and food availability were examined to see if they accounted for any variation detected. Any correlation between larval duration and growth rate will also be discussed. Otoliths from three year classes; 1990, 1997 and 2001 were used to investigate if these early life history traits varied temporally. Archived otolith collections provide an easy and inexpensive source of information on various fish species. They also provide a long term data set that can help scientists to distinguish trends from short-term fluctuations (Hutchinson *et al.* 2003). Information gleaned from the otolith collections in the present study may help to determine stock structure within the ICES areas and provide data to support the sustainable management of plaice fisheries.

2.3 Materials and methods

Three pairs of otoliths are located posteriorly in the head of the fish and these are called the lapilli, sagittae and asterisci. In the present study, all work was carried out using the sagittal otoliths.

Three different areas in western Europe were chosen for the study, ICES area VIIa (Irish Sea), VIIb (West of Ireland) and IVb (North Sea) (*Fig.* 2.1). The otoliths included in the analysis were from three randomly selected year classes: fish hatched in 1990, 1997 and 2001. The fish were 4 years old (as aged by the Marine Institute) on date of capture except in ICES IVb. Some fish from this region were caught at 3 years of age. However they still came from the same year class. Five otoliths from each year class were picked randomly from three different hauls in each ICES area, using random number tables. This gave a total of 135 otoliths for analysis.

***Fig* 2.1: Map of Western Europe showing ICES divisions**

Otolith preparation

The otoliths from CEFAS and the Netherlands were stored dry in envelopes whereas the otoliths from the Marine Institute were stored in "Histokit" single pack resin. These latter samples were removed from the resin by heating on a hotplate. As the resin melted the otolith was picked out using a forceps. Otoliths were stored in "Sterilin" square otolith boxes prior to analysis.

Otoliths were photographed using an Olympus camedia C-3040 digital camera with PC interface attached to an Olympus SZX 7 stereoscopic microscope at 16X magnification. The otoliths were examined and annuli counted to confirm age. Each otolith was then attached to a slide using crystal bond glue. They were polished using wet "Buehler" silicon carbide paper, grit 600µm and 1200µm. The non-sulcus side (concave side) was polished first until the accessory primordium (AP) was visible using an Olympus CX21 compound microscope with an X100 oil immersion objective lens and an overall magnification of X1000. After the first polishing, each otolith was placed in the cap of a 0.5ml micro centrifuge tube and embedded in Buehler Epo-thin epoxy resin. The resin was allowed to set at room temperature over 24 hours. When the resin had set, the otoliths were cut out of the caps using a scalpel and attached to a glass slide using crystal bond glue. Polishing was carried out on the sulcus side with the same grit paper until the microstructure at the otolith core was visible under the microscope.

If daily increments were difficult to discern their contrast was enhanced by etching in a buffered proteinase solution as described in (Shiao *et al.* 1999). Proteinase digests the protein portion of the otolith matrix and retains the major calcified structure to reveal conspicuous daily increments. The otoliths were placed in "Sterilin" square Petri dish containing 25 compartments and covered in the proteinase solution. The Petri dish was then put into a Binder oven at 45°C for 1 hour. The otoliths were taken out periodically over the hour and gently shaken. This allowed the rings to be read with more clarity.

Temperature data were sourced from two locations. "The coastal temperature and ferry route programme: long-term temperature and salinity observations" produced by CEFAS, provided temperature information for ICES area IVb and VIIa. Data for VIIb was obtained from the Marine Institute weather buoy M1 and the National Oceanographic and atmosphere Administration (NOAA) website. (URL: osdpd.noaa.gov/PSB/EPS/SST/sst_and_fields.html). The temperature data time series was averaged. The *Calanus* abundance data were obtained from the continuous plankton recorder (URL: www.dpst@sahfos.ac.uk.) for the years 1990, 1997 and 2001, from January to June. It records the phytoplankton colour index and *Calanus finmarchicus* numbers present, which can be used when assessing food availability for larval plaice. The examination of food availability may help account for any variation in larval duration and growth rate.

Image analysis

Polished otoliths were viewed at 1000X magnification using an Olympus CX41 light microscope. Images of the microstructure were taken using an Olympus camedia C-3040 zoom digital camera. They were processed using Olympus DP- Soft 3.2 image analysis package.

Otolith images were used to estimate the length of larval life and the duration of metamorphosis from counts of daily increments. Increment counts were started from the hatch check which is approximately 10μm from the centre of the otolith (Hovenkamp 1990). Counts were terminated at the first accessory primordium (AP) which demarcates the start of metamorphosis and the end of larval life (Karakiri *et al.* 1989) (Plate 2.1).

Larval increment widths were calculated using the DP-soft programme to provide an estimate of larval growth rates. Each daily growth increment for the fish from each separate ICES area over the three years studied was averaged over a 29 day period. 29 days was the minimum length of larval life observed. Average daily otolith growth is correlated with average daily

somatic growth and the average increment width can be used as an indication of somatic growth (Hovenkamp 1990). Karakiri *et al.* (1989) also showed that daily increment width is growth dependent. For reporting purposes otolith increment width results will represent and be referred to as growth rate.

All measurements were transferred to Microsoft excel for subsequent data analysis.

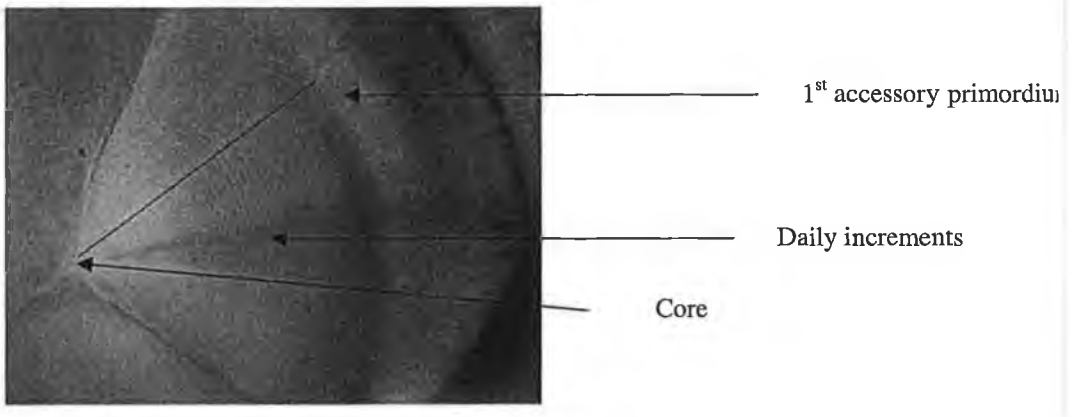

Plate 2.1: Polished otolith microstructure. Measuring line extends out to 1st AP

Data Analysis

All statistical analysis was carried out using MINITAB 15 for windows. Data were tested, where possible, using analysis of variance (ANOVA) which has three assumptions. All data must be independent, the distribution in each of the data groups around the mean must be normal and finally the data must have homogeneity of variance. Normality testing was conducted using the Ryan-Joiner test and for homogeneity of variances Cochran's test was used. Where needed, data were normalised using log transformations. If data could not be normalised, non-parametric tests, Kruskal-Wallis and Mood's median, for significant differences were used (Underwood 1997). When the data proved normal and had equal variances, analysis of variance (ANOVA) was conducted on the balanced set to test 2 hypotheses:

1. There is a difference in plaice larval growth and duration between the three year classes.
2. There is a difference in plaice larval growth and duration between ICES areas VIIa , VIIb and IVb .

The ANOVA design consisted of two orthogonal factors: year and ICES areas and one nested factor: haul. Year and haul were considered to be random factors and region was a fixed factor.

Where ANOVA detected significant differences in the main factors, *post hoc* analysis to compare pairs of means was carried out using Tukey pairwise comparison tests.

Larval growth rates were also examined over a series of five day periods; e.g. 1-5, 6-10, 11-15, 16-20, 20-25 and 26-29 days after hatching. This was to examine how growth rates differed between the three ICES areas over the larval duration.

Correlation analysis was used to investigate the relationship between growth rates at days 20-25 and growth rates from days 1-29, to establish if growth within the five day period (20-25) was representative of growth throughout the larval phase. Daily rings are easier to read around day 20. Therefore, if days 20-25 could be used in analysis instead of the whole larval duration, errors in interpreting the data might be reduced.

Larval duration was calculated by averaging the number of days till metamorphosis in each separate ICES area for each year class. Larval duration was at least 29 days for all fish included in the analysis. Therefore, this 29 day period after hatching was used to calculate mean larval otolith growth rates.

2.4 Results

Overall results revealed that plaice from ICES area IVb had a longer larval duration and a slower growth rate than fish from the other two ICES areas (Table 2.3).

Table 2.3: Table showing mean larval duration and growth rates of plaice in three different ICES areas

Year class	Ices area	mean daily increment width/um	SD(+/-)	mean larval duration/days	SD(+/-)
1990	IVb	1.16	0.22	44	5.3
	VIIa	1.5	0.31	35	6.1
	VIIb	1.3	0.19	38	3.7
1997	IVb	0.97	0.17	50	6.4
	VIIa	1.54	0.24	35	3.2
	VIIb	1.39	0.24	37	4.3
2001	IVb	1.04	0.29	52	9.7
	VIIa	1.32	0.18	38	5.1
	VIIb	1.44	0.25	37	4.1

Larval duration

The mean larval duration of plaice from area IVb in the three year classes studied was 49 days, the average on the east (VIIa) and west (VIIb) coast of Ireland was 36 and 37 days, respectively (Table 2.4 and *Fig* 2.2).

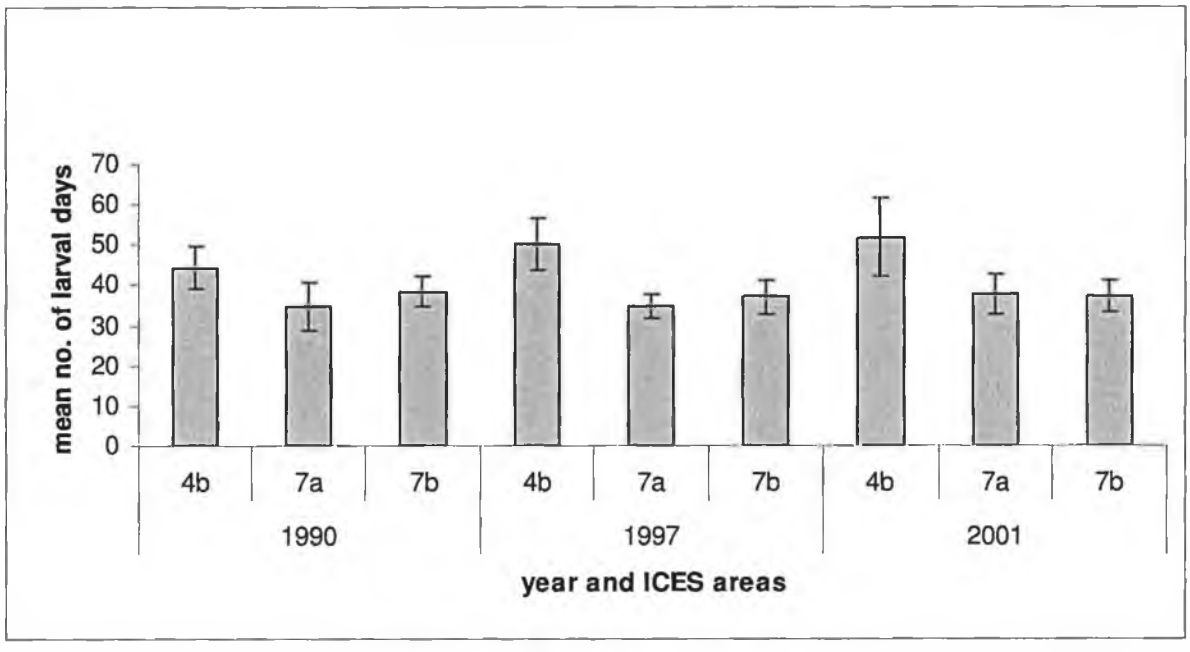

***Fig* 2.2: Graph showing mean larval duration of plaice in three different ICES areas over different time periods. Error bars represent standard deviations**

Table 2.4: Table showing the mean, range of days and standard deviation of larval duration in each ICES area, averaged across three year classes (1990, 1997 and 2001)

ICES Area	Range/days	Mean no. of days	SD (+-)
IVb	37-64	48.8	7.9
VIIa	29-49	35.5	5.0
VIIb	31-48	37.6	4.0

ANOVA showed that there was no significant difference in plaice larval duration between the three years studied ($p>0.05$) within any region. However, analysis did reveal a significant difference in larval duration between regions. There was no significant interaction between year and ICES area (Table 2.5). *Post* hoc analysis showed that the significant differences seen between ICES areas were due to area IVb. The mean larval duration was significantly longer in the North Sea samples than in the other two areas ($p=0.03$).

Table 2.5: Results of a two way ANOVA comparing larval duration between year classes 1990, 1997 and 2001 and ICES areas, VIIa and VIIb

Source	Mean Sq	d.f.	F ratio	p	r^2
Year	0.01	2	1.22	0.39	64.19%
ICES area	0.23	2	28.1	<0.01	
Year/ICES interaction	0.01	4	1.92	0.15	

There was no significant difference in larval duration between areas VIIa and VIIb ($p>0.05$). Years were pooled and a one-way ANOVA was performed to see if any difference in larval duration could be detected between areas VIIa and VIIb with a larger sample size. Results still showed no differences.

Growth rate

Statistical analysis (ANOVA) showed that there was no significant difference in larval growth rates from days 1-29 within any area for any year class. However, analysis did reveal a significant difference in growth rates between ICES areas ($p=0.03$) (Table 2.6). *Post hoc* analysis revealed that ICES IVb had a slower growth rate then the other two ICES areas (*Fig.* 2.3).

Table 2.6: Results of a two way ANOVA comparing larval growth rates between year classes 1990, 1997 and 2001 and ICES areas IVb, VIIa and VIIb from days 1-29

Source	Mean Sq	d.f.	F ratio	p	r^2
Year class	0.03	2	0.17	0.85	53.66%
ICES area	2.03	2	10.43	0.03	
Year/ICES interaction	0.2	4	2.19	0.11	

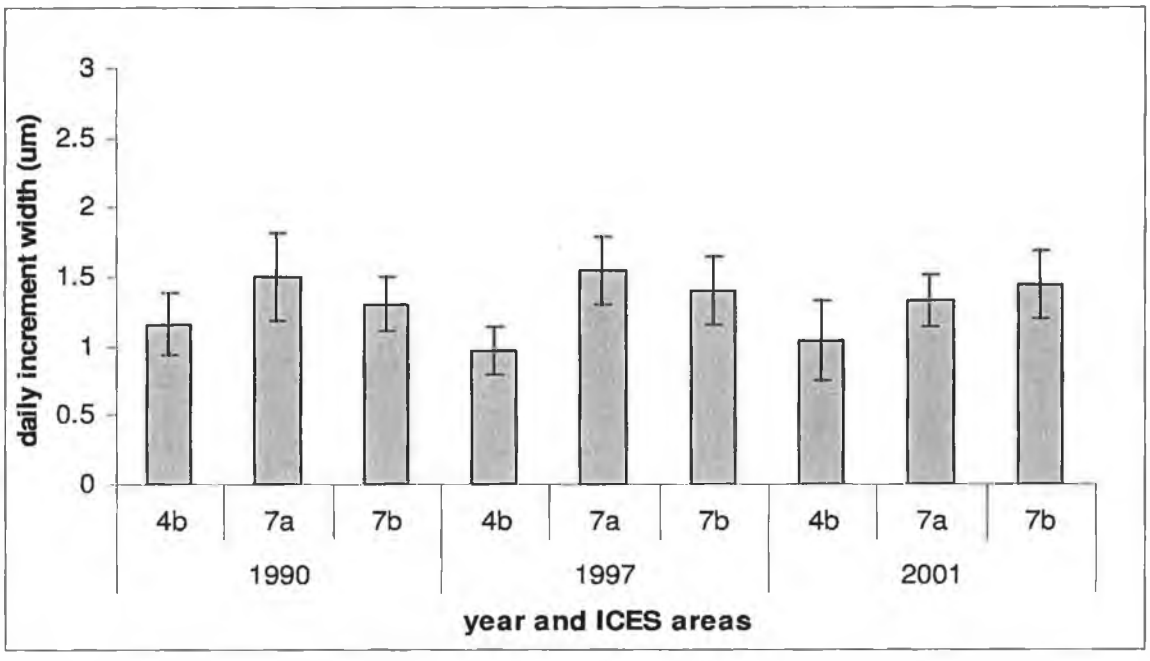

***Fig* 2.3: Graph showing the mean larval growth rates (μm) of plaice over different year classes and from three different ICES areas. Error bars represent standard deviations**

Years were pooled to increase the power of the analysis and a one-way ANOVA was performed to see if any differences in growth occurred between VIIa and VIIb. Results still showed no significant differences between the two areas ($p>0.05$).

When growth rate was examined in 5 day blocks, results showed no significant difference between both years and areas in growth rate until day 16. Significant difference occurred in growth rate at days 16-20 ($p=0.03$). There were also significant differences recorded from days 21-25 and 26-29 (*Fig.*2.4). A Bonferoni correction was conducted because multiple comparisons were performed (Table 2.7). After the correction, only days 21-25 showed significantly growth rate differences when compared to growth rate obtained from the data of the 29 day period. *Post hoc* analysis revealed that ICES area IVb had slower growth rates than VIIa and VIIb. No temporal difference in daily growth rate was found for any ICES area ($p>0.05$).

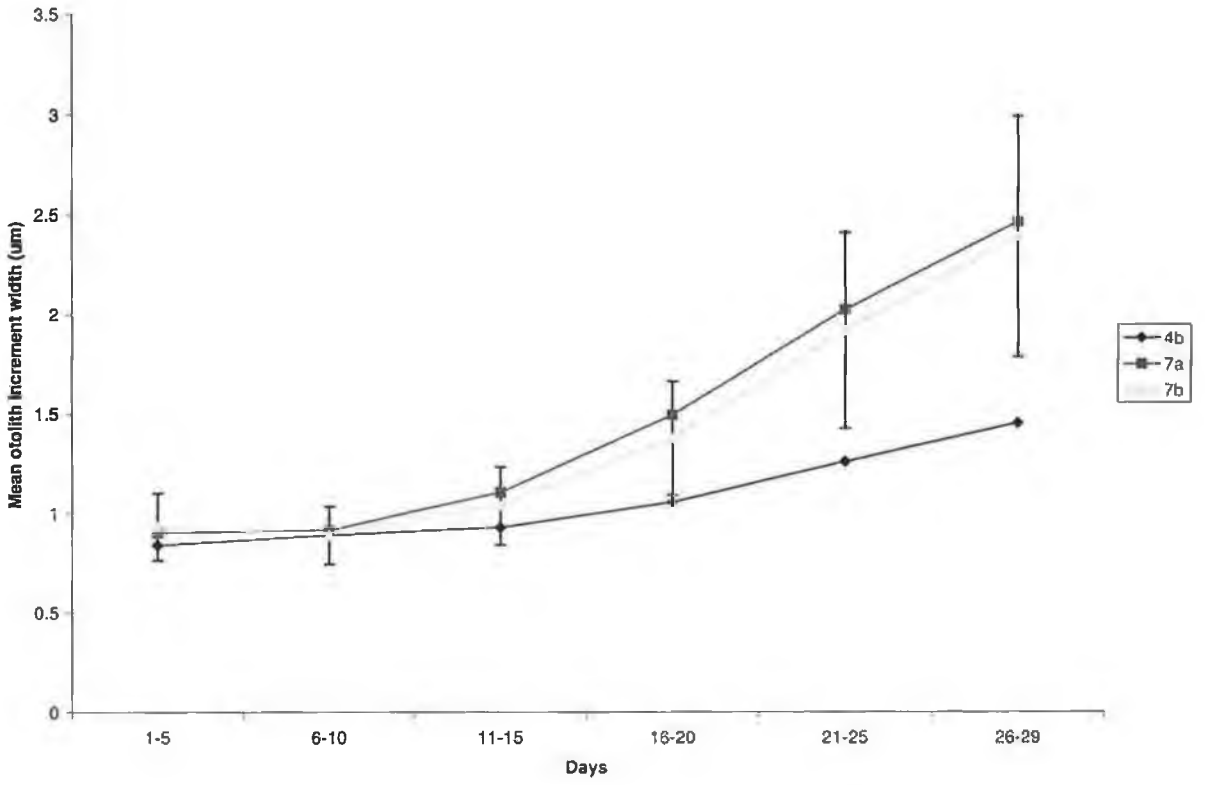

***Fig* 2.4: Mean otolith increment width (days 1-29) of larval plaice in three ICES areas**

Table 2.7: Mean p-value of larval growth rates for year class 1990, 1997 and 2001 in ICES area IVb, VIIa and VIIb in 5 day blocks

Days	p-value
1-5	0.132
6-10	0.584
11-15	0.152
16-20	0.035*
21-25	0.009**
26-29	0.012*

* shows significant difference of 0.05

** shows significant difference after Bonferoni correction

A significant positive correlation was observed between growth at days 1-29 and days 20-25 (correlation coefficient=0.762; p=0.010). Statistical analysis was carried out again for larval growth rates using days 20-25 instead of days 1-29. The same results were observed (*Fig* 2.5);

there was no significant temporal difference in larval growth rate over the study period in any area but there was significant difference between ICES areas (Table 2.8). ICES IVb had a slower growth rate ($p<05$).

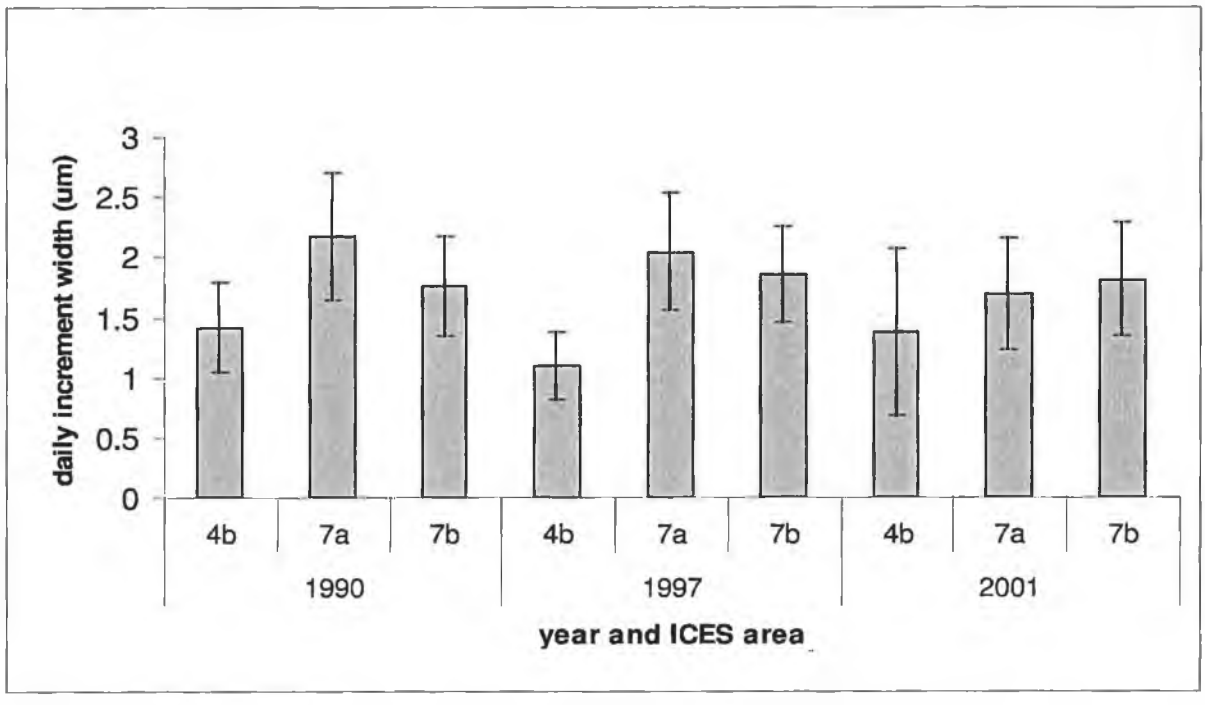

***Fig* 2.5: Graph showing the mean larval growth rates at days 20-25 of plaice from three year classes and from three different ICES areas. Error bars represent standard deviations**

Table 2.8: Results of a two way ANOVA table comparing larval growth rates between year classes 1990, 1997 and 2001 and ICES areas IVb, VIIa and VIIb from days 20-25

Source	Mean Sq	d.f.	F ratio	p	r^2
Year class	0.04	2	1.12	0.41	59.00%
ICES area	0.6	2	18.79	0.01	
Year/ICES interaction	0.03	4	1.28	0.31	

Results showed a strong negative correlation between growth rate and larval duration in the three different ICES areas over time (Table 2.9). As growth rates increase, larval duration decreases (*Fig*. 2.6).

Table: 2.9: Table showing correlation between growth rate and larval duration in three ICES areas over time

ICES area	pearson's correlation coefficient	p value
IVb	-0.754	0.000
VIIa	-0.789	0.000
VIIb	-0.651	0.000

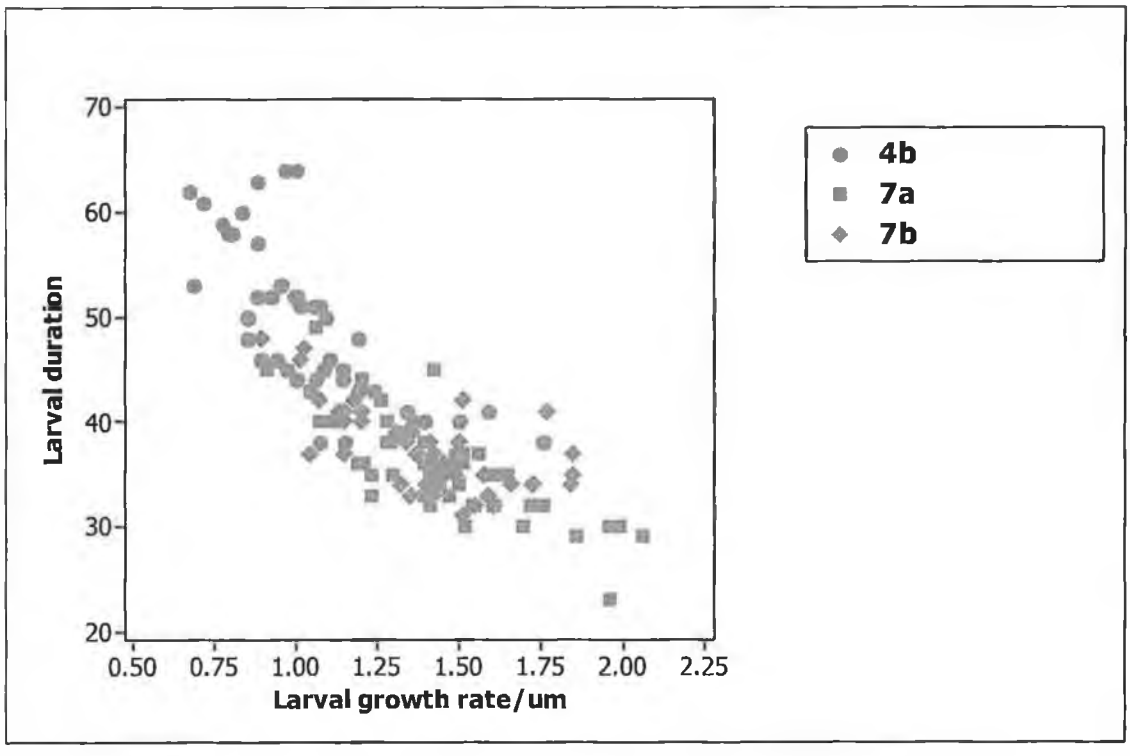

Fig 2.6. **Scatterplot showing strong negative correlation between larval growth rate and duration**

Results from the phytoplankton colour index revealed more phytoplankton present in ICES VIIa and VIIb then ICES IVb (Table 2.10). The *Calanus finmarchicus* data showed ICES area VIIa to have the lowest levels and VIIb with the highest.

Table 2.10: Average phytoplankton colour index and *Calanus finmarchicus* numbers in three ICES areas from Jan-Jun in 1990, 1997 and 2001.

	VIIb	VIIa	IVb
phytoplankton colour	0.5	0.85	1.89
Calanus finmarchicus	0.75	0.24	0.46

Results from the temperature data described average sea temperatures between January and June as coolest in the North Sea, intermediate in the Irish Sea and warmest off the west coast of Ireland (Table 2.11).

Table 2.11: Average sea surface temperatures (°c) from Jan-Jun in three ICES areas in 1990, 1997 and 2001

Year class	IVb	VIIa	VIIb
1990	8.6	9.2	10.3
1997	8.1	8.9	10.7
2001	7.9	9.2	10.2

2.5 Discussion

Larval duration

Results revealed that there was no significant temporal difference in larval duration over the study period in any of the three ICES areas. Other studies have shown that the principal extrinsic factor influencing the rate of development of larval plaice is temperature (Ryland & Nichols 1975). Therefore, results from the current study would suggest that temporal variation in environmental factors such as temperature, over the time period of the study, was not sufficient to produce variation in larval duration. Similar results were also observed in a study conducted by van der Veer *et al.* (1999) in North Sea plaice. They found that despite the interannual differences in temperature (from 6.5°c to 10°c) no differences between years were found with respect to larval size and morphological stage at the time of immigration or the timing of immigration to inshore nursery grounds.

While no temporal variation occurred, spatial differences were observed. Fish in ICES area IVb (North Sea) had a longer larval duration than fish from the other two ICES areas. This would imply that North Sea fish were exposed to different environmental conditions such as temperature and food abundance, as larvae. Plaice from ICES area VIIa and VIIb showed no

significant variation in larval duration. This indicates that the adult fish in these areas were exposed to similar environmental conditions as larvae. This may be because the environment of the Irish Sea is not sufficiently distinct from that of the West coast and larvae developing in both areas have similar development times. Alternatively, larvae from the two areas grow differently, but fish from the two areas mix as adults at feeding and spawning grounds, and so no spatial variation in larval microstructure is detectable in the adults.

The larval duration of plaice from the North Sea observed in this study is shorter than those reported in other studies. In the Wadden Sea (ICES IVb), Karakiri *et al.* (1991) found that larval duration ranged from 50-82 days. For the North Sea Wegner *et al.* (2003) found that the general larval phase of plaice takes from 60 to 90 days. Ryland (1966) conducted tests in a lab and found that larval duration is temperature dependent and is usually between 60 and 70 days at 6.75°c. These larvae were collected as eggs in the southern area of the North Sea. In the current study larval duration for plaice from the North Sea was shorter, ranging from 37 to 64 days. This discrepancy may arise due to movement of plaice from other areas (characterised by shorter larval durations) into the North Sea after the larval stage or inter reader / inter-lab differences. Inter-lab calibration would be required to determine whether reader variation is the source of the observed larval duration differences.

Larval duration for plaice from the Irish Sea was also shorter than other studies, ranging from 29 to 49 days (mean = 35). In the Irish Sea (Al-Hossaini *et al.* 1989) recorded larval duration values of between 42-59 days for plaice with mean larval increments ranging from 50 to 53 days.

Plaice from the west coast had larval durations ranging from 31-48 days (mean = 37). This is a slightly longer larval duration then found in previous studies. A study conducted by Allen *et al.* (2008) found larval duration times of 28-43 days (mean=33) on the west coast of Ireland. (Allard 2006) recorded larval duration in the west coast of Ireland between 21 and 45 days (mean=30).

Results from these previous studies suggest that larvae from the west coast of Ireland reached metamorphosis more quickly then Irish Sea plaice and this would support the theory that larvae from the two areas are spatially separated but mix as adults. In order to confirm this, sampling of juveniles still in the nursery grounds in both areas and before they mix would need to be carried out. If the larval duration of fish was shorter on the west coast, it would confirm that they are from two separate stocks.

Growth rate

Results revealed that there was no significant temporal difference in growth rate over the study period in any of the three ICES areas. However, spatially fish in ICES area IVb had a slower growth rate than fish from the other two ICES areas. Again, this would imply that North Sea fish were exposed to different environmental conditions as larvae. Plaice from ICES area VIIa and VIIb showed no significant variation in larval growth rate. This indicates that the adult fish in these areas were exposed to similar environments.

Daily growth rate for North Sea fish ranged from 0.9µm-1.2µm over the ten year period. A study by Karakiri *et al.* (1991) recorded daily growth rate of 0.2µm-0.8µm in plaice from the Dutch Wadden Sea. This is a slower rate of growth then the results from the current study. However, the above study only measured the first few increments after hatching Another study on North Sea plaice by Hovenkamp (1990) found daily increment widths ranging from 0.7µm-1.5µm, which is similar to the current results.

In the current study increment widths ranged from 1.3µm to 1.44µm over the ten year period for Galway Bay. This is a slower growth rate than that reported by Allen *et al* (2008) who observed growth rates ranging from 1.94µm to 2.79µm in Galway Bay. However, the later study only measured increments representing days 20 to 25 after hatching. As shown in the

results above this period is when a significant amount of growth occurs in plaice larvae (*Fig.* 2.4). and this may account for the differences between the two studies.

No spatial or temporal variation in larval otolith growth was apparent before day 16 in plaice from the three different ICES areas. Larval growth rates, determined from laboratory and field observations, are often slow with little variation during the yolk-sac stages, which persist for some 12 days as seen by Ryland (1966) in lab experiments, but increase rapidly from first feeding until metamorphosis (Nash & Geffen 2005). This would explain the slower growth rate reported by Karakiri as seen above because that study only used the first few days after hatching as a measure of growth rate. Also, growth rates may appear uniform before day 12 because the measurement of increments is not sufficiently precise to detect differences. Because of the difficulties associated with detecting variation early in the larval growth, increments from days 20-25 were used as an index of growth in subsequent analyses. The width of these increments was strongly correlated with increment width over the whole larval phase, justifying the use of this index.

As stated earlier, spatial variation in larval duration and growth rates observed in this study may be related to environmental conditions, such as food abundance. In general, there is a positive relationship between food levels and growth rate (Nash *et al.* 2005). Before metamorphosis, larvae feed on planktonic prey in the water column. In the current study, phytoplankton colour index and *Calanus finmarchicus* numbers were used as an indicator of food availability. Results show that ICES IVb had the lowest level of phytoplankton recorded, followed by VIIa and VIIb. This concurs with observed growth rates in the current study. Fish from the North Sea which had a slower growth rate also had less food available to them then plaice from the other two ICES areas. A study by Folkvord *et al.* (1997) used different feeding environments to determine growth of juvenile herring in the lab. Herring exposed to higher plankton densities had wider otolith increment widths. More data is needed to examine the correlation between food abundance and growth.

A second possible explanation for the differences in growth rate and larval duration between ICES areas is temperature differences between the cooler North Sea and the warmer waters found in VIIa and VIIb (Table 2.8). Fox *et al.* (2000) described average sea temperatures between January and June as coolest (7°c) in the North Sea, intermediate in the Irish Sea (8.9°c) and warmest in the Celtic Sea (10.5°c). Hovenkamp *et al.* (1991) showed that somatic growth rates of plaice larvae were strongly related to water temperatures. Bergman *et al.* (1988) found that growth of plaice in the Wadden Sea depends only on ambient water temperatures. Another study by Hyder *et al.* (1998) found increasing the temperature lowers the development time, increases the numbers at metamorphosis and lowers the pelagic daily instantaneous mortality of plaice in the Irish Sea. Fox *et al.* (2000) found that warmer sea temperatures led to shorter larval durations. The temperature results from the current study concur with the previous studies. Results show that sea surface temperatures were highest in ICES VIIb and fish had the lowest larval duration. Larval duration was longest in the North Sea where the temperatures were the lowest.

Results from the current investigation also revealed a negative correlation between larval duration and growth rate; fish which grew faster as larvae had a shorter larval duration. As discussed above, temperature seems to be the main driving force in relation to growth rate, larval duration and overall year-class strength. An increase in temperature (as with climate change) could affect larval survival and development in many ways. Rapid larval development may increase the chance of larvae reaching the nursery grounds and thus produce a strong year class. An increase could offset the effect of predation in relation to the decreased larval duration and increased growth rates which would make the fish too big to be prayed upon. A study by van der Veer *et al.* (2000) revealed that, after severe winters where the number of predatory crustaceans are reduced, early flatfish juveniles grow to sizes which are too large for recovered crustacean stocks to predate in June. However, higher temperatures may also cause plaice to reach metamorphosis earlier and therefore not reach the safety of the nursery grounds. In the North Sea, there are larger distances between the

spawning grounds and the nursery grounds, unlike the Irish Sea where both spawning and nursery ground are close by (Hyder *et al.* 1998).

Otolith reader effects must also be considered when comparing larval growth and duration differences from different studies. Variation between labs in the preparation or interpretation of increments could produce differences. Are the observed differences greater then differences created by variations between labs or readers? In an overview of otolith studies entering the 21st century, Campana (2005) comments on the developing maturity of otolith microstructure research and the relative scarcity of routine daily increment validation studies. In chapter four, results from an inter-reader experiment from the author's laboratory is conducted to see if inter-reader variation is great enough to explain observed differences in age? If this is the case it would require inter-reader and inter-lab calibration to resolve. However all the otoliths in the current study were read by the same reader so this would negate and inter-reader variation.

Stock identification is an integral component to fisheries stock assessment and is essential for effective fisheries management. The mixing of fish stocks will have a huge effect on how they are managed. If stocks are not detected they could be put under pressure with regards to recruitment. The results in this study indicate that the management units of the North Sea and the other two ICES areas contain distinct populations with limited mixing between them. Therefore, the treatment of the North Sea stock as separate from the others would be an appropriate decision in terms of management. At the moment ICES treat plaice from ICES VIIa and VIIb as distinct stocks, with no mixing. However, different mixing rates that are not detected can yield grossly different predictions of abundance trends (Kell *et al.* 2004). Results suggest the possibility that stocks in ICES VIIa and VIIb are mixing after the larval stage. Additional research is needed to confirm that they are separate stocks at the larval stage. As suggested earlier in the discussion, sampling at the juvenile stage before fish leave

the nursery grounds would help confirm the stock mixing theory. Tagging of these juveniles could also be examined to see if the fish are mixing when they move offshore.

Other aspects of otolith structure can be used in determining different stock or life histories of fish. Trace element composition of otoliths has been examined elsewhere, to determine the relationship between water composition and the resulting composition of the otolith. The following chapter will look at otolith shape as a method of stock identification using the same archived samples as used here. This study may throw some light on the findings from the current study.

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Spatio-temporal variation in adult plaice (*Pleuronectes platessa* L.) determined using otolith shape in ICES area IVb, VIIa and VIIb

3.1 Abstract

Stock identification is paramount in managing fish stocks. In this chapter the otoliths (earstones) of adult plaice were examined to see if fish from different geographical locations could be identified by otolith shape. Otolith shape was characterised using indices calculated from basic manual distance measurements. Archived otoliths from adult fish were used for the investigation. Samples were obtained from collections held by the Marine Institute in Ireland, CEFAS in England and the Animal Science Group in Wageningen University in the Netherlands. Three randomly selected year classes of plaice were examined; 1990, 1997 and 2001. Three areas in Western Europe were chosen for the investigation of spatial variability. These regions were based on international fishing divisions; ICES area VIIa (Irish Sea), VIIb (west of Ireland) and IVb (North Sea). The shape of the first annulus and the whole otolith shape were measured. Statistical analysis of the results showed little variation in otolith shape in plaice from the three ICES areas. Analysis of whole otolith shape shows a small degree of variation in one shape variable (circularity). This study shows that shape indices are not very powerful for discriminating between plaice from different ICES areas. Reasons for lack of discrimination and other phenotypic markers that might be useful are discussed.

3.2 Introduction

Stock structure information provides a basis for understanding fish populations and this knowledge is vital in management. Persistent phenotypic differences, whether environmental or genetic in origin, can indicate limited mixing of fish populations, and may be sufficient cause for stock separation (Swain & Foote 1999). Otoliths, which are structures composed of calcium carbonate are ideal for use in stock identification (Campana & Neilson 1985). The chemical composition of otoliths has been examined as a viable method of stock identification. Changes in concentrations of elements in the environment can influence the concentrations of these elements in the otoliths of fish. The array of elements can characterise different stocks or sub-populations. Geffen, Jarvis *et al.* (2003) looked at spatial differences in the trace element concentrations of Irish Sea plaice. Significant differences in otolith composition were found between sampling sites. The analysis revealed separation between groups of plaice in the eastern Irish Sea indicative of metapopulation structure associated with known spawning grounds. Variation in the growth rate of the fish, produces corresponding variation in otolith microstructure and shape (Gauldie & Jones 2000). Otoliths grow throughout the lifetime of the fish and are less variable than other somatic growth structures such as scales and vertebrate which also lay down growth rings. Once deposited, otolith material is unlikely to be reabsorbed or altered (Campana *et al.* 1985), and is, therefore, unaffected by changes in fish condition which can confound body morphometrics. The shape of fish otoliths can therefore be an ideal marker for fish populations if they maintain distinct environments during some or all of their lives. Variation in otolith shape in fish from different geographical regions is assumed to provide evidence that distinct regions are occupied, at least for part of the life history, thereby representing a phenotypic measure of stock identification (Ihssen *et al.* 1981).

Plaice in the North Atlantic show high genetic homogeneity. Therefore, shape analysis may be useful for discriminating between stocks where genetic methods have failed. If juvenile

plaice from different areas produce otoliths with distinctive shapes from this part of their life history, shape analysis could provide a simple yet accurate method of identifying different fish stocks. Tuset *et al.* (2003) used otolith morphology to identify regional differences in Comber (*Serranus cabrilla)* from the Mediterranean and Atlantic regions. Friedland *et al.* (1994) also used otolith morphology in stock discrimination of Atlantic salmon (*Salmo salar*). They looked at otolith shape as an indicator of both continent of origin i.e. North America or Europe, and country of origin.

Otolith shape can be characterised using indices which are calculated from basic manual distance measurements such as length, width, area and perimeter, or on more complex methods that use mathematical functions such as Fourier descriptors. These are mathematical descriptions of an otolith silhouette that can describe and compare otolith shapes quantitatively (Bird *et al.* 1986). It is also possible to measure the shape of the otolith at a previous point in the fish's life by using the outline of an internal feature such as an annulus (Burke *et al.* 2008). It is thought that a well defined internal annulus corresponds to the shape of the otolith at the time of the annulus formation. Burke *et al.* (2008) assessed the feasibility of using shape parameters generated from internal traces to identify juvenile origin in herring from the Irish and Celtic Sea. They were able to distinguish between populations with a >95% degree of accuracy.

Because otolith growth and shape are linked to fish growth, factors affecting fish growth will also affect otolith growth. While there may be a genetic contribution to otolith shape differences between stocks, environmental factors are considered to be the major determinants of otolith growth (Begg & Brown 2000). Several lab experiments confirm that the relationship between otolith and somatic growth of fish is mediated by temperature and /or modifiers of growth rate (Campana 2005). Campana *et al.* (1993) suggested that environmental effects are generally more influential determinants of otolith shape then genetic effects, because otolith shape changes in response to differences in growth rate. Stock definitions based on

differences in otolith structure depend not only on differential growth rates, but also on the consistency of the environmental conditions encountered during the life history of a fish in each stock (Campana *et al.* 1993). The previous chapter reports spatial variation in juvenile plaice life history parameters over three ICES areas listed above, which appear to be driven by temperature. Spatial variation caused by temperature could also result in growth differences and consequently in the size and shape of the otolith after the first year.

The purpose of this study is to examine shape characteristics of plaice otoliths from three different ICES areas in Europe over three time periods to determine if otolith shape measurements can be used to discriminate between plaice from the different ICES management units. Also, the study uses the shape of the juvenile portion of the otolith to establish if the fish from the three areas are of distinct juvenile origin.

3.3 Materials and methods

Three pairs of otoliths are located posteriorly in the head of the fish and these are called the lapilli, sagittae and asterisci. In the present study, all work was carried out using the sagittal otoliths.

The archived otoliths used in this study were provided from the Marine Institute of Ireland, Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in the UK and the Animal Science Group in Wageningen University in the Netherlands. The samples from the Marine Institute were collected from the commercial fish landings. Samples from CEFAS and Wageningen University were collected during research surveys.

Factors such as age, sex ratios and year class may affect otolith shape. Castonguay *et al* (1991) concluded, from an analysis of Atlantic Mackerel (*Scomber scombrus*) stock structure, that confounding effects of age and year-class on otolith shapes need to be assessed carefully

before drawing any conclusions. For this reason, the otolith samples for this study used the same year class of plaice across the three ICES areas. This eliminated any year class effects. Information on the sex of the plaice used in the present research was unavailable. However, other studies such as Bird *et al.* (1986) looked at shape differences between herring otoliths from Atlantic and Alaskan stocks. They found no differences in the shape of the otoliths arising from sexual dimorphism. Castonguay *et al.* (1991) also came to the same conclusion with respect to Atlantic mackerel.

Three different areas in northern Europe were chosen for the study, ICES area VIIa (Irish Sea), VIIb (West of Ireland) and IVb (North Sea). The otoliths included in the analysis were from three randomly selected year classes: fish hatched in 1990, 1997 and 2001. The fish were 4 years old on date of capture except for those from ICES IVb. Some fish from this region were caught at 3 years old. Five otoliths from each year class were picked randomly from three different hauls in each ICES area, using random number tables. This gave a total of 135 otoliths analysed.

Otolith preparation

The otoliths from CEFAS and Wageningen University were stored dry in envelopes whereas the otoliths from the Marine Institute were stored in "Histokit" single pack resin. These latter samples were removed from the resin by heating on a hotplate. As the resin melted the otolith was picked out using a forceps. Otoliths were stored in "Sterilin" square otolith boxes prior to analysis.

Otolith analysis

Each otolith was placed in a black dish (for best background image) and immersed in water. Images were taken using an Olympus camedia C-3040 zoom digital camera attached to an Olympus SZX 7 stereoscopic microscope. The distance between successive annuli (distinct

bands laid down on the otolith each winter) were used as a measure of annual growth (Plate 3.1).

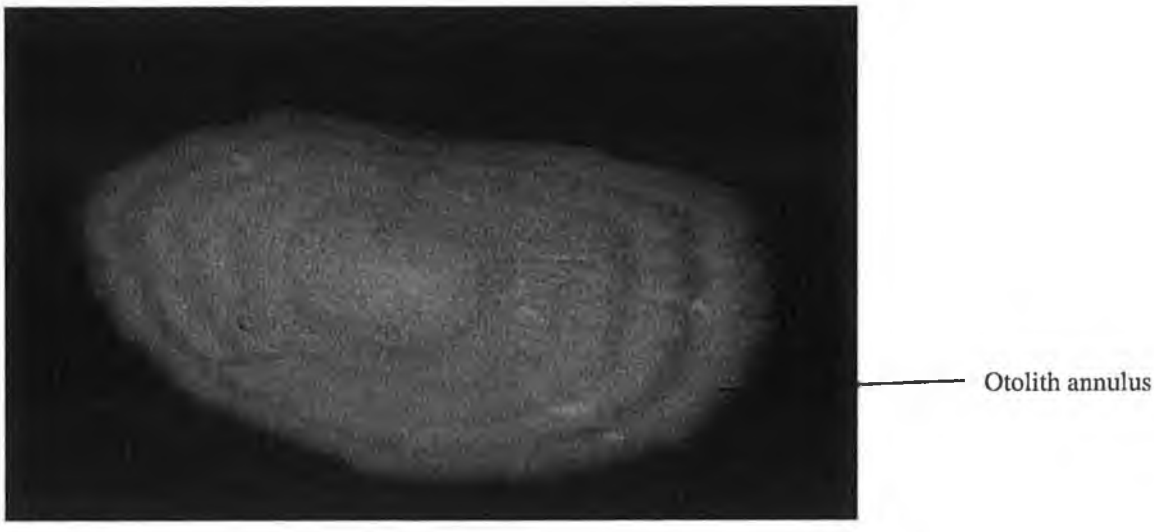

Plate 3.1: Image of a plaice otolith under 1.25x magnification

Measurements were taken from the core to the edge of each annulus along the longest axis using the Olympus Dp-Soft 3.2 image analysis package. This measurement was used as a proxy for fish growth in one year and also confirmed the age of the fish. The outline of the first annulus and the edge of the otolith was traced manually using Olympus Dp-Soft software. A series of two-dimensional measurements was then taken.

For shape analysis, the following size parameters were measured in order to calculate certain shape indices: area (A); perimeter (P); feret width (FW); feret length (FL). Feret length and feret width are the length and width of a box, which encloses the otolith. All measurements were taken using *Olympus* Dp-soft. These size parameters were used to calculate the shape indices. The formulas for the shape indices are described in table 3.1.

Table 3.1: Table showing the size parameters and resulting shape indices calculated for analysis of each otolith

Size parameters	Shape Indices
Area (A)	Circularity = P/A^2
Perimeter (P)	Rectangularity = A/ (FL*FW)
Feret Weight (FW)	Form-factor = $(4\pi A)/P^2$
Feret Length (FL)	Roundness = $(4A) / (\pi FL^2)$
	Ellipticity = (FL-FW) / (FL+FW)

Form-factor is a measure of circumference irregularity, taking values of 1.0 when it is a perfect circle and <0.1 when irregular. Roundness and circularity give information on the similarity of various features to a perfect circle, with minimum value of 1 and 4π (12.57), respectively corresponding to a prefect circle. Rectangularity describes the variations of length and width with respect to the area, 1.0 being a perfect square and values are less than this depending on the ratio of the long to the short side. Finally, ellipticity indicates if the changes in the axes are proportional (Tuset *et al.* 2003).

Statistical analysis

All statistical analysis was carried out using MINITAB 15 for windows. Data were tested using analysis of variance (ANOVA). To ensure that the data met the assumptions of ANOVA, the Ryan-Joiner test was used to test for normality and Cochran's test was conducted to testing for homogeneity of variances. Where needed, data were transformed. If data did not meet the assumptions of normality and equal variances after transformation, non-parametric tests, Kruskal-Wallis and Moods Median were used (Underwood 1997). The analysis of the data tested three hypotheses:

1. There is a significant difference in plaice otolith shape between year classes.
2. There is a significant difference in plaice otolith shape between ICES areas.
3. The otolith size/fish length relationship differs between ICES areas.

The ANOVA design consisted of two orthogonal factors, year and ICES areas, and one factor: haul nested in ICES area. Year and haul were considered to be random factors and ICES area was a fixed factor. Analysis of covariance (ANCOVA) was conducted using MINITAB to test the relationship between otolith size and fish length. ANCOVA is a merger of ANOVA and regression for continuous variables. ANCOVA tests whether certain factors have an effect on the outcome variable after removing the variance for which quantitative predictors (covariates) account. Otolith size is the dependant/response variable, ICES area is the factor and fish length is the covariate.

The data from the trace of the 1st annual ring and the whole otolith outline were treated separately. Form-factor was not normally distributed and did not have equal variance so was omitted from all further analysis. Four shape variables: ellipticity, roundness, rectangularity and circularity were analysed using separate uni-variate ANOVA's. The whole otolith outline data set comprised of a combination of three and four year old fish. Begg *et al.* (2000) found that age is one of the factors inducing large variation in otolith shape within stocks. Therefore, analysis was restricted to the four year old fish. This produced an unbalanced data set. The analysis of unbalanced data using ANOVA can be problematic as the tests for main effects and interactions are not independent of each other. However, computational methods designed for unbalanced data can overcome these issues by the using alternative methods to calculate the sum of squares (Shaw & Mitchell-Olds 1993). This approach is preferable to imposing balance on the data by removing replicates which can greatly reduce the power of the analysis. In this study all data were analysed using unbalanced Type III ANOVA's in Minitab. When significant differences were detected, *Post hoc* analysis to compare pairs of means was carried out using Tukey pairwise comparison tests.

3.4 Results

First annual ring

There were no significant differences in otolith length after year one between hauls, between the three ICES areas or between the three years and there was no significant interaction between the main effects ($p>0.05$). There was also no spatial or temporal significant differences detected for any of the shape indices ($p>0.05$). As the nested factor (haul) was found to be highly insignificant ($p>0.25$), data were pooled across haul to increase the power of the analysis. Again no significant difference could be detected. Data were then pooled across years to detect any spatial differences and no significant differences were seen in any of the indices ($p>0.05$). When data were pooled across ICES areas, a significant temporal difference was seen in roundness ($p<0.01$) and ellipticity ($p< 0.01$). A Tukey pair wise comparison was used to detect where the differences occurred. Fish from 1990 had less rounded and less elliptical otoliths then fish from 1997 and 2001 when one-way ANOVA was conducted (Table 3.2).

Table 3.2: Table showing results of a series of one-way-ANOVA's on shape indices at year 1 (data pooled across ICES areas)

Indices	DF	SS	MS	F	P	r^2
Ellipticity	2	0.03	0.01	7.20	<0.01	9.83%
Roundness	2	0.04	0.02	5.22	0.01	7.33%
Retangularity	2	<0.01	<0.01	1.24	0.29	1.84%
Circularity	2	0.30	0.15	2.08	0.13	3.06%

Whole otolith shape

There was also no spatial or temporal significant differences detected for any of the shape indices ($p>0.05$). Data were pooled across years and one-way ANOVA's conducted to detect spatial differences. Circularity showed a significant difference between ICES areas (Table

3.3). A Tukey pairwise test revealed that plaice otoliths were more circular in fish from ICES IVb compared to fish from ICES VIIa and VIIb.

Table 3.3: Table showing results of a series of one-way-ANOVA's on shape indices at year 4 (data pooled across years)

Indices	DF	SS	MS	F	P	r^2
Ellipticity	2	<0.01	<0.01	1.45	0.24	2.16%
Roundness	2	0.01	<0.01	2.49	0.09	3.64%
Retangularity	2	<0.01	<0.01	0.27	0.78	0.39%
Circularity	2	<0.01	<0.01	10.04	<0.01	13.21%

Data were pooled across ICES areas to detect temporal differences and one-way ANOVA's carried out. A significant difference occurred with ellipticity (Table 3.4). A Tukey pairwise comparison revealed fish from year class 1990 had less elliptical otoliths then 1997 and 2001.

Table 3.4: Table showing results of a series of one-way-ANOVA's on shape indices at year 4 with pooled ICES areas

Indices	DF	SS	MS	F	P	r^2
Ellipticity	2	0.01	0.01	5.30	0.01	7.44%
Roundness	2	0.01	0.01	2.69	0.07	3.92%
Retangularity	2	0.01	0.00	2.49	0.06	4.22%
Circularity	2	0.00	0.00	1.29	0.28	1.92%

ANCOVA showed an interaction between ICES area and fish length (p=0.015). *Post hoc* analysis revealed that fish from ICES IVb had significantly smaller otoliths at a given length then the other ICES areas (*Fig* 3.3). The rate at which otolith size changes with respect to fish size varies between areas.

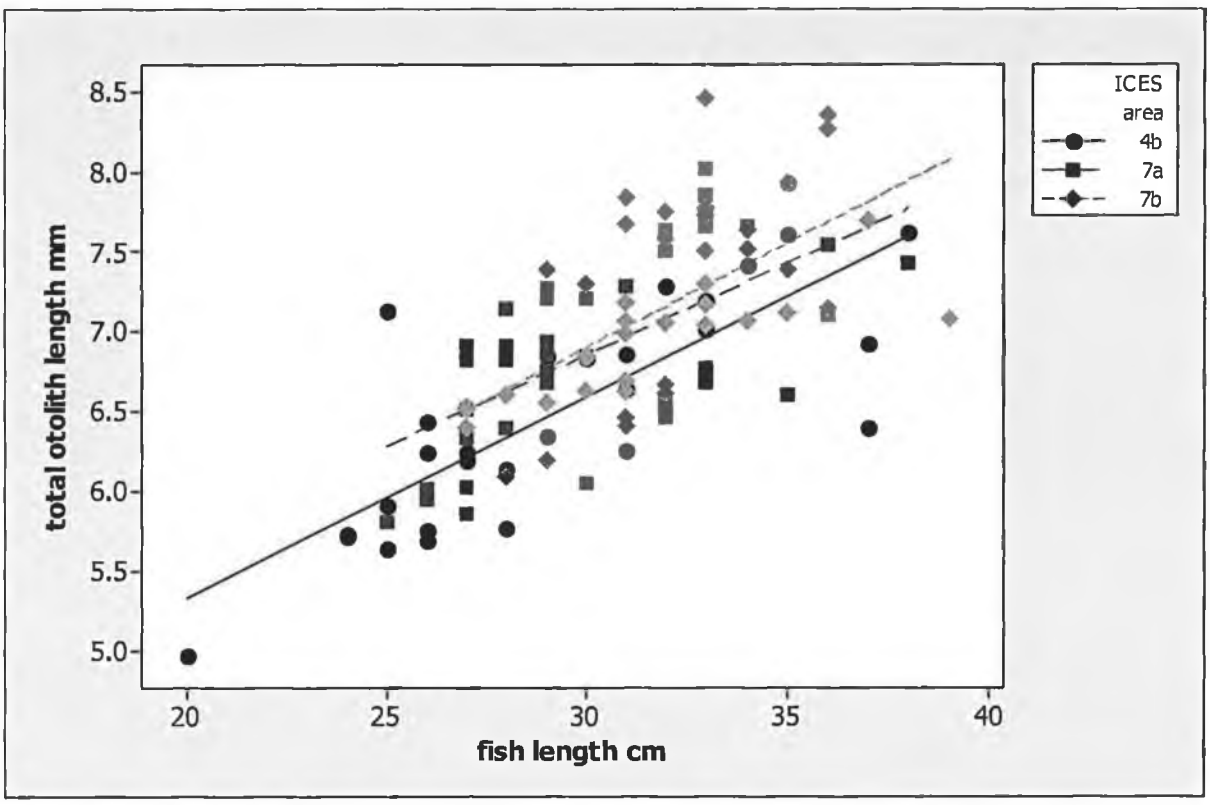

Fig: 3.3 **Scatterplot showing total otolith length vs. plaice length in three ICES areas after year four**

3.5 Discussion

This study has shown that otolith shape indices are not suitable for discriminating between plaice from the three ICES areas studied. Analysis of the whole otolith shape shows a small degree of variation in circularity in fish from the North Sea but this is only when the data were pooled across the three year classes. Thus, otolith shape alone would not be enough to distinguish between fish from different populations at the individual level.

There was also no regional variation in the juvenile otolith shape. Because it is unlikely that all fish were from the same nursery area, the most likely reason that no variation was seen is that there isn't a strong enough regional difference in the environment to produce distinct juvenile signatures. It is also possible that large scale regional differences are overridden by small scale local differences. With the influence of the environment being paramount, the

utility of otolith shape for stock identification would depend on the relative consistency of the environment in a given stock area (Campana *et al.* 1993).

There was some temporal variation in juvenile otolith shape and whole otolith shape when data were pooled over the three ICES areas. These temporal differences are as big as or bigger than regional differences. The variations detected may be due to interannual variations in environmental conditions such as temperature. Campana *et al.* (1993) suggested that environmental effects are influential determinants of otolith shape because otolith shape changes in response to different growth rates. The study also stated that otolith shape will not differentiate well among populations with similar growth rates.

The interaction between ICES area and fish length was significant, showing that the rate at which otolith size changes with respect to fish size varies between areas. This shows that these fish are growing differently and are probably exposed to different environments on average over the life cycle. However, these differences do not have a great impact on otolith shape. This emphasises that for plaice, otolith shape analysis does not have high discriminatory power.

Other studies have been able to distinguish between fish stocks using otolith shape. Burke *et al.* (2008) found that otolith shape could distinguish between migrant and resident components of Celtic Sea winter-spawned herring. A 97% classification success was achieved based on the shape of the 1st winter ring. In Mexico, De Vries *et al.* (2002) found it possible to distinguish individual king mackerel from eastern Gulf and Atlantic stocks and to estimate stock composition in the mixed-stock fishery using otolith shape analysis.

In general, demersal fish live a more sedentary life than pelagic fish. Because of this mode of life, otoliths in demersal fish have a less complex shape (Popper *et al.* 2005). Plaice otoliths are a simple ellipsoid shape when compared to the otoliths of other fish such as herring and

mackerel, which are more complex. Plaice otoliths are rounder in form and lack specific morphological characteristics. Perhaps othliths with more complex shapes (e.g. herring) show more shape variability in response to environmental factors than relatively featureless otoliths (e.g. plaice). However, studies to distinguish cod stocks by otolith shape were successful, even though cod otoliths are also quite simple in shape (Campana *et al.* 1993). But it also should be noted that growth rate differed significantly between the different cod stocks. A study conducted by Stransky *et al.* (2005) found an increasing shape complexity and variation with larger fish or otolith size. Perhaps older fish with larger fish otoliths would have allowed discrimination between stocks in the current study.

Another factor to consider is the degree of stock separation needed to distinguish between populations using otolith shape. Do stocks that can be distinguished on the basis of otolith shape tend to come from more geographically separated and environmentally distinct areas? Friedland *et al.*(1994) were able to discriminate between North American and European salmon using otolith morphometrics but otolith shape appeared to be a weak tool for identification of the country of origin. Turan (2000) observed a direct relationship between the extent of phenotypic divergence and the geographical separation of populations of Northeast Atlantic herring. The study distinguished between Icelandic, Baltic and Trondheimsfjord stocks but was unable to separate out North Sea and Celtic Sea stocks. Perhaps the plaice stocks in the current study did not have sufficient geographical separation to produce distinct otolith shape characteristics.

One method that could potentially be used to improve the discrimination of plaice form different areas based on otolith shape is fourier analysis of otolith outlines. This method is useful for describing more subtle differences in shape as higher order harmonics can be used to measure increasingly complex shapes.

3.6 Conclusion

The results of this study indicate that otolith shape analysis is not a good technique for recognizing stock structure in plaice over the spatial scales involved. It seems in instances where growth rates are similar among groups of fish, even if the groups are widely separated geographically, the prospect for using otolith shape as a stock discriminator is not promising. Further research should focus on other phenotypic markers. Tuset *et al.* (2006) used otolith weight to explain intra-species variation and found that classification percentage increased significantly when otolith weight was considered. Perhaps this factor should be combined with fourier analysis in further studies. Petursdottir *et al.* (2006) detected significant differences in otolith shape between adjacent spawning groups of Icelandic cod. The study showed , when using otolith morphology alone, using shape variables rectangularity and circularity , no significant difference between year class and area were detected However, when combined with otolith size variables (length, weight, width) differences were detected. Future studies should include otolith size data in the analysis.

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Inter-reader precision and bias of daily otolith increments in a Galway-Mayo Institute of Technology laboratory

4.1 Abstract

The scientific and stock assessment literature contains more incorrect age data than people realise. The major cause of these errors is a lack of adequate quality control. This latter process normally involves age validation, tests for bias and measures of precision.

This study examines precision of daily increment counts in plaice otoliths and sets out to determine if inter-reader precision varies between reader combinations and between otolith regions.

Otoliths used in this investigation were from 0+ plaice caught in Skerries Co. Dublin and Galway Bay, Ireland in 2007. Three readers within the fisheries laboratory at the Galway-Mayo Institute of Technology were assigned the letters A, B and C. Otoliths were randomly split into three groups. Each reader was given two groups of otoliths to age.

Counts of daily rings were compared between readers using paired t-tests to determine if readings were affected by systematic bias and differences in precision between readers was measured using coefficients of variation (CV).

There was a high level of systematic bias with certain reader combinations. The larval part of the otolith showed less bias when compared to the metamorphic and the juvenile portions. Results also confirmed that inter-reader precision varied between the portions of the otolith being read. The lowest CV for all readers was for the larval stage. The presence of bias will confound the interpretation of most measures of precision. Before any study is conducted in a laboratory involving ageing fish, inter-reader calibration should be carried out. Reported temporal or spatial variation in growth and larval duration in a fish species may have been due to inter-reader differences rather than reflect actual differences between the populations analysed.

4.2 Introduction

Age information forms the basis for calculations of growth rate, mortality rate and productivity, ranking it among the most influential of biological variables (Campana 2001). However, the process of ageing fish using otoliths can have an element of subjectivity. This subjectivity can occur in the preparation of the otolith or in the reading of them. Stock studies put much effort in discerning differences between otoliths of different fish stocks but very little to resolve the uncertainties of age determination.

Quality control is normally equated with age validation, which can be expensive and time consuming. However, it is a very important factor in ageing projects and should be incorporated into them. A study conducted by Beamish *et al.* (1983) noted that only 66% of 500 publications reporting fish age estimates attempted to corroborate the accuracy of their ages. A mere 3.4% were successful in doing so. A good quality control programme should also test for reader bias and measure precision among age readers.

Other methods of age validation include a statistical program for age validation. Okamura *et al.* (2009) produced a statistical programme for validating annual growth band formation based on edge analysis. Marginal increment analysis is another validation method used. If a growth increment is formed on a daily cycle, the average state of completion of the outermost increment should display a daily sinusoidal cycle when plotted against time of day. However, this is a very hard validation method due to technical difficulties associated with viewing a partial increment affected by variable light refraction (marinebiodiversity.ca/otolith.htm).

The quality of age estimates can be assessed using two measures: age accuracy, the closeness of age estimation to true age, and age precision the closeness of repeated measurements from the same reader (Campana *et al.* 1995). To determine age accuracy, fish of a known age would be required; however, reference collections are not available in many ageing studies.

Measures of precision are valuable for assessing the relative ease of determining the age of an otolith or comparing the skill level of one age reader relative to that of others (Campana 2001). Kimura *et al.* (1979) measured the precision between age readers by random effects analysis of variance. Beamish *et al.* (1981) proposed that an index of "average percent error" (APE) be used. However APE can vary widely among different species and age groups within species and is therefore not widely used. The most commonly used measure of ageing precision now is coefficient of variation (CV). It is a statistical measure of the dispersion of data points in a data series around the mean. The CV is statistically more rigorous and more flexible than other measures (Chang 1982). This is the method used in the current study.

Different age readers can interpret a given otolith in different ways. If the difference is consistent, there is a bias. This is when one reader is consistently assigning a higher or lower age than the other for one or more age groups. Bias may also occur within estimates from the same reader over a period of time. The presence of bias will confound the interpretation of most measures of precision. Therefore, bias should always be addressed before precision (Campana *et al.* 1995).

Repeated age determinations of a sample of fish are conducted for one of two reasons: to determine if there are systematic differences in age estimation between one or more age-readers, methodologies or laboratories or to estimate the reproducibility of age estimates. Campana *et al.* (1995) recommended a combination of a CV to measure precision and an age bias plot to assess the consistency of repeated age determinations.

This study sets out to determine if precision varies between reader combinations and between otolith regions for plaice. Readers were researchers at the Commercial Fisheries Research laboratory at the Galway-Mayo Institute of Technology who were experienced in the techniques involved. Two of the readers were students and the other was a lecturer and project

supervisor. Systematic bias between readers and otolith regions was also investigated. Validation of age estimates was not attempted as the fish studied are not of a known age.

4.3 Materials and methods

Otolith preparation

Otoliths used in this investigation were from 0+ plaice caught in Skerries Co. Dublin and Galway Bay, Ireland in 2007. Fish were hand netted at low tide on sandy beaches. They were bagged and taken back to the lab where they were frozen. The otoliths were removed from the juvenile fish using two dissecting pins under an Olympus SZX 7 stereoscopic microscope. When removed they were stored in "Sterilin" square otolith boxes prior to analysis.

To prepare the otolith for analysis each one was attached to a slide using crystal bond glue. Otoliths were polished using wet "Buehler" silicon carbide paper, grit 600µm and 1200µm. The non- sulcus side (concave side) was polished first until the accessory primordium (AP) was visible using an Olympus CX21 compound microscope with an X100 oil immersion objective lens and an overall magnification of X1000. After the first polishing each otolith was placed in the cap of a 0.5ml micro centrifuge tube and embedded in Buehler Epo-thin epoxy resin. The resin was allowed to set at room temperature over 24 hours. When the resin had set the otoliths were cut out of the caps using a scalpel and attached to a glass slide using crystal bond glue. Polishing was carried out on the sulcus side with the same grit paper until the microstructure was visible under the microscope.

Three readers within the lab were assigned the letters A, B and C. One reader was very experienced and had various published papers that involved reading otoliths. That reader trained the other two in otolith counting. Fifty seven otoliths were randomly split into three

groups of 19 using random numbers table and assigned into groups 1, 2 or 3. Each reader was given two groups of otoliths (Table 4.1).

Table 4.1: Table showing combination which reader looked at which sample

	Reader A	Reader B	Reader C
Sample 1	*	*	
Sample 2		*	*
Sample 3	*		*

Counting daily rings

Each reader counted the otolith daily increments using the same method. Counts were made within three portions of the otoliths: larval, metamorphic and juvenile. Counts within the juvenile and metamorphic regions were made under x 20 objective (with x 10 eyepiece) magnification. The larval region was counted using x 100 oil immersion objective.

Juvenile counts excluded the first complete ring beyond the last 'peak' of all accessory growth centres but included the edge of the otolith. Plaice were considered juvenile until sexual maturity. Where possible, counts were taken along an axis between 4 and 7 o'clock (i.e. anti-rostrum).

Metamorphic counts excluded the metamorphic ring but included the first complete ring beyond the peak of the accessory growth centre nearest the otolith edge. The metamorphic ring is defined as the last complete larval ring before increments are disrupted by accessory growth centres.

Increments within accessory growth centres are often indistinguishable, so counts were normally taken from areas between growth centres. Larval counts included the hatch ring and metamorphic ring. Images of the microstructure were taken using an Olympus camedia C-

3040 zoom digital camera. They were processed using Image-Pro Plus image analysis package.

All measurements were transferred to Microsoft excel, MINITAB 15 and Systat version 11 for subsequent data analysis.

Data analysis

Counts were compared between readers using paired t-tests to determine if readings were affected by systematic bias (consistent over or under estimation of age by one reader compared to a second reader). Prior to the analysis the difference between each reader's counts was tested for normality as the t-test operates under the assumption of normally distributed paired differences. Data which were not normal were analysed using the Wilcoxen test, which is a nonparametric equivalent of a 1-sample t-test. This test computes a Wilcoxen signed rank test on all pairs of variables of the differences between the two.

The coefficient of variation (CV) is a commonly used statistical index of precision. It is expressed as a ratio of the standard deviation to the mean. Here, CV's were computed to obtain measures of inter-reader precision for each reader combination, within each portion of the otolith (larval, metamorphic and juvenile). ANOVA was used to determine if precision varied significantly between the three reader combinations (AB, AC and BC) and between the three otolith regions. Where significant differences were detected, post-hoc analysis was carried out using Tukey pairwise comparisons.

4.4 Results

Systematic bias

There was a high level of systematic bias with reader combinations AB and BC over the total otolith count (Table 4.2). Reader combination AC showed no bias. The paired t-test also showed significant bias when reading the different portions of the otolith.

Table 4.2: Table showing paired t-test results in otolith aging of different reader combinations and different growth stages along with mean pairwise difference (mpwd)

	Sample 1(A&B)			Sample 2(B&C)			Sample3(A&C)		
	p-value	t/z-value	mpwd	p-value	t/z-value	mpwd	p-value	t/z-value	mpwd
Larval counts	0.03	2.36	5.5	0.01	3.15	3.7	0.02	2.23	5.3
Metamorphic counts	<0.05	No bias	4.2	0.01	5.05	3.6	<0.05	No bias	2.3
Juvenile counts	<0.05	No bias	1.4	<0.05	No bias	-1.4	0.01	2.26	14
Total otolith count	0.005	2.819	3.7	0.004	3.28	2	<0.05	No bias	3.7

Paired t-tests showed reader C had consistently higher counts then reader B ($p = 0.004$), up to two days mean differences (*Fig* 4.1).

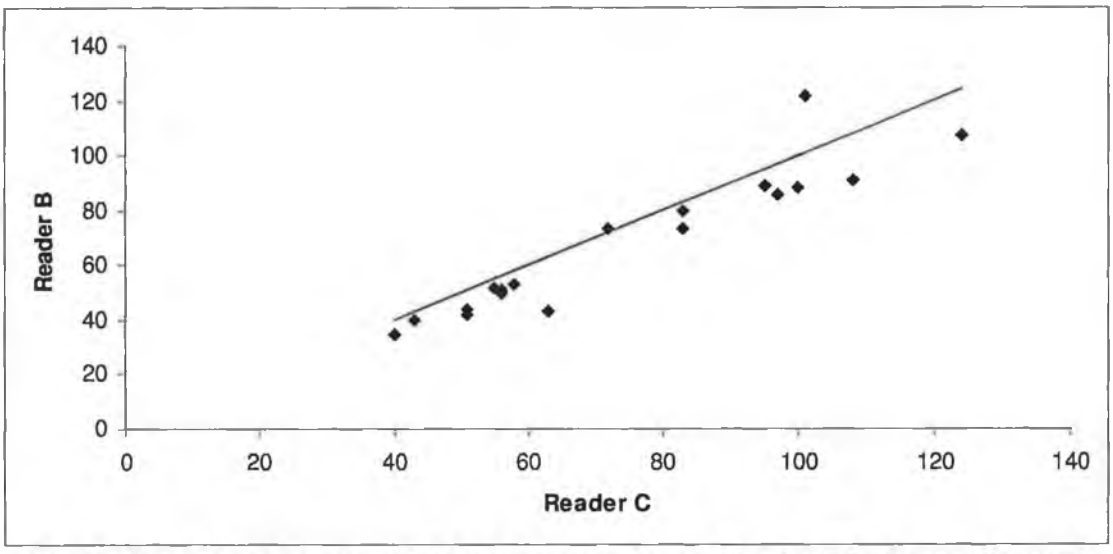

Fig 4.1: Graph showing systematic bias between reader combination BC when reading total otolith increment counts

In the AB combination, reader A had consistently higher counts then B (P = 0.005), up to four days mean paired difference (*Fig*. 4.2).

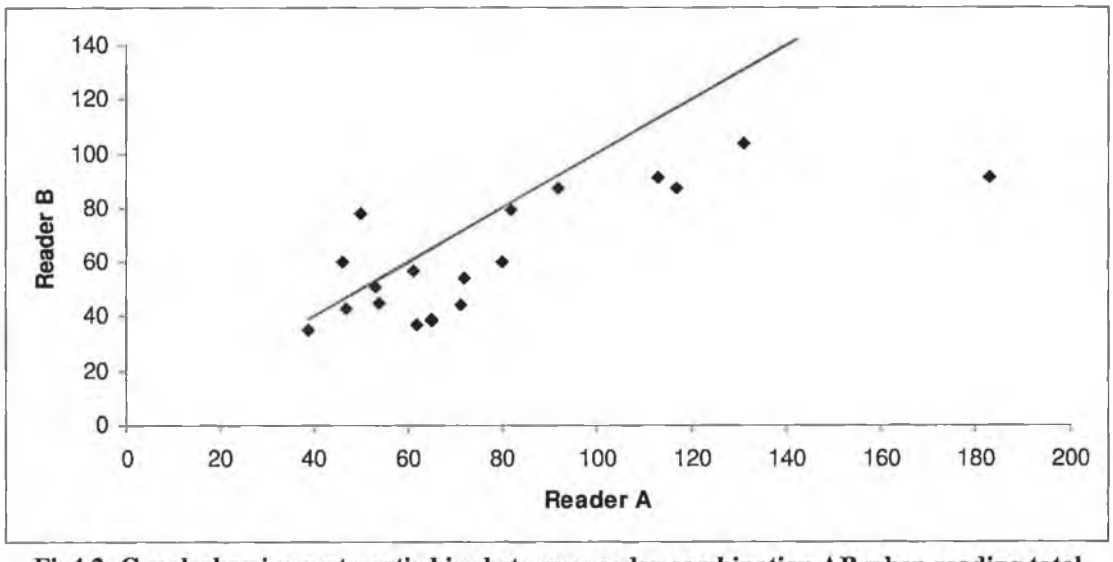

Fig4.2: Graph showing systematic bias between reader combination AB when reading total otolith increment counts

Reader combination AC showed no systematic bias (P>0.05) (*Fig*.4.3). Variation between the readers was randomly distributed.

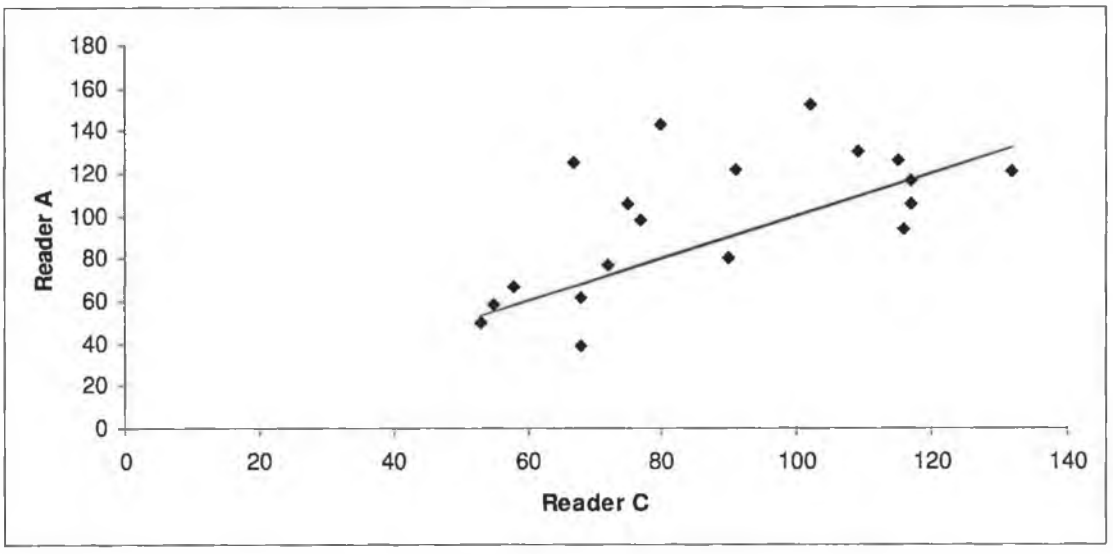

Fig4.3: Graph showing systematic bias between reader combination AC when reading total otolith increment counts

Paired t-tests, combining the data for the three reader combinations, showed significant bias in reading the different portions of the otolith (Table 4.3). The larval part of the otolith showed less bias when compared to the metamorphic (p=0.003) and the juvenile portions (p= 0.024). No significant systematic bias between metamorphic and juvenile regions was detected (p= 0.574) (Table 4.3). The level of systematic bias occurring in the current study depends on the section of the otolith being read.

Table 4.3: Results of a t-test showing inter-reader bias between otolith regions

Otolith regions	P- value
Larval < Metamorphic	0.003
Larval < Juvenile	0.024
Juvenile = Metamorphic	0.574

Precision

Inter-reader precision varies significantly between reader combinations (Table 4.4). To see if these results were driven by any large differences in mean lengths between reader combinations, an ANOVA test was conducted. Results found no significant difference in mean lengths of the fish between the three groups (p>0.05).

Results also confirmed that inter-reader precision varies depending on the portion of the otolith being read. The lowest CV for all readers was the larval stage (Table 4.4), which had the least random error. The results indicate that ageing becomes more difficult as the fish gets older and observer effects become significant at the juvenile stage when the largest number of increments are encountered.

Table 4.4: Table showing mean CV of different reader combination and different growth stages

Samples	Mean larval CV%	Mean metamorphic CV%	Mean juvenile CV	Total otolith countCV%	Mean reader CV%
AB	16.2	34.2	29.3	19.6	24.8
BC	9.7	21.3	8.4	8.9	12.1
AC	18.0	20.3	49.1	15.5	25.7
mean	14.6	25.3	28.9	14.7	

ANOVA results showed no differences in inter-reader precision between the three reader combinations when the larval region was examined (p= 0.138). A high level of imprecision exists between all reader combinations for the metamorphic and juvenile otolith regions (CV= 25.3% & 28.9%, respectively). When the metamorphic region was tested, no significant differences in precision occurred between reader combinations (P=0.141). However, when the juvenile portion was examined a significant difference occurred (P=0.023) Post-hoc analysis showed reader combination CB (CV 8.4%) had significantly less imprecision than combination CA (CV 49.1%) when reading the juvenile portion (Table 4.4).

4.5 Discussion

Systematic bias

The presence of bias will confound the interpretation of most measures of precision. Therefore, bias should always be addressed before precision. Results revealed that reader A

and C over estimated counts in relation to reader B. Reader B had the most experience in reading otoliths and had also trained the other two readers. The fact that reader A and C showed similar bias could be owing to the fact that they are both less experienced then reader B. Inter-reader bias will have an effect on interpretation of age data in the lab as estimates of hatch date and growth rates will vary between readers and between labs. Differences in hatch dates and growth rates between studies/labs could be misinterpreted as regional or temporal variation and this highlights the need for inter-lab calibration. Errors in microstructure data due to reader interpretation could effect models of larval drift. Predictions of larvae metamorphosing early could lead a reader to determine poor recruitment due to juveniles not reaching suitable nursery grounds. Or detection of slow growth rates may lead a reader to conclude that a longer larval duration will lead to higher rates of predation in the water column and again reduced recruitment..

Systematic bias varied with the portion of the otolith being read. Less bias may exist because of an expectation of age at the larval stage. This part of the plaice life cycle has a set and expected duration. Greater bias was detected between readers when reading the metamorphic and juvenile stages of the otolith. However there is also some expectation of duration at the metamorphic stage. To resolve systematic bias, known age fish would have to be aged by all readers, repeatedly and come to some agreement on age. Perhaps further training is required by the readers.

Precision

Results from the current study shows that reader agreement varies between the different reader combinations and between otolith regions. CV was lowest for larval stages. This could be due to the readers having the most experience of aging the larval portion of the otolith. More imprecision occurred with the metamorphic and juvenile stages. This may indicate that the older the fish gets, the harder it is to age. Eklund *et al.* (2000) found variability was highest in older aged herring in a study looking at between-reader variation. However, they

examined yearly, not daily growth. Also, identification of the 1st AP which marks the metamorphic stage can be difficult to identify and any mistakes here can affect subsequent ageing thereafter. This could account for the increase in imprecision for the other two otolith portions. Again, known age fish would help improve skill, accuracy and validate counts.

The above results may have implication for other studies conducted in the same laboratory. Results from previous studies may have reported different ages for populations of fish but this may have only been driven by intra-lab differences rather then actual biological differences. In chapter two, differences observed in the study are valid because only one reader was involved with ageing fish. However, when the results were compared with other studies, variation in growth and larval duration may have been due to inter-reader differences rather then biological differences. To resolve this, the size of the variation should be examined. Are the observed differences in growth and age bigger than inter-reader differences? In this case the reported differences in larval duration, which ranged from 7-15 days were much bigger then the inter-reader variability, which ranged from 3-5 days. Therefore environmental factors account for variation in larval duration.

Power *et al.* (2006) conducted a study to look at precision and bias of blue whiting age estimates within and between age readers. Within reader precision was found to be higher than between reader precision and an experience gradient became evident during the study. Stransky *et al.* (2005) looked at bias and precession of age readers investigating Atlantic redfish. Error in age determination was attributed to interpretation differences between readers and concluded that intercalibration between labs was needed to provide consistent input for stock assessment. While a limited number of studies examine precision of annual age estimates, there is very little formal evaluation of the precision of daily increment counts in the literature. The results of the present study also demonstrate that before any study is conducted in a lab involving fish ageing, especially where results from different readers are to be used, inter-reader calibration should be carried out. When reader agreement is reached,

only then should otoliths be read for a study. Standardising ageing methodologies and investigating variability in age determinations between fisheries laboratories on a large scale also needs to be addressed. When imprecision and bias is sufficiently low between readers in a laboratory, intercalibration with other labs could begin. This would help reduce error in the overall assessment of age for fish, including plaice in Europe, making stock management more reliable. Finally, while the results in chapter 2 do not suffer from inter-reader errors, the possibility that systematic error could evolve over time cannot be excluded. This could be mitigated by ensuring that reading of otoliths was carried out randomly with respect to treatment.

4.6 References

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General Conclusion

Results revealed no temporal differences in larval duration or larval growth rate, over the three year classes examined, in ICES area IVb, VIIa and VIIb, larval plaice development remained constant. Plaice from ICES area VIIa and VIIb showed no significant variation in larval growth rate and larval duration. This indicates that the adult fish in these areas were exposed to similar environmental conditions as larvae (e.g. temperature and food abundance). Alternatively, larvae from the two areas do grow differently, but fish from the two areas mix as adults at feeding and spawning grounds, and so no spatial variation in larval microstructure is detectable in the adults. However, spatial differences were observed. Plaice from ICES IVb did have a reduced growth rate and larval duration when compared to the other two ICES areas. This infers that no mixing of adults from the North Sea and the other two ICES areas occurred. Therefore, the treatment of the North Sea stock as separate from the others would be an appropriate assumption in terms of management. The North Sea basin constitutes a random-mating unit with high gene flow among geographically recognizable stocks (Hoarau *et al.* 2002) and are assessed as one stock.

The shape of these otoliths were examined for temporal and spatial variations. It was hoped that otolith shape could distinguish between separate stocks of plaice. Results revealed otolith shape indices are not suitable for discriminating between plaice from the three ICES areas studied. Analysis of the whole otolith shape shows a small degree of variation in circularity in fish from the North Sea but this is only when the data was pooled across the three year classes. This would not be enough on its own to distinguish between fish from different populations at the individual level. There was some temporal variation in juvenile otolith shape and whole otolith shape when data was pooled over the three ICES areas. These temporal differences are as big as or bigger than regional differences.

A major shift in otolith research and the use of age-based data has been a fundamental requirement to ensure quality control in ageing facilities and data processing (Begg *et al.* 2005). Before any study is conducted in a lab involving fish ageing, inter-reader calibration should be carried out. When imprecision and bias is sufficiently low between readers in the lab, intercalibration with other labs could begin. This would help reduce error in the overall assessment of age for plaice in Europe, making stock management more reliable. When the results from chapter two were compared with other studies, variation in growth and larval duration may have been due to inter-reader differences rather then growth differences. To resole this, the size of the variation should be examined. Are the observed differences in growth and age bigger then inter-reader differences? In chapter four an intralab study involving three readers from GMIT found a high level of systematic bias. The presence of bias will confound the interpretation of most measures of precision. Before any study is conducted in a laboratory involving ageing fish, inter-reader calibration should be carried out.

Over the past decade there have been significant developments in fisheries science, based largely on the technological advances in extracting information from otoliths of fish (Campana 2005). There is no other biological structure that is more important to fishery science than otoliths because of the information they contain. This information includes age and fish growth, movement patterns and habitat interactions. Scientists must continue to develop the appropriate technologies to extract information from otoliths and interpret it accurately in terms of the biology of the fish and the environments they have experienced (Begg *et al.* 2005).

The importance of otoliths is their capability to provide stock identification which is vital for fish stock management: the overall goal. Begg *et al.*(1999a) found that any disregard of stock structure can lead to dramatic changes in the biological attributes and productivity rates of a species. Archived otolith collections provide an easy and inexpensive source of information on various fish species. They also provide a long term data set that can help scientists to

distinguish informative patterns from short-term fluctuations. These otolith collections may help to determine stock structure within the ICES areas and ensure the sustainable management of fisheries.

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