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Investigation into the early life history of the European flounder (*Platichthys flesus* L.) with special emphasis in Galway Bay, Irish west coast

By

Bernadette O'Neill

Ph.D. in Fisheries Biology and Ecology

Supervisors of research

Dr. Deirdre Brophy
Dr. David McGrath

Galway-Mayo Institute of Technology
GALWAY-MAYO INSTITUTE OF TECHNOLOGY
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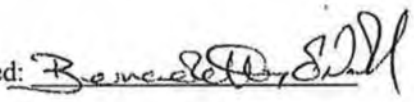
Declaration

I hereby certify that this material, which I now submit for the award of PhD is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: 

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Abstract

European flounder (*Platichthys flesus* L.) economic value is estimated to be worth over €8 million to the Irish economy (mainly from recreational angling activities). Despite such large species value, there is a considerable lack of data for this region, particularly concerning stock structure, timing of life history events and juvenile habitat use patterns. This dissertation addresses these knowledge gaps and describes a number of studies which focus on the understanding of flounder nursery ground ecology. Understanding stock structure and the connectivity between geographically distinct groups of fish is critical for the sustainable management of a species. Meristic analysis revealed a modest separation of juvenile flounder from different regions (west coast of Ireland, east coast of Ireland and the Welsh coast). The variation between regions was subtle and there was a large degree of overlap. The technique is therefore not a powerful method of stock identification on its own but may improve the likelihood of detecting flounder stock structure if combined with other markers in a multidisciplinary approach to stock identification. Little is known of the early life traits of flounder and the affect early benthic life has on habitat use patterns and survival. Otolith microstructure analysis established critical baseline data on the timing and duration of early life history events of juvenile flounder. Peak hatching and settlement occurred in February/March and March/April respectively while the average pelagic larval phase was on average 43 days. Spatial and temporal variability in early life history traits and size were identified which may be related to inter/intra estuarine movement of flounder and/or selective mortality of different settlement cohorts. Flounder sampled from beaches and estuaries within Galway Bay showed different habitat use patterns. Individuals on the beaches used the habitat over a short period of time and were either moving away from the beach habitat or undergoing mortality subsequent to arrival; whereas flounder were continuously found in the estuaries until late summer. There is evidence that biochemical (RNA:DNA) and morphometric condition represent different time scales in fishes' life which can be useful for studying short and long term condition of fish. Under experimental laboratory conditions, post larval flounder held at salinity of 30 had higher RNA:DNA compared to individuals held at salinity of 0 while no difference in morphometric condition was detected. In contrast, in the estuarine environment flounder condition (morphometric and RNA:DNA) increased as salinity decreased. The findings of this thesis provided important information on the dynamics of 0-group flounder which can aid conservation of essential juvenile habitats which in turn can positively affect recruitment of 0-group individuals to the adult spawning population.

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Chapter One

General Introduction

1.1. Overview for fisheries for flounder

In recent years human induced pressure has lead to a reduction in fish stocks, destruction of nursery grounds and shifts in species distributions due to climate change (Myers *et al.*, 1997; Beck *et al.*, 2001; Perry *et al.*, 2005; Courrat *et al.*, 2009; Thurstan *et al.*, 2010). Despite repeated calls from expert advisory groups and ecologists to preserve nurseries and decrease quotas, unsustainable fishing continues and some stocks continue to decline (Anon, 2011a). Thurstan *et al.* (2010) estimated a 94% decrease in benthic fish landings per unit of fishing power by the UK fleet between 1989 and 2007. However, not all demersal fisheries are currently overfished and some species are exhibiting stock recovery such as the Canadian Pacific halibut fishery (*Hippoglossus stenolepis*) (Anon, 2012c). North Atlantic plaice (*Pleuronectes platessa*) and sole (*Solea solea)* have been within safe biological limits for the last two years and plaice spawning stock biomass is currently the highest in recorded history (Anon, 2012a); while the once depleted fishery of yellowtail flounder (*Limanda ferruginea*) on Georges Bank is returning to historical levels (Stone *et al.*, 2004). These increases in recruitment are due to the implementation of the precautionary and maximum sustainable yield approach for plaice (Anon, 2012a) and trip limits, gear restrictions, implementation of Total Allowable Catches and seasonal and permanently closed areas for yellow tail flounder (Stone *et al.*, 2004). However closed fishing areas and seasons are not optimal in all cases (Dinmore *et al.*, 2003; van Keekan *et al.*, 2007). Therefore fishing restrictions should be treated with caution and used on a species by species and stock by stock basis. Nonetheless demersal fisheries can be maintained within safe fishing limits (maximum sustainable yield) where correct management and sustainable fishing are implemented.

Rice and Cooper (2003) suggested that large scale management of flatfish fisheries are more achievable than fine scale management. However, large scale management often requires collaborations and agreements between many governments which can prove difficult. Although not all benthic fish species are presently under threat, most flatfish species have been overfished at some stage in their history (Rice and Cooper, 2003). This emphasises how important consistent monitoring and managing of all flatfish species is.

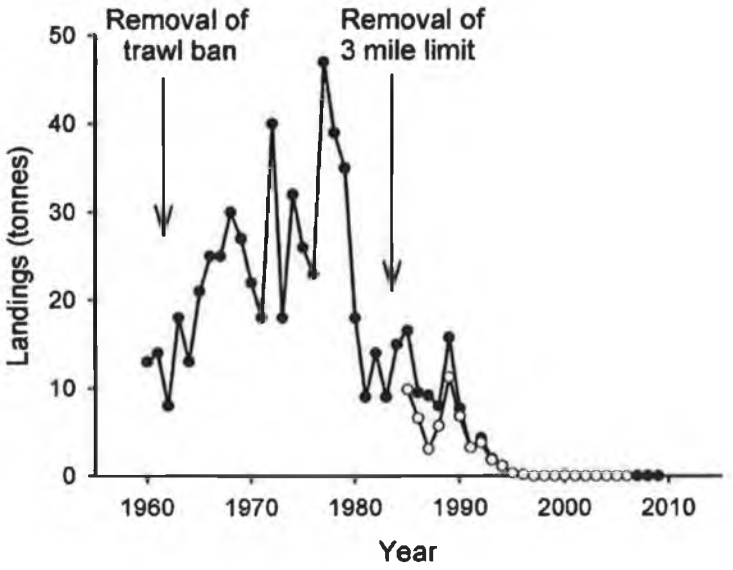

Fig. 1. Landings of flounder. Closed circles indicate landings from the wider Firth of Clyde, and open circles landings from the inner Firth of Clyde (Thurstan & Roberts, 2010).

European flounder (*Platichthys flesus* Linnaeus 1758) is an understudied flatfish species within Irish and European waters. Although target fisheries exist in Danish and Baltic waters, the majority of European countries do not have an active flounder fishery and therefore it is usually caught as by-catch or by recreational fishers (ICES, 2010) by bottom trawling (Anon, 2012d) and rod fishing (Quigley, 2011). Nonetheless revenue from recreational fishing can be large and has been estimated to be worth €8.4 million to the Irish Economy (Keirse, 2008). No minimum landing size has been established for flounder (ICES, 2010).. Although flounder are not as commercially important as other flatfish species such as brill (*Scophthalmus rhombus*), plaice, turbot (*Scophthalmus maximus*) and the common sole (ICES, 2010; Anon, 2012a, 2012b) a significant decline in flounder landings in the Firth of Clyde has been observed since the 1960s (Thurstan and Roberts, 2010; Fig.1.). However, overall landings of flounder throughout European waters have been found to fluctuate with no clear trend (Fig. 2.). These fluctuations may have been influenced by factors such as misreporting of landings, discarding as well as the availability and price or other more commercially important flatfish species (ICES, 2010).

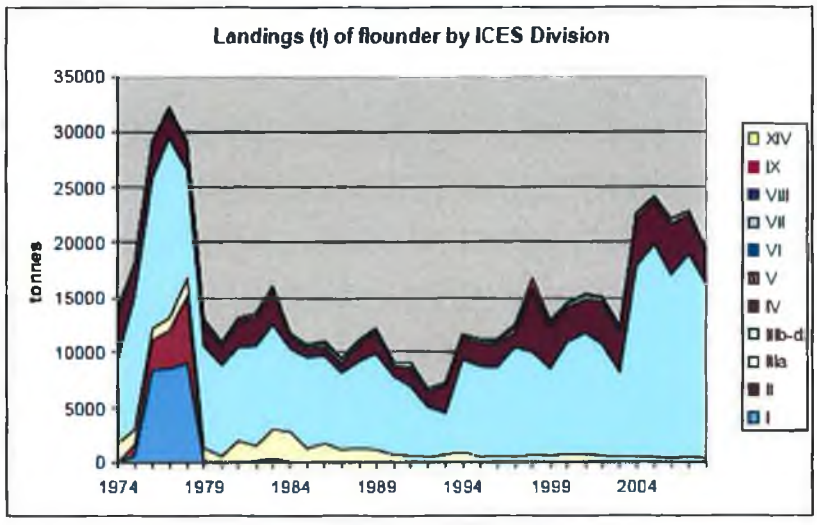

Fig. 2. Landings (t) of flounder by ICES divisions

The lack of commercial interest has lead to a deficit in biological research compared to other commercially important flatfish species such as plaice and the common sole (*Solea solea*). In particular, knowledge of early life history traits, stock structure and spawning stock biomass is deficient in European waters and requires attention. In recent years the research deficit has been recognised in the UK (Skerritt, 2010a) and extensive monitoring and management of flounder habitats and ecosystems has been proposed (Skerritt, 2010b).

1.2. The biology and life history of *Platichthys flesus*

Platichthys flesus belongs to the family Pleuronectidae or right eyed fish and has a wide distribution throughout the coastal waters of Europe (Russell, 1976) (Fig. 3.). Three sub-species have been identified to date (*Platichthys flesus flesus, Platichthys flesus luscus* and *Platichthys flesus italicus*), each inhabiting geographically distinct regions (North Sea/ North Atlantic, Baltic Sea and Adriatic Sea respectively) (Galleguillos and Ward, 1982; Borsa *et al.*, 1997). Although genetically distinct, each sub-species displays similar life history characteristics.

Fig. 3. Distribution and probability of occurrence of the European flounder (Anon, 2011b).

Typically, spawning aggregations of sexually mature flounder occur at sea from late winter to spring (Wheeler, 1969; Sims *et al*, 2005) followed by intensive feeding of spent adults inshore during the summer months (reviewed in Skerritt, 2010a). Eggs hatch 6-7 days following fertilization (Hutchinson and Hawkins, 2004) and larvae then migrate to coastal areas using local currents (Campos *et al*, 1994). Subsequent to fin formation, flounder larvae can alter their vertical position in the water column which allows them to use different currents which may guide them to coastal locations (Grioche *et al.*, 2000). Finally, when flounder reach estuarine nursery grounds they settle (Hutchinson and Hawkins, 1993; Bos, 1999; Hutchinson and Hawkins, 2004). This settling behaviour coincides with metamorphosis (transformation to the typical flatfish form) (Hutchinson and Hawkins, 2004). Once within estuaries, flounder can utilise tidal stream transport to reach optimal microhabitats within the nursery (Bos, 1999; Jager, 1999). As flounder grow and estuarine temperatures decline, 0-group (fish born between January and December of the same year are 0-group until the following January) flounder migrate off shore (Summers, 1979). 1+ group flounder may return to estuaries for summer feeding but do not penetrate as far upstream as 0-group (reviewed in Skerritt, 2010b).

Although offshore spawning and autumn/ winter migration is the typically accepted life history strategy of flounder, geographic variability has been found. Estuarine spawning cohorts (Morais *et al.*, 2011) and overwintering of young of the year flounder in estuaries (Martinho *et al.*, 2007; Martinho *et al.*, 2008) have recently been described. These anomalies were found within estuaries at flounder's southern distribution (Portugal). Martinho *et al* (2008) attributed estuarine overwintering to a temperature induced extended

growing season while Morais *et al.* (2011) hypothesised that predetermined features such as estuary size and flow may support estuarine spawning females (sea run mothers). Furthermore, compared to other European populations, northern Baltic Sea flounder populations produce demersal eggs (Nissling *et al.*, 2002) and spawn relatively close to the coast which encourages successful recruitment to nursery grounds in the absence of strong currents (Florin *et al.*, 2009).

Habitat overlap between flounder and other pelagic and flatfish species has been well documented; however, variation in spawning period, prey type and fish size has limited competition between them (Jager *et al.*, 1993; Aarnio and Mattila, 2000; Martinho *et al.*, 2008; Mariani *et al.*, 2010; Martinsson and Nissling, 2011). Reduced salinity, increased temperature and a muddy/silty substrate are typical characteristics of flounder nursery habitat (Kerstan, 1991; van der Veer *et al.*, 1991; Jager *et al.*, 1993; Zucchetta *et al.*, 2010). However, deciphering which of these factors exerts the most influence on flounder distribution and abundance is difficult as they are often correlated (Gibson, 1994). Nonetheless flounder are the only flatfish known to penetrate into freshwater and salinity remains one of the best predictors of juvenile flounder distribution. It has been suggested that salinity may act as a physiological trigger in flounder movement into and within estuaries (Bos and Thiel, 2006). This may be influenced by an increase in food quality and quantity and a decrease in predation and competition in low salinity upstream sites (Beaumont and Mann, 1984; Bos, 1999).

1.3. Stock identification

Fish stocks generally refer to management units which may be composed of spatially and/or temporally separated self-sustaining population units (Reviewed in Begg and Waldman, 1999; Kell *et al.*, 2004). However, the definition of fish stock can vary and the terms fish stock and population are regularly used interchangeably. In this thesis, a stock is referred to as a group of fish which are largely self-reproducing and display different life history patterns. Understanding stock structure is essential in order to make good management decisions (Begg and Waldman, 1999). Numerous stock identification techniques such as genetics (Hemmer-Hansen *et al.*, 2007; Florin and Hoglund 2008), otolith chemistry (Jónsdóttir *et al.*, 2007; Swan *et al.*, 2004), otolith shape analysis (Burke *et al.*, 2008), mark recapture (Fritsch *et al.*, 2007) and parasitology (Moore *et al.*, 2003) have successfully been used to identify different stocks, populations and spawning cohorts of fish species. In particular, phenotypic characters are more appropriate for studying short term environmentally induced differences compared to genetic variation that looks at

evolutionary differences between stocks (Chittenden *et al.*, 2010). Phenotypic features such as meristics (fin rays) are affected by local environmental conditions (Colman, 1976) and set during the larval phase (Begg and Waldman, 1999; Swain and Foote 1999). Consequently, fish living in geographically distinct areas can exhibit differences in fin ray numbers (Turan *et al.*, 2006) which can prove useful in distinguishing fish from different nursery grounds and aid the determination of juvenile origin in adult fish.

Numerous studies have attributed geographic variation in meristic characters to differences in environmental factors (Tåning, 1952; Lindsey, 1953; Fahy, 1980; Cloutier *et al*, 2010). Temperature has repeatedly be found to be an important factor in fin ray segmentation and final counts (Lindsey, 1953; Colman 1976; Fahy, 1980; Kinoshita *et al.,* 2000; Georgakopoulou *et al.,* 2007) while Cloutier *et al* (2010) found that water velocity significantly affects fish locomotion and consequently the timing and development of bone and cartilage. Although inter-annual differences in fin ray counts have been reported (Lindsey, 1953; Hulme, 1995) relatively consistent environmental influences have the potential to aid stock discrimination in the absence of genetic discreetness (Begg and Waldman, 1999)

1.4. The role of nursery grounds in flounder ecology

A habitat is a nursery if juveniles of a particular fish species occur at high densities. It provides shelter from predation/ competition, enhances growth and survival and may be critical for sustaining adult populations (Gibson, 1994; Beck *et al.*, 2001; Dahlgren *et al.*, 2006). Near shore habitats such as estuaries are recognised as essential nursery areas for numerous fish species (Le pape *et al.*, 2003; Able, 2005). However not all nurseries are of equal quality and this can result in variation in fitness, growth and survival. Furthermore, the suitability of a habitat as a nursery can vary from species to species and is determined by a number of interacting biotic (Nissling *et al.*, 2007; Hampel *et al.*, 2005) and abiotic factors (Hutchinson & Hawkins, 1993; Attrill & Power, 2002; Andersen *et al.*, 2005; Bos & Thiel, 2006; Freitas *et al.*, 2009; Zucchetta *et al.*, 2010) which can vary both spatially and temporally (Cabral *et al.*, 2007; Ramos *et al.*, 2009). Gibson (1994) produced a comprehensive illustration of the environmental factors which can affect habitat quality, fish growth, survival and recruitment (Fig. 4.). However anthropogenic influences can further compromise nursery area quality. Since the beginning of the industrial revolution in the late 18th century there has been a shift in coastal ecology globally due to the destruction, degradation and pollution of these sensitive habitats (reviewed in Howarth, 2008). Eutrophication due to human activity (agriculture, aquaculture, discharge from

wastewater treatment plants and discharge from industries) can change the structure and functioning of an ecosystem and reduce biodiversity (Ætebjerq *et al.*, 2003). The addition of nutrients can initially increase primary production (phytoplankton) and subsequently zooplankton and fish abundances (Nixon and Buckley, 2002). However problems arise when primary production is increased above the capacity the system can absorb it (Rabalais *et al.*, 2009). When the excess plankton and aquatic plants die they are decomposed by bacteria which use and sometimes deplete the dissolved oxygen which is necessary for the growth and survival of resident fish species (reviewed in Boesch *et al.*, 2001). Consequently, nurseries that have different human impacts can vary in their quality (Ramos *et al.*, 2011), and nurseries located in areas with high human impact may not provide high quality habitats for juvenile fishes (Vasconcelos *et al.*, 2007; Amara *et al.*, 2009; Courrat *et al.*, 2009; Ramos *et al.*, 2011).

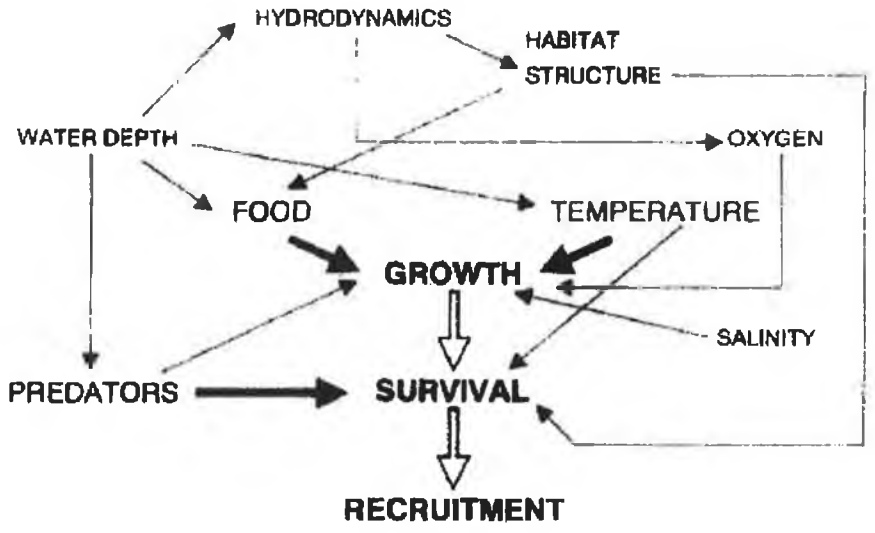

Fig. 4. Diagram illustrating inter-relationships between factors contributing to habitat quality that influence growth, survival and recruitment of juvenile flatfishes. The size of the lettering and the thickness of the black arrows indicate the relative importance of the factors. Not all possible inter-relationships are shown (Gibson, 1994).

Numerous techniques have been used in the assessment of flatfish nursery quality such as fish size, growth (somatic and otolith), condition, fish densities, sediment contamination and biomarker response (Goksøyr *et al.*, 1996; Le Pape *et al.*, 2003; Martinho *et al.*, 2007; Amara *et al.*, 2009; Vasconcelos *et al.*, 2009; Haynes *et al.*, 2010; De Raedemaecker *et al.*, 2011; De Raedemaecker *et al.*, 2012). Although no single technique has been established as being the ultimate indicator of habitat quality, fish growth and condition have been extensively used due to their consistent reliability as indirect indicators of the past and present environment. Both biochemical and

morphometric condition indices are useful proxies in determining the growth, health and energy status of individual fish (Amara *et al.*, 2009; Tanner *et al.*, 2009; Vasconcelos *et al.*, 2009). Morphometric condition indices are a direct measure of body shape and size while biochemical indices such as RNA: DNA ratios measure protein synthesis which is responsible for the execution and regulation of anatomical functions. RNA: DNA ratios and morphometric indices respond to their environment over short and long time periods respectively. The difference in the response of the two indices can be useful as it allows a comprehensive view of fish condition over time.

1.5. Critical events during the early life of the European flounder

Morphological features which can measure fish age and growth such as otoliths, vertebrae and scales are useful for interpreting and understanding life history dynamics, Otoliths have been consistently used in aging fish using annual rings since 1899 (Ricker, 1975) while daily increment analysis techniques were first developed in the 1970s (Brothers *et al.*, 1976). Since their discovery, circadian increments (produced through the deposition of calcium carbonate and protein) have been used in estimating critical life history events such as hatching and settlement dates (Karakiri *et al.*, 1991; Fox *et al.*, 2007; Gunnarsson *et al.*, 2010) and growth rates (Karakiri and von Westernhagen, 1989) of many flatfish species. Otolith growth is continuous (Campana and Thorrold, 2000) and is related to somatic growth; therefore it is a useful proxy in determining fish growth during specific life history stages (Campana and Neilson, 1985).

Identifying life history events, durations and growth and the processes controlling them can reveal information on larval drift patterns, recruitment success and stock, population, sub-population and cohort structure. However in order to understand the processes affecting growth, survival and recruitment we must first understand the life history patterns.

1.6. Overview of the study area

In chapter two sites from the Irish west coast, east coast and Welsh coast were examined while in the remaining data chapters (three, four, five and six) all sites (two estuaries/rivers and two beach habitats) were within inner Galway Bay. Galway Bay consists of a diverse shoreline ranging from rocky terraces, shingle, sandy beaches, salt marshes and intertidal sand and mudflats (Anon, 2008). The Galway Bay complex (000268) and Inner Galway Bay (0004031) are considered Special Areas of conservation largely due to the range and number of wintering wetland birds, common seals and otters

(Anon, 2012e). River runoff and shallow habitats also make this bay a suitable nursery ground for young-of-the-year (YOY) flatfish.

Thesis outline and objectives

The overall aim of this thesis was to examine the early life history of European flounder (*Platichthys flesus).* Understanding patterns in the timing of life history events, population structure and subsequently the driving factors behind these dynamics is of importance in understanding year class strength and adult recruitment. These complex subjects are examined in the present thesis which is intended to increase our understanding of flounder ecology and biology both in Irish waters and beyond. Each data chapter is presented in the format of a scientific publication. Full details of the manuscripts associated with each chapter are shown below:

Summary of chapters

Chapter 2:

Scales of variability in fin ray counts of flounder, *Platichthys flesus* L. on Irish and Welsh coasts.

Published as: O'Neill, B., Keirse, G., McGrath, D., Brophy, B. 2012. Scales of variability in fin ray counts of flounder, *Platichthys flesus* L. on the Irish and Welsh coasts. Biology and Environment: Proceedings of the Royal Irish Academy 112b, 1-7.

Fin ray characters were used to differentiate between flounder from geographically distinct locations. Variation in phenotypic features such as fin rays can indicate that the larval phase of fish were separated for a prolonged period of time and therefore may be useful in recognising stocks and populations while incidentally enabling the identification of larval origin.

The main objectives were:

- Investigate variability in fin ray counts in juvenile flounder from the west coast of Ireland, west Irish Sea and East Irish Sea.
- Investigate the potential for using fin rays to determine the nursery origin of adult fish

Chapter 3:

Investigation of early life events of European flounder (*Platichthys flesus* L.) within Galway Bay, west Ireland, as described by otolith microstructure.

A detailed examination of early life history events of flounder within Galway Bay was carried out using otolith microstructure examination. Understanding life history patterns is essential prior to understanding the processes which affect fish growth, mortality and recruitment.

The objectives were:

- Identify the timing of early life events, larval duration and larval growth rate of flounder within Galway Bay
- Assess spatial and temporal variability in the early life traits of flounder

Chapter 4:

Habitat utilisation in 0-group European flounder *Platichthys flesus* (L.) within Galway Bay, Ireland.

Numerous environmental and anthropogenic factors can affect fish growth and development which may lead to variation in survival and year class strength. Nursery habitat quality is a key factor affecting the success of individual fish. Therefore, identifying factors/ habitats which affect the growth and development of fish is valuable in understanding recruitment variability

The objectives were:

- Investigate if the metamorphic onset is size dependant.
- Establish if habitat type affects fish metamorphosis (deposition of accessory primordia).
- Determine seasonal trends in flounder abundance in beach and estuarine habitats.

Chapter 5:

An experimental investigation of salinity effects on growth, development and condition in the European flounder (*Platichthys flesus*. L.).

Published as: Bernadette O'Neill, Fien De Raedemaecker, David McGrath, Deirdre Brophy. 2011. An experimental investigation of salinity effects on growth, development and condition in the European flounder (*Platichthys flesus*. L.). Journal of Experimental Marine Biology and Ecology 410, 39-44.

Salinity is an important factor in flounder migration and development and therefore can also affect individual condition. Under suboptimal conditions, such as high salinity,

flounder may delay development which may have serious consequences on survival. Therefore, the effects of salinity on post larval growth, development and condition (morphometric and RNA: DNA) was assessed under controlled environmental conditions.
The objectives were:

- Investigate the effect of salinity on flounder growth, development and condition.
- Examine the relationship between morphometric condition and RNA: DNA ratios.

Chapter 6:

Spatio-temporal variability in the condition of juvenile flounder (*Platichthys flesus* L.) within Galway Bay, West Ireland.

The quality of nursery habitats was examined using two condition indices, morphometric and RNA: DNA ratios. Spatial (inter and intra location) and temporal fluctuation in both condition indices and fish size were examined and explored in relation to estuarine characteristics and salinity.
The objectives were:

- Examine the scale of variability in flounder size, age and condition both spatially and temporally.
- Investigate the relationship between the condition indices.

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Chapter Two

Scales of variability in fin ray counts of flounder, *Platichthys flesus* L. on Irish and Welsh coasts

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Author contribution

O'Neill, B: Processing and analysis of samples, data analysis and writing of paper

Keirse, G: Sample collection

McGrath, D: Secondary project supervisor

Brophy, D: Primary project supervisor

Abstract

Meristic variation within a fish species can arise due to environmental factors and can aid investigation between fish stocks and closely related juvenile fish species. The aim of this study was to investigate spatial variation in meristic characteristics for juvenile flounder *Platichthys flesus* from Irish and Welsh coasts. Five meristic fin ray counts were taken from 120 fish from nursery grounds in three regions (west Irish coast, east Irish coast and Welsh coast). Statistical analysis revealed regional variability for two of the meristic counts (dorsal and anal fin rays). A discriminant function analysis revealed a weak separation of the three regions. This separation improved when samples from the east Irish region were eliminated, with 65% of the fish from the Irish west coast and 73% of the fish from Wales correctly classified.

Key words: Meristic; *Platichthys flesus*; adult stock separation; taxonomy; nursery ground importance.

1. Introduction

The European Flounder *Platichthys flesus* (L.) has a wide distribution and supports a commercial fishery in Baltic and Danish water (FAO, 2011). Although *P. flesus* generates estimated revenue of €8.4 million for shore angling in Ireland (Keirse, 2008), it is not regarded as an important commercial species. Therefore, little research has been conducted on flounder early life history in Irish and north Atlantic waters. ICES has recently recognised this knowledge deficit and have suggested an increased research intensity in its northern distribution (ICES, 2010).

Spawning in flounder primarily occurs between January and February (Skerritt 2010). Historically *P. flesus* have been found to spawn at sea; however, Morais *et al.* (2011) recently described an estuarine spawning population in the Minho estuary, Portugal, where larval mixing of estuarine and marine spawned larvae occurred in coastal areas. Following spawning, flounder eggs and larvae, drift and migrate inshore (Wheeler, 1969; Campos *et al.*, 1994; Koubbi, *et al.*, 2006), where the pelagic duration can take up to two months to complete (Grioche *et al.*, 2000). After the juvenile phase, flounder can remain inshore or migrate off shore during their adult stages (ICES, 2006) which may lead to isolation or mixing of flounder from different nursery grounds.

Identifying fish stocks is fundamental for effective fisheries management (Begg and Waldman, 1999) where consistent progress and evolution of identification methods are necessary as management and conservation requirements change (Begg *et al.*, 1999). Genetic techniques such as the analysis of microsatellite markers are valuable in this regard (Hemmer-Hansen *et al.,* 2007; Florin and Hoglund, 2008) and have successfully discerned between discreet populations of *P. flesus* in European waters. However, for management purposes, a 'stock' is not necessarily represented by genetic discreteness, but is often represented by a range of definable features which can be used to distinguish between groups of fish (Begg and Waldman, 1999). While minimal mixing of populations may result in genetic homogeneity (due to the sensitive nature of molecular techniques), information from other techniques can potentially increase differentiation between environmental stocks (Coyle, 1998). Begg and Waldman (1999) found that phenotypic variation can indicate prolonged separation of post larval fish under different environmental regimes and so is more appropriate for studying short term environmentally induced differences compared to genetic variation that looks at evolutionary differences between stocks (Chittenden *et al.*, 2010). Techniques such as meristic analysis (Hemmer-Hansen *et al.,* 2007; Florin and Hoglund, 2008), otolith chemistry (Jónsdóttir *et al.,* 2007; Swan *et al.,* 2004), otolith shape analysis (Burke *et al.*, 2008), mark recapture (Fritsch *et al.,* 2007) and parasitology (Moore *et al.,* 2003) have successfully been used to identify and separate different stocks, populations and spawning cohorts of fish species.

Fin ray and meristic variability have been used successfully, usually in combination with other methods, to separate adult and juvenile fish populations (Nielsen *et al.,* 1998; Gröger and Gröhsler, 2001) and sub species (Galleguillos and Ward, 1982), discern between similar juvenile fish species (Haynes *et al.,* 2008; Haynes *et al.,* 2010), and indicate which stock-specific nursery environment juvenile fish come from (Pawson and Jennings, 1996). Meristic characteristics are quantative features occurring in series (scales, myomeres, vertebra and fin rays). Their numbers are influenced during early development predominately by temperature and can also be affected by salinity, dissolved oxygen and light (Colman 1976; Lindsey 1988). Fin ray counts can be both positively (Lindsey, 1953; Kinoshita *et al.,* 2000) and negatively correlated (Georgakopoulou *et al.,* 2007; Fahy, 1980) with water temperature depending on species. After early larval development is completed fin ray numbers remain stable and constant into adulthood and are therefore representative of the environment during their pelagic larval phase (Swain and Foote, 1999). Consequently, fish living in geographically different areas can exhibit variation in the segmentation of their fin rays. Although significant morphological differences cannot

prove restricted gene flow, it can suggest a lack of mixing between fish groups (Turan *et al.*, 2006).

The present study aims to investigate the variability of fin ray counts in juvenile European flounder from three regions in the north Atlantic and Irish Sea. The potential for using meristic characteristics to assist the discrimination of flounder from different nursery grounds and to aid the determination of juvenile origin in adult fish is assessed.

2. Materials and methods

2.1 Sample collection

0-group flounder were sampled in 3 regions; west of Ireland, east of Ireland and west of Wales. Two sites within each region were examined (Fig. 1). Although the two sites within the east of Ireland region were far apart (over 200km), no significant differences in any of the variables were observed ($p<0.05$) justifying their grouping within the same region. Sampling was carried out from May to June 2006 using a fyke net and a beach seine. Sea surface temperature data from the eastern Atlantic and Irish Sea was obtained from the Irish Marine Institute data buoys. The monthly means and standard deviations of the temperature measurements were calculated for the Irish Sea and Atlantic data buoys from January to May which covered the spawning and larval pelagic phase of flounder (Fig. 2.)

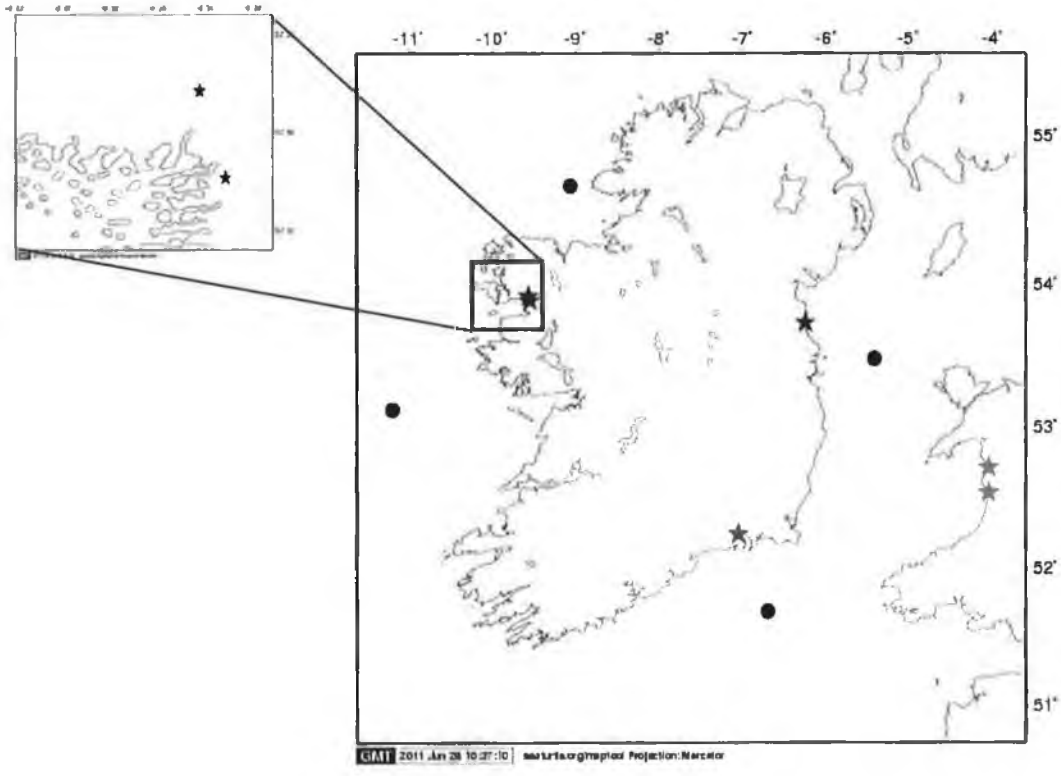

Fig. 1. Sampling locations in the Atlantic and Irish Sea nursery grounds in 2006 including temperature data buoy positions. ★ Indicates sampling sites, ● indicates data buoy locations. Mapping was generated using www.seaturtle.org.

2.2. Meristic analysis

The dorsal, caudal, anal, right and left pectoral fin rays were counted on all of the 120 fish collected (n= 40 per region) using a Leica zoom 2000 dissecting microscope. Random recounts were carried out throughout the analysis to assess precision.

2.3 Data analysis

Statistical analysis was carried out using Minitab 15 and SYSTAT 11. Normality was assessed using the Anderson-Darling test. All meristic variables did not follow a normal distribution, however they were still subjected to parametric analysis of variance, as this procedure is considered robust to departures of normality (Underwood, 1997). Bartlett's and Levene's test of equal variance were used to test for homogeneity of variances where all variables were found to have equal variances. Nested ANOVAS were carried out on all five meristic measurements with region included as a fixed factor and site as a random factor nested within region. When significant differences were detected Tukey's post-hoc test was used to determine where these differences lay. Three variables were found to be uncorrelated to each other (caudal, anal and the right pectoral fin; $p>0.05$) and were included in subsequent multivariate analysis. Nested MANOVAS (n=40 per region) analysed multivariate differences between regions and sites within regions while Discriminant Function Analysis (DFA) determined their discriminatory power.

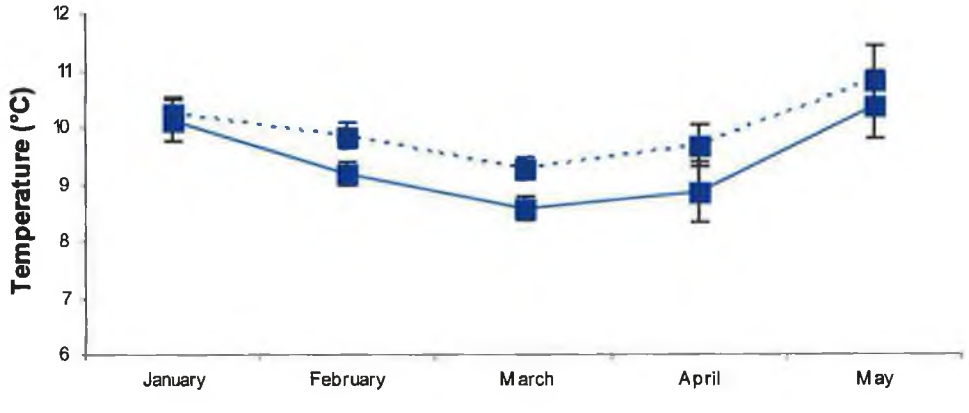

Fig. 2. Average monthly sea surface temperatures from the Irish Sea (continuous line) and Atlantic (dashed line) data buoys where the vertical lines denote standard deviation

3. Results

3.1. Univariate analysis

Fin ray counts are summarised in Table 1. Nested ANOVAs found no significant differences in caudal, right and left pectoral fin ray counts between sites or regions. Dorsal and anal fin ray counts showed differences between regions ($p<0.05$) with no differences between sites ($p>0.05$). Fish caught on the Irish west coast had higher mean counts for dorsal and anal fins than fish from the Welsh coast (Fig. 3).

Region	Caudal	Dorsal	Anal	P Right	P Left
West Ireland	18	62 (57-68)	44 (37-47)	11 (9-12)	10 (8-11)
East Ireland	18	61 (55-65)	43 (40-47)	11 (8-13)	10 (6-12)
Wales	18 (17-18)	60 (54-65)	42 (40-45)	10 (8-12)	10 (9-11)

Table 1. Mean and range of flounder fin ray counts (caudal, dorsal, anal and right and left pectoral) for the west and east Irish and Welsh sites.

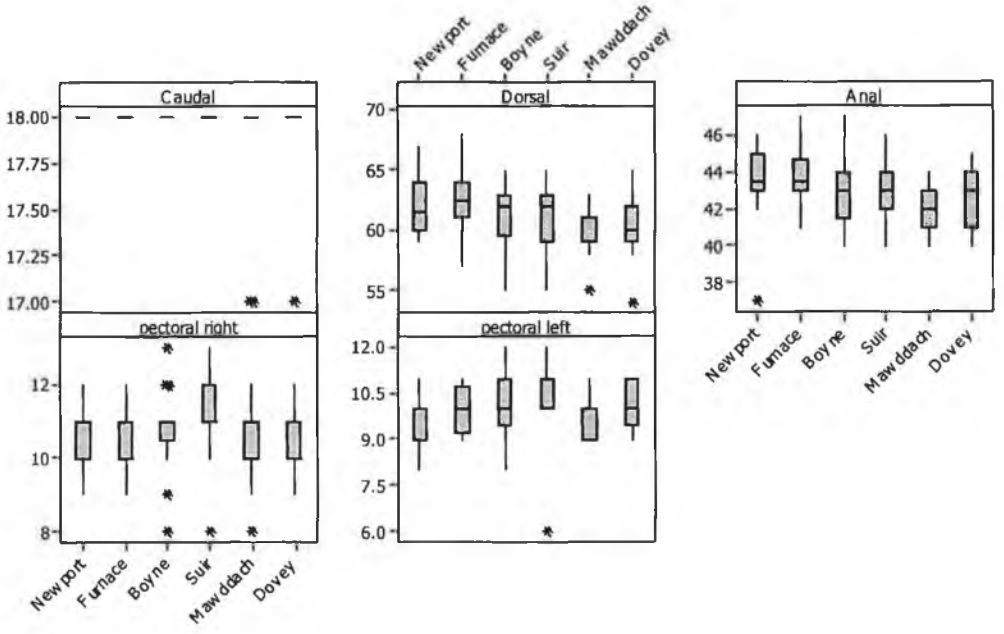

Fig. 3. Box plots displaying spatial trends in caudal, dorsal, anal, right pectoral and left pectoral fin ray. Newport and Furnace represent samples taken from the west of Ireland, Boyne and Suir from the east of Ireland while samples taken from Mawddach and Dovey represent the Welsh coast.

3.2. Multivariate analysis

A nested MANOVA of caudal, right pectoral and anal fin ray counts showed a significant difference between regions ($p < 0.05$) and no significant difference between sites nested within regions ($p > 0.05$). A DFA based on the same variables revealed a relatively poor separation when all three regions where analysed (Table 2a), with a high degree of overlap between samples. The separation between regions increased considerably when a second DFA was carried out excluding the East of Ireland samples (Table 2b). The canonical scores from the DFA containing all three regions are illustrated in Fig 4.

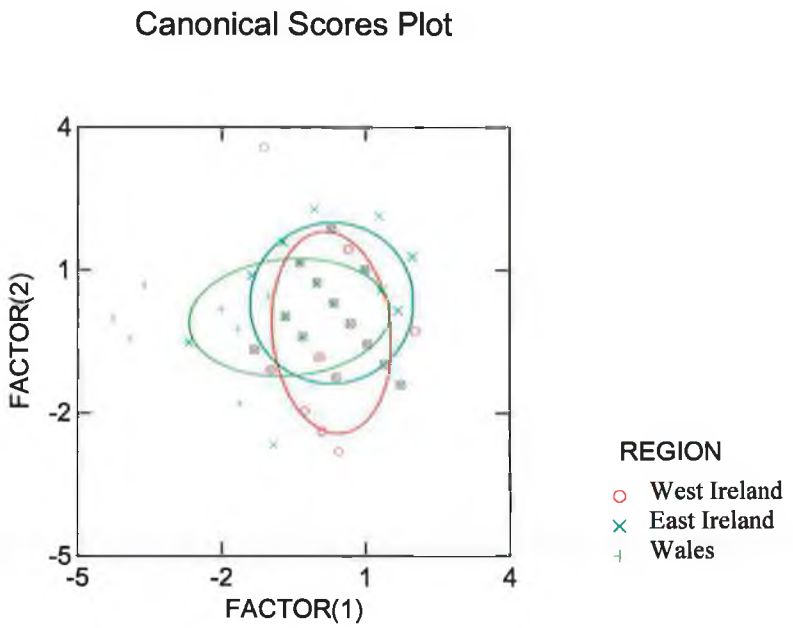

Fig. 4. Canonical Scores plot displaying flounder meristic data from west Ireland, east Ireland and Wales.

(a)

Region	West	East	Wales	% correct
West Ireland	20	13	7	50
East Ireland	10	21	9	53
Wales	6	12	22	55
Total	36	46	38	53

(b)

Region	West	Wales	% correct
West Ireland	29	11	73
Wales	14	26	65
Total	43	37	69

Table 2 (a). DFA showing the percentage and number of correctly assigned flounder for the three regions, west Ireland (n=40), east Ireland (n=40) and Wales (n=40).

Table 2 (b). DFA showing the percentage and number of correctly assigned flounder for the west of Ireland and Wales samples.

4. Discussion

The fin ray counts reported in this study predominantly fall within published ranges (n=115 out of n=120) with the exception of anal fin ray counts for flounder on the west and east coast of Ireland and dorsal fin ray numbers on the west (Wheeler, 1969; Russell, 1976). Where anomalies in fin ray counts were found, it was established that each fish (n=5) had a single fin ray outside of expected ranges. Variation in fin ray counts for other flatfish species have been previously reported (Bañón, 2008; Haynes *et al.*, 2010) and related to temporal and spatial variation in environmental factors such as temperature. This discrepancy may be due to geographic or temporal variation in the environmental conditions that influence meristic traits, such as temperature. Understanding the scales of variation in fin ray counts is important where such counts are used to differentiate between fish species. When adult traits are not yet visible they can be used for the discrimination of closely related species which can co-inhabit nursery grounds (Haynes *et al.*, 2008, 2010).

This study revealed within region homogeneity and between region heterogeneity in flounder fin ray counts where regional variability was observed for dorsal and anal fin ray counts between the west of Ireland and Welsh samples. These results may be indicative of ecological (but not necessarily genetic) stock structuring as has been identified by Marques *et al.* (2006) for *P. flesus* on the Portuguese coast.

Overall, spatial variation in fin ray counts was low and the classification success of the discriminant function analysis was only moderately higher than would be expected by chance. This may be due to a lack of sufficient temperature difference between the two areas during the pelagic larval phase. A temperature controlled experiment revealed that a 5°C difference in temperature induced fin ray differences in European sea bass *Dicentrarchus labrax* (Georgakopoulou *et al.*, 2007). Temperature information from the marine data buoys suggests a difference of less than 1°C during larval development in 2006. This relatively small temperature difference may not be great enough to induce a strong separation in fin ray counts between the regions analysed. An experimental design examining the response level of fin ray development and segmentation to various temperature levels could pinpoint the critical temperature causing variation in flounder.

Alternatively, mixing during the larval phase may reduce regional variation in meristic traits. Given that flounder eggs drift with water current (Campos *et al.*, 1994), migration and mixing within the Irish Sea prior to arrival at the nursery grounds on the Welsh and east Irish coasts cannot be ruled out. Grioche *et al.* (1997) also described current induced larval drift of *P. flesus* along the French coast which may lead to mixing of populations (if they exist). In the Irish Sea, plaice larvae are subject to planktonic mixing (Watts *et al.*, 2004) and therefore the juveniles within a nursery area may have dispersed from more than one spawning area. Due to the large distance and slow active swimming speed of larval flatfish (Fukuhara, 1988), mixing between juveniles sampled on the Irish west coast and those from the Irish Sea is less likely. Therefore, a continued effort to describe the temporal and spatial variability in flounder meristic characters throughout their European distribution is essential in providing accurate information on flounder ecology and morphology.

The results of this study suggest that discrimination based on fin ray counts alone is not sufficiently powerful to accurately determine nursery ground origin for flounder. However, given that low levels of spatial variation in meristic counts was observed, the method could potentially be useful for classifying flounder from nursery areas when used in combination with other techniques. Begg and Waldman (1999) suggested a combination of at least one genetic and one phenotypic character be employed when attempting to decipher stocks. In general the use of two or more stock identification techniques is desirable as it gives a more comprehensive picture of stock structuring and ecology of a given area. Therefore, future investigations of nursery ground origin and stock structuring of *P. flesus* on the Irish and Welsh coasts should employ a combination of techniques as well as meristics, such as genetic analysis, otolith shape analysis, otolith chemistry and parasitology.

It is important to consider whether the fin ray counts recorded at each site are entirely representative of the regions or reflect more local patterns. In the case of the east coast of Ireland region, the two sites were well separated (120km) and showed no variation in fin ray counts. Therefore the meristic characteristics of flounder in this region appear to be well characterised. However, the sites located on the west coast of Ireland and Wales were separated by just 4 and 20km respectively and therefore may not be representative of the region over a broader spatial scale. Haynes *et al.* (2008) report no variation in fin ray counts of flounder on the west coast of Ireland over a spatial scale of 150km. It is therefore likely that flounder fin rays are homogenous in this region. Nonetheless, given that variation in fin ray counts can occur on both large (O'Reilly and Horn, 2004) and small

(Haynes *et al.,* 2008) spatial scales a more comprehensive sampling program would be needed to assess the extent of the variability within and between regions and to confirm if true regional differences exist. Due to the lack of temporal sampling in the present study, it is suggested that future studies should be conducted over a number of years and therefore increase analytical power.

The significant correlation between the anal and dorsal fin rays and the right and left pectoral fin rays indicates a similar environmentally induced response within each fin ray pair. A similar correlation was found for dorsal and anal fin rays in previous studies of juvenile Japanese flounder (*Paralichthys olivaceus*) (Kinoshita *et al.,* 2000) and European flounder (Haynes *et al.,* 2008). The fact that the response in pectoral fin ray numbers is independent of variation in dorsal or anal counts suggests that fin ray development and segmentation may be influenced by more than one environmental factor during the pelagic phase or may be determined during different stages of development. This study provides useful information to support taxonomic identification based on fin ray counts. The results highlight that flounder fin ray counts can vary between nursery areas and may therefore be useful in tracing flounder population movements and distribution when combined with other stock identification techniques. Further investigation into the temporal and spatial stability of flounder fin ray counts is warranted.

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Chapter Three

Investigation of early life events of European flounder (*Platichthys flesus* L.) within Galway Bay, west Ireland, as described by otolith microstructure

Abstract

Otolith microstructure was used to describe 0-group flounder (*Platichthys flesus* L.) early life history events (timing of hatching and settlement, size at settlement, duration of the larval phase and larval growth rates) within Galway Bay, west Ireland. Sampling was conducted in four sites in April over three years (2005, 2008 and 2009), and also included two sites in May and June in 2008. Hatching and settlement periods occurred in February/March and March/April respectively. This was earlier than the hatching and settlement periods reported in other studies conducted on other European populations (except for settlement on the Portuguese coast which was similar to this study). Intra-annual variation in flounder life history traits (hatch period and pelagic larval duration) were identified which may be attributed to selective mortality of specific cohorts and/or movements of flounder between and within estuaries. Larval otolith growth rates and hatching times varied between years which could reflect inter-annual differences in local environmental conditions. The present study is the first to use otolith microstructure in estimating early life history events from juvenile European flounder and therefore provides essential baseline information.

Keywords: *Platichthys flesus*, early life history traits, otolith microstructure, Ireland, Galway Bay

1. Introduction

European flounder (*Platichthys flesus*) is a widely distributed euryhaline flatfish species (Family: Pleuronectidae) ranging from the Eastern Atlantic, Mediterranean, White Sea and Black Sea (Anon, 2011a). Adult flounder aggregate at sea to spawn from late winter to spring (Wheeler, 1969; Sims *et al.*, 2005) and produce eggs which hatch 6-7 days after fertilisation (Hutchinson & Hawkins, 2004). Near shore habitats are recognised as essential nursery areas for flounder (Able, 2005), supporting high numbers of juveniles, with enhanced survival and growth rates relative to other habitats (Beck *et al.*, 2001; Le Pape *et al.*, 2003). Both eggs and larvae rely on water currents for transportation (Campos, 1994) prior to the development of fins which can help larvae direct their onshore migration to some extent (Grioche *et al.*, 2000). The perception of tidal rhythms can assist the migration to specific areas within an estuarine nursery (Bos, 1999; Jager, 1999), where the

larvae eventually settle (Hutchinson and Hawkins, 1993; Bos, 1999), and transform into the well known benthic flatfish form.

Otoliths can provide valuable information on the growth and early life history dynamics of fish. The analysis of daily increments in otoliths has been widely used to estimate age, the timing of life history stages and growth rates in larval and juvenile fish (Beaumont and Mann, 1984; Karakiri *et al.*, 1991; Fox *et al.*, 2007; Allen *et al.*, 2008; De Raedemaecker *et al.*, 2010; Gunnarsson *et al.*, 2010; Haynes *et al.*, 2011). The deposition of diurnal increments has been confirmed for numerous flatfish species including flounder (Bos, 1999). In flatfish, the pelagic larval duration is indicated by a series of increments circling the otolith hatch check. These increments may be difficult to interpret due to the narrowing of increments when fish are exposed to unfavourable low temperature (<5°C) conditions (Karakiri and von Westernhagen, 1989). The transition from a pelagic to benthic lifestyle is associated with behavioural and morphological changes termed metamorphosis which often coincides with settlement onto nursery grounds (Geffen *et al*, 2007; Hutchinson and Hawkins, 2004). During metamorphosis daily increment deposition is disturbed and a number of checks known as accessory primordia are formed in the otoliths (Modin *et al.*, 1996; Stevenson and Campana, 1992).

Variations in otolith growth rates, and the timing and duration of early life history stages have been associated with environmental variables such as temperature, salinity, light conditions (Ryland and Nichols, 1975; Karakiri and Von Westernhagen, 1989; Rankin and Sponaugle, 2011) and turbulence (Gallego *et al.*, 1996). Early life history traits (ELHTs) are also influenced by biological factors including size at hatching (Kennedy *et al.*, 2007) and the abundance of predators and prey (Winemiller and Rose, 1993; Allen *et al.*, 2008). Factors which affect the timing of ELHTs, growth and survival of eggs, larvae and juveniles can have a direct effect on the likelihood of individuals reaching nursery habitats and eventually joining the sexual mature population. Therefore, otolith microstructure analysis may provide useful information regarding the selective mortality of young fish (Folkvord *et al.*, 2010).

Year class strength and recruitment of fishes are attributed to variation in mortality during the early life stages (van der Veer *et al.*, 1994; Caley *et al.*, 1996; Nash and Geffen, 2012). Although the majority of fish mortalities occur in the egg and larval phase (Houde, 1987; Chambers *et al.*, 2001), effects which occur during the pelagic phase can also affect subsequent life stages (Sponaugle and Pinkard, 2004; Raventos and Macpherson, 2005; Hamilton *et al.*, 2008; Smith and Shima, 2011). Variation in larval growth and condition, pelagic larval duration (PLD) and size at age can all result in differential survival during

the settlement and juvenile phase of fishes (Searcy and Sponaugle, 2001; Hoey and McCormick, 2004; McCormick and Hoey, 2004; Raventos and Macpherson, 2005). As a result the ELHT's of surviving juveniles represent a subset of those displayed by the original settlers (Sponaugle and Grorud-Colvert, 2006). When hatching and settlement occurs over an extended period, multiple sampling events are needed to detect all cohorts and determine how life history events can change over time. In addition, microhabitat choice can change with fish growth and development (Ramos *et al.*, 2010) Failing to sample both spatially and temporally may produce bias in the calculation of ELHTs as certain cohorts may be not be observed.

There is no specific fishery for flounder in many European countries, with the exception of Baltic and Danish waters where it is a target species (Anon, 2010; Anon, 2011a). Nonetheless, global catches have been increasing since the 1950s, exceeding 24,000 tonnes since the early 2000's (Anon, 2011a). Although not considered commercially important in Ireland, Keirse (2008) estimated that flounder recreational fishing is worth 8.4 million euros annually to the Irish economy. Compared to more economically important flatfish species such as plaice, flounder has attracted little scientific study (Skerritt, 2010) and early life history dynamics derived from otolith microstructure have not been well described (with the exception of Bos, 1999).

The aim of the present study was to estimate the timing of hatching and settlement events, pelagic larval duration, size at settlement and larval growth of 0-group flounder by means of otolith microstructure analyses and also to examine spatial (sites) and temporal (seasonal and inter-annual) variation in these traits. Spatial variation in ELHTs could indicate underlying stock structure if cohorts/ populations inhabited different nurseries while, seasonal variation could reflect selective mortality or movements of cohorts after settlement. Timing of hatching and larval growth rates can have consequences for subsequent survival of juvenile and adult fish. Therefore, information on how these variables vary inter and intra-annually can provide insight into recruitment variability.

2. Materials and methods

2.1. Fish sampling

Galway Bay is a large bay located on the east Atlantic Ocean on the west coast of Ireland (Fig. 1). The bay is 62km long and its mouth ranges from 10 to 33km wide (Anon, 2011b). River runoff and shallow habitats make this bay a suitable nursery ground for 0-group flatfish.

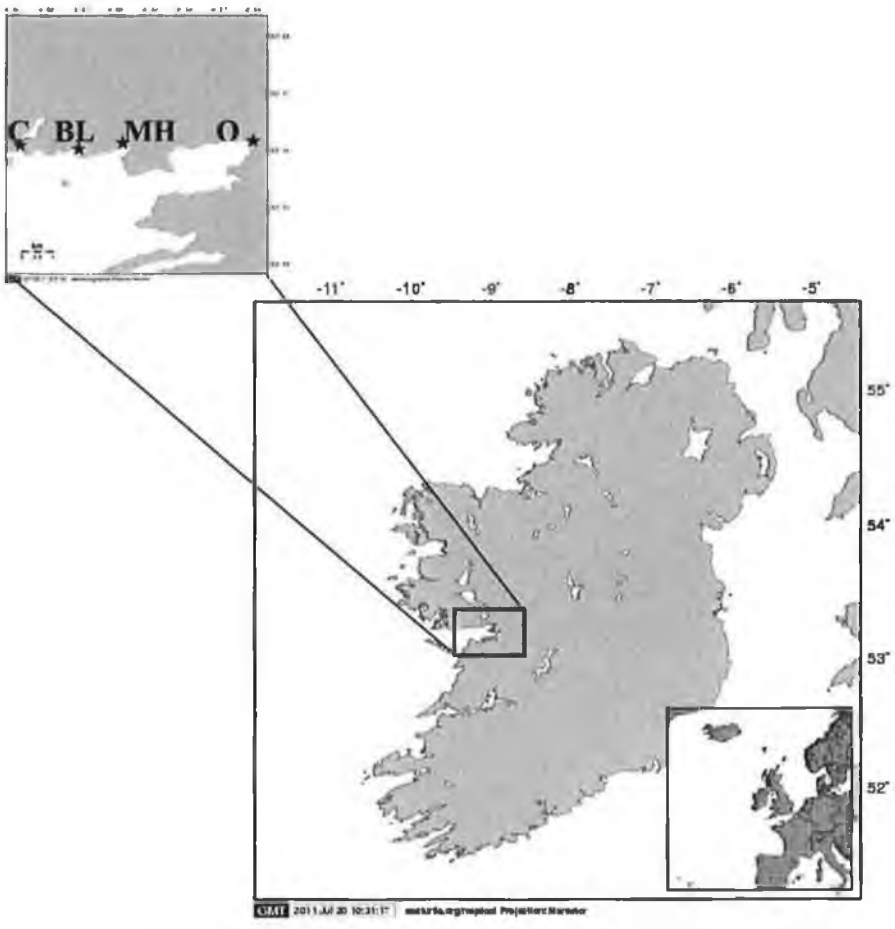


Fig. 1. Sampling sites used to assess *P. flesus* early life history located within Galway Bay, Republic of Ireland: C, Corrib; O, Oranmore; MH, Murrough house; BL, Ballyloughaun.

Sampling occurred in April over three years (2005, 2008 and 2009) on two beaches (Murrough House and Ballyloughaun) and two river estuaries (Corrib and Oranmore) (Fig. 1) within Galway Bay. In 2008, sampling was also carried out in May and June in order to assess how the measured variables could change intra-annually. Due to the presence of muddy and rocky substrate in the sampled estuarine locations, the most efficient method of catching recently settled flounder was by hand net, whilst push nets were used on the beaches due to the more homogenous substrate. All captured 0-group fish were frozen until processing. Fish were randomly sub-sampled prior to processing. A frequency distribution of all fish sampled revealed that each size class was sufficiently represented in the subsample (Fig. 2)

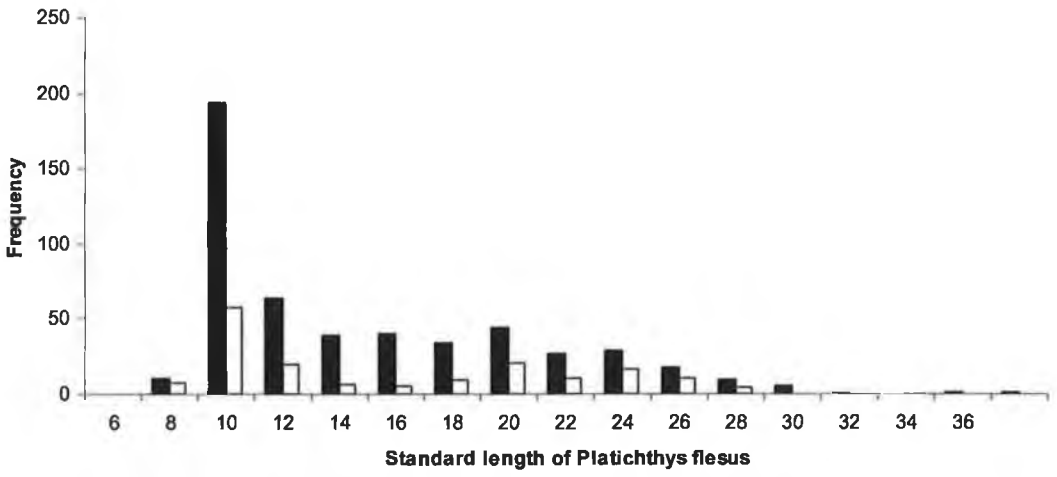


Fig. 2. Frequency distribution of fish size over two years (2008 & 2009) where the closed columns represent all fish sampled and open columns a subsample used for otolith analysis.

2.2. Otolith preparation and interpretation

Sagittal otoliths were removed from 0-group flounder, cleaned and placed in a mounting medium and attached to glass slides. Otoliths from 2005 were mounted in TAAB transmit resin while the otoliths from 2008 and 2009 were mounted in crystal bond (for larger otoliths which required polishing) or clear nail varnish (for very small easily read otoliths). Samples and data were collected in 2005 as part of a separate project. Otoliths were polished when necessary to improve the visibility of growth increments (Stevenson and Campana, 1992) using 2000 and 4000 grit silicon carbide paper. Otoliths extracted from the 2005 samples were examined using an Olympus Camedia C-3040 attached to Olympus CX41 light microscope and DP-Soft 3.2 image analysis software. All other otoliths (2008 and 2009) were examined using an Olympus BX51 interfaced with a cooled mono 12 bit Q Imaging camera, PC and Image Pro 6.3 image analysis system.

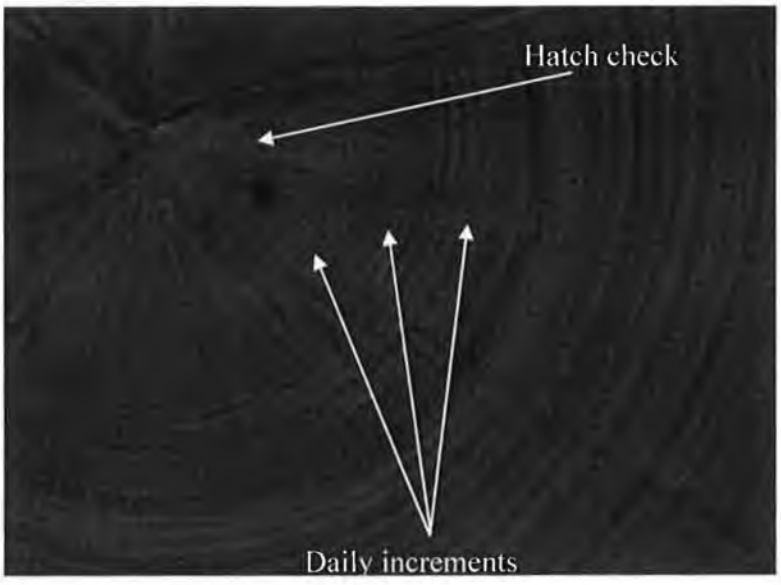


Fig. 3. Flounder otolith at 100x magnification. Arrows indicate observed hatch check and daily increments.

The total number of presumed daily rings (all recognisable pairs of concentric light and dark bands) were counted on each otolith starting at the hatch check. In the absence of hatch check validation for flounder sagittal otoliths it was assumed that the hatch checks were laid down similarly to that of plaice; characterised by a dark band around 10μm from the otolith core (Hovenkamp, 1990; Fig. 3). It is assumed that the presence of secondary growth (Accessory primordia) is indicative of benthic settlement and that increments after this represent post-settlement growth. Post settlement age was estimated by counting the daily increments from the first AP out to the tip of the last AP and continued out to the otolith edge. The pelagic larval duration (PLD) is described as the area between the hatch check and first accessory primordia (AP). The PLD was estimated by counting the number of rings from the hatch check to the last clear ring prior to the first AP. The hatch date of each fish was determined by subtracting the total increment number from the catch date while the settlement date was back-calculated from the date of capture to the increment prior to the first AP. Larval increment widths (days 1-27) were used as a proxy for larval growth (LG) and measured to the nearest 0.01μm. Individual LG was estimated by averaging the first 26 increment widths after the hatch check. No length or LG data was available from fish sampled in 2005. To assess reader precision, 29 otoliths were randomly selected and subjected to recount. All recounts of larval increment widths and increment number had a co-efficient of variation <12%. Initially right otoliths were chosen for examination; however, a number of right otoliths had to be discarded as they were cracked, over polished or unreadable. In such cases left otoliths were analysed. In a comparison of

10 sagittal pairs no significant difference in hatch day, larval duration, total age and larval growth was observed between the right and left otoliths (paired t-test, $p > 0.05$).

Fish length at settlement was back-calculated employing the biological intercept method as described in Stevenson and Campana (2002) using the formula: $L_a = L_c + (O_a - O_c)(L_c - L_i)(O_c - O_i)^{-1}$, Where L_a is length at settlement; L_c is length at capture (SL); O_a is otolith size at settlement; O_c is otolith size at capture; L_i is length at hatching, O_i is otolith size at hatching. Length at hatching was taken to be an average of 2.8mm (2.3-3.3) (Anon, 2011c).

2.3. Data analysis

In order to determine if the otolith growth rate reflected somatic growth, standard length and otolith radius were tested for correlation. In addition, both spatial (sites) and temporal (months and year) variability in early life variables were assessed (Fig. 4 and 5).

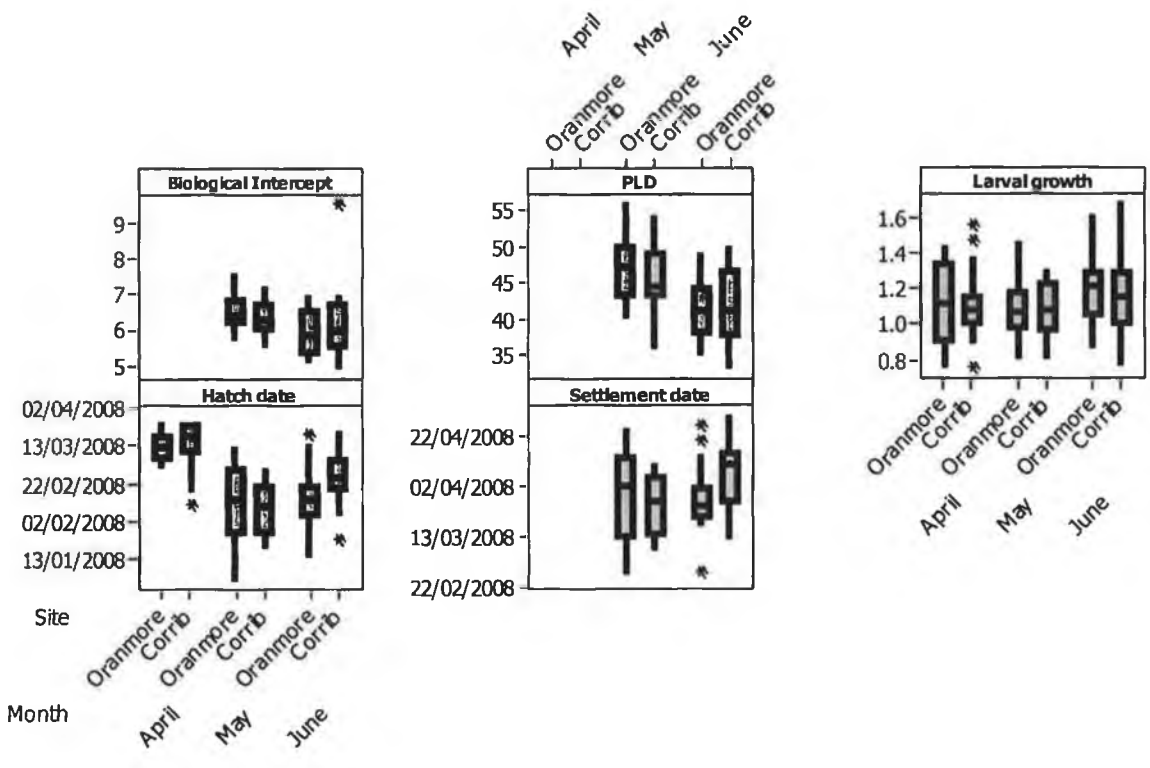


Fig. 4. Box plots displaying spatial and temporal variability in biological intercept (mm), pelagic larval duration (days), larval growth (μm), hatch date and settlement date of *P. flesus* in 2008 (May and June).

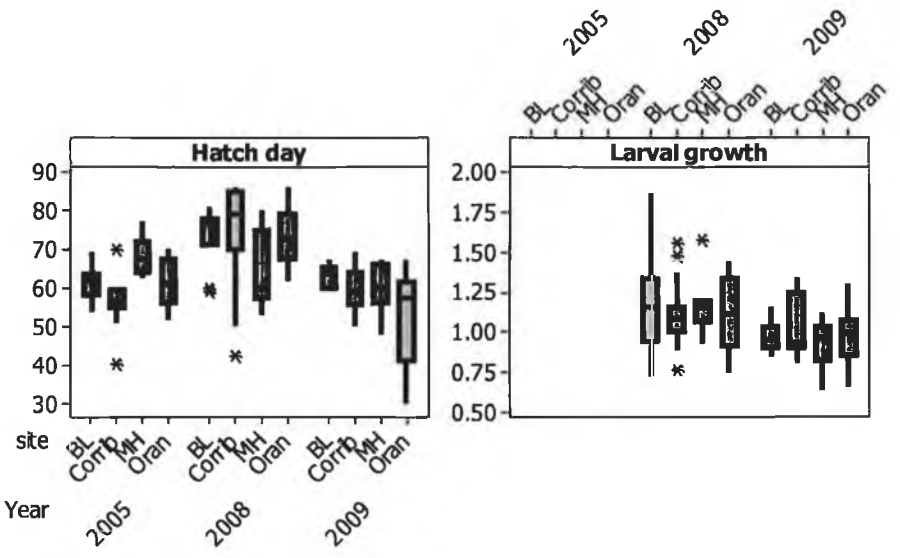


Fig. 5. Box plots displaying spatial and temporal variability in the hatch day (day of the year) and larval growth (μm) of *P. flesus* sampled in April over three years (2005, 2008 and 2009).

2.3.1. Seasonal variation

By sampling flounder over a number of months and sites, flounder early life history can be comprehensively described. A single sampling event may not capture earlier or later hatchers, while the potential influence of post-settlement mortality on the various hatching cohorts may not be accounted for. Therefore, monthly samples from more than one site are more representative of the 0-group population.

ANOVA's were used to examine variation in the five measured variables (L_a, LG, PLD, hatch date and settlement date) between two sites (Oranmore and Corrib) and between months (April, May and June) in 2008. The main effects and interactions were included in each model and all factors were treated as random. Flounder were not present in samples from the two beach sites after April, so seasonal comparisons could not be conducted for these sites. In April, settlement had just commenced and so many flounder had no accessory primordia on their otoliths and subsequently settlement dates, PLD and L_a could not be estimated; therefore these variables were compared only between fish collected in May and June.

2.3.2. Inter-annual variation

For settling founder collected in April (2005, 2008 and 2009), ANOVA's were used to compare LG and hatch dates between the four sites and between years, treating all factors as random. The main effects and interactions were included in each model. As

many of the flounder did not display accessory primordia; settlement, PLD and L_a could not be estimated and therefore, inter-annual variation in these variables could not be investigated. In addition, larval increment widths and standard length were not available for flounder sampled in 2005 so these variables were compared only between 2008 and 2009 sampled flounder.

Prior to analysis all data were subject to tests of normality. Bartlett's, Levene's and F-test (depending on the distribution of the data) were used to test for equal variances. Most of the data was suitable for parametric analysis. When data did not follow assumptions for parametric analysis non parametric analysis (Kruskal-Wallis) was carried out. This was the case for the comparison of L_a between months in 2008 (May and June). Where significant differences were detected these were further interrogated using Tukey's post hoc test (ranked in the case of non-parametric analysis). All statistical analysis was carried out in Minitab 15 with a significance level set at 0.05.

3. Results

Variable	Month	Site	n	Mean	Range (Days)	St Error
Hatch date						
	April					
		Oranmore	10	13 March	24	2.3
		Corrib	19	15 March	44	2.9
	May					
		Oranmore	20	12 February	71	4.8
		Corrib	20	09 February	42	3.0
	June					
		Oranmore	19	15 February	65	3.6
		Corrib	18	26 February	57	3.1
Mean larval otolith growth (µm)						
	April					
		Oranmore	10	1.1	0.8	0.1
		Corrib	19	1.1	0.7	0.0
	May					
		Oranmore	20	1.1	0.6	0.0
		Corrib	20	1.1	0.5	0.0
	June					
		Oranmore	19	1.2	0.7	0.0
		Corrib	18	1.1	0.9	0.1
Pelagic Larval Duration (days)						
	May					
		Oranmore	20	47	16	1.0
		Corrib	20	46	18	1.0
	June					
		Oranmore	19	42	14	1.0
		Corrib	20	42	17	1.3
Settlement date						
	May					
		Oranmore	20	30 March	58	4.3
		Corrib	20	25 March	34	2.7
	June					
		Oranmore	19	07 April	59	3.2
		Corrib	18	28 March	50	3.1
Biological Intercept (SL: mm)						
	May					
		Oranmore	20	6.5	1.8	0.1
		Corrib	20	6.4	1.7	0.1
	June					
		Oranmore	19	5.9	1.8	0.1
		Corrib	18	6.2	4.6	0.2

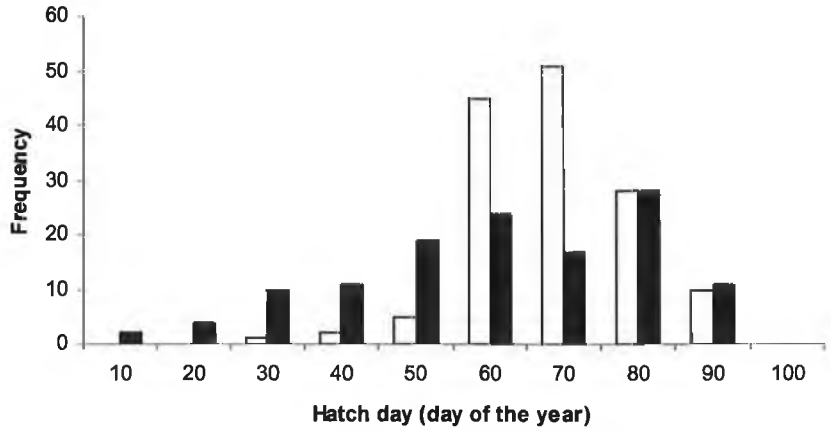
Table 1. Summary of the number of otoliths analysed, the mean, range and standard error of the mean for hatch day, settlement day, pelagic larval duration, larval growth and biological intercept for each site (Oranmore and Corrib) within each month (April-June 2008).

Variable	Year	Site	n	Mean	Range (days)	St Error
Hatch date						
	2005	BL	20	03 March	15	0.9
		MH	14	10 March	14	1.3
		Oranmore	9	02 March	18	2.1
		Corrib	10	25 February	30	2.4
	2008	BL	12	14 March	22	2.1
		MH	9	05 March	27	3.3
		Oranmore	10	13 March	24	2.3
		Corrib	19	15 March	44	2.9
	2009	BL	10	04 March	7	0.8
		MH	10	01 March	19	1.9
		Oranmore	10	22 February	37	4.0
		Corrib	9	01 March	19	1.9
Mean larval otolith Growth (µm)						
	2009	BL	12	1.2	1.1	0.1
		MH	9	1.2	0.6	0.1
		Oranmore	10	1.1	0.7	0.1
		Corrib	19	1.1	0.8	0.0
	2009	BL	10	1.0	0.3	0.0
		MH	10	1.0	0.5	0.0
		Oranmore	10	1.0	0.6	0.1
		Corrib	9	1.0	0.5	0.1

Table 2. Summary of the number of otolith analysed, the mean, range and standard error for hatch day and larval growth for each site (BL, Ballyloughaun; MH, Murrough House; Oranmore; Corrib) in April over three years (2005, 2008 and 2009).

The early life history variables derived from the otolith microstructure analysis are summarised in Table 1and 2. Examination of frequency distribution plots for settlement and hatching dates (all data combined) revealed no distinct pulses in hatching or settlement (Fig. 6a and b). A total of 219 individuals were used to estimate the hatching period of flounder within Galway Bay. Hatch day varied from early January to late March, peaking in late February/early March (Fig. 6a). A total of 110 juvenile flounder were used to estimate flounder settlement and PLD. Settlement dates ranges from February 27th - May 1st, peaking in March/April (Fig. 6b), whilst the average PLD for all fish examined was 43 days (s.d. 6).

(a)



(b)

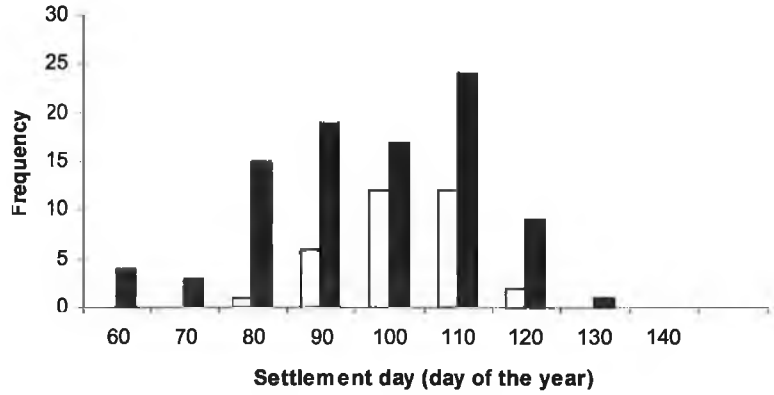


Fig. 6. Frequency distribution of hatch (a) and settlement (b) day where the open columns denote fish sampled from April 2005, 2008 and 2009 and the closed columns April, May and June 2008.

A strong positive correlation between flounder size (SL) and age ($n=176$; $p<0.001$; $r^2=0.86$) and otolith radius ($n=176$; $p<0.001$; $r^2=0.92$) shows that otolith growth is proportional to somatic growth in juvenile flounder. Fig. 7 shows a consistent increase in otolith increment width with age, indicating a corresponding increase in somatic growth.

3.1 Seasonal and spatial variation in 2008

Seasonal variation in ELHTs was found in flounder sampled in 2008 (Fig. 4; Table 3). There was a significant difference in hatch dates between months; fish sampled in April hatched later than those sampled in May (average 33 days) and June (average 23 days), whilst fish from samples taken in June hatched later (average 10 days) than fish sampled in May. This suggests that all hatching cohorts (early and late hatchers) were not represented in each sampling event. There was no significant difference ($p>0.05$) between sites and no interaction between site and month.

Juvenile flounder sampled in May 2008 had a significantly longer larval stage duration ($p<0.001$; average 4 days) and settled at a significantly larger size (L_a) ($p<0.05$) compared to those sampled in June which may reflect selective mortality. There was no consistent spatial or temporal variation in settlement dates; no significant differences were detected between months or between sites although the interaction between site and month was significant ($p<0.05$).

There were no significant differences ($p>0.05$) in larval otolith growth between sites or between months and no significant interaction between site and month.

Variable	Source	DF	F	P	r^2 (adj)
Hatch day					
	Month	2	40.09	0.000	44.03
	Site	1	1.11	0.294	
	Month*Site	2	1.95	0.148	
	Total	104			
MDLG					
	Month	2	1.76	0.178	0.00
	Site	1	0.64	0.426	
	Month*Site	2	0.11	0.899	
	Total	105			
PLD					
	Month	1	19.65	0.000	18.59
	Site	1	0.56	0.457	
	Month*Site	1	0.42	0.517	
	Total	76			
Settlement					
	Month	1	0.57	0.590	6.01
	Site	1	0.18	0.747	
	Month*Site	1	4.59	0.036	
	Total	75			
L_a					
Kruskal-Wallis					
	Month	1		0.003	
	June	1			
	May	1			
May					
	May	1	0.62	0.435	0.00
	Total	39			
June					
	Site	1	1.22	0.276	0.62
	Total	36			

Table 3. Statistical results from 0-group *P. flesus* otolith microstructure analysis (April-June 2008).

3.2. Inter-annual differences

Flounder sampled in April 2008 hatched later than those sampled in April 2005 and 2009 ($p<0.001$, Fig. 5; Table 4). However, this trend was not consistent across all sites over the three years and a significant interaction ($p<0.05$) between year and site was found.

Variable	Source	DF	F	P	r^2 (adj)
Hatch day					
	Year	2	6.30	0.033	39.89
	Site	3	0.27	0.847	
	Year*Site	6	4.90	0.000	
	Total	141			
MDLG					
	Year	1	12.98	0.001	8.60
	Site	3	0.13	0.941	
	Year*Site	3	0.80	0.499	
	Total	88			

Table 4 Statistical results from 0-group *P. flesus* otolith microstructure analysis (April 2005, 2008 and 2009).

Fish sampled in 2008 grew more quickly (as indicated by their otolith increment widths) compared to those sampled in 2009 ($p<0.05$, Fig. 7). There was no significant difference in otolith growth between sites and no interaction between site and year.

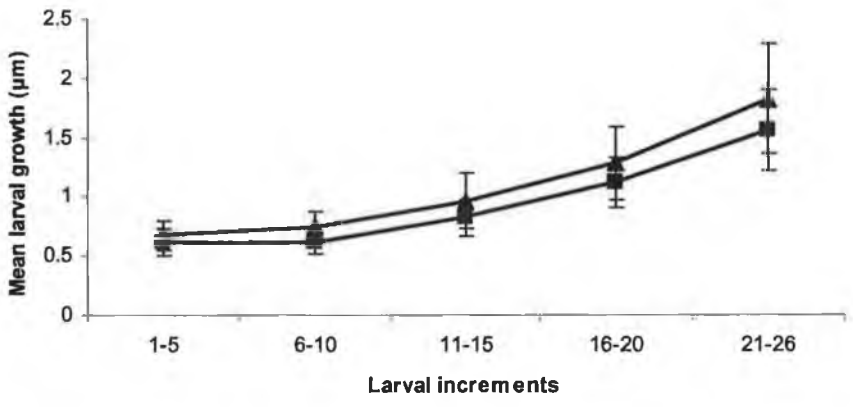


Fig. 7. Average width of otolith increments from day 1 to 27 for April 2008 (n=50) (▲) and 2009 (n=39) (■).

4. Discussion

The estimated hatching and settlement period for 0-group flounder within Galway Bay was from January to March and late February to early May with a peak in late February/early March and March/ April respectively. Bos (1999) found a slightly later hatching period in the River Elbe, (early March to early May), while Grioche *et al.* (1997) found that the flounder hatching period finished in May in the eastern English Channel. Settling juveniles were first found in May in the western Dutch Wadden Sea (van der Veer *et al.*, 1991) and July in the Baltic Sea area (Martinsson and Nissling, 2011) while settlement occurred in April in the Minho estuarine nursery grounds in Portugal (Freitas *et al.*, 2009). Regional and population differences in ELHTs are to be expected as changes in water temperature/currents and other biotic factors (prey and predator type and concentration) can have a profound affect on life history dynamics. In addition, differences in sampling strategies (spatial and temporal) can further confound comparisons between studies. Although a number of studies have examined the dynamics of flounder early life (Grioche *et al.*, 1997 and 2000; Jager, 1999; Martinho *et al.*, 2007; Martinsson and Nissling, 2011; O'Neill *et al.*, 2011), only Bos (1999) previously used otolith microstructure to determine the age of larvae. Therefore, the present manuscript is the first to the authors' knowledge that uses post larval flounder otolith microstructure to estimate the early life variables and as a result provides important baseline information on this understudied flatfish species.

The positive relationship between flounder standard length and otolith radius and fish age suggests that somatic growth is reflected in the growth of the otolith and therefore supports the use of otoliths in calculating the somatic growth in 0-group flounder. However, there is a certain amount of error associated with daily age and growth estimations due to the limitations of light microscopy (Fox *et al.*, 2003) as well as the difficulties of interpreting increments laid down during periods of slow or disturbed growth (metamorphosis) (Sogard, 1991; Fox *et al.*, 2003) and during adverse environmental conditions (Karakiri and von Westernhagen, 1989). In the present study, the timing of peak settlement estimated from the otolith microstructure analysis (March/ April) was consistent with the authors' observations of settling flounder in the estuaries in March/April. This suggests that the otolith readings were reasonably accurate.

A strong link between larval life (growth and condition) and post settlement mortality has been established for a number of marine fish species (Raventos and Macpherson, 2005; Grorud-Colvert and Sponaugle, 2006; Hamilton *et al.*, 2008).

Differential post-settlement survival of specific cohorts with different larval histories can produce variation in ELHTs between sampling events. In the present study, the otolith microstructure results suggest that flounder which spent longer in the pelagic environment and settled at a larger size were selectively removed from the population over time (from May to June 2008). A similar phenomenon was observed in newly settled bluehead wrasse (*Thalassoma bifasciatum*) (Grorud-Colvert and Sponaugle, 2010). Fish which have a longer PLD and settle at a larger size can be in poorer condition at settlement; this may decrease their chance of survival for a number of reasons (Searcy and Sponaugle, 2001; Grorud-Colvert and Sponaugle, 2006). Due to the low condition, fish take more risks and actively search for prey in the presence of predators while a reduction in swimming ability due to the lack of energy reserves decreases their ability to evade predators quickly (Grorud-Colvert and Sponaugle, 2006).

Alternatively, the observed differences in hatch dates and PLD between months may reflect movements of cohorts within and between estuaries. Low connectivity between estuarine nursery grounds has been identified for a number of juvenile flatfish which is probably due to the weak swimming ability of larval and juvenile flatfish (Gibson, 2005; Vinagre *et al.*, 2008a). Therefore, the movement of post larval flounder between different estuaries is unlikely. However, larvae and juveniles can undergo extensive migrations within an estuary (up to 7km; Ramos *et al.*, 2010) with the help of selective tidal stream transport (Jager, 1999; Gibson, 2005). As flounder grow and develop their choice of microhabitat changes (Ramos *et al.*, 2010); smaller individuals concentrate in the upper estuarine sites and larger individuals in the lower sites (Vinagre *et al.*, 2008b; Pers obs). The early hatching fish, which were absent from samples collected in April 2008, but appeared in catches later in the sampling season (May and June), may have moved from elsewhere in the estuary, downstream to the sampling area after April due to an ontogenetic shift in microhabitat use. Further investigation incorporating tagging of juvenile flounder could elucidate ontogenetic shifts in flounder distribution within the estuaries. As well as highlighting the importance of the larval phase for post-settlement survival these results highlight the importance of sampling over an extended period in order to fully describe settlement dynamics for fish with extended hatching and settlement periods.

Inter-annual differences were observed in flounder larval growth rate and time of hatching in Galway Bay. The observed variation could reflect a real difference across the whole population over the three years which may be due to a change in temperature and/or prey availability during egg development and onshore migration (von Westernhagen, 1970;

Karakiri and von Westernhagen, 1989; Feet *et al.*, 2002; Otterlei *et al.*, 2002; Aldanondo *et al.*, 2008). Irish Marine Institute data buoy M3 (Irish west coast) revealed a higher water surface temperature in the first three months of 2008 compared to 2009. This difference may help explain the inter-annual difference in growth, however, growth data of more than two years is required to test this further. Alternatively, selective mortality processes during larval immigration and/or initial settlement may have changed between the years producing differences in ELHT's of the surviving juveniles. Perhaps in 2008 survival of the late hatchers was enhanced relative to other cohorts whereas in 2005 and 2009 the early hatching individuals did better. According to the growth-mortality hypothesis (reviewed in Anderson, 1988), faster larval growth can result in increased survival, therefore inter-annual variation in hatching time and associated variation in growth rates may lead to variable recruitment in flounder. However, a longer time series of data is required to investigate further how the timing of hatching and larval growth can affect flounder recruitment.

In summary, this study describes the early life history of *P. flesus* on the Irish west coast using otolith microstructure analysis. Seasonal variation in hatching and PLD is detected which may reflect differential survival of hatching cohorts or ontogenetic movements within an estuary. Larval otolith growth rates were shown to vary between years, possibly due to environmental factors. Further research into the mechanisms controlling the observed variation may provide insight into recruitment variability in flounder. The seasonal and annual differences in ELHTs highlight the importance of looking at multiple sampling events in order to grasp the full picture of flounder early life.

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Chapter Four

Habitat utilisation in 0-group European flounder, *Platichthys flesus* (L.), in Galway Bay, Ireland

Abstract

Otolith microstructure and abundance estimates were employed to examine possible habitat induced differences in the early life of flounder. Flounder sampled from two habitats of different salinities (rivers and beaches) were from a homogenous hatching cohort. The presence of accessory primordia was strongly related to standard length which corroborates previous studies that metamorphosis is size dependant. Flounder sampled on the high salinity beach habitats did not delay development as expected and were younger than those in the rivers. This difference in age may be due to increased mortality of new recruits in the higher salinity habitat or the movement of older flounder out of the beach habitats. In addition, trends in abundance showed differences between the beach and river habitats. Abundance peaked in both habitats in April and then decreased dramatically in May and June on the beaches while a steady seasonal decline was observed in the rivers. Overall, flounder were found to use river habitats to a greater extent compared to beaches while overwintering in the rivers was unlikely. The results suggest that although post-larval flounder are delivered to beaches, rivers are a more important habitat for settling flounder either due to enhanced survival or active migration to lower salinity areas.

Keywords: *Platichthys flesus*, habitat utilisation, Otolith microstructure, Galway Bay, Ireland.

1. Introduction

The European flounder, *Platichthys flesus* (Linneaus 1758), family Pleuronectidae, is a migratory species found in coastal and estuarine habitats (Russell, 1976; Summers, 1979; Morais *et al.*, 2011) throughout Europe (Wheeler, 1969). Flounder are largely a catadromous species although recent studies have revealed some exceptions (Morais *et al.*, 2011). Adult flounder move off shore in winter (Russell, 1976) and spawn in deeper waters in spring (Wheeler, 1969). On hatching, the larvae drift with currents to coastal locations (Campos, 1994) and have the ability to perform vertical migration subsequent to fin formation (Grioche *et al.*, 2000). Once within estuarine systems flounder can actively migrate to optimal sites using selective tidal stream transport (Bos, 1999; Jager, 1999, 2001). Flounder first appear in estuaries in spring (Summers, 1979; Hutchinson and Hawkins, 1993; Martinho *et al.*, 2008) where they grow and become accustomed to a benthic orientated life style (Kerstan, 1991) prior to seaward dispersal in autumn (Skerritt,

2010). At the southern limit of their distribution range, flounder have been found to spawn in estuaries and coastal areas (Morais *et al.*, 2011) and have the ability to spend the first year over wintering in estuaries (Martinho *et al.*, 2007, 2008).

Estuaries and coastal habitats are recognised as important flounder nurseries, often supporting large numbers of larvae and juveniles (Rasmussen, 2005; Freitas *et al.*, 2009; Ramos *et al.*, 2009; Vasconcelos *et al.*, 2011). Larvae drifting from the spawning grounds to nursery grounds are subject to very high mortality due to predation, starvation and transport to unsuitable habitat, which can affect the overall recruitment success of a species (Houde 1987). Surviving larvae are delivered to nursery habitats, the quality of which can vary. High quality nursery habitats support high rates of growth and survival of juvenile fish, whereas settlement onto suboptimal nursery areas can lead to less successful recruitment (Gibson, 1994).

To date, studies of flatfish early life history have focused on either estuarine or coastal nurseries and comparisons between habitat types are scarce. Environmental factors can vary significantly between these habitat types which can ultimately affect growth, survival, development and eventual recruitment to the adult populations (Power *et al.*, 2000). Evidence suggests that salinity influences growth, development and migration of flounder (Gutt, 1985; Bos and Thiel, 2006; Hutchinson and Hawkins, 2004) and flounder that settle in high salinity sites can have reduced rates of growth, development and survival. Due to the weak swimming abilities of flounder larvae prior to fin formation (Grioche *et al.*, 2000), the delivery of larval flounder to optimal (low salinity) nursery sites is largely dependent on dispersal by favourable wind and water currents (van der Veer *et al.*, 2000; reviewed in Houde, 2008). Flounder which are carried to suboptimal (high salinity) coastal habitats are at an immediate disadvantage as metamorphosis is likely to be delayed (Hutchinson and Hawkins, 2004) and vulnerability to predation will subsequently increase (Houde, 1987). Therefore, whether coastal or estuarine habitats are used during the first year of life has important consequences for recruitment to the adult stocks.

Daily increments deposited on otoliths have proved particularly useful for estimating early life history variables such as hatch dates, metamorphic and settlement dates and larval growth rates in many pelagic and flatfish species (e.g. Stevenson and Campana, 1992; Brophy and King, 2007; Gunnarsson *et al.*, 2010; Geffen *et al.*, 2011; Haynes *et al.*, 2011). Metamorphosis involves dramatic morphological and physiological changes and is associated with the transition from the pelagic to the benthic dwelling life stage in the majority of flatfish (Karakiri *et al.*, 1991; Modin *et al.*, 1996; Geffen *et al.*, 2007). Upon exposure to low salinity, flounder sink/ settle and metamorphic onset is

immediate (Hutchinson and Hawkins, 2004). During this transitionary period, accessory primordia (AP) are typically formed on the sagittal otoliths (Karakiri *et al.*, 1989). Consequently, the presence of AP on recently settled fish can be used to estimate the timing of settlement and metamorphic onset.

The ecology and economic importance of *P. flesus* differs throughout the species' distribution range (Cabral *et al.*, 2007; Martinho *et al.*, 2008; Anon, 2010a). Although considered commercially important in Baltic and Danish waters where it is targeted for fishing, flounder is considered a non-commercial species in most of Europe and is a by-catch in mixed demersal fisheries (Anon, 2010a, 2011a). Keirse (2008) identified substantial value in flounder shore angling in Ireland by both national and international recreational fishermen. The economic value was calculated as €8.4 million annually, which exceeds the value of many commercially important species. However, the economic worth of flounder recreational fishing in tourism has largely been overlooked and consequently no management or conservation measures have been established. Although a number of recent studies have been conducted on continental European populations (Nissling *et al.*, 2007; Franco *et al.*, 2010; Vasconcelos *et al.*, 2010; Morais *et al.*, 2011), little published literature on the ecology of flounder in Irish waters exists (Haynes *et al.*, 2008; Mariani *et al.*, 2010; O'Neill *et al.*, 2011). The present study is the first to elucidate early life history patterns in *P. flesus* using otolith microstructure in Irish waters while the spatial location of the sampling sites (west Ireland) can further increase our knowledge on flounder early life dynamics in this understudied geographic area.

The aim of the present study was to describe early life dynamics of flounder at sites with contrasting salinity regimes within Galway Bay on the west coast of Ireland. Otolith microstructure analysis was used to establish if flounder in river and beach habitats originated from the same cohort and to establish if flounder were settling in both habitats. The timing of metamorphic onset and the size dependent nature of metamorphosis were compared between the habitats to explore if settlement was delayed at higher salinity habitats. Seasonal changes in abundance in each habitat type were examined to determine seasonal trends and explore how flounder were using each habitat during the first year of life.

2. Materials and methods

2.1. Study area and sampling procedure

Flounder were collected from four sites in inner Galway Bay (Fig. 1). The four sites were categorised as either beach or river habitats. Galway Bay is a semi enclosed body of water, where freshwater from rivers mixes with oceanic saltwater. Therefore, the whole of Galway Bay can be considered an estuary. Two high salinity beaches (Ballyloughaun and Murrough House) and two low-medium salinity river habitats (Oranmore and Corrib) were sampled over five years (2005, 2006, 2008 and 2009). Samples and data collected in 2005 and 2006 were collected as part of a separate project. The four sites were chosen as flounder had been recorded there during previous unpublished surveys conducted by the authors. Salinity was measured on each sampling occasion and it was established that the upper river zones were representative of low salinity, lower river zones as medium salinity and the beaches as high salinity zones. Flounder from both river zones (upper and lower) were used to represent the river habitat.

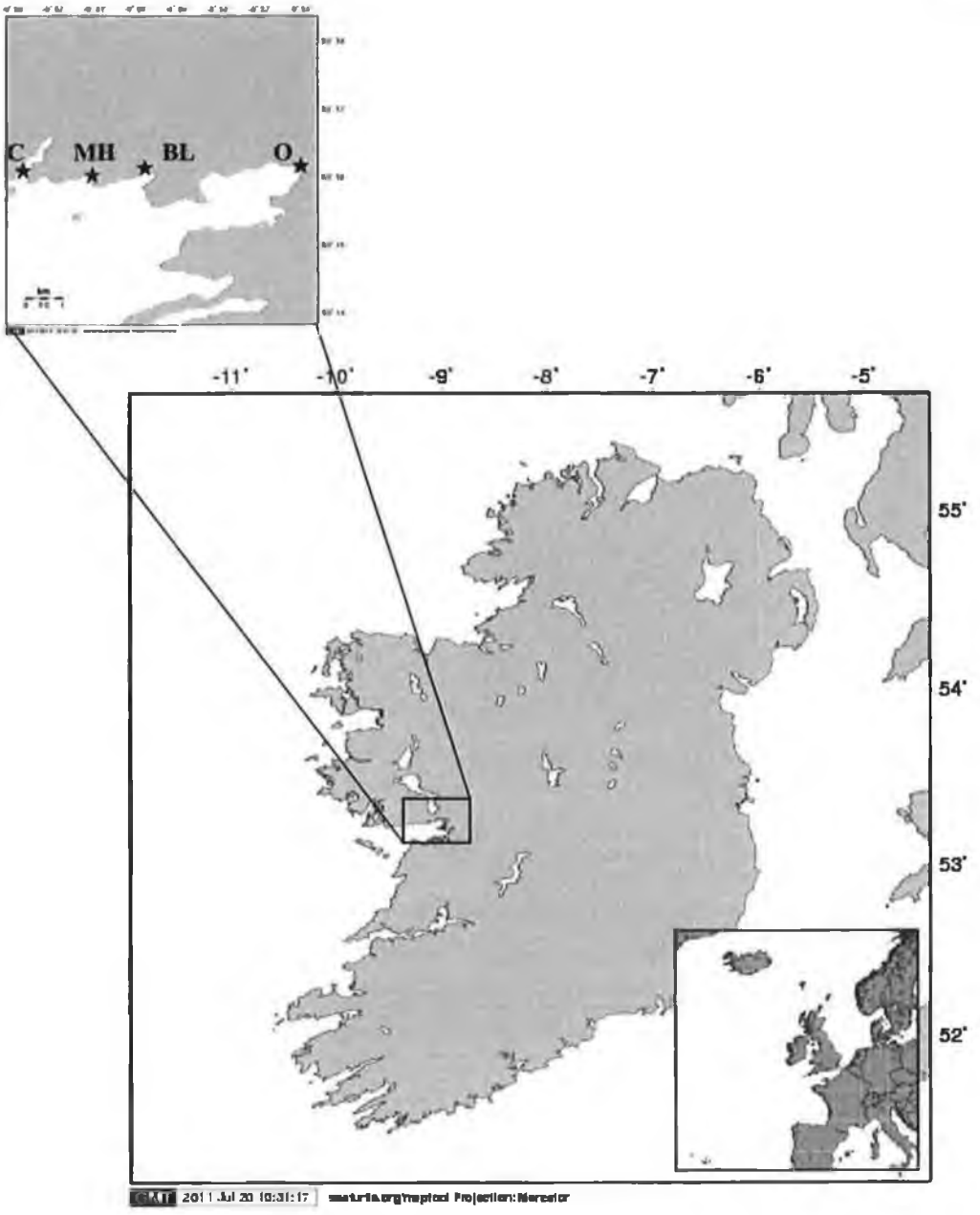


Fig. 1. Locations of the four sampling sites located within Galway Bay, Republic of Ireland. C, Corrib; O, Oranmore; MH, Murrough House; BL, Ballyloughaun.

Sampling took place fortnightly at all locations, from March to August in 2005 and 2006 and from April to August in 2008. Additional samples were collected from both beaches in September and October 2008 and from both rivers and both beaches in April 2009. Relative abundance (number of fish per number of hauls) was calculated for each sampling occasion for the two rivers over three years (2005, 2006 and 2008) and the two beach sites within one year (2008). Numbers of plaice (*Pleuronectes platessa* Linneaus 1758*)* were also recorded at the beaches (this species was absent from the river sites) and relative abundance was calculated for both plaice and flounder sampled on beaches in

2008. It's assumed that if larval/juvenile plaice were successfully sampled by push net and beach seine, then if present, larval/juvenile flounder should also be detected. As flounder larval transport can vary temporally and spatially and is highly dependent on tidal cycles (Jager, 2001), all sampling was carried out within two hours of low tide to remove possible variation associated with tidal cycle effects.

Hand nets were used to sample the river locations as they proved to be the most efficient method of catching recently settled flounder from a muddy and rocky substrate. The hand net had a depth of 12cm and mesh size of 1mm and was swept along a 3m transect. A Riley push net was used to sample flounder on the beaches as its mesh size and design is intended for such use. The push net had 1.5m x 0.3 frame made from 4cm box iron. The net was a 10mm square mesh shrimp net, lined with 2mm heavy duty curtain mesh and was pushed 50m parallel to the beach. A Danish style beach seine (5.5m long and 2m deep, with a 5mm mesh size) was used to collect larger individuals on the beach locations later in the season in 2008 (August, September and October), therefore decreasing the chance of net avoidance by the larger juveniles. Net size and swept area should be considered when comparing abundances between habitats as both the beach seine and push net were larger and had a larger swept area compared to the hand nets which were used in the rivers. The swept area (net width * pushed area) of each hand net was $0.36m^2$ and push net was $75m^2$. The swept area of the beach seine was more difficult to calculate due to the variation in the recovery of each net. Nonetheless it's suggested that the swept area is similar to that of the push net. Seasonal trends in flounder relative abundance within each habitat were examined.

2.2. Otolith microstructure examination

Sagittal otoliths were placed in a mounting medium and attached to glass slides. Otoliths from 2005 were mounted in TAAB transmit resin while the otoliths extracted from 2008 and 2009 sampled individuals were mounted in crystal bond. Otoliths were polished until the hatch check was visible. In the absence of hatch check validation for flounder sagittal otoliths it was assumed that the hatch checks were laid down similarly to that of plaice; characterised by a dark band around 10μm from the otolith core (Hovenkamp, 1990). When polishing was not required otoliths were mounted in clear nail varnish for immediate microstructure examination. Otoliths extracted from the 2005 samples were examined using an Olympus Camedia C-3040 attached to Olympus CX41 light microscope and DP-Soft 3.2 image analysis software. All other otoliths (2008 and 2009) were examined using an Olympus BX51 interfaced with a cooled mono 12 bit Q

Imaging camera, PC and Image Pro 6.3 image analysis system. Cracked or unreadable otoliths were eliminated from analyses. A subsample of 10 fish were chosen to compare all measured variables between the right and left otolith and no significant difference was observed (paired t-test, $p > 0.05$). This consistency in readings between right and left otoliths justified the use of either otolith in analysis.

The total number of presumed daily rings (a pair of concentric light and dark bands) were counted on each otolith and used as an estimation of fish age. The larval period is described as the area between the hatch check and first accessory primordia (AP). In the closely related Pleuronectidae species, plaice settlement (transition to the bottom dwelling phase) and metamorphosis (transformation to the typical flatfish appearance) has been associated with the formation of accessory primordia on sagittae otoliths (Karakiri *et al.*, 1989; Modin *et al.*, 1996). An estimated hatch date of each fish was determined by subtracting the total increment number from the catch date.

Otolith increment widths were used as a proxy for larval growth rates (LG) and were calculated by measuring the increment widths along the longest readable axis from day 1 to 27. The average larval growth rate for each individual was estimated by calculating the average width of the 26 increment widths.

29 otoliths were re-analysed to assess the precision of estimated larval duration, larval growth rate and total age. The co-efficient of variation for repeat readings was <12%. Therefore, original readings were considered reliable for estimating life history variables.

2.3. Statistical analysis

All statistical analysis was carried out using Minitab 15 with the significance level set at $p \leq 0.05$. Prior to analysis, all data were subject to tests of normality. Bartlett's, Levene's and F-test (depending on the distribution of the data) were used to test for equal variances. ANOVA's or the non-parametric Kruskal Wallis test was used to compare early life history traits between habitats as appropriate. When significant differences were detected, Tukey's post hoc test was used to establish the origin of these differences.

Due to unequal variances, a Kruskal-Wallis was used to test if fish with AP present in their otoliths (indicating that metamorphosis and settlement has commenced) were bigger and older than fish without AP. The purpose of this analysis was to establish if metamorphosis is size/age dependant in wild flounder.

Metamorphic onset has been linked to salinity in experimentally reared flounder (Hutchinson and Hawkins, 2004). This study investigated the influence of salinity on the

rate of metamorphic development in wild caught flounder. The otolith microstructure data was examined for evidence of delayed metamorphosis in the higher salinity beach habitats. A chi-square analysis was used to determine if samples collected from low salinity river habitats contained a higher proportion of flounder with AP in their otoliths (indicating that metamorphosis had commenced) compared to samples collected from higher salinity beach habitats. An ANOVA examining the age of individuals from both habitats which had not yet deposited AP was carried out (Table 1). If flounder on the beaches were delaying metamorphosis those without AP should be older on the beaches compared to the rivers.

Variables	**df**	**Larval growth** 2008&09		**L_s** 2009		**df**	**Hatch day** 2005,08&09		**Age of fish without AP** 2005,08&09	
		F	P	F	P		F	P	F	P
Year	1	12.01	**	-	-	2	30.26	***	0.52	ns
Habitat	1	0.08	ns	2.55	ns	1	2.00	ns	4.47	*
Site (Habitat)	2	0.16	ns	2.89	ns	2	0.44	ns	0.49	ns

Table 1. Summary of ANOVA statistical results examining spatial variability between habitats. *P<0.05, **P<0.01, ***P<0.001, ns non-significant. Variability in the number of fish analysised exists between sites, habitats and years. 2005: BL=20; MH=14; Oran=9; Corrib=19. 2008: BL=12; MH=9; Oran=10; Corrib=9. 2009: BL=10; MH=10; Oran=10; Corrib=9. Of the 141 fish analysed 109 didn't display Ap.

Flounder spawned at certain times or locations may be transported to beach habitats while those from other cohorts disperse to rivers due to variation in circulation patterns. Otolith microstructure analysis was used to establish if flounder found in both habitats in April originated from the same spawning cohort. Nested ANOVAs were used to test for variation in hatch date and larval growth between flounder in the different habitats (Table 1). Year and site were treated as random factors while site was nested within the fixed factor habitat type (beach/river). Where the nested level was not significant it was removed and a one-way ANOVA was performed. The standard length data from 2008 showed unequal variances between habitats and sites. Therefore, data from 2008 and 2009 were analysed separately. A nested ANOVA was used to compare standard length between habitats and between sites nested within habitats in 2009. Kruskal-Wallis tests were used to test for variation in standard length between sites and habitats in 2008.

3. Results

A significant difference (Kruskal- Wallis; $p<0.001$) in standard length (n=89) and fish age (n= 142) was observed between flounder with and without AP. Individuals with AP were significantly larger and older than those without AP (Fig. 2). Flounder without AP had an average standard length of 9.2mm (s.d. 1.0) and age of 37 days (s.d. 5) while those displaying AP were on average 10.9mm (s.d. 1.3) and 57 days old (s.d. 10). Despite the fact that flounder from the two habitat types showed no difference in standard length, a significantly higher (chi-square; $p<0.001$) proportion of flounder sampled from the river habitats displayed AP compared to those sampled on the high salinity beaches. There was no evidence of delayed metamorphosis on the beaches; examination of fish without AP only, showed that individuals on the beaches were significantly younger than those in the rivers.

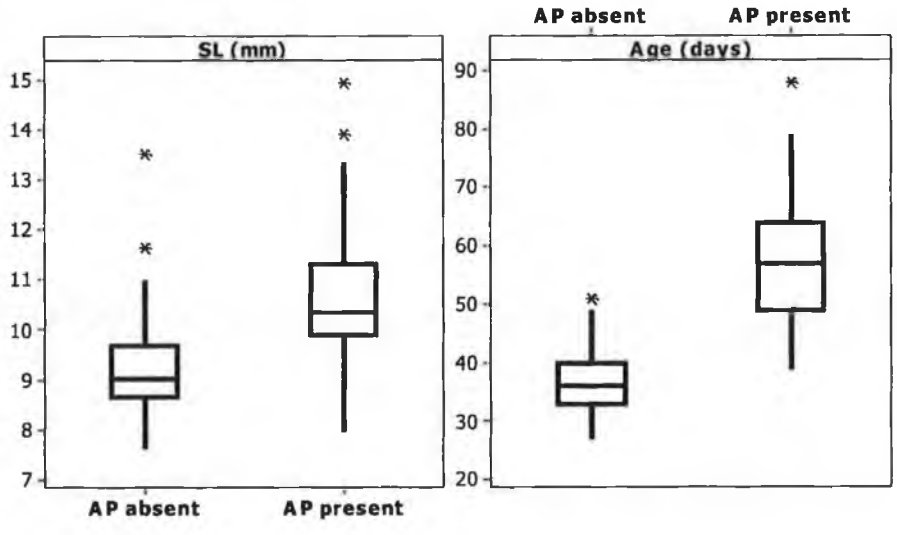


Fig. 2. Box-plot showing variation in standard length (L_s) (n=117) and age (days) (n=170) between flounder with and without accessory primordia on their sagittal otoliths. The horizontal line represents the median value and * symbolises extreme observations. The boxes signify the inter-quartile ranges while the whiskers (vertical lines) are values which extend from the box to adjacent values.

No significant variation in hatch day, larval growth rate or standard length was found between habitats and sites nested within habitats (Table I). High abundances of flounder were found on beach and river habitats in April (Fig. 3 and 4). A steady seasonal decline in abundance was subsequently observed in the rivers from March to August (Fig. 4) while flounder abundance decreased drastically in beaches, subsequent to April sampling (Fig. 3). Due to the difference in gear size and swept area the abundance of

flounder in the rivers in April were higher than those sampled on the beaches per unit of effort. It is also worth noting that flounder abundance increases in August with the use of a beach seine (Fig. 3) most likely due to its larger size and swept area.

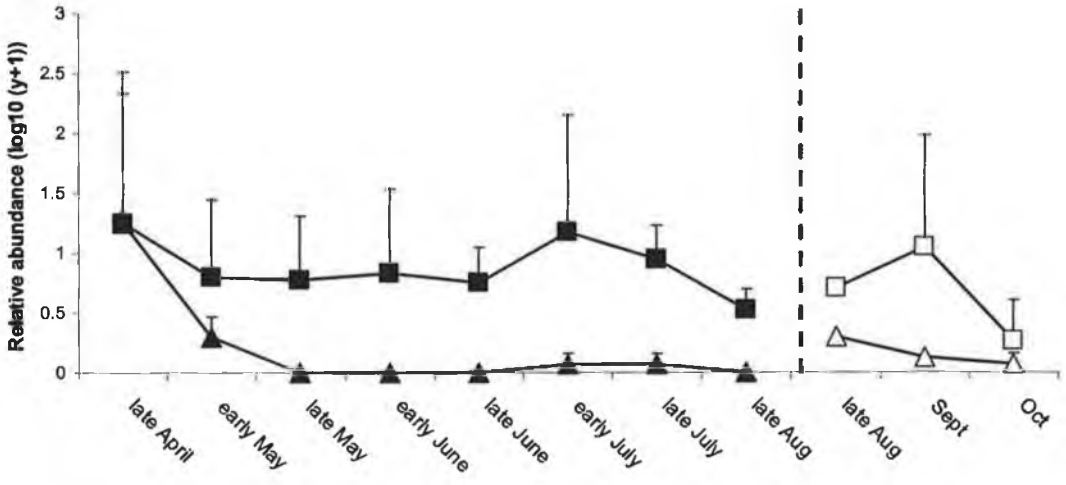


Fig. 3. Plot showing patterns of temporal variation in relative abundance of plaice (■) and flounder (▲) using push net (closed symbols) and beach seine (open symbols) on beach habitats in 2008. The solid vertical lines denote standard deviation of abundance while the dashed vertical line represents the change from push net to beach seine.

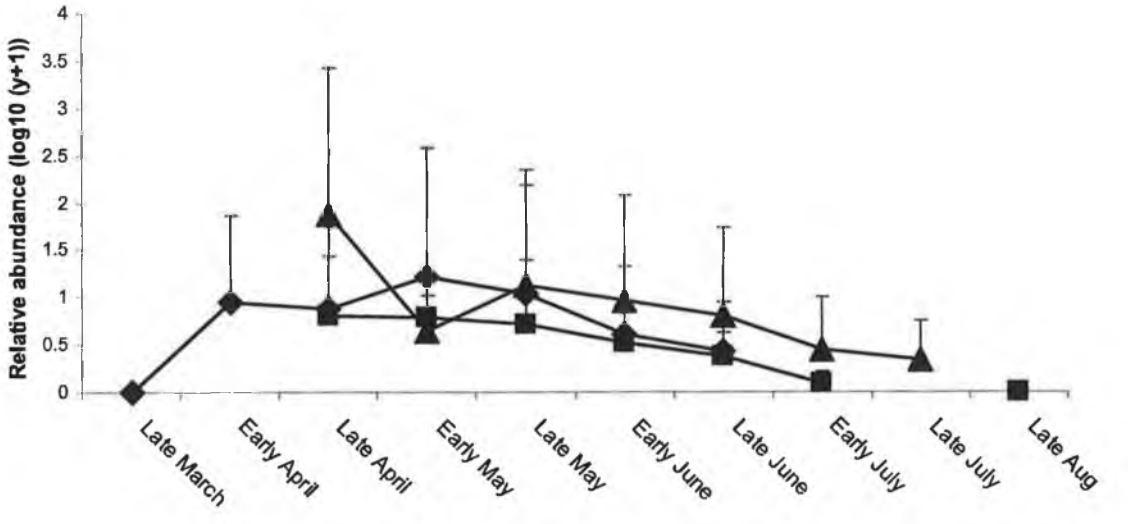
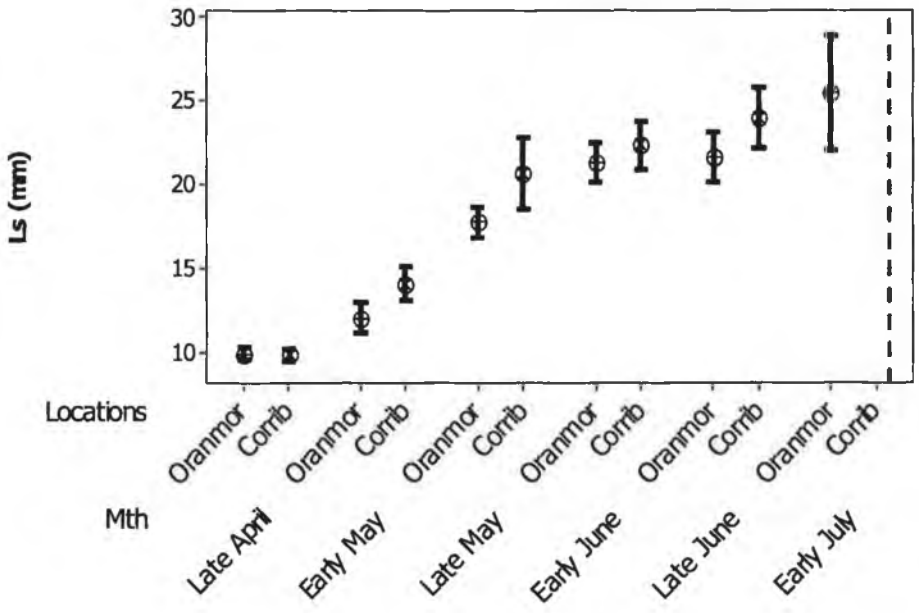


Fig. 4. Relative abundance of flounder within the river habitats over three years, 2005 (▲), 2006 (◆) and 2008 (■), where the vertical lines denote the standard deviation.

The decline and low abundances of flounder on beach habitats corresponded to the presence of plaice (Fig. 4) in the samples. A slight rise in flounder abundance was observed in July and August which may be due to an ontogenetic shift in habitat preference. Species overlap was not analysed in the rivers as the presence of other flatfish species besides flounder were rare. A considerable increase in flounder size was observed in the river and beach habitats over the 2008 sampling season (Fig. 5a and b). Subsequent to July flounder were undetectable in the rivers and therefore are not represented on Fig. 5a.

(a)



(b)

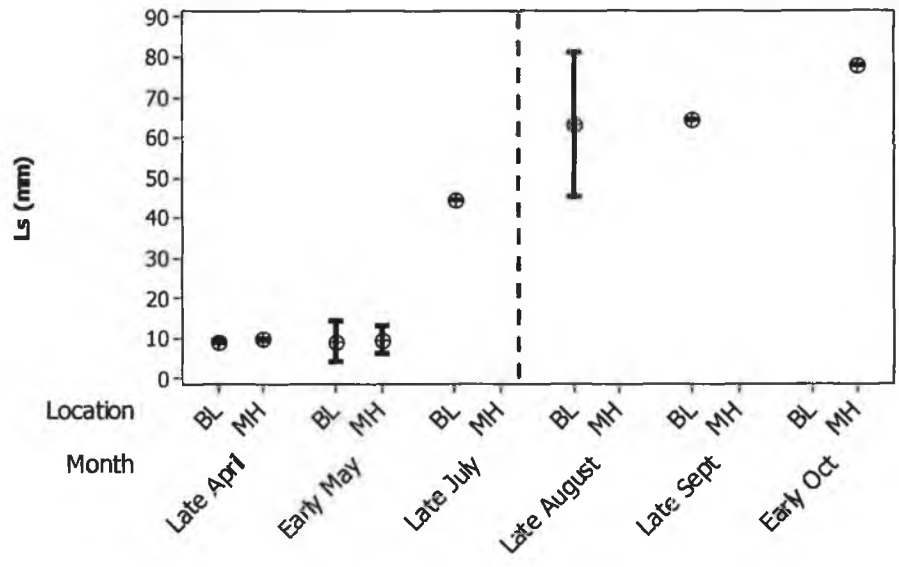


Fig. 5a (river) and 5b (beach). Plots showing seasonal patterns in the standard length of flounder in the estuarine (Oranmore and Corrib; n=367) and beach habitats (Murrough house, MH and Ballyloughaun, BL; n=85) in 2008. The dashed vertical line on fig 5a and b distinguishes between early (left of the line) and late season (right of the line) sampling. Flounder were undetectable in estuaries subsequent to July and therefore are not represented on the figure 5a.

4. Discussion

The present study revealed that metamorphosis (as indicated by the presence of AP on sagittal otoliths) is size dependent in wild flounder. Although flounder with AP were also older than flounder without AP, metamorphosis and settlement have been attributed to fish size rather than age (Policansky, 1983; Gavlik *et al.*, 2002; Hutchinson and Hawkins, 2004). Previous experimental studies found that flounder had to reach a critical length of 8.14mm total length (±0.61) in order to metamorphose (Hutchinson and Hawkins, 2004). However, in the present study the minimum size of flounder with AP was above this size threshold (9.43 L_s). This suggests that the minimum size necessary for metamorphosis is larger in wild flounder compared to experimentally reared individuals. This may be due to differences in environmental factors. Metamorphosis is an energy demanding period in fish development (Gwak *et al.*, 2003) and slight changes in rearing conditions such as prey type/quantity (variable vitamin and nutrient contents), temperature and salinity can affect the development and growth of flounder and subsequently size and timing of metamorphosis (Hutchinson and Hawkins, 2004; Pinto *et al.*, 2010; Fernández and Gisbert, 2011). In addition, the sample size used in the present study may have failed to capture

flounder settling at a smaller size, which suggests that a larger sample size may be more appropriate at detecting a true minimum settlement size.

This study found no evidence of delayed metamorphosis on the beaches. The presence of flounder with AP on their otoliths, albeit in low numbers, indicated that metamorphosis could occur in the higher salinity habitats. In addition, flounder without AP on the beaches were younger compared to those in the rivers suggesting that flounder on the beaches were not delaying settlement but instead were newer younger recruits. The absence of older flounder may be a consequence of local differences in larval dispersal, predation or movement of flounder from the beaches subsequent to arrival. Larval flounder can make minor adjustments to the direction of their onshore migration (Grioche *et al.*, 2000; Sentchev and Korotenko, 2007), while wind and water currents can further alter onshore movements (Grioche *et al.*, 1999; Sentchev and Korotenko, 2007). Due to the small spatial window available for direct transportation to rivers it is inevitable that some flounder will arrive on suboptimal habitats such as the beaches described in this study. Once within an estuarine system flounder can use selective tidal stream transport to migrate towards more favourable microhabitats (Bos, 1999; Jager, 1999) such as oligohaline and mesohaline sites (Vinagre *et al.*, 2005; Ramos *et al.*, 2009; Vasconcelos *et al.*, 2010), which can offer protection from predation and competition. Plaice are potential compeditors for juvenile flounder (Mariani *et al.*, 2010) while brown shrimp can compete with or predate upon (van der Veer *et al.*, 1991) flounder depending on their stage of ontogenetic development. Both are known to be less tolerant to low salinity environments compared to flounder (Wheeler, 1969; Freitas *et al.*, 2009). The lower numbers of older flounder on the beaches may reflect greater levels of predation and competition on new recruits in this habitat. 0-group flounder generally distribute and settle in low salinity, muddy and turbid habitats (Kerstan, 1991; Vinagre *et al.*, 2005; Cabral *et al.*, 2007; Ramos *et al.*, 2009; Zucchetta *et al.*, 2010). This preference for estuarine sites may be driven by predation and competition avoidance.

There were no differences in hatch date and larval growth between flounder from the river and beach habitats in April (2005, 2008 and 2009). This suggests that in each year, flounder which were delivered to both optimal and sub-optimal sites in inner Galway Bay originated from a common spawning cohort prior to reaching the nursery grounds. The transportation of pre-settlement flounder to sub optimal sites can directly affect development and survival which plays a crucial role in the year class recruitment (Houde, 2008). Therefore, understanding the biological (e.g. adult spawning locations) and

environmental (e.g. wind, water currents) factors that determine larval flounder transport, dispersal and distribution could help understand and predict recruitment.

The fate of flounder that are transported to sub-optimal beach habitats is uncertain. Three possible outcomes are suggested: (1) flounder complete metamorphosis on the beach habitats, develop and grow and eventually rejoin estuarine flounder during off shore migration in autumn/ winter, (2) due to increased stress on the beaches (predation/ competition), flounder die, (3) flounder actively migrate from the beach habitats to the other sites, possibly the lower salinity estuarine sites. Further investigation using natural (otolith chemistry) or anthropogenic (tagging devices) markers and an increased sampling effort is required to test these hypotheses. The decline in the abundance of flounder on the beaches relative to plaice suggests that they are not using the habitat to any great extent and are either suffering high mortalities or are migrating from the beaches to other areas.

The trends in relative abundance observed in the present study indicate that juvenile flounder do not overwinter in river habitats in Galway Bay. Flounder were absent from hand nets after July in 2008 which suggests that numbers were either too low to be detected or that flounder which were present migrated out of the rivers. The estimates of fish abundance may have been confounded by the efficiency of the hand nets; as fish grow and develop greater swimming capabilities the ability to avoid gear increases (Kuipers, 1975; Walsh, 1984; Kanou *et al.*, 2004). Although the employment of larger nets may have captured larger juveniles this was not possible in the present study due to environmental constraints within the rivers (substrate and obstacles). The push net and beach seine successfully caught plaice throughout the 2008 sampling season and there was no evidence of net avoidance by the larger plaice. It is therefore likely that flounder can also be caught using these methods if present at a sufficiently high abundance. Abundance estimates of both flounder and plaice differed considerably on the beaches, indicating that both species use the beach habitats differently. The slight increase in flounder abundance on the beach habitats in July and August may reflect the migration of juvenile flounder from the rivers to coastal habitats. The assessment of gear efficiency is suggested for future work as it would help elucidate if indeed fish were leaving the rivers or if the fish were actively avoiding the nets. Overwintering of 0-group *P. flesus* has been described in the Mondego estuary (southernmost region of flounder's distribution) and has been related to the high local water temperature (Martinho *et al.*, 2007). However, in northern regions of flounder distribution migration into offshore/coastal areas in autumn/winter is customary (Summers, 1979; Hutchinson and Hawkins, 1993; Anon, 2010a). The abrupt lowering of estuarine temperatures in autumn/winter generates a metabolic advantage for coastal/ offshore

movement (Able *et al.*, 2006). The estuarine dependence of flounder appears to apply only to the first few months of juvenile development which may be due to a decrease in predation and/or competition with increase size and development.

In the present study, very young flounder (< 26 days old) were absent from the river sites. This indicates that adults were not spawning in the rivers studied. Bos (1999) came to a similar conclusion for flounder sampled in the river Elbe in Germany. Although Morais *et al* (2011) described an estuarine spawning group of flounder in the Minho estuary, Portugal; coastal/offshore spawning is generally accepted for European flounder. The location of spawning grounds has not been established for flounder on the Irish west coast, six years (2004-2009) of biological surveys carried out by the Irish Marine Institute between February-April failed to capture a single adult flounder in the ICES zones Viib, Viij and Viig (Gerritsen *pers comm.*). This suggests that adult flounder were either not using these areas to spawn or spawned earlier in the year and left the sampling area. A dedicated sampling program with the help of a modelled projection of the spawning area is required to determine flounder spawning grounds.

Although the recruitment of juveniles to the adult population was not assessed, the high relative abundance of juvenile flounder observed in the rivers in April and the consistent presence of juvenile flounder in the rivers until July demonstrate the importance of these rivers as nursery grounds. The function of the rivers as effective juvenile habitats can however be affected by anthropogenic activities such as habitat destruction and pollution (Gilliers *et al.*, 2004; Amara *et al.*, 2007; Le Pape *et al.*, 2007; Courrat *et al.*, 2009). Therefore, the importance of these river sites as flounder nurseries should be considered in the future management plans.

The present study is the first to identify flounder nursery grounds in Ireland. The results show that metamorphosis in flounder is size dependant. Although post-larval flounder are delivered to beaches, rivers are a more important habitat for settling flounder either due to enhanced survival or active migration to lower salinity areas. The rivers in the present study appear to be superior juvenile habitats compared to the beaches. In Galway Bay juvenile flounder are likely to migrate out of the rivers in the autumn/winter of their first year rather than spend the winter there. Information on the location of flounder spawning grounds and the factors that influence the dispersal of flounder larvae to coastal areas may improve our understanding of recruitment variability for this species. It is suggested that natural resource such as estuaries need to be protected and monitored, as degradation of essential fish habitats can significantly affect recruitment to the adult spawning population.

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Chapter Five

An experimental investigation of salinity effects on growth, development and condition in the European flounder (*Platichthys flesus*. L.)

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Author contribution

O'Neill, B: Sampling, processing and analysis of samples, maintaining live specimens, data analysis and writing of manuscript.

De Raedemaecker, F: Established RNA:DNA protocol.

McGrath, D: Secondary project supervisor.

Brophy, D: Primary project supervisor

Abstract

European flounder (*Platichthys flesus*. L.) is a euryhaline flatfish species which can actively migrate towards and cope with low salinity environments. A laboratory experiment was undertaken to analyse the effect of salinity on condition and growth of metamorphosing European flounder. The working hypothesis was that flounder, which preferentially settle in low salinity habitats, would display accelerated development and/or enhanced growth and condition at lower salinities. The fish used in the experiment were in the late stages of metamorphosis. At the end of the 21 day laboratory rearing period no significant difference in ontogenetic development was found between exposures (salinity of 0, 10, 20 and 30). No significant differences in somatic growth rate, somatic condition or standard length were observed between treatments. There was no correlation between RNA: DNA ratio and somatic condition. Contrary to expectations, mean RNA: DNA ratios (measure of short-term well being) tended to increase with salinity and were significantly higher in the 30 salinity exposure compared to the 0 salinity exposure. The working hypothesis was, therefore, rejected. The results demonstrate that laboratory observations can fail to capture the complex ecological interactions at play in field environments. The preference for low salinity environments may be driven by other environmental factors such as predator/competition avoidance and food supply.

Key words: European flounder; salinity; RNA: DNA; somatic condition; SGR.

1. Introduction

Estuarine and shallow marine habitats are important nurseries for the larval and juvenile stages of many flatfish species (Cabral *et al.*, 2007; Vasconcelos *et al.*, 2011). Here species distributions overlap (Ramos *et al.*, 2009) and species specific microhabitat use can occur (Ramos *et al.*, 2010). Estuarine nurseries can offer a number of advantages for young fish such as predator and competition avoidance, high food availability and rapid growth and development (Beck *et al.*, 2001, 2003; Le Pape *et al.*, 2003). However, estuaries can also impose physiological challenges on developing fish as the levels of certain abiotic factors can vary greatly between and within estuaries (Cabral *et al.*, 2007). Whilst intermediary salinity conditions can infer an advantage in terms of growth (Boeuf and Payan, 2001), low and variable salinity conditions (due to river run off and tidal

fluctuations) are a common feature of estuarine systems which can result in an increase in osmotic pressure (Hutchinson and Hawkins, 1990).

Salinity influences energy expenditure in fish; there is a significant energetic cost associated with the mechanisms used by fish to maintain osmotic balance (Boeuf and Payan, 2001). Osmoregulatory cost is generally lowest under isosmotic conditions (Jobling, 1994; Likongwe *et al.*, 1996) and can increase when moving from stable to variable salinity environments (Hutchinson and Hawkins, 1990). However, an isosmotic environment is not preferential for all species where optimal salinity in terms of growth and condition can vary during ontogenetic development (Cardona, 2000; Partridge and Jenkins, 2002; Allen & Cech, 2007). From a review of the literature, Deacon and Hecht (1999) showed that in general, marine spawned fish grew better at salinities higher than the isosmotic level whilst fresh water spawned fish had optimal growth below the isosmotic level. Information on species specific salinity tolerances and their interaction with ontogenetic development is useful for maximising growth rates, condition and development rates in aquaculture whilst recognition of environmental conditions promoting enhanced growth, survival and recruitment can aid the identification of high-quality nursery habitats.

P. flesus is an estuarine dependant flatfish during its juvenile phase (Martinho *et al*, 2010) and can therefore be exposed to more variable abiotic factors compared to species in other coastal habitats. Benthic settlement concludes the pelagic larval phase (Van der Veer *et al.*, 1991) and is associated with metamorphosis in the majority of flatfish (Geffin *et al.*, 2007). Low salinities can induce immediate metamorphosis in mature *P. flesus* larvae (Hutchinson and Hawkins, 2004). In the absence of sufficiently low salinity, flounder may delay settlement prolonging their sensitive larval phase (Hutchinson and Hawkins, 2004). Once settled, the post larval flounder exhibit vertical migrations and use estuarine tidal stream transport (Bos, 1999; Jager, 1999), to actively migrate to oligohaline sites (Bos and Thiel, 2006). Kerstan (1991) found that within estuaries, densities of juvenile *P. flesus* significantly increased with decreasing salinity. Salinity is therefore thought to be a steering factor in flounder transport, migration (Jager, 1998) and development (Hutchinson and Hawkins, 2004).

The overall quality of nursery areas can affect flounder growth, condition (Amara *et al.*, 2009), survival and eventual recruitment to the adult populations (Power *et al.*, 2000). Biochemical and morphometric indices are representative of the health and energy status of individual fish and are therefore reflective of overall habitat quality (Amara *et al.*, 2009; Tanner *et al.*, 2009; Vasconcelos *et al.*, 2009). RNA:DNA ratios (a nucleic acid

derived condition index) were first used to examine the nutritional status and general well being of fish in the 1960's and since then have become more widely used (Imsland *et al.*, 2002; Peck *et al.*, 2003; Gilliers *et al.*, 2004; Mercaldo-Allen *et al.*, 2006). This biochemical index reflects variation in protein synthesis rates where it is assumed that the amount of DNA (an index of cell number) is stable under changing environmental conditions whereas the amount of RNA (an index of the protein synthetic capacity of a cell) varies (Bulow, 1970, 1987).

Given that *P. flesus* migrate towards and develop in low salinity environments (Hutchinson and Hawkins 2004; Bos and Thiel 2006), it is hypothesised that post larval flounder will display enhanced growth, condition and rates of development under these conditions. Gutt (1985) showed that at a size of 4.2 to 5.3cm (3-4 months after settlement) food conversion rates, growth and condition of *P. flesus* are highest at intermediate salinities. The aim of the present study was to assess the overall affect of salinity on the development, growth and condition of *P. flesus* during and shortly after metamorphosis under controlled experimental conditions. Somatic growth rates and RNA: DNA ratios are used as indices of individual condition. Although experimental studies are not truly reflective of the natural environment, the assessment of environmental variables in isolation may provide an understanding of their effects upon fish life history traits.

2. Materials and Methods

2.1. Biological sampling

Post larval flounder were sampled during low tide from a known flounder nursery within Galway Bay on the 16th of April 2010. It is therefore assumed that all flounder sampled were from the same cohort. The site was situated within an estuary on the Oranmore River where salinity frequently falls below 0.5 salinity during low tide. Previous sampling expeditions recorded high abundances of flounder in this particular habitat where the presence of other flatfish species was rare. Flounder were sampled using hand nets. 200 fish were transferred alive to buckets containing water collected from the estuary whilst 40 fish (start control) were immediately frozen in liquid nitrogen on site. Subsequent to the experimental period fin rays of all fish were counted to confirm species identification (Russell, 1976; Wheeler 1969). Due to the high mortality and stress associated with fish handling it was decided to use the 40 control fish as a base line for comparison with post experimental fish, using the increase in mean length and weight measurements to estimate growth.

2.2. Experimental design

Four separate blue plastic re-circulation tanks were used for each salinity exposure. Each tank contained four separate compartments and fish were completely contained within each compartment and were unable to move between compartments. Three compartments were used as treatment replicates where each replicate held ten fish and the fourth held extra flounder. Where mortality occurred within a treatment, replacement fish were added from the fourth compartment to maintain constant densities in each replicate. Initially the water in each tank (dechlorinated tap water) was held at 0 salinity which was comparable to their natural environment in the estuary. After a period of acclimatisation (7 days) each tank had its salinity increased steadily (over 5 days) by gradually adding a concentrated mixture of dissolved peacocks sea salt and dechlorinated tap water until the desired salinity exposure was reached. The treatment conditions during the 21 day experimental period were held at the following salinities: Tank 1: 0; Tank 2: 10; Tank 3: 20; and Tank 4: 30. Temperature was maintained at around 11°C which closely matched the temperature in the natural environment. All fish were killed in liquid nitrogen after the experimental period and were subsequently stored at -80 for further analysis. Standard length (SL) (mm) and weight (g) of all fish were measured.

2.3. Feeding and rearing conditions

Nutritionally deficient *Artemia* were enriched with a homemade enrichment procedure described by Tamaru *et al.* (2003). Post larvae were fed live enriched *Artemia* nauplii twice daily. *Artemia* concentrations were increased steadily as the fish grew, from 300 *Artemia*/fish/day to 500 *Artemia*/fish/day. The unit used to house the experiment was devoid of external/natural light, instead a simulated natural light regime 14L: 10D was used. The water was gently aerated and nitrite, ammonia, salinity, temperature, pH and dissolved oxygen were monitored daily. A 15% - 25% water change was carried out every 3-5 days to maintain ammonia, nitrite and pH levels.

2.4. Otolith and eye migration examination

Sagittal otoliths (n=116) were removed from each fish and mounted in crystal bond. The presence/absence of accessory primordia (AP), which indicates the start of settlement (Karakiri *et al*, 1989) and metamorphosis (Modin *et al.*, 1996), was assessed on the right otolith under a compound microscope at 200x and 400x magnifications. All fish examined (n=116) displayed at least one AP on their sagittal otoliths. The ontogenetic stages of *P. flesus* were determined upon examination of eye migration, using appropriate

keys (Keefe and Able, 1993; Hutchinson and Hawkins, 2004). All fish examined fell into three developmental categories: stage IV, most of the left eye visible from the left side; Stage V, entire left eye is past the dorsal mid-line; and Stage VI, eye completely translocated.

2.5. Analytical protocol

RNA: DNA ratios were determined for each individual following a method described by Caldarone *et al.* (2001) and Clemmesen (1993). Essential trials were carried out (detection limits, standard calibration curves of RNA (Bakers yeast) and DNA (calf thymus) and spike recovery of homogenates) prior to routine use of the procedure as suggested by Caldarone *et al.* (2001). New standard curves were created for each 96 well plate.

Fish heads, caudal fins and gut contents were excised prior to analysis, therefore ensuring that gut content did not contribute to RNA: DNA ratio. Dissecting tools were rinsed with de-ionised water after each fish dissection to avoid cross contamination. Tissue was homogenised using glass beads and TEN-SDS 0.01% buffer and vortexed (15 min) using the pulse option and subsequently centrifuged (6000rpm for 10 min at 4°C). The supernatant was collected and stored in microtubes. Replicate samples of each supernatant were analysed to ensure acceptable reproductability. Nucleic acids concentrations were determined using an Ascent microplate fluorometer at the excitation wavelength of 355nm and emission wavelength of 592nm. Fluorescence was measured using ethium bromide. Total fluorescence was measured initially to determine total fluorescence within each sample. RNase (5µl) was subsequently added to each sample, incubated for 30 min (37°C) to facilitate the breakdown of RNA. The samples were then cooled to room temperature and a second fluorescence reading was taken. The RNA content was calculated by subtracting the 2nd reading (minus blanks) from the 1st reading (minus blanks) whilst total DNA was determined from the fluorescence remaining after the addition of the RNase solution (minus blanks).

2.6. Somatic condition and growth rate

Individual morphological condition was measured using the residuals of a regression model where the log standard length was fitted against log weight. It assumes that fish in bad condition have a large negative residual.

The specific growth rate (SGR) was estimated as $SGR = (e^{g} - 1)*100$ where $G = \ln (m_1/ m_0)/t$; m_1 = average weight of fish from the start control (n=40); m_0 = weight of

individual fish subsequent to the experimental period; t= experimental period (days). Due to the high mortality associated with handling, the mean weight of the control group (instead of the individual fish within the experiment) was used in calculating the SGR.

2.7. Data analysis

Data exploration was carried out as suggested by Zuur *et al.* (2010). True outliers were visualised using a Cleveland dotplot (R 2.13.0) and removed prior to statistical analysis. Homogeneity of variance was investigated using Bartlett's and Levene's test.

A one-way ANOVA was used to compare mean SGR between exposures. Nested ANOVA's were used to compare standard length and somatic condition between salinity exposures and between replicates (nested within exposures). A nested ANCOVA, with SL included as a covariate was used to determine if RNA: DNA ratio varied between exposures, or replicates (nested within exposures). In each model, replicate tanks were treated as a random factor and exposure as a fixed factor. When no significant difference was observed between replicates within each exposure, the nested level was eliminated and a one-way ANOVA was performed between exposures. Whenever the null hypotheses were rejected post hoc tests (Tukey's) were performed. A Pearson correlation was used to examine relationships between the measured variables. Chi square analysis was used to test for a significant difference between observed and expected frequencies in ontogenetic development between fish exposed to different salinities. Except where otherwise stated, all statistical analysis was carried out in Minitab 15 with a significance level set at $p \leq 0.05$.

3. Results

Mortalities during the tank acclimatisation and experimental period were low. During the first 2 days of tank acclimatisation, 52 mortalities occurred. Two mortalities were observed subsequent to acclimatisation, on day two and five in 20 and 30 salinity exposures respectively. Tank parameters (salinity, temperature, dissolved oxygen and pH) were maintained within safe and desired limits (Table 1).

Exposure	Temperature (°C)	Salinity	DO(%)	pH
0 Salinity	10.86 (s.d. 0.199)	0.00 (s.d. 0.00)	91.52 (s.d. 1.53)	8.15(s.d. 0.13)
10 Salinity	10.77 (s.d. 0.129)	10.16 (s.d. 0.06)	91.81 (s.d. 0.85)	8.00 (s.d. 0.09)
20 Salinity	10.85 (s.d. 0.137)	20.17 (s.d. 0.16)	92.02 (s.d. 0.59)	7.96 (s.d. 0.07)
30 Salinity	10.95 (s.d. 0.150)	30.10 (s.d. 0.16)	92.20 (s.d. 0.50)	7.92 (s.d. 0.09)

Table 1. Mean and standard deviation of daily measurements (temperature, salinity, dissolved oxygen and pH) within each salinity exposure.

RNA: DNA ratio and standard length of fish increased considerable over the experimental period (Fig. 1). Overall there was a weak but significant relationship between RNA: DNA ratio and standard length (n=116. P=0.036. r=0.195) whilst no significant relationship existed between RNA: DNA ratio and weight (n=116, p=0.055, r=0.179). No significant correlation was found between the RNA: DNA ratio and somatic condition (n=115, p=0.851, r=-0.018). Somatic condition was independent of standard length (n=120, p=0.957, r=0.005) and therefore comparisons of fish condition between tanks were not biased by standard length variation. No significant correlation was observed between the exposure means of SGR and RNA: DNA ratio (n=4, p=0.633; r=-0.367) and between SGR and SL (n=4, p=0.180; r= 0.820).

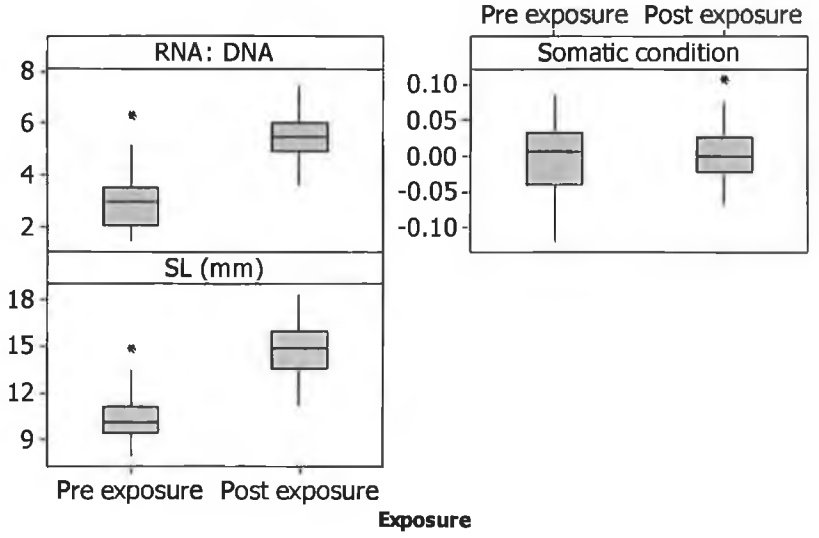

Fig. 1. Box-plot displaying RNA: DNA, Somatic condition and standard length (SL) of juvenile *P. flesus* pre and post experiment (fish combined from all exposure groups: 0, 10, 20 and 30). The horizontal line represents the median value and * symbolises extreme observations. The boxes signify the inter-quartile ranges whilst the whiskers (vertical lines) are values which extend from the box to adjacent values.

At the start of the experiment 60% of the control group (n=30) were found to be at stage IV, 33% at stage V and 7% at stage VI. At the end of the experiment 93% of *P. flesus* (n=120) were at stage V and 7% at stage VI showing that the flounder continued through metamorphosis during the experimental period. Chi–Square analysis established that there was no significant difference between the observed and expected values in flounder developmental stage between the salinity exposures (n=120, p=0.078).

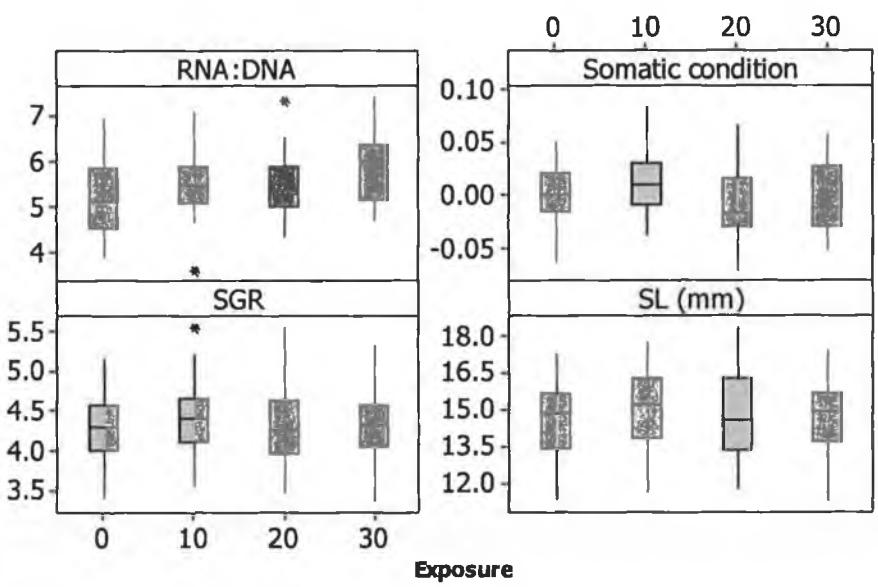

Fig. 2. Box-plot displaying patterns in RNA: DNA, somatic condition, specific growth rate (SGR) and standard length (SL) in each salinity exposure (0, 10, 20, 30). The horizontal line represents the median value and * symbolises extreme observations. The boxes signify the inter-quartile ranges whilst the whiskers (vertical lines) are values which extend from the box to adjacent values.

Nested ANCOVA revealed no significant difference in RNA: DNA ratio between replicates. Replicates were therefore pooled within each exposure. One-way ANCOVA revealed significant difference between exposures ($p<0.05$). Post hoc analysis revealed that the RNA: DNA ratio in flounder trunk muscle was higher in fish exposed to salinity of 30 compared to those in the 0 salinity exposure. No significant difference was found for somatic condition, SRG and SL. However, due to the use of mean weight rather than true weight per fish in calculating SRG, caution should be taken when interpreting results. Fig.2. displays the range and mean of each variable examined whilst Table 2 shows a summary of the ANOVA results.

Variable	Source	DF	F	P	R^2 (adjusted)
RNA: DNA					
	Co-variate: SL	1	6.26	0.014	11.10%
	Exposure	3	4.10	0.049 x	
	Replicate (Exposure)	8	1.07	0.392	
	Total	115			
	Co-variate: SL	1	5.16	0.025	10.67%
	Exposure	3	4.27	0.007	
	Total	115			
Somatic condition					
	Exposure	3	1.22	0.365 x	8.69%
	Replicate (Exposure)	8	1.88	0.071	
	Total	118			
	Exposure	3	2.27	0.084	3.12%
	Total	118			
Specific Growth Rate					
	Exposure	3	0.64	0.592	0.00%
	Total	119			
Standard Length					
	Exposure	3	0.66	0.601	0.00%
	Replicate (Exposure)	8	0.42	0.906	
	Total	119			
	Exposure	3	0.29	0.834	0.00%
	Total	119			

Table 2. Summary of statistical analysis of variance for RNA: DNA, somatic condition, specific growth rate and standard length.

4. Discussion

The results of present study show that under experimental conditions salinity influenced the biochemical condition of flounder during metamorphosis. Mean RNA: DNA ratios tended to increase with salinity and were significantly higher in the 30 salinity exposure compared to the 0 salinity exposure. Intermediate salinity conditions (10-20) did not appear to be optimal in terms of nutritional condition. This is in contrast to a previous experimental investigation which showed that in larger juvenile flounder (4.2 to 5.3cm), food conversion rate, growth and condition were highest at intermediate salinities (Gutt, 1985), suggesting that salinity responses can vary with ontogeny. The results of the current study are surprising given that *P. flesus* in the natural environment display a preference for low salinity sites during early development, actively seeking low salinity immediately prior to metamorphosis (Hutchinson and Hawkins, 1993; Bos and Thiel, 2006).

The lower RNA: DNA ratios observed at 0 salinity may reflect the physiological demands of living in freshwater; at low salinities, the higher levels of enzyme activity required to maintain plasma osmo- and ionoregulatory balance increase the demand for metabolic energy (Sampaio and Bianchini, 2002). As the results of the present study indicate, isosmotic conditions are not necessarily optimal for fish growth and condition. Euryhaline fish whose isosmotic point is generally between salinities of 10 and 13 (Sampaio and Bianchini, 2002) often show increased growth rates and enhanced condition under hypoosmotic conditions (Watanabe *et al.,* 1988; Sampaio and Bianchini, 2002; Moustakas *et al.*, 2004; Klaoudatos and Conides, 1996). An interactive effect between salinity and temperature has previously been found to affect feeding and growth of fish (Likongwe *et al.*, 1996; Imsland *et al.*, 2001; Wuenschel *et al.*, 2004). In particular an interaction effect with salinity and temperature has previously been shown to affect flounder egg and embryonic development (von Westernhagen, 1970). In this study, the effects of salinity were studied in isolation. Whilst intermediate salinities did not enhance growth, condition or development at 11°C, these conditions may become more favourable at different temperatures. Further experimental investigation, incorporating additional measurements (hormones, plasma, osmotic analysis etc) and a range of temperature regimes at each salinity exposure would be needed to test this hypothesis in post larval flounder. It is also worth noting that growth-related effects of salinity are not necessarily restricted to the metabolic costs of osmoregulation (Imsland *et al.*, 2002). Spontaneous activity and swimming behaviour (Boeuf and Payan, 2001) as well as food consumption, digestion and absorption of prey can be altered under different salinity regimes (Boeuf and

Payan, 2001; Jobling, 1994). These processes can affect energy expenditure and therefore fish condition.

Metamorphosis in flounder can be triggered by a reduction in salinity (Hutchinson and Hawkins, 2004). However, the results of the present study show that once metamorphosis has commenced, salinity does not affect development (as indicated by eye migration). An increased tolerance to short term changes in salinity during late metamorphosis has been observed in other flatfish species (Hiroi *et al.*, 1997; Schreiber and Specker, 1999); this may account for the continued development in the high salinity treatment and the absence of developmental differences between exposures.

Discrepancies between these experimental results and the behaviour and distribution of settling flounder in their natural environment highlight the difficulties associated with using laboratory experiments to understand complex ecological processes. Many studies in the literature have reported responses to salinity under laboratory conditions that are at odds with observations from wild populations. For example, three estuarine dependant species, the dusky kob (*Argyrosomus japonicus*), Brazilian flounder (*Paralichthys orbignyanus*) and spotted grunter (*Pomadasys commersonnii*) did not show enhanced growth rates under hyperosmotic conditions, and in all cases appeared to do better at higher salinities (Deacon and Hecht, 1999; Sampaio *et al.*, 2001; Bernatzeder *et al.*, 2010). Although laboratory experiments are not representative of the natural environment, studying the effects of an environmental variable in isolation can complement the interpretation of field studies. The growth and mortality responses of flounder vary between stable and fluctuating salinity conditions (Andersen *et al.*, 2005); therefore, an experiment with varying salinity exposures would more closely simulate the natural tidal estuarine environment and may provide a better indication of the optimal salinity conditions for growth, condition and survival of flounder during metamorphosis.

The active migration of flounder to freshwater areas, whilst possibly not metabolically advantageous, may lead to higher survival due to the decrease in predation and competition between and within species. Excessive competition (space and food), food availability, and high predation may act as a cue for the upstream migration of flounder (Beaumont and Mann, 1984; Bos, 1999) with salinity acting as the physiological trigger (Bos and Thiel, 2006). Habitat choice is governed by a combination of interacting environmental factors which ultimately affect the flatfish assemblage patterns (Ramos *et al.*, 2009). Habitat trade-offs have been described for many fish species, where fish choose less than optimal growing conditions to avoid predation and competition (Halpin, 2000; Camp *et al.*, 2011). Malloy *et al.* (1996) described the selection of suboptimal habitats for

growth by juvenile stone flounder (*Kareius bicoloratus*) due to the decreased predation risk. The brown shrimp (*Crangon crangon*), which inhabits marine and brackish habitats, is a common predator of flounder (Anon, 2011-FAO). Small post larval flounder are more vulnerable than larger juvenile to *C. crangon* predation (van der Veer *et al.*, 1991).. The movement of *P. flesus* outside of *C. crangon* habitat range may therefore be an environmentally induced survival mechanism. The re-colonisation of high salinity habitats by large (38-68mm) juvenile *P. flesus* in July (personal observation) may coincide with a decreased predation risk due to increased size. The influence of low salinity on metamorphosis in *P. flesus* may therefore be indicative of an evolutionary induced survival mechanism which ensures that flounder do not settle until they have reached a suitable nursery habitat (Hutchinson and Hawkins, 2004).

A review of the literature revealed that numerous studies found no relationship between RNA: DNA and somatic condition (Gilliers *et al.*, 2004; Tanner *et al.*, 2009; Vasconcelos *et al.*, 2009; De Raedemaecker *et al.*, 2012). Suthers (1998) proposed that a time delay in the response of a fish to the immediate environment (latency) may account for poor correlation between condition indices such as those used in the present study. Furthermore, the lack of variation in somatic condition between exposures in the present study may indicate that morphometric indices are less sensitive to environmental changes than biochemical indices such as the RNA: DNA ratio. RNA: DNA concentration can fluctuate over short temporal scales (Stierhoff *et al.*, 2009) whereas morphometric condition indices reflect well-being over a longer time period. Therefore, RNA: DNA ratios are likely to respond more rapidly to sub-optimal growing conditions compared to somatic indices. A number of studies report variation in growth, condition and osmoregulatory ability with experimental duration and stage of ontogeny (Imsland *et al.*, 2002; Sampaio and Bianchini, 2002; Trippel and Neil, 2003; Walther *et al.*, 2010). Therefore, experimental duration should be considered prior to experimental set up. Consequently, a longer experimental period (> 21 days) may have been more appropriate for detecting differences in somatic condition and specific growth rate. Although outside the scope of the present study, determination of RNA: DNA ratios at regular intervals over an experimental period would allow a more detailed examination of the response of condition to salinity and help to determine critical periods during metamorphosis when salinity exerts an influence on growth and condition.

A wide range of factors can affect variation in post larval growth and condition, therefore the understanding of such factors is important for assessment of the habitat quality of nursery areas. The results of the present study indicate that large differences in

salinity (0 – 30) can affect RNA: DNA ratios in metamorphosing post larval *P. flesus*. Over the relatively short experimental period, no differences were observed in development, somatic condition and growth between salinities. This finding suggests that although sensitive protein based indices are affected by salinity, post larval flounder are, overall, very well adapted for survival and growth in a wide range of stable salinities without being adversely affected. It is also suggested that salinity in the natural habitat, rather than directly impacting on growth of flounder, may act as a cue for predator and competition avoidance.

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Chapter Six

Spatio-temporal variability in juvenile flounder (*Platichthys flesus* L.) condition, age and size composition within Galway Bay, west of Ireland

Abstract

The present study aimed to investigate the effect of habitat characteristics on juvenile flounder in Galway Bay, west of Ireland, by analysing condition (RNA: DNA and morphometric condition), size (standard length) and age (days) at multiple spatial (estuaries and zones within estuaries) and temporal (years and months) scales. Based on surveys carried out in May and June 2008 correlation analysis established that flounder condition increased (RNA:DNA and morphometric) as surrounding salinity decreased. However, RNA:DNA showed no difference between upper and lower estuarine sites which were, in most cases (not always) representative of low and medium salinity conditions respectively. Nonetheless, individuals in low salinity zones were smaller and in better morphometric condition compared to individuals in the medium salinity. The spatial variation in size structure indicates that flounder move along a salinity gradient as they grow which may have had a knock on affect on the morphometric condition of individuals. The shift in microhabitats may be due to a number of factors such as prey type and availability, predator and competition avoidance. The significant decline in RNA:DNA from May to June (2008) is indicative of a reduction in growth rate which may be due to environmental and/or physiological factors. There was no correlation between the two condition indices and they did not show the same patterns of spatial and temporal variation. This is probably because RNA:DNA is more sensitive to environmental change compared to morphometric condition and therefore represents fish condition over a relatively short period of time. These findings aid our understanding of how flounder use estuaries as nursery habitats and how these use patterns can affect fish condition.

1. Introduction

Estuaries are recognised as essential habitats for many fish species worldwide (Whitfield, 1997; Able, 2005; Cabral *et al.*, 2007; Wasserman and Strydom, 2011). The dynamic environment within an estuary can lead to the formation of microhabitats (Allen and Baltz, 1997; Ramos *et al.*, 2009). The preference for specific microhabitats is the result of individual responses to environmental factors such as salinity, temperature, depth, sediment type, and prey and predator abundance and can vary with fish development and age (Wennhage, 2000; Gibson *et al.*, 2002; Bos and Thiel, 2006; Vinagre *et al.*, 2008a; Ramos *et al.*, 2010). In turn, spatial or temporal variation in exposure to varying environmental factors during early life stages can lead to differences in metabolism,

growth and survival, which can ultimately affect year-class strength and recruitment to the adult population (Houde, 1978; van der Veer *et al.*, 1994).

The overall quality of a nursery area can be evaluated by assessing and monitoring habitats by means of bio-indicator responses (Gilliers *et al.*, 2004), whilst examination of fish age and size structure within the nursery can give an indication of juvenile habitat use as fish grow and develop. Both biochemical (RNA: DNA) and morphometric condition indices have been successfully used in assessing nursery habitat quality (Fonseca *et al.*, 2006; Amara *et al.*, 2009; Vasconcelos *et al.*, 2009). Morphometric condition indices are widely used in fisheries research and are derived from a length-weight relationship (Blackwell *et al.*, 2000). RNA:DNA is a nucleic acid based condition index which assumes that the amount of DNA within a cell is constant and the amount or RNA varies in relation to protein synthesis (Bulow, 1970, 1987). Protein synthesis is responsible for the execution and regulation of anatomical and physiological functions and can account for 11-42% of the metabolic energy budget in a range of animal species (Fraser *et al.*, 2002). Individual growth rate and nutritional status have been directly linked to RNA: DNA, which has been identified as a short term indicator of fish condition (reviewed in Buckley *et al.*, 1999). Both condition indices are affected by a number of biotic (Islam and Tanaka, 2005; Walther *et al.*, 2010), abiotic (Imsland *et al.*, 2002; Stierhoff *et al.*, 2009; De Raedemaecker *et al.*, 2012) and anthropogenic (Amara *et al.*, 2009) factors, which makes them good indicators of habitat quality. Assessing nursery quality and describing patterns of estuarine use by juvenile fish can enhance understanding of early life history dynamics and subsequently of recruitment variability to the adult stocks which may encourage conservation and management of high-quality nursery habitats.

In this study, 0-group juvenile flounder were collected from upstream/ low salinity and downstream/ medium salinity zones in two estuarine systems in Galway Bay to investigate whether flounder habitat use was size and/or age dependent and to determine if differences in microhabitat use affected flounder condition (morphometric and biochemical). Variability in condition between estuaries was examined to establish if differences in habitat quality existed. The influence of season and individual size and age on flounder condition was also investigated. Finally, the relationship between both condition indices was tested to ascertain if they responded similarly to ecological conditions.

2. Materials and Methods

2.1. Field sampling

Sampling was carried out in inner Galway Bay on the west coast of Ireland. The sampling area was divided into two zones; low and medium salinity. Two upper estuarine/low salinity sites and two lower estuarine/medium salinity sites were sampled within the Corrib and Oranmore estuaries (Fig. 1). Sampling took place over three months (April, May and June) in 2008. Flounder were scarce in the medium salinity zones in April, so only fish from the low salinity zones in that month were included in the analysis. Fish from the low salinity sites within the two estuaries were sampled in April 2009 to determine if similar spatial patterns were observed in different years. Sampling of fish and measurement of water salinity took place within two hours of low tide. Salinity readings from late April to early July 2008 showed that the low salinity sites had consistently lower salinity levels than the medium salinity sites, with the exception of one occasion in the Corrib estuary (Fig. 2a and 2b). Due to the relatively large tidal range in Galway Bay each of the estuarine zones has different salinity concentrations at different tidal stages. Measuring the salinity of the estuaries over the tidal cycle was not possible due to the fast flow of water out of the estuaries. Nonetheless it's suggested that sites closest to the mouth of the estuary had higher salinity concentrations compared to sites further upstream irrespective of tide. Due to this natural gradient flounder in the lower estuarine sites would be exposed to consistently higher salinities than individuals further up the estuary.

All fish were frozen using dry ice or liquid nitrogen and subsequently stored at -80°C until processing. Standard length (SL) and weight of all fish were measured to the nearest 0.01mm and 0.001g respectively. Overall, 114 (112 included in aging) flounder were used in this study, 95 (93 included in aging) from 2008 and 19 from 2009.

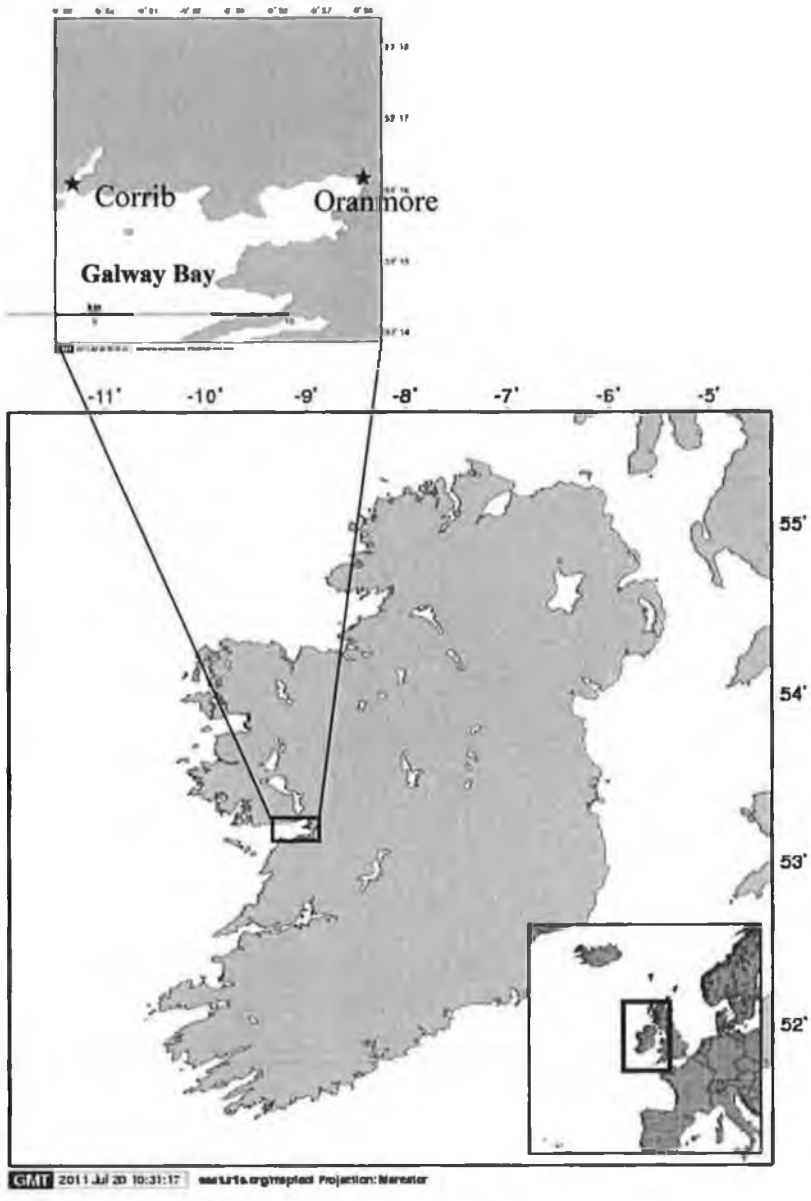

Fig. 1. Map showing the locations of the sampling sites on the north-west Galway Bay, west of Ireland.

2.2. Aging technique

Sagittal otoliths were mounted on glass slides using a suitable medium; small otoliths which required no polishing to reveal the increments were placed in clear nail varnish, larger otoliths were mounted in crystal bond and polished until the hatch check was visible using 2000 and 4000 grit silicon carbide. Otoliths were examined using an Olympus BX51 interfaced with a cooled mono 12 bit Q Imaging camera, PC and Image Pro 6.3 image analysis system. The total number of presumed daily rings (a pair of concentric light and dark bands) was counted on each otolith to estimate fish age and included both the larval and post larval (where present) phase.

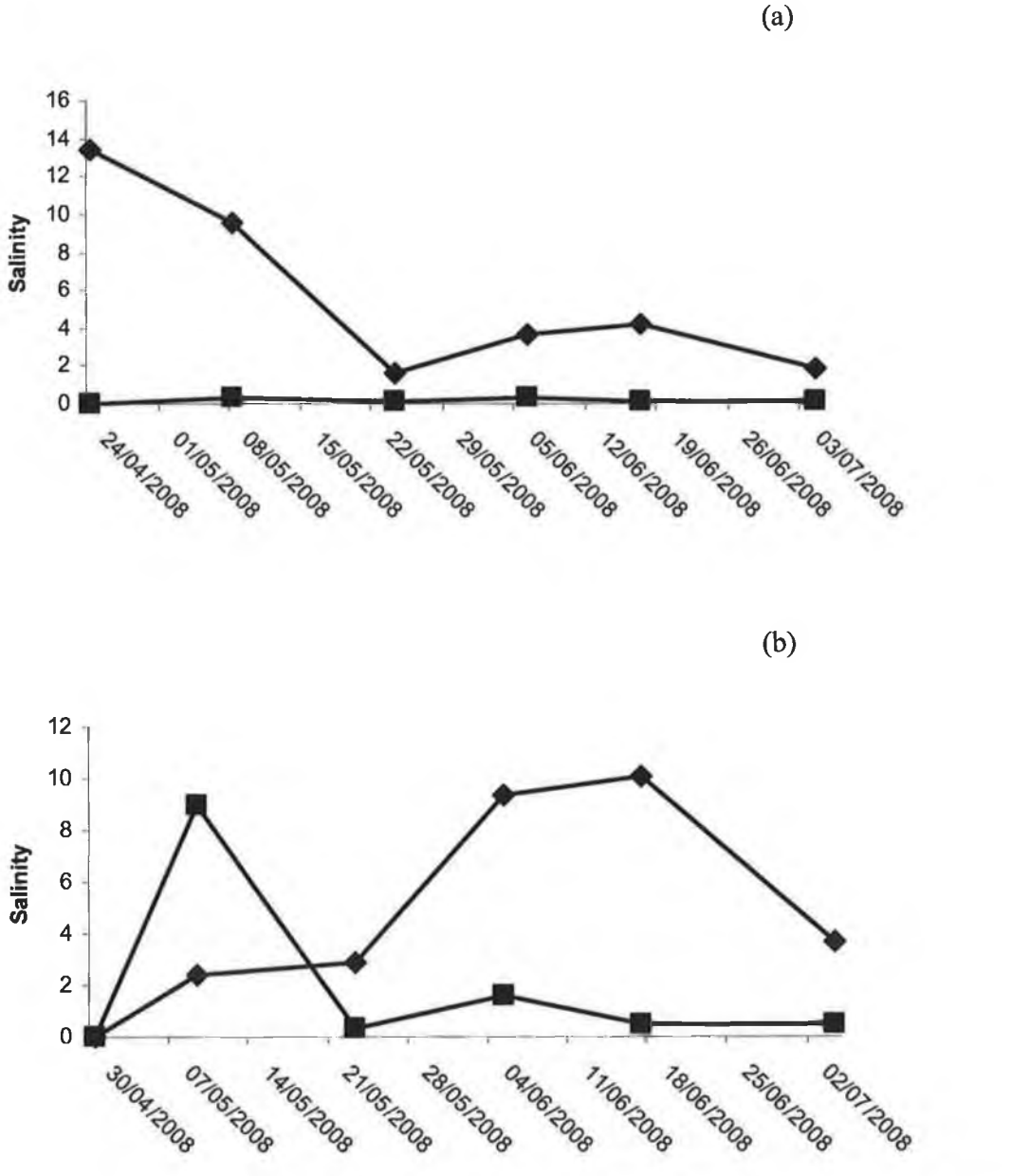

Fig. 2. Temporal salinity trend in the medium (♦) and low (■) salinity zones in the Oranmore (a) and Corrib (b) estuaries.

2.3. Condition Indices

Individual morphometric condition was measured using the residuals from a regression of $\log_{10}$ standard length on $\log_{10}$ weight model. This method is useful for assessing fish condition from a range of fish sizes (Blackwell *et al.*, 2000). The residuals capture the deviation of predicted weight from a common weight-length relationship. Fish in poorer condition are assumed to have a larger negative residual. This relative condition index is hereafter referred to as morphometric condition.

RNA: DNA ratios were determined from trunk muscle tissue for each individual fish following a method described by Caldarone *et al.* (2001) and Clemmesen (1993). Essential trials were carried out (detection limits and spike recovery of homogenates) prior to routine use of the procedure, as suggested by Caldarone *et al.* (2001). New standard calibration curves of RNA (Baker's yeast) and DNA (calf thymus) were created for each 96 well plate. Dissecting tools were rinsed with de-ionised water after each fish dissection to avoid cross contamination. Tissue samples were macerated and homogenised using glass beads and TEN-SDS 0.01% buffer in a pulsating vortex mixer (for 15 minutes) and subsequently centrifuged (6000 rpm for 10 minutes at 4 °C). The supernatant was collected and stored in microtubes on ice. When needed, supernatants were diluted to fall within the standard curve range. Replicate samples of each supernatant were analysed to ensure acceptable reproductability. Four wells in every microplate were used to determine fluorescence in the absence of tissue homogenate (Blank). The fluorescent dye, ethidium bromide, was used which binds to double and single stranded nucleic acids allowing both DNA and RNA to fluoresce. Nucleic acid concentrations were determined using an Ascent microplate fluorometer at 355nm excitation wavelength and 592nm emission wavelength. Total fluorescence was measured initially to determine total fluorescence within each sample. Five µl RNase (RNase purified from bovine pancreas, 20 U/ml) was subsequently added to each sample, incubated for 30 minutes (37°C) to facilitate the breakdown or RNA. The samples were then cooled to room temperature and a second fluorescence reading was taken. The RNA content was calculated by subtracting the 2nd reading (minus mean blank) from the 1st reading (minus mean blank) while total DNA was determined from the fluorescence remaining after the addition of the RNase solution (minus mean blank). Two replicates of control homogenates prepared from fresh mussel (*Mytilus edulis*) tissue were used in every microplate to verify accurate reproducibility of the method. When the co-efficient of variation of replicate samples exceeded 10% samples were re-run to obtain more precise results. In addition, the RNA:DNA slope ratio was calculated for each microplate (mean=1.99; s.d. = 0.11; n=8 microplates) to allow direct inter-calibration with other studies (Caldarone *et al.*, 2006).

2.4. Statistical analysis

All statistics was carried out in Minitab 15 (unless otherwise stated) with the significance level set at $p \leq 0.05$. All data were tested for outliers using a Cleveland dotplot (R Development Core Team, 2008), normality and homogeneity of variance. Where assumptions were not met a $\log_{10}$ (RNA: DNA for May and June sampled fish) or Box-

Cox transformation (morphometric condition for May and June sampled fish) was used to ensure equal variances. Data which was not normal was ranked prior to correlation analysis.

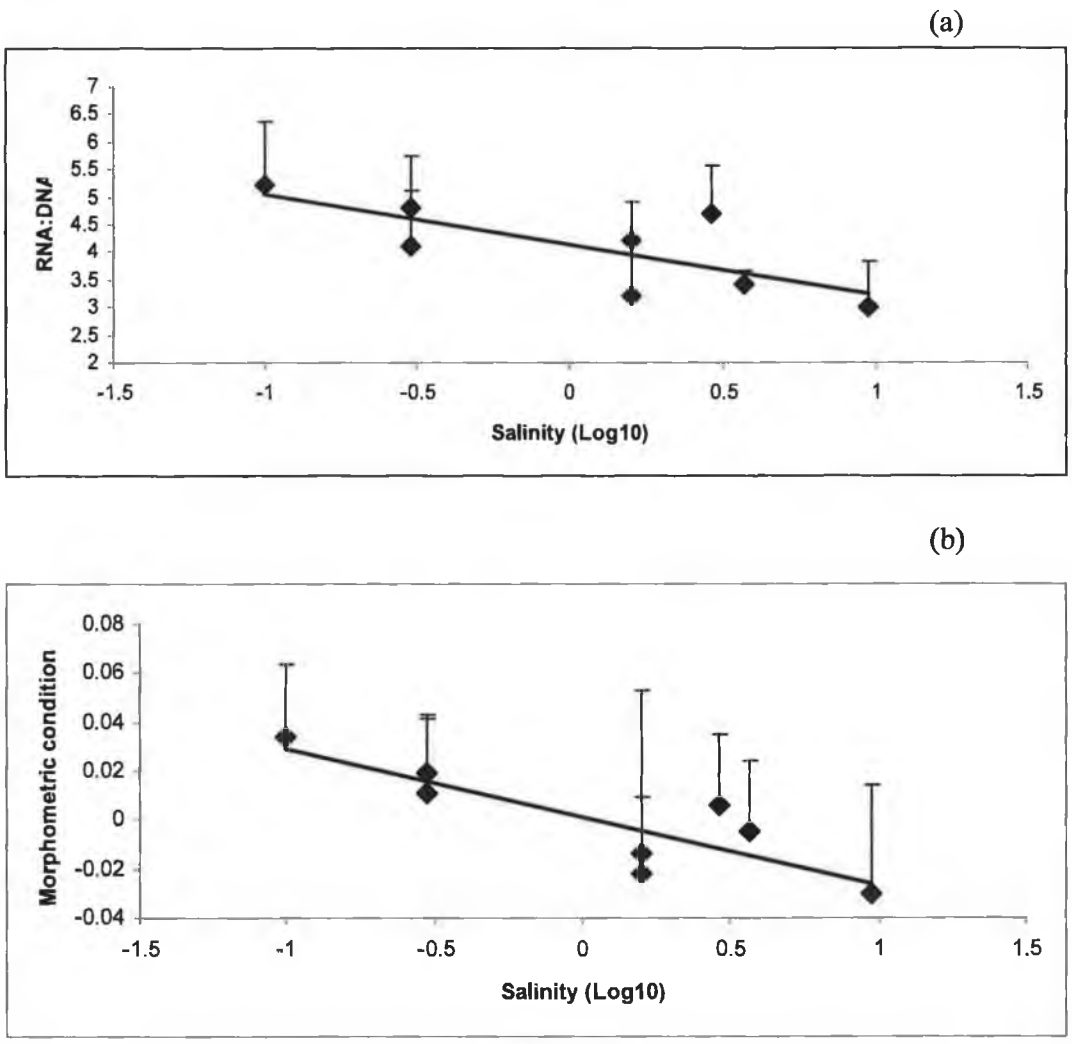

Fig. 3. Relationship between site salinity and mean RNA:DNA (a) and morphometric condition (b); horizontal lines denote standard deviation. Fish were collected from May and June 2008 (n=77).

A Pearson correlation was performed to test for a significant relationship between RNA: DNA and morphometric condition for all fish pooled (2008 and 2009, n=114). Additional correlations were performed to determine if there was a size effect for either condition indices in May/June 2008 and April 2008/2009. Correlation analyses were performed to test for a relationship between site salinity and mean RNA:DNA and morphometric condition on each sampling occasion.

ANOVAs were used to test for spatial and temporal variation in RNA: DNA, morphometric condition, age and standard length of flounder. One set of ANOVAs examined monthly variation in the measured variables by comparing data from May and

June 2008. Data from April 2008 was excluded from that analysis due to the lack of flounder in samples taken from the medium salinity zones in that month. Month and salinity zone were included as fixed orthogonal factors in the analysis and the random factor site was nested within salinity zone. The month*salinity zone and the month*site (salinity zone) interactions were also examined. A second set of ANOVAs used data from the low salinity zone in April 2008 and 2009 to explore inter-annual variation. Year was included as a random orthogonal factor and the random factor site was nested within year. In all cases, when no significant difference was detected at the lowest level of the analysis, the ANOVA was re-run excluding that level. When significant differences were detected, Tukey's post hoc test was used to establish the origin of these differences.

3. Results

Exploratory analysis revealed no significant correlation ($p>0.05$; $n=114$) between morphometric condition, RNA: DNA and standard length when fish from all years (2008 and 2009), sites (Corrib and Oranmore) and months (April, May and June) were pooled. For fish sampled in May and June 2008 ($n=77$) and April 2008/ 2008 ($n=37$) no significant correlation ($p>0.05$) were found between either condition indices (RNA: DNA and morphometric condition) and standard length ($p>0.05$). As there was no correlation between fish size and condition indices it was not necessary to adjust the data for size effect prior to ANOVA analysis. A significant correlation was observed between both condition indices (RNA:DNA, $p<0.05$, $r^2=0.56$; Morphometric, $p<0.05$, $r^2=0.73$) and site salinity for fish sampled in May and June 2008 (Fig 3a and b). When data from April 2008 and 2009 was included the relationship was no-longer significant.

Morphometric condition differed significantly between salinity zones. Individuals from the low salinity zone were in better morphometric condition compared to those sampled from the medium salinity zones. There were no significant difference in morphometric condition between months or sites nested within salinity zones and no significant interactions. Flounder sampled in June had a lower RNA:DNA compared to individuals sampled in May (Fig 4). A significant difference in RNA: DNA was observed between sites nested within salinity zones; flounder from the low salinity site in Oranmore were in better condition than those from the low salinity site in the Corrib. No significant interaction or salinity zone effects were found for RNA: DNA. In the analysis of fish age there was a significant interaction between month and site nested within salinity zone. An interaction plot revealed that flounder from the low salinity site in the Oranmore estuary

increased in age from May to June while the average age of flounder in the low salinity site in the Corrib estuary decreased slightly from May to June. The main age effects cannot be sensibly interpreted in the presence of a significant interaction. Flounder were significantly smaller (SL) in the low salinity sites compared to the medium salinity sites and were larger in June compared to May. A summary of the ANOVA results is displayed in Table 1.

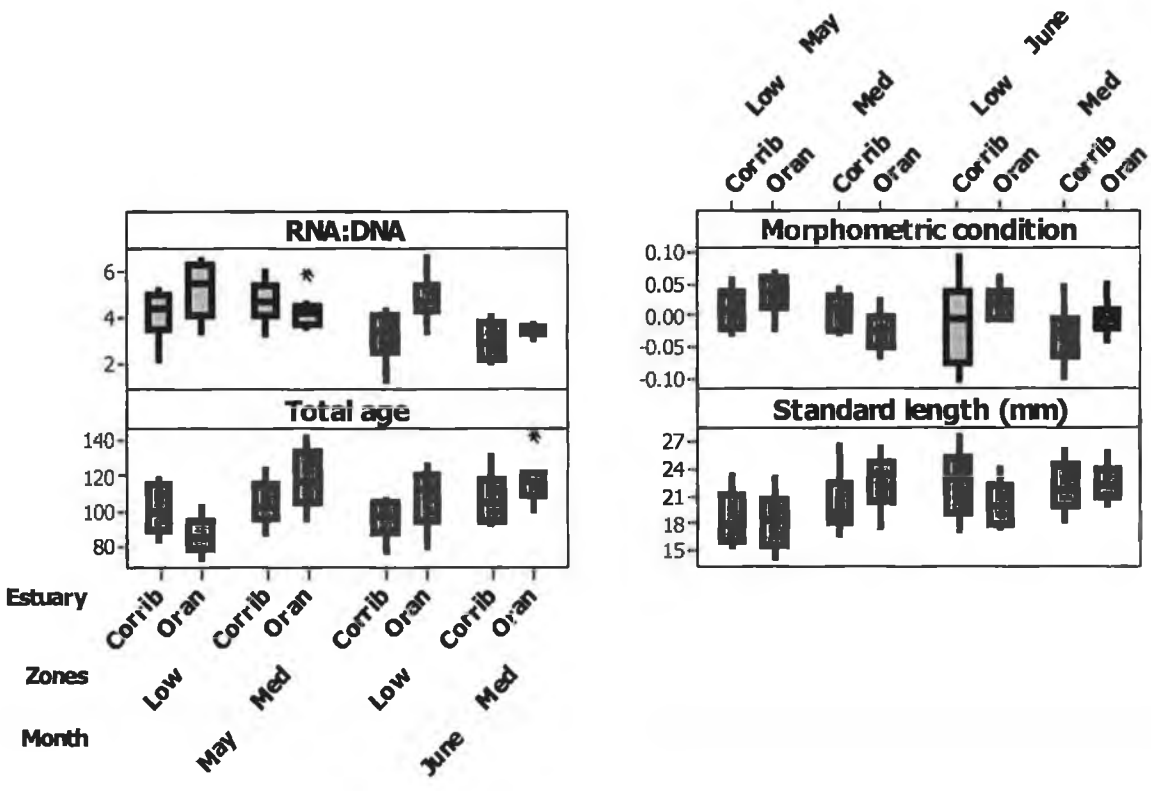

Fig. 4. Temporal and spatial variation of RNA: DNA (n=77), morphometric condition (n=77), total fish age (n=75) and standard length (n=77) of *P. flesus* over two months within the 2008 sampling season.

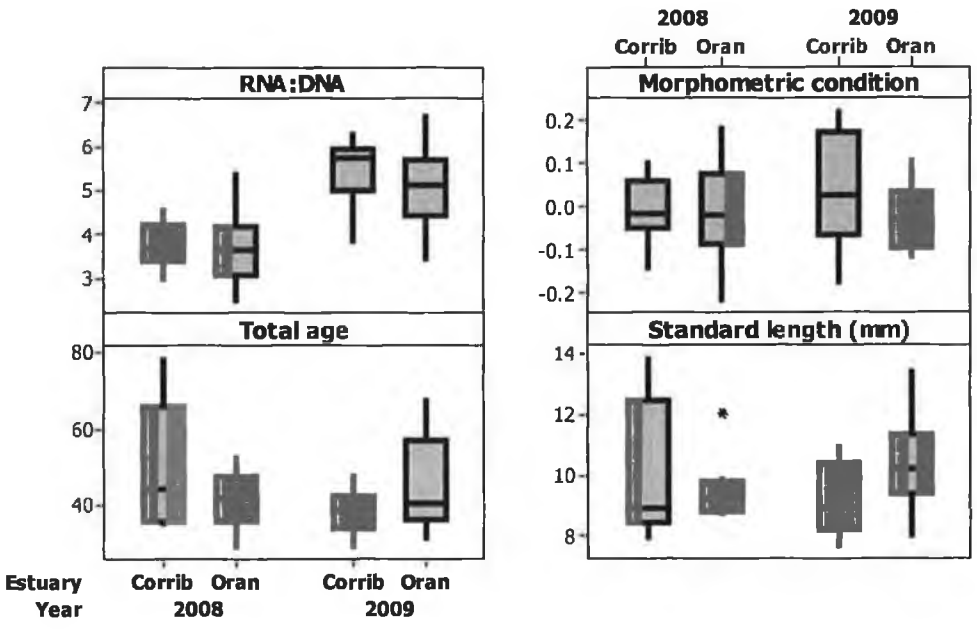

Fig. 5. Temporal and spatial variation of RNA: DNA (n=37), morphometric condition (n=37), total fish age (n=37) and standard length (n=37) of *P. flesus* within Galway Bay (2008 and 2009).

Examination of inter-annual variability revealed that flounder sampled in April 2009 had a significantly higher RNA: DNA ratio compared to individuals sampled in April 2008 while no significant differences between sites within each salinity zone were detected. There was no significant variation between years or sites for morphometric condition, fish age or standard length (Table 1; Fig 5).

Variables	df	RNA:DNA		Morphometric Condition		Total age		Standard length	
		F	P	F	P	F	P	F	P
May & June 2008									
Month	1	19.22	***	2.43	ns	0.33	ns	7.46	**
Zone	1	0.37	ns	9.47	**	6.44	ns	8.82	**
Site(Zone)	2	9.32	***	2.47	ns	0.61	ns	2.32	ns
Month*Zone	1	1.88	ns	0.03	ns	0.27	ns	2.17	ns
Month*Site(Zone)	2	2.14	ns	2.03	ns	5.47	**	1.48	ns
April 2008 & 2009									
Year	1	70.30	*	0.03	ns	0.76	ns	0.00	ns
Site(Year	2	0.46	ns	0.78	ns	1.82	ns	1.81	ns

Table. 1. Results of ANOVA comparisons of RNA: DNA, somatic condition, total fish age and standard length in juvenile *Platichthys flesus* from Galway Bay, west of Ireland. *P<0.05, **P<0.01, ***P<0.001, ns non-significant.

4. Discussion

In the present study, spatial (salinity zones and sites) and temporal (months and years) differences were observed in the condition (morphometric and RNA: DNA), age and size of juvenile flounder in two estuarine systems within Galway Bay. No significant difference in RNA:DNA was observed between designated salinity zones however, mean RNA:DNA was found to increase with decreasing salinity. Although each salinity zone was identified to represent low and medium salinity concentrations, salinity in each salinity zone fluctuated and was on occasion similar or inverted (higher salinity in the low salinity zone and vice versa). As RNA:DNA can vary over relatively short temporal scales (Stierhoff *et al.*, 2009; hours to days) recent salinity conditions rather than fish distribution are likely to have affected RNA:DNA either directly (energy cost) or indirectly (shift in prey type and quantity with salinity). In addition, mean morphometric condition also had a negative relationship with salinity and a significant difference in morphometric condition was detected between the upper estuarine sites and the lower estuarine sites.

These results are not consistent with previous experimental results. O'Neill *et al.* (2011) detected higher RNA: DNA in post larval flounder under high salinity (30) compared to individuals exposed to low salinity (0), while Gutt (1985) found that older juveniles had an increase in condition (morphometric) under medium salinities (5 and 15). Any physiological advantages associated with living at medium or high salinities may be off set by environmental factors in the wild, such as temperature, predation, competition and food availability. Although several cues simultaneously influence flounder behaviour and distribution (Bos, 1999; Vasconcelos *et al.*, 2010), salinity is commonly referred to as the main driving factor (Kerstan, 1991; Bos and Thiel, 2006). It is worth noting that salinity tolerances can change with ontogenetic development (Schreiber and Specker, 1999; Partridge and Jenkins, 2002) which can affect comparisons between studies that use fish of different developmental stages. Although isosmotic conditions are generally considered to reduce metabolic rate and enhance growth rate (Boeuf and Payan, 2001), this is not always the case and depends on a number of factors such as food ingestion and conversion efficiency which can vary at different salinities (Gutt, 1985; Deacon and Hecht, 1999; Imsland *et al.*, 2001a). In addition metamorphosing and recently settled flounder can use tidal stream transport to actively migrate towards low salinity environments (Jager, 1999; Bos and Thiel, 2006). These tidally induced microhabitat movements and fluctuations in external salinity concentrations may help explain the lack of variation in RNA: DNA between salinity zones. Discrepancies between experimental results and the

behaviour and distribution of euryhaline fish within their natural environment have been previously identified in a range of species (Deacon and Hecht, 1999; Bernatzeder *et al.*, 2010). Therefore, a combination of both field and laboratory studies can improve our understanding of how fish use and are affected by nursery ground habitats.

Flounder sampled in the low salinity zones were smaller and in better condition (morphometric) than those in the medium salinity zones suggesting that there was a size dependant shift in flounder habitat use patterns. Low salinity environments can offer shelter from predation and competition during early juvenile fish development (Tomiyama and Omori, 2008). Brown shrimp (*Crangon crangon)* can predate on small flounder (up to 30mm; Van der veer *et al.*, 1991) while habitat overlap between flounder and other flatfish species such as plaice can increase competition for preferred prey items (Mariani *et al.*, 2011). However, both brown shrimps and plaice are less tolerant of fresh water compared to flounder (Wheeler, 1969; Freitas *et al.*, 2009). In the present study it was observed that brown shrimp and plaice did not occur in the low salinity zones but were in low numbers in the medium salinity zones (Pers obs). Therefore, the smaller flounder in the low salinity zones may have enhanced protection from predation and competition compared to the larger fish found in the medium salinity zones. Although the larger individuals in the medium salinity zones are less vulnerable to predation they may still exhibit predator avoidance techniques (shelter more and consume less food) which can impair condition (Gibson *et al.*, 2002; Lemke and Ryer, 2006; Maia *et al.*, 2009). Similarly, a decrease in predation risk of the smaller juveniles in the low salinity zones may have led to a reduction in fish metabolic rate and energy demands (Howell and Canario, 1987) and an increase in morphometric condition.

Size dependant spatial segregation of juvenile flounder may be due to an ontogenetic shift in foraging success (Vinagre *et al.*, 2008a) which can reduce intra-specific competition (Amara *et al.*, 2001; Aarnio *et al.*, 2006). A change in prey type with estuarine gradient (Islam *et al.*, 2006; Vinagre *et al.*, 2008a) can influence flatfish size class structure due the foraging success of both prey and predator (Andersen *et al.*, 2005; Vinagre *et al.*, 2005, 2008a). As juvenile flounder grow they broaden the size range of prey (Andersen *et al.*, 2005; Vinagre *et al.*, 2008a). However, a varied diet rather than specialised diet can negatively affect fish condition as was observed in juvenile plaice in Galway Bay (De Raedemaecker *et al.*, 2010). If this is true for juvenile flounder the consumption of a wider range of prey by the larger juveniles in the medium salinity zones may negatively affect their morphometric condition. Given that the present study did not examine predator presence and abundance or prey type, the mechanisms underlying the

observed variation in juvenile flounder size, age and condition can only be speculated upon.

The decrease in flounder condition (RNA: DNA) from May to June 2008 is indicative of a reduction in individual growth rate which may be due to environmental and physiological factors. Several studies have described an increase in RNA and RNA: DNA with a decrease in water temperature (Buckley *et al.*, 1999; Imsland *et al.*, 2001b; Ramírez *et al.*, 2004; Walther *et al.*, 2010) which may be a compensatory mechanism in order to achieve constant growth rate (Goolish *et al.*, 1984). In addition, a decrease in RNA: DNA has also been associated with an increase in fish age, size and development (Gwak *et al.*, 2003; Fonseca *et al.*, 2006; Vinagre *et al.*, 2008b; Ciotti *et al.*, 2010; Tong *et al.*, 2010). Upon initial settlement flatfish are at high risk from predation (Ellis and Gibson, 1995), however, fast growth and high condition during this critical time can improve survival (Houde, 1987; Gibson *et al.*, 2002; Grorud-Colvert and Sponaugle, 2010) and therefore, may be more important for young post larvae compared to later juvenile stages. Other locally occurring factors such as prey availability and predation risk can also affect RNA: DNA of young fish (Skajaa *et al.*, 2003; Buckley and Durbin, 2006; Amara *et al.*, 2009) and have been shown to vary both spatially and seasonally (Gibson *et al.*, 2002; Vinagre *et al.*, 2008b; Amara *et al.*, 2009; De Raedemaecker *et al.*, 2012). Experimental investigation into how specific environmental variables (temperature, prey and predator type and abundance) can affect flounder RNA: DNA at different developmental stages is recommended for future work.

Despite the inter-annual variation in RNA:DNA homogenous morphometric condition indicates that the estuaries were of comparable quality over the two years. As discussed earlier, fish condition (RNA:DNA) can vary for a number of environmental reasons, however, rainfall and prey availability have been highlighted among the main causes of inter-annual variability in previous flatfish studies (Vasconcelos *et al.*, 2009; De Raedemaecker *et al.*, 2012). Due to the relationship between nutritional status and fish condition, future studies should also incorporate gut content analysis to help explain differences in individual condition.

Overall, the morphometric and biochemical condition indices showed different patterns of spatial and temporal variation. This is consistent with some previous studies (Vasconcelos *et al.*, 2009; Walther *et al.*, 2010; De Raedemaecker *et al.*, 2012). The response of fish to environmental change is reflected primarily in fish RNA: DNA and secondly in somatic growth and condition, which are short and long term indicators of fish condition respectively (Ferron and Leggett, 1994; Ramírez *et al.*, 2004; Stierhoff *et al.*,

2009; Tanner *et al.*, 2009). Consequently, the faster response of RNA: DNA implies that its relationship with morphometric condition indices can change rapidly and as a result, may not necessarily be correlated. Therefore, a combination of both morphometric and nucleic acid-based condition indices provides greater insight into the long and short term condition of juvenile flounder.

In conclusion, juvenile flounder displayed spatial and temporal variability in condition (morphometric condition and RNA: DNA), size and age. It was also established that flounder condition increased as exogenous salinity decreased which may be due to a number of environmental and physiological factors. The higher morphometric condition of fish in the upper estuarine (low salinity) sites is indicative of increased habitat quality (feeding and growth). The difference in size between flounder in the lower and upper estuaries suggests that flounder select different microhabitat conditions depending on their size, preferring the low salinity zones upon initial immigration and moving to the medium salinity zones as they grow. Finally, due to the variation in the response level in both condition indices, each of these two indices represent different ecological information and when combined are a useful proxy in assessing habitat quality.

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Chapter Seven

General Discussion

CHAPTER 7

This present chapter will summarise and review the previous topics covered in Chapters two-six, discuss them collectively and also in a broader ecological and geographic context. Although interest in flounder ecology has increased in the last number of years, research effort has been largely restricted to flounder sampled on the Portuguese coast and southern North Sea. However, in order to fully understand the life history of a species, sampling and research must be carried out throughout its distributional range. Identifying the environmental factors which affect survival, growth and condition at each life phase (egg-larvae-Juvenile-Adult) is important in understanding the overall recruitment success of a population. Identifying patterns and processes in the sensitive early life history stage can aid our understanding of flounder biology and ecology which is relevant to both fisheries assessments and aquaculture applications. The thesis presents important baseline information on the early life history of flounder and insight into microhabitat preferences and the mechanisms influencing growth and survival during and after settlement.

The main findings of the thesis are summarised below:

- In Chapter two, spatial and temporal variation in fin ray counts of flounder from nursery grounds on the east and west coasts of Ireland and the coast of Wales was presented. The potential for using meristic techniques for the identification of flounder stocks was discussed.
- In Chapter three, baseline information on early life history traits (hatch and settlement dates, pelagic larval duration and larval growth rate) of flounder was presented. Spatial and temporal variability in hatch date and larval duration of recently settled individuals may be indicative of selective mortality operating on larval traits and/or movement of flounder out of the sampling sites.
- In Chapter four, the use of beach and estuarine habitats by flounder during and after metamorphosis and settlement was described. It was suggested that flounder were either suffering enhanced mortality on the beaches compared to individuals sampled in the estuaries or that flounder quickly moved away from the beaches following initial arrival.
- In Chapter five, morphometric condition, biochemical condition (RNA:DNA), development and growth of flounder held at different salinity regimes (0, 10, 20, 30) was examined. Flounder held at 30 salinity had higher mean RNA: DNA than flounder from the other salinity treatments. This may be due to a change in energy necessary for osmoregulation and growth at higher salinities. None of the other measured variables

varied between treatments. These experimental results are contrasted with observations from wild populations.

- In Chapter six, condition (morphometric and RNA: DNA) was found to increase with decreasing salinity. This may be due to salinity related changes in community composition: e.g. the abundance of predators, competitors and prey items. As flounder grew they were found to move down stream perhaps to reduce intra-specific competition.

6.1. Stock plasticity

Fish stocks and populations are identified on the basis that there are differences in their characteristics due to environmental and/or genetic factors (Begg and Waldman, 1999). For flatfish, population complexity can vary from species to species. Changes in bathymetry, eddies, fronts, currents and swimming ability can all play a part in separating or mixing fish which originated from different spawning and nursery grounds (Bailey, 1997; Nielsen *et al.*, 1998; Hoarau *et al.*, 2004; Garcia-Vazquez *et al.*, 2006; Chapter one). The efficiency in delineating fish stocks can vary depending on the methods of detection used (genetic, phenotypic, otolith chemistry, parasitology). Genetic techniques are increasingly used in stock separation; however multiple classes of markers may be required to obtain reliable stock identification (Hoarau *et al.*, 2004). In addition, the choice of technique depends of the resolution required and therefore should be considered on a case by case basis (Begg and Waldman, 1999). Although genetic variation is suitable for examining evolutionary induced differences (Begg and Waldman, 1999), phenotypic variation is more appropriate to studying short term environmentally induced differences between fish stocks (Chittenden *et al*, 2010) and have been used to differentiate adult and juvenile fish populations (Gröger and Gröhsler 2001; Nielsen *et al.*, 1998). Therefore, phenotypic features such as meristics which can very depending on the exogenous fish larval environment (Colman 1976; Lindsey 1988) are suitable for detecting geographic origin (Chapter one).

Traditionally stock structure has been examined on large spatial scales, however, there is an increasing interest in small scale stock structure due to the failure of current management to sustain and manage fish stocks (Stephenson, 1999; Feeney and La Valley, 2011). Sub populations which partly depend on recruitment from their off spring for survival can be negatively affected by overfishing when they are not managed as separate entities (Rice and Cooper, 2003). Such collapses can affect the overall phenotypic and genetic diversity of a species (Hauser *et al.*, 2002; Hutchinson *et al.*, 2003; reviewed in Kenchington and Heino, 2003).

The programme of 8th International Flatfish Symposium (IJmuiden, The Netherlands, 2011) focussed on connectivity, underlining the current relevance of this topic. Two keynote speakers (Felipe Volckaert and Mike Sinclair) emphasised the importance of stock structure and connectivity to flatfish ecology and management. The degree of stock complexity will depend on migration patterns and tactics such as transport vs. retention, migration vs. residency, philopatry vs. vagrancy and homing vs. random movements. Therefore, a full understanding of life history dynamics throughout a species life and distribution (over both small and large spatial scales) is required to make comprehensive management decisions. In this thesis, only modest separation was achieved using meristic techniques. However, the findings presented in Chapter one highlight the potential use of fin ray counts as part of a multidisciplinary approach to flounder stock identification and determination of nursery ground origin.

6.2. The importance of the larval phase

It is widely recognised that growth and development during the early life stages in fish are critical for determining year class strength and recruitment to the adult spawning population (Houde, 1987; Gibson, 1994; van der Veer *et al.*, 1994). Hjort first recognised the importance of the larval stages in determining recruitment variability over 100 years ago. He developed a number of theories such as the 'critical-period' (the importance of first feeding) and 'aberrant drift' hypothesis (dispersal of eggs/larvae by unfavourable currents) (reviewed in Houde, 2008). Although these theories explain recruitment variability in some stocks (Theilacker *et al.*, 1996; Bergenius *et al.*, 2002) they do not sufficiently describe the fluctuations across all species. Therefore additional theories have been proposed. It is suggested that fish which grow fast (growth-mortality; Anderson, 1988), spend less time at the larval phase (stage-duration hypothesis, Houde, 1987; Anderson, 1988) and are larger at age (bigger is better; Miller *et al.*, 1988) have an increased chance of survival (reviewed in Houde, 2008). In order to study the mechanisms affecting recruitment one must first understand the life history patterns and the extent to which they can vary both temporally and spatially. In addition, determining life history patterns may be useful in helping to discriminate between environmental and fisheries driven variability in year class strength and recruitment.

Although estuarine spawned flounder have been described in the Minho estuary, Portugal (Morais *et al.*, 2011), it is generally accepted that most flounder populations and cohorts reproduce in the marine environment (Wheeler, 1969; Bos, 1999; Ramos *et al*, 2010). The findings presented in Chapter four also suggest a marine rather than an

estuarine spawning population. Spawning of flatfish occurs over an extended period of time (Gibson, 2005) which may result in the match/ mismatch of cohorts and populations to peaks in food availability (Cushing, 1990). Fish that are spawned and hatched during suboptimal conditions such as strong off-shore winds, low temperature, high predation and low prey abundances may be unsuccessful in reaching coastal nursery grounds (Houde, 1987, 2008), while those that spend longer in the pelagic environment and grow slower are less likely to survive the first year of life (Houde, 1987, 1989; Anderson, 1988; Grorud-Colvert and Sponaugle, 2010).

Though larvae experience the highest mortalities, predation during settlement can also cause considerable losses (Gibson *et al.*, 2002). Vulnerability to predation during this period can be influenced by effects carried over from the larval phase (Raventos and Macpherson, 2005; Grorud-Colvert and Sponaugle, 2006, 2010; Fontes *et al.*, 2011). As discussed in Chapter three, certain larval traits such as fast growth, enhanced condition and short larval durations are advantageous for juvenile survival whilst individuals with contrary larval histories can be removed from the population over time (Gibson, 1994; Takasuka *et al.*, 2007; Islam *et al.*, 2010). As a result, juvenile survivors may represent a subset of the life history traits displayed by the original population. In Chapter three, individuals with long pelagic larval durations were observed to have been removed from the population over time. Identifying characteristics subject to selective mortality can inform restocking programs and can enhance our understanding of how and why recruitment may fluctuate.

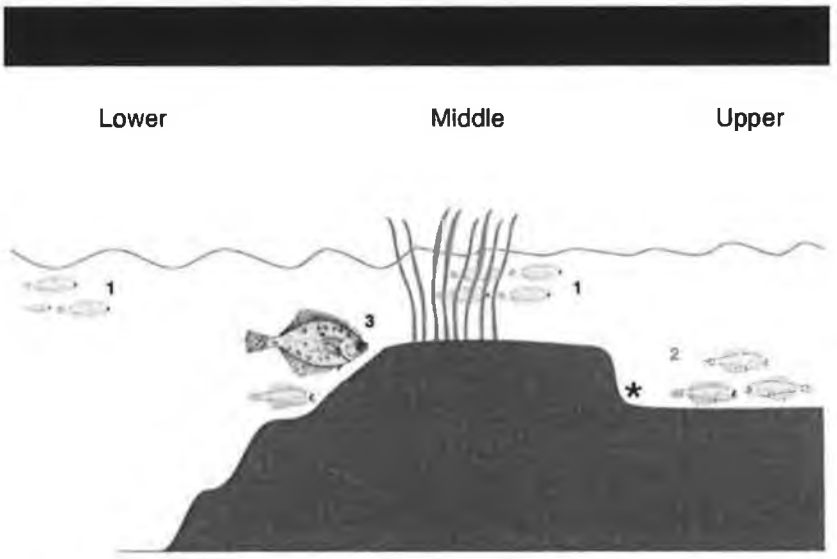

Fig. 1. Summary of the early life of *P. flesus* in the lower, middle and upper Lima estuarine sections. 1—metamorphosing larvae; 2—new settled juveniles and 3—adults; *—possible settlement area (Ramos *et al.*, 2010).

6.3 Microhabitat use in juvenile flatfish

Subsequent to inshore movement of larvae, flounder can be found in a number of diverse habitats which may vary in quality as observed in Chapter four. Flounder were present on both beach and estuarine habitat in spring; however individuals on the beaches appear to suffer high mortalities and/or move out of the beaches after initial arrival. A different pattern was observed in the estuaries which the flounder continued to occupy until late summer/autumn. The results presented in Chapters four and six suggest that flounder do better in low salinity estuarine habitats compared to beach habitats. Due to the relatively small spatial window available for direct transportation of flounder to optimal habitats, it is probable that some flounder will arrive on suboptimal sites, which can have consequences for growth, development and mortality.

Ontogenetic movement of juveniles has been identified in a number of flatfish species including flounder (Ramos *et al.*, 2010, Fig. 1; Chapter 6) and allows fish to use microhabitats suitable for each size class and developmental stage (Allen and Baltz, 1997; Martinho *et al.*, 2007; Ramos *et al.*, 2010). Upon entering estuaries 0-group flounder migrate to middle and upper estuarine sites (Bos, 1999; Ramos *et al.*, 2010) which offer both suitable prey items and a reduced risk of predation during the vulnerable settlement period (Beaumont and Mann, 1984; Bos, 1999). As flounder grow they are less susceptible to predation (van der Veer *et al.*, 1991), can feed on a broader range of prey items (Andersen *et al.*, 2005; Nissling *et al.*, 2007, Fig. 2) and shift their distribution down stream (Ramos *et al.*, 2010).

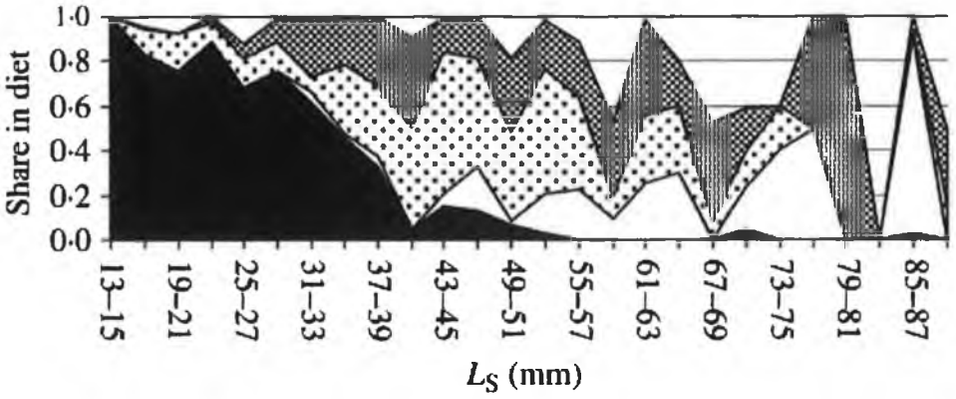

Fig. 2. The contribution of some major food items (■, copepods; , amphipods; ⁘, Oligochaeta; ▩, Chironomidae) to the diet of flounder in relation to length (samples from 2004) (Nissling *et al.*, 2007).

Flounder, plaice and dab (*Limanda limanda* L.) are found on near shore habitats along the Irish coastline (Allen *et al.*, 2008; De Raedemaecker, 2012; this thesis - Chapter four). Although juvenile dab, plaice and flounder distributions can overlap (Gibson *et al.*,

2002; Mariani *et al.*, 2011), peak densities of plaice and dab occur between 1-3m and 3-5m respectively while juvenile flounder are typically distributed in shallower water (<1.4m depth) (Bolle *et al.*, 1994; Pihl *et al.*, 2000; Andersen *et al.*, 2005) reflecting the different life strategies adopted by the species. In addition, salinity can strongly influence flatfish distribution; flounder preferentially inhabit low salinity environments during the juvenile phase (Kerstan, 1991; this thesis - Chapters four and six) while both dab and plaice are less tolerant of reduced salinities (Elliott *et al.*, 1990; Mariani *et al.*, 2011). Intra-specific habitat partitioning has also been described for each species where microhabitat preference changes as fish grow and develop (Gibson *et al.*, 2002; this thesis - Chapter six). The inter and intra specific resource partitioning exhibited by the three flatfish species may prevent competition for prey items (Aarnio *et al.*, 1996; Amara *et al.*, 2001; De Raedemaecker *et al.*, 2011; Mariani *et al.*, 2011) consequently increasing survival. To further understand such resource partitioning a multispecies approach could reveal the factors that affect the ability of flatfish species to utilise low salinity habitats (e.g. as a consequence of predator pressure).

An essential fish habitat (EFH) has been defined as "those waters and substrate necessary to fish for spawning, breeding, feeding or growing to maturity" (Magnuson-Stevens Fishery Conservation and Management Act). Degradation of EFH due to land reclamation and pollution may reduce the ability of species to partition the available habitat and increase overlap and competition. Furthermore, human induced change in the environmental factors which determine flatfish dispersal (water currents) and distribution (temperature, salinity, prey availability) could affect their chances of finding optimal nursery habitats and subsequently influence survival and recruitment. EFH have been extensively studied in the United States where EFH have been described for around 1000 managed species under the federal fishery management plan (Anon, 2012). Identifying and describing EJH can be useful in assessing threats to habitats which fish rely on. However, descriptions of EFH have been limited to commercially important fish and do not address the impact of ecosystem processes such as trophic level interactions (LoSchiavo, 2005). In recent years the ecosystem approach to fisheries management has become increasing popular which takes the whole ecosystem (including socio-cultural and economic factors) and their interactions into account when making management decisions, ensuring the long-term health and diversity of an ecosystem (Reviewed in Garcia and Cochrane, 2005).

6.4. The effects of microhabitat structure and salinity on juvenile flounder condition

Microhabitat selection is driven by species specific needs (Allen and Baltz, 1997; Baltz *et al.*, 1998) which can vary during ontogenetic development (Partridge and Jenkins, 2002; Adams *et al.*, 2004; Allen & Cech, 2007; Ramos *et al.*, 2010; Chapter six) and can result in variability in growth, condition and mortality (Islam *et al.*, 2006; this thesis - Chapter six). All fish must osmoregulate in order to maintain osmotic pressure which can be an energy demanding process (Boeuf and Payan, 2001). When fish are outside an isosmotic environment the energy needed to maintain osmotic balance generally increases (Jobling, 1994; Gaumet *et al.*, 1995; Likongwe *et al.*, 1996) although this is not definitive (Boeuf and Payan, 2001). Therefore, a shift in microhabitat preference with ontogenetic development such as that described in Chapter six may represent a trade-off between the energy necessary for osmotic regulation and the presence of predator, prey items and competitors.

Overall, fish growth and condition can vary for a number of environmental (salinity, temperature, prey type and concentration) and physiological (age, size and development) reasons (Buckley *et al.*, 1999; Imsland *et al.*, 2001, 2002; Gwak *et al.*, 2003; Fonseca *et al.*, 2006; Vinagre *et al.*, 2008b; Ciotti *et al.*, 2010; Tong *et al.*, 2010; Chapter five and six). Interestingly Imsland *et al.* (2001) found that although temperature affected turbot growth and food conversion there was also an interaction affect between temperature and salinity. This suggests that the effect of salinity on growth and condition can change as temperature changes. In addition, prey type can also vary with salinity (Islam *et al.*, 2006) subsequently affecting food conversion rate (Gutt, 1985). As a result, any physiological advantages associated with living at specific salinities can be offset by other environmental factors in the wild, such as predation, competition, food availability, temperature and substrate which can vary on relatively small spatial scales (Martino and Able, 2003; Zucchetta *et al.*, 2010). Therefore, it is suggested that salinity alone may not be the driving factor determining flounder distribution and abundance, but may co-vary with more important regulating factors such as those discussed above. Interactions between environmental variables may help explain the contrasting results found in Chapters five and six. This study highlights the difficulty in asking real world questions in a controlled experimental environment. Nonetheless, experiments are useful for examining the effect of specific experimental factors in isolation which can be interpreted relative to the natural dynamics at play on the wild population in the natural environment. Further investigation into how each environmental factor can influence fish condition and growth is warranted. Nonetheless, since fish presumably grow faster and have enhanced condition in good

quality habitats, condition and growth rate are suggested as useful proxies for determining habitat quality.

The relationship between morphometric and biochemical (RNA:DNA) condition indices can vary and a number of studies found no relationship between the two (Gilliers *et al.*, 2004; Tanner *et al.*, 2009; Vasconcelos *et al.*, 2009; De Raedemaecker *et al.*, 2012; Chapter 5 and 6). Although both indices reflect the health and energy status of a fish they are representative of different time scales (Stierhoff *et al.*, 2009) which may be due to a time lag in the response of each index (Suthers, 1998). Morphometric condition showed less variation compared to RNA:DNA (Chapter 5 and 6) which suggests that it is more stable and less prone to short term influences compared to RNA:DNA.

6.5. Threats to flatfish nursery grounds.

6.5.1. Pollution

In recent years human impact has influenced ecosystem viability and condition. Near shore habitats such as beaches, estuaries, reefs, mangroves and salt marshes are most at risk from human interference due to the close proximity to urbanised human settlements (Beck *et al.*, 2003; Coleman *et al.*, 2008; Courrat *et al.*, 2009; Defeo *et al.*, 2009; Rochette *et al.*, 2010). Pollution and degradation/loss of coastal habitats is a cause for concern as many flatfish species rely on these areas as nursery grounds (Gilliers *et al.*, 2006; Rochette *et al.*, 2010) which can have a knock on effect on the overall recruitment and sustainability of stocks (Peterson, 2003; Rochette *et al.*, 2010). Rivers and estuaries are particularly sensitive to anthropogenic affects and are some of the most modified and threatened aquatic environments in the world where chemical (toxic compounds, PCBs and heavy metals), thermal (power plants) and eutrophication (organic compounds) pollution can affect the community structure and quality of these ecosystems (Jones *et al.*, 1996; Hall *et al.*, 1997; Whitfield and Elliott, 2002; Gilliers *et al.*, 2006; Courrat *et al.*, 2009).

Flatfish are particularly sensitive to heavy metal pollution as they spend a large proportion of their lives living on and feeding from the benthos where many contaminants accumulate (Johnson *et al.*, 1998; Bolton *et al.*, 2003). High levels of heavy metals can impose considerable stress and energy demands on individuals (Hopkins *et al.*, 2000) and negatively influence juvenile flatfish density, growth and condition (Gilliers *et al.*, 2006; Amara *et al.*, 2007, 2009). In addition, the introduction of organic matter into the river can significantly increase the growth and reproduction of plankton and macrophyte vegetation which can consume large quantities of oxygen from the water. The tolerance for dissolved oxygen is species and life stage specific (Breitburg *et al*, 1991; Marshall and Elliott, 1998;

Tallqvist *et al*, 1999; Yamashita *et al*, 2001) and low levels can limit food digestion, conversion, metabolic rate and consequently growth rate (Neill and Bryan, 1991; Jobling, 1994; Stierhoff *et al.*, 2006; Del Toro-Silva *et al.*, 2008). Flounder are especially vulnerable to anthropogenic influences given that they often inhabit estuaries that are close to human settlements and sources of pollution.

Although a waste water treatment plant caters for the Galway City and Oranmore area, no municipal waste water treatment is available for residents surrounding these areas including further upstream from the Corrib estuary (Lough Corrib) (Anon, 2007). Consequently, run-off from farms, residents and businesses can affect the river and estuarine ecosystem (Neal and Jarvie, 2005; Toner *et al.*, 2005) and the benthic community that rely on them (Hall *et al.*, 1997). Although the effects of pollution on flounder habitat quality use were not examined in the present thesis, it is an important topic and future studies should address how flounder microhabitat preferences, growth and condition are influenced by structural alterations and organic and chemical pollution within estuaries. Pharmaceuticals in sewage waste are of particular concern as they can contain a cocktail of endocrine disrupting chemicals such as oestrogen which when present in high quantities can affect development and reproduction (Janssen *et al.*, 1997; Desbrow *et al.*, 1998; Routledge *et al.*, 1998; reviewed in Vos *et al.*, 2000).

6.5.2. Global climate change

It is the generally held consensus among scientists that the world's oceans are warming due to a combination of natural and anthropogenic influences (Levitus *et al.*, 2000; Spielhagen *et al.*, 2011). Climatic influences on the marine and estuarine environment can cause shifts in the distribution, abundance and habitat use patterns of fish species (Cabral *et al.*, 2001, 2007; Attrill and Power, 2002; Sims *et al.*, 2004; Perry *et al.*, 2005). Although warmer waters are generally advantageous for fish growth (Karakiri and von Westernhagen, 1989; Grorud-Colvert and Sponaugle, 2010) flounder egg viability are compromised when temperatures exceed 12°C (von Westernhagen, 1970) and considerable mortality can occur when flatfish are exposed to exceedingly high temperatures (Berghahn *et al.*, 1993). If, as predicted, Irish air temperature increases by 1.25-1.5 by 2060 (McGrath *et al.*, 2005) it will undoubtedly have an affect on coastal ecosystems and subsequently flounder ecology and recruitment. McGrath *et al* (2005) also predicted a 10-25% increase in summer precipitation and 10% decrease in winter precipitation which may have further consequences for aquatic life. Given the euryhaline nature of flounder and its attraction to fresh water, fluctuations in salinity and river flow can have a knock on effects on how

flounder use estuarine habitats during early development. Therefore, continuous monitoring of nursery ground functioning is suggested to detect any fluctuations in habitat use patterns. A detailed understanding of how different life stages are affected by anthropogenic influences (climate change, pollution and habitat degradation) could improve our understanding of the mechanisms driving recruitment variability.

6.6. Concluding remarks

From a management point of view understanding the entire life cycle of a fish and how and why aspects of their life can vary over time and space is important for making good management decisions. This work provides baseline information on early life history traits and habitat use patterns of flounder which can be beneficial in the conservation and management of essential juvenile habitats and fish stocks. Nonetheless further research is necessary to fully understand all aspects of flounder life history. Identification of flounder spawning grounds off the Irish west coast, larval transport mechanisms and the juvenile contribution of each nursery ground to the adult populations is necessary in understanding recruitment variability and how recruitment can change due to anthropogenic pressures such as pollution and climate change.

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